DEVELOPMENT AND EVALUATION OF siRNA LOADED GELATIN NANOCARRIERS FOR THE TREATMENT OF ASTHMA

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAI‘I AT HILO IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN PHARMACEUTICAL SCIENCES

MAY 2016

By

Susanne R. Youngren-Ortiz

Dissertation Committee:
Mahavir B. Chougule, Chair
Kenneth R. Morris, Co-Chair
Aaron Jacobs
Mazen Hamad

Keywords: Asthma, Gelatin, Nanoparticles, siRNA, Parenteral, Intravenous, Inhalation
DEDICATION

This dissertation is dedicated to my mother, without whom none of my accomplishments would have been possible.

This dissertation is also dedicated to my husband and son who, despite experiencing sacrifices and challenges during the time I was completing this work, had everlasting support, patience, and love.
ACKNOWLEDGEMENTS

This dissertation would not have been possible without the support and encouragement from my family, friends, classmates, research advisors, committee members, and all of the faculty and staff of the Daniel K. Inouye College of Pharmacy. I thank the PhD Program Directors, Dr. Anthony Wright, Dr. Anthony Otsuka, and Dr. Ghee Tan who took their time to guide the PhD program to promote graduate student success. I thank Dr. John Pezzuto, the founding Dean, and Dr. Carolyn Ma, the interim Dean of the Daniel K. Inouye College of Pharmacy for supporting the presence of a vigorous research program that consists of PhD research graduate students.

I have immense gratitude to my research advisor, Dr. Mahavir Chougule, for his guidance and mentorship during my graduate studies. He has given me the opportunities to challenge myself in my knowledge and skillset in pharmaceutics and molecular biology, and by doing so, sparked my progress as a scientific researcher. I extend my sincere gratitude for my dissertation co-Chair, Dr. Kenneth Morris, and my dissertation committee members, Dr. Aaron Jacobs and Dr. Mazen Hamad, who provided key constructive criticism and encouragement during my dissertation research and writing.

I send my sincere thanks to my lab mates both present and past for their advice and encouragement, including Dr. Rongbing Yang, Dr. Laura Espana-Serrano, Dr. Rakesh Tekade, Mr. Nishant Gandhi, and Mr. Micah Glasgow. In addition, I would like to thank Ms. Tina Carvalho for performing the scanning electron and transmission electron microscopy at the Biological Electron Microscope Facility (BEMF) of the University of Hawaii at Manoa.

I have had the privilege of serving as a graduate teaching assistant at the Daniel K. Inouye College of Pharmacy of the University of Hawaii at Hilo throughout my tenure as a graduate student, which funded my tuition and living expenses all while having the opportunity to gain experience in teaching at the college level. Being a teaching assistant was a wonderful opportunity that connected me with amazing professors, instructors, and students of the College of Pharmacy. I thank the Graduate Assistant Coordinators, Dr. Edward Fisher, Dr. Linda Connelly, and Dr. Dana-Lynn Ko’omoa-Lange, Cara Suefuji, as well as my supervisors, Mrs. Mimi Pezzuto, Dr. Tamara Kondratyuk, Dr. Ken Morris, Ms. Xinyan Wang, Dr. Deborah Taira, and Dr. Anthony Otsuka.

This work would not have been possible without the financial support provided by the Leahi Fund to Treat & Prevent Pulmonary Disease of the Hawai’i Community Foundation, Honolulu, HI, USA, the Daniel K. Inouye College of Pharmacy, University of Hawaii at Hilo, and the Research Corporation of the University of Hawai’i at Hilo, HI, USA.
ABSTRACT

Asthma is a chronic inflammatory disease that impacts about 300 million people worldwide and affects over 8% of Americans. Existing asthma treatments, such as anti-histamines or steroids, do not address the underlying cellular processes responsible for asthma pathogenesis and pathophysiology and therefore, more targeted approaches are needed for mitigating the disease. There is a continued need for drug or oligonucleotide delivery systems which could selectively deliver anti-asthmatic therapeutics directly to their site of action. T helper 2 (Th2) cell-mediated asthma progression plays a major role in inflammatory mediator production and airway mucus hyper-secretion associated with chronic atopic (allergic) asthma. The signal transducer and activator of transcription 6 (STAT6) is an essential transcription factor in asthma pathogenesis and progression via Th2 cells. RNAi is a naturally occurring gene silencing process that has a high degree of specificity and the potential to silence genes of interest. However, the clinical utility of RNAi therapy has been hampered due to poor cell penetration, nonspecific effects, rapid degradation, and short half-life. The central hypothesis of this work is that receptor-guided STAT6 siRNA loaded gelatin nanocarriers will selectively deliver STAT6 siRNA to T cells and silence STAT6 mRNA and protein expression thereby inhibiting the transcription of master gene GATA3 involved in the initiation and progression of asthma and attenuate allergic asthma. The overall objective of this research work was to formulate and characterize gelatin nanocarriers for the targeted delivery of STAT6 siRNA to T cells and to evaluate their effects in vitro. The development and evaluation of receptor-targeted STAT6 siRNA loaded gelatin nanocarriers represents the first critical step in the pre-clinical development of a novel nanotherapeutic for asthma therapy.

To establish the effectiveness of gelatin nanocarriers of STAT6 siRNA for the targeted cellular delivery of siRNA for the treatment of asthma, a proof-of-concept formulation was evaluated. The objective was that to develop a gelatin based STAT6 siRNA loaded nanocarrier (S6S-GNC) and to evaluate if it provided biostability and delivery of STAT6 siRNA to cancer cells to exert anti-cancer activity through STAT6 mediated anti-proliferative and pro-apoptotic effects in a human lung cancer cell line. The prepared nanoformulation was characterized for size, charge, loading efficiency, release kinetics, stability, as well as in vitro cytotoxicity and protein downregulation in A549 cells treated with S6S-GNC. This investigation inferred gelatin polymer-based nanocarriers to be a robust, stable and biocompatible strategy for the delivery of STAT6 siRNA.

The main objective of this study was to prepare, optimize, and characterize the interleukin receptor targeting peptide conjugated STAT6 siRNA loaded gelatin nanocarriers (S6S-GNC-P) and to evaluate their in vitro efficacy under cell binding, cell internalization, antiproliferative, and STAT6 protein and mRNA downregulation assays following treatment in a mouse Th2 cell line. The formulation was optimized using Taguchi orthogonal array design of experiments, regression analysis, and desirability index. The S6S-GNC were PEGylated using a carbodiimide crosslinking of maleimide terminated PEG5000. The PEGylated S6S-GNC were peptide conjugated through a stable thioether bond between the maleimide functionalized PEG and the cystamide containing interleukin targeting peptide. The optimized S6S-GNC-P formulation contained approximately 4800 PEG5000 in a brush-border configuration and 720 peptide molecules per gelatin nanocarrier and had a particle size of 98 ± 3.6 nm, PDI of 0.66 ± 0.02, zeta potential of +6.3 ± 0.54, and STAT6 siRNA loading efficiency of 19%. The S6S-GNC-P underwent a sustained diffusion-based STAT6 siRNA release through a matrix-based spherical system, as confirmed with kinetic modeling. The formulation was found to be stable in the presence of simulated physiological media and under
electrophoretic mobility assays in the presence of RNAse. The S6S-GNC dose dependency studies found that 100 nM STAT6 siRNA equivalent doses were superior than other concentrations in the range of 25-200 nM siRNA for maximal STAT6 protein downregulation compared to the scrambled siRNA loaded GNC control. The S6S-GNC-P did not impart any significant cell cytotoxicity in Th2 cells in the concentration range of 25-250 nM STAT6 siRNA, but did significantly (p<0.001) reduce STAT6 protein expression in Th2 treated cells compared to the medium, the STAT6 siRNA positive, and scrambled siRNA-GNC-P controls, as well as the PEGylated S6S-GNC formulation. The S6S-GNC-P treated Th2 cells had a significant decrease in STAT6 mRNA expression levels when compared to the medium control (p<0.01) and the PEGylated S6S-GNC (p<0.001).

Controlled release Gem-GNC with particle size of approximately 180 nm, EE% >90%, and LE% of ~9% were successfully developed for pulmonary delivery by the inhalation route of administration. The target particle size value was 150 nm, so the employed predicted equation generated via linear regression modeling for particle size led to the volume ratio of 7:10 of 90% v/v ethanol, which was selected by fixing the gelatin and genipin concentrations to 1% w/v and 0.02% w/w, respectively. The SEM and TEM images had shown that the developed Gem-GNC were uniform in particle size and were of a smooth spherical morphology with particle size of approximately 200 nm. The Taguchi analysis determined that genipin concentration was the most influential parameter on particle size since it had the largest S/N ratio range. DSC of lyophilized Gem-GNC indicated that the Gem and excipients where molecularly dispersed. PXRD analysis of the lyophilized Gem-GNC found that Gem within the Gem-GNC had an amorphous configuration. The formulation was found to be stable in the presence of pH 6.4-8.4 DPBS solutions. The release mechanisms of Gem from the Gem-GNC were found to be non-Fickian diffusion and erosion from a matrix-based nanocarrier. The Gem-GNC exhibited a controlled release of Gem, which is highly desirable for the long term constant delivery of the formulation with reduced dosing intervals. The nebulized Gem-GNC exhibited a mass median aerodynamic diameter (MMAD) of 2.0 ± 0.16 μm, geometric standard deviation (GSD) of 2.7 ± 0.16, and fine particle fraction (FPF) of 75.2 ± 2.4%. A549 cells treated with Gem-GNC obtained IC_{50} of 0.023 μM, however the Gem solution obtained an IC_{50} of 0.013 μM at 72 hr. The H460 cells treated with Gem-GNC obtained 5-fold lower IC_{50} at 48 hr when compared to the Gem solution, whereas at 72 hr the IC_{50} for Gem-GNC was 10-fold lower than the IC_{50} of Gem solution (** p<0.01). The developed Gem-GNC was found to be effective in protecting Gem from degradation and was able to delivery Gem within the tumor cells to exert anticancer activity. The development and evaluation of the Gem-GNC provides evidence that an aerosolized GNC approach may be useful for the delivery of therapeutics to the lungs, possibly for lung cancer treatment. More studies are warranted to fully illustrate the safety profile in order to form risk and benefit comparisons.

The novel formulation of S6S-GNC-P was intended to be delivered by the intravenous route of administration. However the third objective, which was focused on the development and evaluation of aerosolized gemcitabine-loaded gelatin nanocarriers (Gem-GNC), depicted the potential for inhalation delivery of S6S-GNC-P by nebulization. A gemcitabine loaded gelatin nanocarrier (Gem-GNC) formulation was optimized and evaluated for their aerodynamic properties following nebulization. Future studies are necessary to evaluate the effects of administered gelatin nanocarriers, because they have potential for antigenic or immunogenic effects in the highly sensitive lungs of asthmatic patients.
# TABLE OF CONTENTS

DEDICATION .................................................................................................................. iii

ACKNOWLEDGEMENTS .............................................................................................. iv

ABSTRACT .................................................................................................................. v

LIST OF TABLES .......................................................................................................... xvi

LIST OF FIGURES ....................................................................................................... xviii

LIST OF ABBREVIATIONS AND SYMBOLS ............................................................... xxvi

1. INTRODUCTION ...................................................................................................... 1

   1.1. Motivation and Research Background .............................................................. 1

       1.1.1. The Impact of Asthma and Critical Barriers to Medical Care ..................... 1

       1.1.2. STAT6 Short Interfering RNA (STAT6 siRNA) Therapy for the Effective
               Management of Asthma ........................................................................... 3

           1.1.2.1. Up-regulation of STAT6 in asthma .................................................... 6

       1.1.3. Gelatin-based Therapeutic Nanoparticles for siRNA Delivery .................... 7

       1.1.4. Nanocarriers for STAT6 siRNA Delivery and Asthma Therapy ............... 12

       1.1.5. IL4Rα Targeting ..................................................................................... 13

2. HYPOTHESIS, OBJECTIVES, AND RATIONALE ................................................... 17

3. LITERATURE REVIEW ........................................................................................... 26

   3.1. Introduction to Asthma Nanotherapeutics and Aerosolized siRNA Nanoparticle
        Delivery Systems ............................................................................................. 26

   3.2. Recent Progress and Advances in Nanoparticle Drug Delivery Systems for Asthma
        Therapy .............................................................................................................. 27

           3.2.1. Rationale for Nanoparticulate Drug Delivery Systems for Asthma
                   Therapy ................................................................................................. 27

           3.2.2. Asthma Pathogenesis and Pathophysiology .......................................... 28

                3.2.2.1. Cells Mitigating Asthma ................................................................. 31

                        3.2.2.1.1. Airway Epithelial Cells .......................................................... 32

                        3.2.2.1.2. Dendritic Cells ................................................................. 33

                        3.2.2.1.3. T Lymphocyte Subsets ...................................................... 33

                        3.2.2.1.4. B Lymphocytes ................................................................. 33

                        3.2.2.1.5. Innate Lymphoid Cells ...................................................... 34
3.2.2.1.6. Eosinophils…………………………………………………34
3.2.2.1.7. Mast Cells…………………………………………………34
3.2.2.1.8. Neutrophils…………………………………………………34
3.2.2.1.9. Macrophages and monocytes…………………………35
3.2.2.1.10. Basophils…………………………………………………35

3.2.3. Current FDA Approved Therapeutic Agents and Investigational Drugs…………………………………………………………………………………35

3.2.3.1. Currently Approved Therapeutic Agents…………………..36
3.2.3.2. Investigational Drugs in Clinical trials and Preclinical Studies…………………………………………………………………………………..41

3.2.3.2.1. Small Molecules………………………………………..41
3.2.3.2.1.1. Battling Corticosteroid Resistance………………….41
3.2.3.2.1.2. Bronchodilators……………………………………..43
3.2.3.2.1.3. Novel Receptor Antagonists and Agonists for Asthma Therapy…………………………………………………………………47
3.2.3.2.1.4. Chemokine Receptor Inhibitors…………………..48
3.2.3.2.1.5. Kinase Inhibitors…………………………………….49
3.2.3.2.1.6. Macrolides……………………………………………51

3.2.3.2.2. Biologics…………………………………………………..52
3.2.3.2.2.1. Targeting IL-4/IL-13……………………………….52
3.2.3.2.2.2. Targeting IL-5……………………………………….54
3.2.3.2.2.3. Targeting IL-9……………………………………….55
3.2.3.2.2.4. Targeting IL-2……………………………………….55
3.2.3.2.2.5. Targeting IL-17…………………………………….56
3.2.3.2.2.6. Targeting Other Asthma Pathways………………..56

3.2.3.2.3. Oligonucleotide-based Asthma Therapy……………….57

3.2.4. Nanoparticle-Based Therapeutic Delivery Systems Currently Under Pre-Clinical Investigation for the Treatment of Asthma…………………………..61

3.2.4.1. Polymeric Nanoparticles………………………………….61
3.2.4.1.1. Gelatin………………………………………………….61
3.2.4.1.2. Albumin……………………………………………….63
3.2.4.1.3. Poly lactic-co-glycolic acid (PLGA) .......................... 64
3.2.4.1.4. Dendrimers .................................................. 67
3.2.4.1.5. Chitosan ..................................................... 70
3.2.4.1.6. Other Polymers ............................................. 79
3.2.4.2. Lipid-Based Nanoparticles .................................. 85
  3.2.4.2.1. Liposomes ................................................. 85
  3.2.4.2.2. Solid Lipid Nanoparticles and Nanostructured Lipid
              Carriers ...................................................... 89
  3.2.4.2.2. Lipid Nanomicelles ................................... 91
  3.2.4.2.3. Lipid Nanocapsules .................................. 92
3.2.4.3. Carbon-based Nanoparticles ............................. 94
  3.2.4.3.1. Fullerenes ............................................... 94
3.2.4.4. Metal-based Inorganic Nanoparticles .................... 96
  3.2.4.4.1. Super-paramagnetic Iron Oxide (SPIO)
              Nanoparticles ............................................. 97
  3.2.4.4.2. Gold .................................................... 99
  3.2.4.4.3. Silver ................................................. 100
3.2.4.5. Nanocrystals .............................................. 101
3.2.4.5. DNA and RNA Nanoparticles .......................... 106
3.2.4.6. Microparticle-based Carriers for Nanoparticle
              Delivery ....................................................... 107
3.2.5. Summary and Future Directions .......................... 108
3.3. Aerosol Delivery of siRNA to the Lungs- Rationale
         for Gene Delivery Systems ....................................... 109
  3.3.1. Rationale for Aerosol Delivery of siRNA to the Lungs .... 109
  3.3.2. Pulmonary Route of Administration ...................... 110
    3.3.2.1 Inhalation Route ......................................... 111
      3.3.2.1.1 Inhalation Aerosol Delivery Devices .............. 112
    3.3.2.2 Intratracheal Route ..................................... 114
      3.3.2.2.1 Intratracheal Aerosol Delivery Devices .......... 116
    3.3.2.3 Intranasal Route ......................................... 116
3.3.2.3.1 Intranasal Aerosol Delivery Devices
3.3.2.4 Passive Inhalation Exposure Chambers for Animal Studies
3.3.3. Evaluation of Pulmonary Drug Delivery Systems
3.3.3.1 In vitro Characterization
3.3.3.2 Ex vivo Characterization
3.3.3.3 In vivo Characterization
3.3.4. siRNA Delivery to the Lungs
3.3.4.1 Barriers of Pulmonary Delivery of siRNA
3.3.4.2 Barriers of Intracellular siRNA Delivery
3.3.5. Summary of the Rationale for Gene Delivery Systems for Aerosol Delivery of siRNA to the Lungs
3.4. Nanocarrier-based Delivery Systems for Aerosol Delivery of siRNA to the Lungs
3.4.1. Rationale for Nanocarrier-based Delivery Systems
3.4.2. Non-viral Delivery of siRNA to the Lung
3.4.2.1 siRNA Delivery
3.4.2.1.1 Introduction to siRNA Delivery
3.4.2.1.2 RNA Modifications & Preparation Methods
3.4.2.1.3 Examples of siRNA Delivery
3.4.2.2 Lipid-based Delivery Systems
3.4.2.2.1 Introduction to Lipid-based Delivery Systems
3.4.2.2.2 Preparation Methods
3.4.2.2.2.1 Cationic Lipoplexes
3.4.2.2.2.2 Liposomes
3.4.2.2.2.3 Solid Lipid Nanoparticles and Nanostructured Lipid Carriers
3.4.2.2.3 Examples of Lipid-based Delivery Systems
3.4.2.3 Polymer-based Delivery Systems
3.4.2.3.1 Introduction to Polymer-based Delivery Systems
3.4.2.3.2 Preparation Methods
3.4.2.3.2.1 Emulsion and Diffusion Techniques
3.4.2.3.2 Solvent Evaporation .........................................154
3.4.2.3.2.3 Nanoprecipitation .........................................155
3.4.2.3.2.4 Salting Out ..................................................156
3.4.2.3.2.5 Dialysis .......................................................156
3.4.2.3.2.6 Supercritical Fluid Technology (SCF) ..........156
3.4.2.3.2.7 Interfacial Polymerization ..............................157
3.4.2.3.2.8 Controlled/Living Radical Polymerization (C/LRP) .................................................................158
3.4.2.3.2.9 Hydrophilic Polymer Ionic Gelation/Coacervation .................................................................159
3.4.2.3.3 Examples of Polymer-based Delivery Systems .........159
3.4.2.4 Peptide-based Delivery Vectors ..................................162
  3.4.2.4.1 Introduction to Peptide-based Delivery Vectors .........162
  3.4.2.4.2 Preparation Methods .........................................163
  3.4.2.4.3 Examples of Peptide-based Delivery Vectors .........164
3.4.2.5 Inorganic-based Delivery Systems ..........................165
  3.4.2.5.1 Introduction to Inorganic-based Delivery Systems ....165
  3.4.2.5.2 Examples of Inorganic-based Delivery Systems .......165
3.4.3. Ongoing clinical trials on aerosolized siRNA based medicines ......166
3.4.4. Summary and Future Directions of Pulmonary siRNA Delivery ....167

4. MATERIALS AND METHODS .................................................169
  4.1. Materials ...............................................................169
  4.1.1. Cell Culture .......................................................169
  4.1.2. Chemicals .........................................................170
  4.2. Methods ...............................................................172
    4.2.1. Selection of Gelatin Molecular Weight Fraction by Controlled Desolvation ......................................172
    4.2.2. Formulation and Optimization of gelatin nanocarriers using Design of Experiments (DoE) .................................................................173
      4.2.2.1. Development of S6S-GNC using a Full Factorial DoE .............173
4.2.2.2. Development of Targeted STAT6 siRNA loaded Gelatin Nanocarriers .......................................................... 175
4.2.2.3. Development of Inhalable Gemcitabine Loaded Gelatin Nanocarriers using the Taguchi Orthogonal Array Method .... 181
   4.2.2.3.1. Preparation and Purification of Gemcitabine Loaded Gelatin Nanocarriers (Gem-GNC) .................................. 185
4.2.3. Particle Size and Zeta Potential Measurements ........................................ 185
4.2.4. Determination of Entrapment Efficiency (EE%) and Loading Efficiency (LE%) ............................................................... 187
4.2.5. Evaluation of Inhalable Gelatin Nanocarriers Containing Gemcitabine (Gem-GNC) ..................................................... 188
   4.2.5.1. In vitro Release of Gem from Gem-GNC .............................. 188
   4.2.5.2. pH Stability of Gem-GNC ..................................................... 189
   4.2.5.3. Differential Scanning Calorimetry (DSC) of Gem-GNC and Excipients ................................................................. 189
   4.2.5.4. Powder X-ray Diffraction (PXRD) of Gem-GNC and Excipients .......................................................... 189
   4.2.5.5. In vitro Aerosol Characterization of Gem-GNC .................. 190
   4.2.5.6. Cell Proliferation MTT Assay on A549 and H460 NSCLCs Treated with Gem-GNC ..................................................... 191
4.2.6. Preparation of S6S Loaded Gelatin Nanocarriers (S6S-GNC) and S6S-GNC conjugated with IL4R targeting peptide .......... 192
4.2.7. Determination of Surface Morphology and Particle Size via Scanning Electron Microscopy and Transmission Electron Microscopy ................................................................. 194
4.2.8. Determination of the Number of Surface Bound Peptides per GNC .... 195
4.2.9. In vitro Release Profile of S6S from S6S-GNC and S6S-GNC-P and Kinetic Analysis ................................................................. 197
4.2.10. Stability Studies under Serum and pH medium .......................................... 198
4.2.11. S6S Stability Study: Agarose Gel Electrophoretic Mobility Assay .............................. 199
4.2.12. In vitro Cytotoxicity of S6S-GNC in A549 Adenocarcinoma Cells and S6S-GNC and S6S-GNC in Mouse Th2 Cells

4.2.13. Cellular Internalization Assay of S6S-GNC in A549 adenocarcinoma cells using inverted fluorescence microscopy

4.2.14. Upregulation of IL4Rα in Asthmatic Mouse Lung Tissue and Th2 cells

4.2.15. Determination of Optimum Surface Peptide Density Using STAT6 ELISA Assays

4.2.16. Flow Cytometry Experiments for Cell Binding and Cellular Internalization using FITC-S6S-GNC and FITC-S6S-GNC-P

4.2.17. Protein and mRNA Downregulation by S6S-GNC-P via Western Blot and RT-PCR

4.2.18. Statistical Analysis

5. RESULTS AND DISCUSSION

5.1. STAT6 siRNA Loaded Gelatin Nanocarriers

5.1.1. Development of STAT6 siRNA loaded Gelatin Nanocarriers

5.1.2. In vitro Release of STAT6 siRNA from STAT6 siRNA loaded GNC (S6S-GNC)

5.1.3. Serum and pH Stability of STAT6 siRNA Loaded Gelatin Nanocarriers

5.1.4. STAT6 siRNA Loaded Gelatin Nanocarrier Agarose Gel Electrophoretic Mobility Assay

5.1.5. In vitro Cytotoxicity of STAT6 siRNA Loaded Gelatin Nanocarriers

5.1.6. Cellular Internalization Assay of STAT6 siRNA and Nile Red Loaded Gelatin Nanocarriers

5.1.7. STAT6 Protein Downregulation by STAT6 siRNA Loaded Gelatin Nanocarriers

5.2. Gemcitabine Loaded Gelatin Nanocarriers (Gem-GNC) for Pulmonary Delivery

5.2.1. Preparation and Purification of Gem loaded Gelatin Nanocarriers (Gem-GNC)
5.2.2. Preparation and Evaluation of Lyophilized Gem-GNC

5.2.3. Gem-GNC Stability

5.2.4. In vitro release from Gem-GNC

5.2.5. Gem-GNC Imaging with Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)

5.2.6. Differential Scanning Calorimetry (DSC)

5.2.7. Powder X-ray Diffraction (PXRD)

5.2.8. Cell Viability Assay of Gem-GNC treated A549 and H460 NSCLC Cell Lines

5.2.9. In vitro Aerosol Characterization

5.3. STAT6 siRNA loaded Gelatin Nanocarriers for Asthma Treatment

5.3.1. Upregulation of IL-4Rα in Asthmatic Mouse Lung Tissue and Th2 cells

5.3.2. Optimization of RNAiMax and STAT6 siRNA Concentrations

5.3.3. Preparation of S6S Loaded Gelatin Nanocarriers (S6S-GNC) and S6S-GNC conjugated with IL4R targeting peptide (S6S-GNC-P)

5.3.4. In vitro Release of STAT6 siRNA from S6S-GNC and S6S-GNC-P

5.3.5. Stability of S6S-GNC and S6S-GNC-P

5.3.6. Optimization of IL4Rα Peptide Concentration Conjugated onto the Nanocarrier Surface

5.3.7. Agarose Gel Electrophoretic Mobility Shift Assay for Stability Assessments

5.3.8. S6S-GNC and S6S-GNC-P Safety Determination by MTT Cell Proliferation Assay

5.3.9. STAT6 Protein and mRNA Downregulation by S6S-GNC-P by Western Blot Protein Analysis and RT-PCR

6. SUMMARY AND FUTURE DIRECTIONS

6.1. Summary

6.2. Future Directions

APPENDIX A: PUBLICATIONS

APPENDIX B: Data for Objective 1
LIST OF TABLES

Table 1.1. Type A gelatin amino acid content.

Table 3.1. Summary of biological molecular targets for siRNA-mediated asthma therapies. Used with permission from Ref. [553]. Copyright John Wiley and Sons 2015.

Table 3.2. Parameters to consider in siRNA formulation design for inhalation.

Table 3.3. Techniques for evaluating pulmonary drug delivery systems.

Table 3.4. USP and Ph.Eur. Cascade Impactors for orally inhaled dosage forms.

Table 3.5. Pulmonary barriers to lung siRNA delivery [743].

Table 4.1. Full Factorial 3³ design for the optimization of S6S-GNC using APAP as a model drug.

Table 4.2. Taguchi orthogonal array of APAP-GNC design of experiments parameters.

Table 4.3. Predicted full factorial data for APAP-GNC.

Table 4.4. Experimental matrix and responses from the L⁹ orthogonal array and the S/N ratios of experimental results for particle size the Taguchi Orthogonal Array Gem-GNC batches. Particle size was determined with PBS pH 7.4 at 25°C by dynamic light scattering as described within the methods section. The gelatin concentration was expressed as a weight volume percent (% w/v) of gelatin weight to batch volume. The desolvating agent addition was expressed as volume ratio of 90 % v/v ethanolic aqueous solution to volume of gelatin batch. The genipin concentration was expressed as weight percent (% w/w) of genipin to gelatin weight. Experiments were performed in triplicate (n=3).

Table 5.1. Gem-GNC particle size, zeta potential, Entrapment Efficiency (EE%) and Loading Efficiency (LE%). The Gem-GNC and Placebo GNC batches were subjected to dynamic light scattering and electrophoretic mobility analysis to determine their particle size and zeta potential before lyophilization using the prepared nanoparticle suspension and after lyophilization using 10 mg of freeze dried nanocarriers redispersed in 10 ml of distilled deionized water and equilibrated for 1 hr.

Table 5.2. In vitro release models showing correlation coefficient (R²) values for Gem release from the Gem-GNC within DPBS and SLF (pH 7.4). Variables are defined in the text.

Table 5.3. DSC determination of endothermic events of the Gem-GNCs, physical mixture of actives and excipients, Placebo GNCs and unentrapped Gem, Placebo GNCs, lactose monohydrate, gelatin, genipin, and the active ingredient Gem.

Table 5.4. Particle size, zeta potential, entrapment and loading efficiencies of the non-PEGylated S6S-GNC, PEGylated S6S-GNC, and S6S-GNC-P.
Table 5.5. *In vitro* release models showing correlation coefficient (R2) values for STAT6 siRNA release from the S6S-GNC and S6S-GNC-P within DPBS (pH 7.4). Variables are defined in the text.

Table 5.6. S6S-GNC suspension characteristics.
LIST OF FIGURES

**Figure 1.1.** Normal mouse and asthmatic OVA-challenged mouse lung tissue. (A) Increased expression and phosphorylation of STAT6 and (B) Histological analysis of inflammatory cells in airways. The sections shown in panel B were stained with a standard periodic acid-Schiff method and the dark red-purple staining represents the presence of mucus and highlights the goblet cell hyperplasia.

**Figure 1.2.** IL4Rα mediated targeted delivery of S6S-GNC-P. Schematic representing selective targeting of S6S-GNC-P to IL-4Rα expressing T immune cells. Interaction of S6S-GNC-P with IL-4Rα followed by endosomal uptake of S6S-GNC-P inside the T cells, endosomal escape of positively charged GNC via proton-sponge effect and release of free S6S inside cytoplasm to exert the silencing effect by formation of S6S complex. This results in the silencing of STAT6 by S6S-RISC on STAT6 followed by cleavage of STAT6, which leads to blockade of phosphorylation and activation of STAT6.

**Figure 2.1.** Cellular uptake and intracellular mechanism of action (MOA) of targeted S6S-GNC.

**Figure 2.2.** IL4Rα targeted S6S-GNC-P, (A) S6S-GNC-P internalization via IL4R endocytosis, (B) STAT6 siRNA release and formation of STAT6 siRNA-RISC complex, and (C) STAT6 silencing in Th2 cells.

**Figure 3.1.** Host responses in the pathogenesis of asthma. Several asthma drugs in pre-clinical and clinical trials target the cytokines or receptors that are central to these pathways, including IL-4, IL-5, IL-13, IL-17. TSLP=thymic stromal lymphopoietin. Reprinted with permission from Ref. [270]. Copyright 2014 BMJ Publishing Group Ltd.

**Figure 3.2.** Susceptibility of genes within asthma-associated cells. Representation of the most robust asthma candidate genes identified through association studies or positional cloning in a cell-based framework. Reprinted with permission from Ref. [301]. Copyright 2008 Nature Publishing Group.

**Figure 3.3.** Stepwise approach for managing asthma. Used with permission from the Global Initiative for Asthma. Copyright 2015 Global Initiative for Asthma.

**Figure 3.4.** Representative image of the gelatin polymer structure.

**Figure 3.5.** Pre-treatment with NP-CpG reduces mucus production in lungs of allergic mice. Mice were pre-treated with CpG and then sensitized and challenged with HDM. (A) Representative periodic acid-Schiff (PAS)-stained lung sections showing mucus-producing goblet cells (dark magenta) within bronchi (scale bar, 100 µm). (B) Quantification of mucus-producing goblet cells per unit length. (C) Representative PAS-stained lung sections showing leukocyte accumulation (blue) around blood vessels (Scale bar, 100 µm). (D) Inflammation score, defined as area of leukocyte infiltration and normalized to the average values in naïve mice. Data shows mean ± SEM from three independent experiments, 14 mice per group (5 mice in naïve group). *P
< 0.05, **P < 0.01; ##P < 0.01 compared to naïve mice. Reprinted with permission from Ballester et al. 2015 under Creative Commons Attribution 4.0 International License (CC BY 4.0) [615].

**Figure 3.6.** Noninvasive lung MRI. (a) Representative MR images of mouse lung for control mice, ovalbumin-challenged mice (i.e. OVA), and OVA mice following intrapulmonary administration of either SPIONs (upper row) or SPIONs–IL4Rα (lower row) acquired at 2 h, 48 h, and 7 days post administration. White arrows highlight the presence of void signal dots related to SPIONs (with or without IL4Rα conjugation). SPIONs–IL4Rα were found to preferentially colocalize with the inflammatory (hyper-intensity) regions in the asthmatic lung (blue circles) following ovalbumin challenge. (b) Quantitative assessments of inflamed lung volume (ILV) for control versus OVA mice at 2 h, 24 h, 48 h, and 7 days post administration of either SPIONs or SPIONs–IL4Rα. Error bars are standard deviations of triplicates. Reprinted with permission from Ref. Al Faraj et al. 2015 [676]. Copyright © 2015 John Wiley & Sons, Ltd.

**Figure 3.7.** siRNA pathway schematic. Reprinted with permission from Ref. [704, 705]. Copyright: (2007) Nature Publishing Group.

**Figure 3.8.** Intratracheal instillation and bronchoalveolar lavage on rat.

**Figure 3.9.** InExpose™ (SCIREQ®) nose-only exposure system.

**Figure 3.10.** Schematic representation of the (A) Andersen 8-stage cascade impactor, (B) Marple-Miller impactor, and (C) Multi-stage liquid impinger. Reprinted with permission from Ref. [796]. Copyright 2013 Elsevier

**Figure 3.11.** Isolated perfused rat lung preparations: (a) horizontally positioned isolated perfused rat lung with a scheme of forced solution instillation, (b) vertically positioned isolated perfused rat lung with a scheme of nebulization catheter dosing. Reprinted with permission from Ref. [744]. Copyright 2003.

**Figure 3.12.** Aerosol siRNA delivery to the lungs. (A) pulmonary delivery of naked or siRNA nanocarriers, (B) Carrier-based delivery of siRNA cellular uptake. The aerosolized formulation of siRNA and use of appropriate device delivers siRNA by inhalation, intratracheal, or intranasal delivery to the human or animal lung. Once the siRNA carriers reach the lower respiratory tract, they may reach target cells and become internalized through endocytosis. Carriers capable of endosomal escape avoid degradation in the lysosome and release free siRNA within the cytoplasm. The RNAi pathway may then be initiated, ultimately leading to mRNA degradation and gene silencing.

**Figure 3.13.** Schematic of different non-viral siRNA delivery vectors.

**Figure 3.14.** Cationic liposome and anionic siRNA interaction forming a lipoplex. The cationic lipoplex interacts with the negatively charged cellular membrane leading to enhanced delivery within the cells via endocytosis.
Figure 3.15. Avanti Mini-Extruder (A), Avanti Mini-Extruder internal schematic (B), and Nano DeBEE high-pressure homogenizer (C).

Figure 3.16. Schematic of SLN preparation with encapsulated siRNA. Feed solutions 1 and 2 pictured in (a) are sequentially added to the receiver solution pictured in (b). SLNs form following evaporation of organic solvents, as shown in (c). Alternatively, siRNA/DOTAP complex and tristearin are solubilized in CHF, as shown in (d), and then added to the aqueous receiver solution. A schematic drawing of SLN is presented in (e). Reprinted with permission from Ref. [935]. Copyright 2011 American Chemical Society.

Figure 3.17. Diagram showing the preparation of polymeric nanoparticles using the coacervation (desolvation) technique.

Figure 3.18. Co-delivery of siRNA and anticancer agents using mesoporous silica nanoparticles (MSN) The surface engineered approach consists of surface bound siRNA and PEG-LHRH.

Figure 4.1. Preparation of S6S-GNC. The 1% w/v aqueous gelatin solution was incubated with the STAT6 siRNA for 10 min at 35°C, then ethanol and crosslinker was added dropwise at a stirring rate of 600 rpm at 35°C for 1 hr, at which point the stirring rate was reduced to 200 rpm. After approximately 4 h, the ethanol was completely evaporated and STAT6 siRNA loaded gelatin nanocarriers remained in a colloidal suspension in water or PBS pH 7.4. The resultant nanoparticles were collected by centrifugation and re-suspended for subsequent characterization or lyophilization in the presence of 1% w/w lactose monohydrate.

Figure 4.2. Particle size analysis report for GNC formulated at 600 rpm stir rate (magnetic stir bar method). The bars and dots and error bars represent the mean ± standard deviation (n=3).

Figure 4.3. APAP-GNC Preparation by the 2-step desolvation method and purification by centrifugation and dialysis.

Figure 4.4. Taguchi orthogonal array design of experiments for APAP-GNC preparation as a model for S6S-GNC. This Figure indicates the batch particle size, polydispersity index, zeta potential, and loading efficiency results. Bars represent mean and error bars are standard deviations (n=3).

Figure 4.5. Schematic showing the formulation of Gem loaded gelatin nanocarriers (Gem-GNC).

Figure 4.6. Experimental analysis of Taguchi designed experiments: (A) Taguchi main effects plot for mean particle size and (B) main effects plot for particle size S/N ratios of the Gem-GNC.

Figure 4.7. Formulation of PEGylated S6S-GNC conjugated with IL4Rα targeting peptide (S6S-GNC-P).

Figure 4.8. PEG standard curve obtained using the Barium/Iodine colorimetric method measured at 535 nm. Data are presented mean ± standard deviation as the dots and error bars and the line is the best linear fit. Samples were run in triplicate (n=3).
Figure 4.9. BCA protein assay representative standard curve of absorbance at 562 nm versus BSA concentration. Dots show the mean ± standard deviation with n=3.

Figure 5.1. Interaction Plot for the dependent variable particle size with Stir rate, concentration of ethanol and glutaraldehyde using the full factorial experimental design for the formulation development of gelatin nanocarriers. Stirring rate levels were 300, 600, and 600 rpm, the ethanol volume was 7, 8, 9 ml corresponding to 41, 44, and 47% v/v added, and the 10% GTA volume was 100, 150, and 300 µl.

Figure 5.2. Particle size and zeta potential of the S6S-GNC and placebo-GNC batches. The bars represent the mean particle size (nm), the square markers represent the zeta potential (mV), and the error bars represent their standard deviation. Particle size measurements were conducted in PBS pH 7.4 medium at a concentration of 25% v/v nanoparticle dispersion to buffer medium concentration. The zeta potential measurements were conducted in water containing 10µM NaCl at a concentration of 5% v/v nanoparticle dispersion. Measurements were conducted in triplicate (n=3).

Figure 5.3. In vitro STAT6 siRNA release profile for the S6S-GNC formulation compared to the STAT6 siRNA solution. Lyophilized formulation was re-suspended in PBS pH 7.4 and filled inside dialysis membrane bags with MWCO of 300 kDa (Sigma, USA). The membrane bags were placed in 50 ml of PBS medium maintained at a temperature of 37±2°C with continuous gentle stirring at 300 rpm on a magnetic heating and stirring plate. At specific time intervals, 0.5 ml aliquots of dissolution medium were withdrawn and analyzed using a Biospek UV spectrophotometer. Results are represented as mean ± standard deviations (where n=3).

Figure 5.4. Stability of S6S-GNC formulation. Outcome is as expressed by size (nm) and zeta potential (mV) under the influence of varying pH between 5.4 to 8.4 and 10 %v/v FBS at physiological pH 7.4 to mimic the serum found in human blood. Results are represented as mean ±SD (n=3).

Figure 5.5. Agarose Gel Electrophoretic Mobility shift assay. The scrambled siRNA control, scrambled siRNA treated with RNAse control, S6S-GNC, S6S-GNC treated with RNAse, filtrate, filtrate treated with RNAse, and the placebo-GNC treated with RNAse were loaded onto a 1% w/v agarose gel and electrophoresed at a constant voltage of 70 V. This study was performed to examine the stability of the encapsulated siRNA due to preparation conditions and the stability in the presence of RNAse. The arrow head indicates the distance traveled by the cleaved siRNA fragments.

Figure 5.6. Cytotoxicity of the developed S6S-GNC and STAT6 siRNA with Lipofectamine on A549 lung cancer cells. The graph shows the percent cell viability observed after 24 and 48 hr following treatment. Cell viability was performed using 5×10³ A549 (human adenocarcinoma cell line) cells in F12-K medium supplemented with 10% FBS and an antibiotic solution of penicillin (5000 U/ml), streptomycin (0.1 mg/ml) and neomycin (0.2 mg/ml) (PSN). Cell incubation was conducted within a humidified atmosphere of 5% CO₂ at a temperature of 37±0.5°C. The formulation and STAT6 siRNA Lipofectamine complexes were applied as freshly prepared solutions between 0 to 15 nM concentrations. The absorbance of the formazan crystals dissolved
in DMSO was read at 540 nm on a Biospek Synergy H1 plate reader. Values are represented as Mean ± SD (where n=3).

**Figure 5.7.** Fluorescence Images of control and Nile-red loaded-GNC (Nile-GNC) taken after 15 min and 1 h of treatment in A549 cells (at 40x magnification). For this assay, lung cancer A549 cells (5 × 10^4) were seeded on 24 well plates and incubated at 37±0.5°C under 5% CO₂ for 24 h. The cells were treated with the Nile Red solution (control) or Nile Red loaded formulations for 15 min and then treated for an addition 45 min for a total treatment time of 1 hr. After 15 min or 60 min, media was removed and the resulting cells were washed with PBS.

**Figure 5.8.** Effect of nanocarriers on the STAT6 protein expression by Western Blot. (A) image of Western Blot and (B) Densitometry of the bands normalized to beta actin. The effect of STAT6 siRNA-GNC on the expression of STAT6 in A549-treated lung cancer cells was shown. A549 cells were pre-incubated with S6S-GNC, STAT6 siRNA -Lipofectamine Complex, and without any treatment (control). The cells were lysed and STAT6 protein expression was analyzed by Western blot of whole cell lysates. The β-Actin expression was analyzed as a loading control. Bars show mean ± standard deviation (n=3). ***p<0.001.

**Figure 5.9.** Stability of Gem-GNC at pH 6.4, 7.4 and 8.4 in DPBS as assessed by the (A) Particle size and (B) polydispersity index after reconstitution of the three independent Gem-GNC batches. Time after reconstitution of 0 days refers to 1 hr after rehydration with normal saline solution. Results are shown as mean ± SD (n=3). Statistical significance was determined at p<0.05.

**Figure 5.10.** In vitro release of Gem solution and Gem-GNC within DPBS (pH 7.4) and SLF (Gamble’s Solution; pH 7.4). Lyophilized formulations were resuspended in distilled deionized water and placed into a dialysis membrane bag with molecular weight cut-off of 12-14 kDa. The membrane bags were then placed into 100 mL of DPBS or Gamble’s SLF medium maintained at a temperature of 37°C with continuous stirring at 300 rpm. At specified time intervals, 0.5 mL dissolution medium was sampled and analyzed for Gem content using HPLC with a UV diode array detector. The amount of Gem-GNC filled into the membrane bag was 10 mg/mL at 0.48% w/w Gem content. The Gem solution filled into the membrane bag was 0.1 mg/mL. Total volume of the Gem-GNC suspension and the Gem solution was 0.5 ml. Results are represented as mean ± SD (n=3).

**Figure 5.11.** SEM and TEM Micrographs of the Gem-GNCs. (A) SEM micrograph with a scale bar of 3.0 μm and (B) TEM micrograph with a scale bar of 500 nm. The Gem-GNC particle size measured from the SEM micrograph is 229 ± 68 nm, while the particle size measured from this TEM micrograph is 197 ± 18 nm. SEM: HV= 5.0 kV. TEM: 0.002427 um/pixel, HV=100.0 kV, Direct Magnification 6000X.

**Figure 5.12.** DSC thermograms of the Gem-GNCs, physical mixture of actives and excipients, Placebo GNCs and unentrapped Gem, Placebo GNCs, lactose monohydrate, gelatin, genipin, and the active ingredient Gem. The samples were placed into the pinholed 40 μL aluminum crucibles,
sealed, and the change of heat flux was analyzed with increasing temperature from 25-300 °C compared to an empty control crucible.

**Figure 5.13.** PXRD Diffraction Patterns. (A) Gelatin, (B) Lactose monohydrate, (C) Gem, (D) Genipin, (E) Physical mixture of actives and excipients compared to the placebo GNC and Gem, (F) Gem-GNC compared to the placebo GNC and Gem mixture.

**Figure 5.14.** Cell viability over time and concentration of A549 cells (A) and NCI-H460 cells (B) treated with Gem-GNC and Gem solution. Non-treated cells were used as controls. A549 cells treated with Gem-GNC had an IC$_{50}$ value of 0.023 µM after 72 hr, whereas the Gem solution had an IC$_{50}$ of 0.013 µM. The H460 cells treated with the Gem solution achieved IC$_{50}$ values of 230 µM after a 48 hr and 59 µM after 72 hr. Results are shown as mean ± SD (n=3).

**Figure 5.15.** Log cumulative probability plot of particle size versus cumulative weight percent (%) frequency of Gem-GNC impaction within an 8-stage non-viable Andersen cascade impactor. Data markers represent particle diameter (µm) of the nebulized nanocarrier containing droplets at a given cumulative probability less than stated size and the blue line is best fit linear curve.

**Figure 5.16.** IL4Rα relative protein expression in Th2 cells, normal lung tissue protein lysates, and OVA treated allergic asthma mouse lung protein lysates. A) Western blot analysis and B) quantitative densitometry of the IL4Rα Western Blot bands normalized with β-Actin. Data are shown as mean ± standard deviation (n=3). **p<0.01, ***p<0.001.

**Figure 5.17.** IL4Rα mRNA expression profile by array [1207, 1208]. Bars show mean ± standard deviations (n=3).

**Figure 5.18.** Cell viability of Th2 cells (TIB-224) following treatment with non-complexed RNAiMax cationic liposomes. Non-treated cells were used as a control. Results are shown as mean ± SD (n=4).

**Figure 5.19.** STAT6 siRNA concentration was optimized using Western blot protein analysis following treatment at varying doses. Western blot of Th2 cell lysates which were treated with varying levels of STAT6 siRNA in complex 0.17% v/v or 0.25% v/v RNAiMax (A), (B-C) shows the densitometric relative STAT6 protein expression in the 0.17% v/v RNAiMax STAT6 siRNA complexes normalized to the medium or RNAiMax treated controls respectively, and (D-E) shows the densitometry relative STAT6 protein expression in the 0.25% v/v RNAiMax STAT6 siRNA complexes normalized to the medium or RNAiMax treated controls respectively. Western blot band density was determined by densitometry using ImageJ software and was normalized to the beta actin loading control and normalized relative to either the medium control or the RNAiMax control as noted above. Bars show means ± standard deviations, with n=3.

**Figure 5.20.** MTT cell viability assay on Th2 cells treated for 24 hr with STAT6 siRNA RNAiMax complex using 0.25% v/v Lipofectamine RNAiMax reagent per well. Results are shown as mean ± SD (n=4).
Figure 5.21. Representative SEM and TEM images of S6S-GNC-P. (A) S6S-GNC-P non-dispersed freeze dried without cryoprotectant and imaged with SEM, (B) S6S-GNC-P non-dispersed freeze dried with lactose and imaged with TEM, (C) S6S-GNC-P dispersed and imaged with TEM, (D) S6S-GNC dispersed and imaged on SEM with tilted stage, (E) TEM image of dispersed S6S-GNC-P using Formvar grid, and (F) TEM image of dispersed S6S-GNC-P using a lacy grid.

Figure 5.22. *In vitro* release of STAT6 siRNA from S6S-GNC (red), S6S-GNC-P (green), and STAT6 siRNA solution (blue) in DPBS pH 7.4 medium at 37°C using a 100 kDa dialysis membrane (STAT6 siRNA MW 13.3 kDa).

Figure 5.23. Stability of S6S-GNC and S6S-GNC-P in DPBS pH 7.4 (A), DPBS pH 7.4 containing 10% FBS (B), and water containing 10% v/v FBS (C). Results are shown as mean ± SD (n=5).

Figure 5.24. Kinematic viscosity of gelatin nanocarriers as measured by a Cannon Fiske viscometer.

Figure 5.25. STAT6 protein quantification using ELISA assay following treatment with 75 nM STAT6 siRNA equivalents within S6S-GNC-P containing varying concentrations of surface conjugated peptide. Data represented as mean ± standard deviation. *p<0.05, **p<0.01, ***p<0.001 with n=4.

Figure 5.26. Binding assay flow cytometry relative fluorescent intensities following incubation at 4°C with the various S6S-GNC and S6S-GNC-P formulations. (A) Cellular fluorescence intensity following 4°C incubation for 1 hr (n=3), (B) Quantification of cellular fluorescence intensity following 4°C incubation for 1 hr (n=3), (C) cellular fluorescence intensity following 4°C incubation for 4 hr (n=2), and (D) quantification of cellular fluorescence intensity following 4°C incubation for 4 hr (n=2).

Figure 5.27. Internalization assay by flow cytometry. Fluorescent internalization comparison between medium control cells, plain gelatin nanocarriers (GNC plain), S6S-GNC, 7.5% mol ratio S6S-GNC-P, and 15% mol ratio GNC-P under 37°C incubation for 5 min (A), 15 min (C), 30 min (E), and 1 hr (G). The fluorescent means and standard deviations are plotted for the time points of 5 min (B), 15 min (D), 30 min (F), and 1 hr (H). * p<0.05, **p<0.01, ***p<0.001. Results are shown as mean ± SD (n=3).

Figure 5.28. Agarose gel electrophoretic mobility shift assay of cationic S6S-GNC-P. This data demonstrated that the gelatin nanocarriers entrap the STAT6 siRNA effectively.

Figure 5.29. Agarose gel electrophoresis shift assay to determine the stability of STAT6 siRNA entrapped within the gelatin nanocarrier matrix. This data demonstrates that the gelatin nanocarrier binds the siRNAs and effectively prevents them from RNAse degradation.

Figure 5.30. Cell viability of Th2 cells treated with different levels of STAT6 siRNA equivalents of S6S-GNC (A) and S6S-GNC-P (B) conducted at 48 hr post-treatment. No significant
differences exist, p>0.05. Results are shown as mean ± SD (n=3). No statistically significant differences exist between the medium control and the gelatin nanocarrier formulations.

**Figure 5.31.** S6S-GNC and S6S-GNC-P dose dependent downregulation of STAT6 protein in Th2 cells. Representative Western blots of S6S-GNC treated Th2 cell lysates (A) and S6S-GNC-P treated Th2 cell lysates (B), as well as, densitometry analysis of the S6S-GNC bands (C) and of the S6S-GNC-P bands (D) are shown. Densitometry was performed by first normalizing each band with their corresponding beta actin loading controls. Densitometry results are shown relative to the scrambled siRNA control. *p<0.05, **p<0.01. and ***p<0.001. Data are reported as mean ± SD (n=3).

**Figure 5.32.** Western blot analysis of STAT6 in Th2 cells after 100 nM STAT6 siRNA treatment (A), quantification by densitometry of STAT6 protein expression normalized using β-actin (B), and STAT6 relative mRNA expression in Th2 cells following 50 nM STAT6 siRNA treatments. Data are presented as mean ± SD (n=3). *** p<0.001.

**Figure 5.33.** RT-PCR on total RNA extracts from Th2 cells treated with 100 nM equivalents of S6S-GNC and S6S-GNC-P using medium and scrambled siRNA controls were normalized to beta actin mRNA expression. ** p<0.01 and *** p<0.001. Data are presented as mean ± SD (n=3).
LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>*</td>
<td>p&lt; 0.05</td>
</tr>
<tr>
<td>**</td>
<td>p&lt; 0.01</td>
</tr>
<tr>
<td>***</td>
<td>p&lt; 0.001</td>
</tr>
<tr>
<td>2D</td>
<td>Two dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>Three dimensional</td>
</tr>
<tr>
<td>3D SPECT</td>
<td>Three dimensional single-photon emission computed tomography</td>
</tr>
<tr>
<td>Å</td>
<td>Angstrom</td>
</tr>
<tr>
<td>AHR</td>
<td>Airway hyper-responsiveness</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>AuNP</td>
<td>Gold nanoparticles</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>BALF</td>
<td>Bronchoalveolar lavage fluid</td>
</tr>
<tr>
<td>Bec</td>
<td>Beclamethosone</td>
</tr>
<tr>
<td>BLT1</td>
<td>LTB4 receptor</td>
</tr>
<tr>
<td>CCR</td>
<td>CC-chemokine receptor type</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>Cmax</td>
<td>Maximum plasma concentration</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine-phosphate-guanine motif</td>
</tr>
<tr>
<td>CpG-ODN</td>
<td>Cytosine triphosphate deoxyribonucleotide connected to a quinine triphosphate deoxyribonucleotide by phosphodiesterase linkage oligonucleotides</td>
</tr>
<tr>
<td>CRA</td>
<td>Cockroach allergen</td>
</tr>
<tr>
<td>CXCL11</td>
<td>Eotaxin</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DCs</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>Der f2</td>
<td>Dermatophagoides farinae mite group 2 allergen</td>
</tr>
<tr>
<td>Der p2</td>
<td>Dermatophagoides pteronyssinus-2 antigen</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s phosphate-buffered saline</td>
</tr>
<tr>
<td>DPI</td>
<td>Dry powder inhaler</td>
</tr>
<tr>
<td>ENO</td>
<td>Exhaled nitric oxide</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>Gem</td>
<td>Gemcitabine</td>
</tr>
<tr>
<td>Gen</td>
<td>Genipin (Crosslinking agent)</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
</tr>
<tr>
<td>GNC</td>
<td>Gelatin nanocarrier</td>
</tr>
<tr>
<td>HDAC2</td>
<td>Histone deacetylase-2</td>
</tr>
<tr>
<td>HDM</td>
<td>House dust mite</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat-shock protein</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>i.t.</td>
<td>Intratracheal</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>ICS</td>
<td>Inhaled corticosteroid</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL4R</td>
<td>Interleukin 4 Receptor</td>
</tr>
<tr>
<td>Jak</td>
<td>Janus Kinase</td>
</tr>
<tr>
<td>Ka</td>
<td>Absorption rate constant</td>
</tr>
<tr>
<td>Ke or Kelim</td>
<td>Elimination rate constant</td>
</tr>
<tr>
<td>LABA</td>
<td>Long-acting beta2-agonist</td>
</tr>
<tr>
<td>LAMA</td>
<td>Long acting muscarinic antagonists</td>
</tr>
<tr>
<td>LTB4</td>
<td>Leukotriene B4</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
</tr>
<tr>
<td>MART</td>
<td>Maintenance and Reliever Therapy</td>
</tr>
<tr>
<td>Acronym</td>
<td>Term</td>
</tr>
<tr>
<td>---------</td>
<td>------</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NiMS</td>
<td>Nanoparticles in microparticle system</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>Nrf2</td>
<td>Nuclear factor erythroid 2-related factor 2</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-small cell lung cancer</td>
</tr>
<tr>
<td>OCS</td>
<td>Oral corticosteroid</td>
</tr>
<tr>
<td>p</td>
<td>Statistical p value</td>
</tr>
<tr>
<td>pAb</td>
<td>Polyclonal antibody</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PDE</td>
<td>Phosphodiesterase</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene) glycol</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>Ph.Eur.</td>
<td>European pharmacopeia</td>
</tr>
<tr>
<td>PGA</td>
<td>Polyglycolides</td>
</tr>
<tr>
<td>PGD2</td>
<td>Prostaglandin D2</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>P-gp</td>
<td>P glycoprotein</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PLA</td>
<td>Polyactides</td>
</tr>
<tr>
<td>PLGA</td>
<td>Polyactide co-gluco-lides</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>RAO</td>
<td>Recurrent airway obstruction</td>
</tr>
<tr>
<td>rHG</td>
<td>Recombinant human gelatin</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>------</td>
<td>------------</td>
</tr>
<tr>
<td>S6S</td>
<td>STAT6 siRNA</td>
</tr>
<tr>
<td>S6S-GNC</td>
<td>PEGylated STAT6 siRNA Loaded Gelatin Nanocarrier</td>
</tr>
<tr>
<td>S6S-GNC-P</td>
<td>PEGylated and peptide conjugated STAT6 siRNA Loaded Gelatin Nanocarrier</td>
</tr>
<tr>
<td>SABA</td>
<td>Short-acting beta2-agonist</td>
</tr>
<tr>
<td>SH2</td>
<td>Src-homology 2 domain</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering Ribonucleic acid</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducer and Activator of Transcription</td>
</tr>
<tr>
<td>Syk</td>
<td>Spleen tyrosine kinase</td>
</tr>
<tr>
<td>t½</td>
<td>Half-life</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>Th</td>
<td>T helper lymphocytes</td>
</tr>
<tr>
<td>Th0</td>
<td>T naïve lymphocytes</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper lymphocytes subset 1</td>
</tr>
<tr>
<td>Th2</td>
<td>T helper lymphocyte subset 2</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TSE</td>
<td>Transmissible spongiform encephalopathies</td>
</tr>
<tr>
<td>TSLP</td>
<td>Thymic stromal lymphopoietin</td>
</tr>
<tr>
<td>US FDA</td>
<td>United States Food and Drug Administration</td>
</tr>
<tr>
<td>USP</td>
<td>United States Pharmacopoeia</td>
</tr>
<tr>
<td>Vd</td>
<td>Volume of distribution</td>
</tr>
<tr>
<td>γc</td>
<td>Common gamma chain</td>
</tr>
<tr>
<td>ψ</td>
<td>Surface (Zeta) potential</td>
</tr>
</tbody>
</table>
CHAPTER 1. INTRODUCTION

1.1. Motivation and Research Background

1.1.1. The Impact of Asthma and Critical Barriers to Medical Care

Asthma is a chronic inflammatory disease incorporating various phenotypes and mechanisms of pathogenesis that impacts approximately 300 million people worldwide and affects over 8% Americans [1]. There is a continual rise in the proportion of asthmatic patients [2]. Approximately 18.7 million adults and 6.8 million children in the U.S. have asthma [3]. Asthma is the leading chronic disease among children and accounts for approximately 13 million missed school days per year [3]. Furthermore, asthma is the third leading cause of hospitalization among children under the age of 15. Although asthma is more common in children than in adults, most deaths from asthma are in adults [4]. Despite the use of current therapies, each day 9 Americans die from asthma [4]. Asthma prevalence is highest among children, women, and Native and African Americans [5, 6]. Asthma costs the U.S. $56 billion each year and impacts of this disease on human health and well-being are devastating [2].

Despite of advances in the understanding of immunological mechanisms initiating and mediating allergic airway response, current asthma treatments still rely on therapies that aim to relieve symptoms rather than targeting pathogenically relevant elements of asthma [7-11]. Recent national asthma surveys found that asthma healthcare is suboptimal and that the disease remains poorly controlled [12-14]. Existing treatment options such as antihistamines or steroids are nonspecific, therefore more targeted approaches that act on the underlying causes of asthma are needed for the mitigation of the disease state [15, 16]. Initial approaches to treat asthma emphasized on the relief of bronchoconstriction with β2-adrenergic bronchodilators but the discovery of airway inflammation as an important pathophysiological component of asthma has led to the use of inhaled corticosteroids as another asthma therapy [17, 18]. Concerns about the long-term detrimental effects of high-dose inhaled corticosteroids therapy include cataracts, anxiety, osteoporosis, hyperglycemia, and stunting growth in children [19-22]. Aside from anti-immunoglobulin E (IgE) and anti-IL5 biological therapies for severe asthma, there are no major new drug classes available for the treatment of asthma in the last 20 years [11, 23]. The IL4, IL5, IL13, IL9, GM-CSF and tumor necrosis factor-α antibodies are under clinical trials for asthma [11, 24]. Antibody therapies require high doses and are associated with high production costs, poor tissue penetration, and recent evidence has suggested that resistance to immunotherapeutics can
An IgE blocking antibody, omalizumab, has been approved by FDA, even though 40% of allergic asthma patients failed to obtain clinical response [10, 26]. These therapies mainly relieve symptoms rather than targeting underlying pathogenic elements of the disease [9, 10, 26]. A recent national asthma survey revealed that asthma healthcare is suboptimal and the disease still remains poorly controlled. Eighty-five percent of asthma patients who were on asthma controller medications showed uncontrolled persistent asthma [27]. Additionally, uncontrolled eosinophil inflammation occurs in most asthmatic patients and these patients poorly respond to conventional medications. Therefore, there is an unmet need for newer therapies targeting elements of asthma pathogenesis to better control the disease and improve the quality of life.

Typical clinical features of asthma are recurrent episodes of wheezing, shortness of breath, chest tightness, and coughing. Many patients with asthma—especially those with early onset—are atopic, meaning they are genetically predisposed to generate IgE antibodies upon exposure to environmental allergens, such as house dust mite, pollen, and animal dander [18]. Repeated inhalation of such allergens can subsequently induce an IgE-mediated hypersensitivity reaction, which is characterized by increased vascular permeability, vasodilatation, smooth muscle contraction, bronchoconstriction, and airway inflammation. Asthmatic airways also undergo structural changes (airway remodeling), with epithelial changes, increases in smooth muscle mass, deposition of extracellular matrix proteins, and goblet cell hyperplasia [18]. The development of allergic asthma can be divided into two phases: (1) an allergic sensitization phase and (2) a phase of disease development characterized by airway inflammation and remodeling. Allergic sensitization occurs in susceptible individuals, when allergen exposure and subsequent allergen processing and antigen presentation by dendritic cells induces the differentiation of naïve CD4+ T cells into effector Th2 cells which produces the IgE antibody and other key facilitators of asthma [28-30]. Interleukin (IL) 4 plays an important role in this Th2 differentiation stage [31, 32]. In this regard, certain transcription factors are also of particular interest because they are activated specifically in response to IL4 and IL13 cytokines secreted by T helper (Tho and Th2) cells upon their presentation to foreign allergen [28, 33, 34]. One of the most prominent transcription factors regulating the differentiation of Th2 cells, production of Th2 cytokines and effector functions mediated by Th2 cytokines is a signal transducer and activator of transcription 6 (STAT6) [34, 35]. Therefore, STAT6 specific therapies represent a key method towards the development of an effective clinical therapeutic against atopic asthma.
1.1.2. STAT6 Short Interfering RNA (STAT6 siRNA) Therapy for the Effective Management of Asthma

STAT6 is a member of the Signal Transducer and Activator of Transcription protein family that is present in the latent form within the cytoplasm of T helper cells, B cells, myeloid cells and certain non-immune cells, such as epithelial cells and fibroblasts. Once activated by IL-4 or IL-13 binding to their cognate receptors, conserved tyrosine residues on the receptor are promptly phosphorylated by the Janus Kinase (Jak) family of the Tyrosine Kinases following the interaction of IL4 and IL13 with the IL4 receptor alpha (IL4Rα) and IL13 receptor alpha (IL13Rα) respectively [33, 34, 36-38]. The phosphorylation allows the docking of STAT6 protein via a Src-homology 2 (SH2) domain and the Jaks may phosphorylate the conserved tyrosine-641 on the STAT6 protein, which then allows for the formation of STAT6 homodimers. This activated phosphorylated STAT6 dimer then translocates into the nucleus where it binds directly to DNA via a DNA-binding domain, giving it the ability to regulate transcription [39, 40]. IL13 can induce AHR, eosinophilia, and eotaxin production without the presence of IL4Rα, indicating that IL13 can signal independently of this receptor in a STAT6 mediated mechanism [41]. The alternative signaling by IL13 occurs through the MAPK pathway [42]. Fujisawa et al. had shown that IL13 induces mucus cell metaplasia via the STAT6 dependent p38 MAPK pathway [43]. Therefore, inhibition of IL4 or IL13, or targeting the shared receptor IL4Rα, would be less effective than targeting STAT6 expression, since this would block activities of IL4, IL13, IL4Rα, and by other pathways like MAPK [42].

STAT6 is required for the expression of transcriptional gene GATA3, which is the primary master transcription gene that mediates differentiation of Th2 cells and production of IgE antibody and other key facilitators of asthma such as macrophage, mast cell, and eosinophilis [44-48]. STAT6 is also responsible for positive feedback for Th2 cell differentiation under allergen exposure and the amplification of the asthma process [34, 35, 49, 50]. The silencing of STAT6 not only helps in alleviating asthma by inhibition of GATA3 gene transcriptional pathway via STAT6 silencing but also by the subsequent loss of the positive feedback mechanism towards conversion of Th2 cells from naïve T cells [49, 50]. Investigators have demonstrated the use of S6S to decrease STAT6 messenger RNA and protein which was an effective approach to rapidly achieve attenuation of ongoing IL4 and IL13 mediated events [51]. Thus, STAT6 represents a promising therapeutic target for the management of asthma.
Currently no STAT6 inhibitors have been approved by the FDA, and those within preclinical studies (Leflunomide, Tofacitinib, and AZD1480) have poor efficacy, undesirable pharmacokinetic properties and associated adverse effects, thus highlighting the need to develop alternative STAT6 targeting approach for effective asthma therapy [52, 53]. Leflunomide, an isoxazol derivative, N-(4-trifluoromethylphenyl)-5-methylisoxazol-4-carboxamide, was reported to inhibit IL13 induced STAT6 phosphorylation in human bronchial smooth muscle cells, but with a relatively weak potency (250 μM) [52]. The Jak inhibitor, tofacitinib, showed significant efficacy in rheumatoid arthritis and is FDA-approved for this indication [53]. Through the inhibition of Jaks, tofacitinib abrogated anti-CD3 induced IFN-γ, IL4, IL17 production in CD4+ T cells isolated from the peripheral blood of healthy volunteers [53]. Tofacitinib was also shown to inhibit STAT1, STAT3, STAT4, STAT5, and STAT6 activation in cultured anti-CD3-stimulated T cells, which suggests that targeting the cytokine signaling pathway with tyrosine kinase inhibitors may be an approach for immunosuppression [53]. Tofacitinib does have a major disadvantage, in that, it was shown that low dose tofacitinib accelerated the onset of experimental autoimmune encephalomyelitis at a concentration that suppressed collagen-induced arthritis [54]. The pyrazolyl pyrimidine AZD1480, which potently inhibited Jak1 (IC50 value of 1.3 nM) and Jak2 (IC50 value of 0.4 nM) [55], differentially suppressed STAT1, STAT3, STAT5, and STAT6 activation at 0.1-5 μM concentration in prostate, ovarian, glioma, and breast cancer cells in vitro [56, 57]. The risk-benefit balance for tyrosine kinase inhibitors is greatly skewed towards risk outweighing the benefits when taking into account the side effects associated with these Jak inhibitors, such as thrombocytopenia, anemia, metabolic abnormalities, and hyperacute relapse of symptoms when treatment is discontinued [58].

Other STAT6 inhibitors that have not reached clinical trials, but show some promise in the treatment of asthma and are small molecules are: AS1517499, niflumic acid, YM-341619 (IC50 value of 0.70 nM) [59], AS1810722 (IC50 value of 2.4 nM) [60] and TMC-264. Both AS1810722 and YM-341619 are potent, orally active STAT6 inhibitors that could be developed for the treatment of STAT6-dependent allergic asthma. Treatment of the human B lymphoma cell line DND39 with TMC-264, an agent identified from the fermentation broth of Phoma spp., inhibited IL-4-dependent tyrosine phosphorylation and activation of STAT6 [61]. At low micromolar concentrations (1.6 uM), TMC-264 selectively inhibited the phosphorylation of STAT6 over that of STAT5 or STAT1, and it also blocked STAT6 DNA binding activity in a dose-dependent
manner [61]. Its derivative, AS1517499, showed a significantly improved potency against STAT6 activity (IC50 of 21 nM) and inhibited IL-13 induced STAT6 phosphorylation and subsequent Th2 differentiation without affecting Th1 polarization [42, 62]. AS1517499 inhibits phosphorylation of STAT6 and upregulation of RhoA, which was verified with murine asthma model [42]. However, no effect was seen in IgE levels, indicating that the inhalation delivery of AS1517499 was blocking pathways downstream of Th2 differentiation, possibly because the inhibitor was delivered to airway bronchial tissue instead of immune cells [42, 62]. These STAT6 inhibitors are still being studied in laboratory pre-clinical experiments, therefore true insight into their potential adverse drug reactions and pharmacokinetic parameters are unknown. Other drug classes for inhibiting STAT6 activity include oligodeoxynucleotide decoys, antisense oligonucleotides, peptides and natural products [42, 63].

The use of short interfering RNA (siRNA) for asthma therapy represents a promising alternative for STAT6 silencing due to their efficient knockdown of targeted genes and more rapid bench-to-bedside development compared to conventional anti-asthmatic drugs [64-67]. Importantly, mRNA and protein expression levels of IL4 and IL13 in lung tissues of STAT6 knockout mice were significantly diminished compared with challenged controls [49, 50, 68]. Previous studies demonstrated the targeting of STAT6 by STAT6 siRNA (S6S) administered intranasal route, effectively blocked the development of cardinal features of allergic airway disease such as allergen-induced airway inflammation and airway hyper responsiveness (AHR) [51]. This local delivery of unprotected siRNA may detrimentally impact its kinetics due to nuclease degradation of the siRNA in vivo. Investigators have observed allergen-induced alterations in lung tissues (goblet cell hyperplasia, peribronchial inflammation with eosinophils and CD4+ T cells) to be significantly reduced after S6S treatment formulated within commercially available untargeted cationic liposomes (Lipofectamine 2000) or via intranasal route [51, 68]. These data clearly illustrate that targeting STAT6 by S6S effectively blocks the development of cardinal features of allergic asthma, like allergen-induced airway inflammation and AHR. Therefore, the targeting STAT6 has a high potential approach for the treatment of allergic asthma. Conversely, despite this huge therapeutic potential the clinical utility of siRNA therapy has been greatly hampered due to its poor cell penetration, rapid degradation, short half-life and non-specific effects [69, 70]. Also the delivery of S6S is complicated by its intracellular location. Hence, a suitable delivery system must be developed to mediate site-specific delivery of S6S to the desired site of action i.e. T (T
naïve, Th0 and Th2) cells and this step is a prerequisite to successfully explore their anti-asthmatic potential. To circumvent this difficulty, it is herein proposed to formulate S6S as a nanotherapeutic by encapsulating them within the gelatin nanocarrier (GNC) and conjugation with IL4Rα targeting peptide for targeted delivery of S6S to T cells.

1.1.2.1. Up-regulation of STAT6 in asthma

In collaboration with Dr. Hoffmann (UH Manoa JABSOM), it has been demonstrated that the up-regulation of STAT6 is associated with the progression of asthma in an acute ovalbumin (OVA) model of allergic asthma [71]. Using the mouse model of asthma that involves OVA-challenge or PBS-challenge (as a negative control), increased expression and phosphorylation of STAT6 was detected in the lungs from asthmatic mice (Figure 1.1 A). An important step in the signaling pathways that differentiate Th cells toward the Th2 phenotype and subsequent allergic airway inflammation is the phosphorylation of STAT6 [72, 73]. In addition, lungs from asthmatic mice exhibited increased infiltration of inflammatory cells by histological analyses of tissue surrounding lower lung airways (Figure 1.1 B) as shown by the standard periodic acid-Schiff method staining where the dark purple staining highlights the presence of mucus and helps identify goblet cell hyperplasia. Hence, targeted delivery of S6S using natural biopolymer based S6S-GNC-P will selectively deliver S6S to T immune cells thereby silence STAT6 and downregulate the phosphorylation of STAT6 and downstream GATA3 transcriptional gene expression. The GATA3 transcription factor is a mediator of the production of IgE, mast cells, eosinophils and basophils, which are chief mediators of asthma, by promoting the excretion of IL-4, IL-5, and IL-13 from Th2 cells. IL4 and IL13 share a common α-chain with IL-4Rα for activation of the STAT6-dependent signal transduction pathway and therefore, the targeted delivery of S6S via S6S-GNC-P should theoretically help in blocking both IL4 as well as IL13-mediated asthma progression. The encapsulation of S6S within the GNC will further help to overcome the limitation associated with S6S delivery and will also avoid peripheral silencing problem (off-target silencing) commonly associated with the delivery of siRNA’s.
Figure 1.1. Normal mouse and asthmatic OVA-challenged mouse lung tissue. (A) Increased expression and phosphorylation of STAT6 and (B) Histological analysis of inflammatory cells in airways. The sections shown in panel B were stained with a standard periodic acid-Schiff method and the dark red-purple staining represents the presence of mucus and highlights the goblet cell hyperplasia.

1.1.3. Gelatin-based Therapeutic Nanoparticles for siRNA Delivery

Various nanocarrier systems have been approved by FDA for human diseases and several others are undergoing clinical trials [74-76]. Nanocarriers (NC) have generated strong attention for siRNA delivery owing to their ability to overcome the siRNA delivery related issues [64, 77-80]. A variety of NC systems including nanoparticles, nanogels, microspheres, dendrimer, carbon nanotubes and various others are currently being explored for delivery of therapeutic agents for asthma therapy [81-85]. However, the siRNA delivery using currently reported NC based approaches suffer major limitations such as instability, complexity of preparation, toxicity, and biocompatibility [86-89].

The use of synthetic polymer based nanocarriers has been limited due to toxicity and longer path to reach clinic [90]. An ideal nanocarrier system for asthma therapy must be safe, biodegradable, site-specific, and overcome limitations associated with naked siRNA delivery [91]. Polymeric nanoparticles formed from biodegradable proteins or polysaccharides make up a growing field of drug delivery systems for targeted and controlled release, with the goal to enhance therapeutic effects while reducing side effects of formulated active pharmaceutical agents [92]. The ideal polymeric nanoparticle should be composed of a material that is biodegradable, nonimmunogenic, renewable, possess high binding capacity, low reticuloendothelial system
uptake via aqueous steric barriers, and sufficient chemical and/or physical stability in vivo and during storage [93].

Gelatin is a safe and biodegradable polymer and hence, the developed gelatin nanocarriers are expected to take a shorter path to reach the clinic. Gelatin has been used previously within intravenously administered injection containing 1.4-3.48% gelatin solutions [94]. Therefore, considering the already established safety profile of gelatin and expected biocompatibility, it was selected for this project. Gelatin is derived from collagen, a natural biodegradable polymer. While collagen has some advantages such as biocompatibility, nontoxicity, and efficient isolation and purification, it is known to cause immunogenic responses, thus limiting its use [95]. Atellocollagen, a variant of collagen that has the telopeptide removed, has been shown to have decreased immunogenicity. However, collagen and atellocollagen have poor mechanical strength and variable release kinetics [96, 97]. Therefore, the product of collagen hydrolysis, gelatin, is widely used because of its wide availability, low antigen profile, low binding to drug molecules, and reproducible release profiles [95]. Gelatin has modifiable amine groups which allow conjugation of targeting ligand on the surface of nanocarriers. An established desolvation method to prepare nanocarriers using cationic type A gelatin was selected [98, 99]. Type A gelatin, or cationic gelatin, was found to be superior for siRNA entrapment and delivery compared to type B gelatin due to its positive charge [100]. In addition, cationic gelatin is expected to enable endosomal escape via proton sponge effect at endosomal pH 6.4 and thus delivers the STAT6 siRNA in the cytoplasm [101, 102]. The i.v. route was selected over the pulmonary inhalation route to screen this novel asthma therapy because of the obstacles of mucociliary clearance of nanocarriers, an inefficient nanocarrier delivery to spleen, and poor mucus penetration associated with inhaled nanomedicines.

Gelatin is denatured collagen that results from either a partial acid or alkaline hydrolysis of collagen. It is a generally recognized as safe (GRAS) substance according to the United States Food and Drug Administration (FDA) and has a long history of use in food products, cosmetics, and pharmaceuticals [93]. Clinically, gelatin has been used in parenteral formulations, namely as a plasma expander, stabilizers in protein formulations, vaccines, and gelatin sponge [103]. Gelatin is biocompatible and biodegradable and has the added advantage of having low antigenicity when sufficiently denatured [93]. Since gelatin is a collagen derivative, the enzymatic degradation of gelatin does not produce harmful byproducts. Although collagen requires collagenases for enzyme
hydrolysis and is resistant to other proteases, gelatin is degraded by most proteases [104]. While collagen shows high antigenicity that makes pulmonary delivery by the inhalation route of administration inappropriate, gelatin undergoes extensive hydrolysis, denaturation, purification, followed by heat treatment sterilization which leads to a polymer with non-antigenic and immunogenic properties [104-107]. Gelatin contains many different accessible functional groups due to its protein structure, therefore there is a high potential for modifications via crosslinkers, targeting ligands, and pegylation. The amino acid structure of gelatin contains cationic, anionic, and hydrophobic groups at a ratio of about 1:1:1 where the molecule is 13% positively charged due to lysine and arginine, 12% negative charged due to glutamic and aspartic acid, and 11% hydrophobic due to the leucine, isoleucine, methionine, and valine, at or around neutral pH. One third of the amino acids in gelatin are glycine, another third are proline and hydroxyproline, and the rest are other amino acids as shown in Table 1.1 [108]. The structure of gelatin contains triple helical motifs that are composed of (Gly-X-Pro)\textsubscript{n} where X is lysine, arginine, methionine, or valine [108]. Due to the structure of gelatin, amine (pKa 9.5) and carboxylic acid (pKa 3.75) groups may be exposed to impart charge characteristics. There are two forms available commercially, cationic type A gelatin, prepared from an acid hydrolysis of pig skin type I collagen which has a isoelectric point (pI) between 7-9 or anionic type B gelatin, prepared from an alkaline hydrolysis of bovine collagen that has a pI between 4.8-5 without exogenous chemical modifications [109].
Table 1.1. Type A gelatin amino acid content [104].

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>9</td>
</tr>
<tr>
<td>Arginine</td>
<td>8</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>6</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>10</td>
</tr>
<tr>
<td>Glycine</td>
<td>21</td>
</tr>
<tr>
<td>Histidine</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Hydroxylysine</td>
<td>1</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>12</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1</td>
</tr>
<tr>
<td>Leucine</td>
<td>3</td>
</tr>
<tr>
<td>Lysine</td>
<td>4</td>
</tr>
<tr>
<td>Methionine</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>2</td>
</tr>
<tr>
<td>Proline</td>
<td>21</td>
</tr>
<tr>
<td>Serine</td>
<td>4</td>
</tr>
<tr>
<td>Threonine</td>
<td>2</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Valine</td>
<td>2</td>
</tr>
</tbody>
</table>

Methods for the preparation of gelatin nanocarriers (GNCs) include desolvation, coacervation-phase separation, emulsification-solvent evaporation, reverse phase microemulsion, nanoprecipitation, self-assembly due to chemical modification or from simple mixing, and layer-by-layer (LbL) coating [108]. Crosslinking of GNCs is necessary to produce particles with adequate stability, shape, and longer circulation in vivo versus uncrosslinked particles [110]. Genipin is a covalent amine crosslinker extracted from the gardenia fruit. It is a desirable crosslinker of gelatin because it is roughly 10,000 times less cytotoxic than glutaraldehyde, however, it requires a longer crosslinking time [111, 112]. The crosslinking reaction mechanism of proteins with Genipin was proposed to involve the two free amino groups of lysine residues on the protein chains crosslinking with one molecule of genipin [112].

Although gelatin nanocarriers have great potential advantages in the field of targeted siRNA delivery, there are inherent issues with the use of animal-origin gelatins. These gelatins carry the risk of contamination with synthetic growth hormones and transmissible spongiform encephalopathies (TSEs), but the risk is minimized due to the acid, alkaline, and heat treatments that occur during manufacturing processes that minimize the risk in drug products [113, 114].
Commercially available gelatins for pharmaceutical development have low-endotoxin levels. Modern manufacturing processes with washing and filtrations steps, final ultra-heat treatment sterilization step, and a drying of the gelatin solution, allows for the preparation of gelatin with the highest quality of chemical, physical, virological, and bacteriological safety [104]. For example, GELITA MedellaPro® has high purity levels, low allergenic potential, high tolerance by the body, low affinity for tissue or cell non-specific interaction. Gelatin is used as a vaccine preservative and stabilizer, allowing for increased stability under varying temperatures. Gelatin in vaccines does lead to one case of anaphylaxis in every two million vaccine injections in patients with gelatin allergies. Current amounts of gelatin in vaccines ranges from 0.0015 mg per dose to 14.5 mg per dose [115-117]. Additionally, recombinant human gelatin (rHG) is currently available (FibroGen South San Francisco, CA, USA). The rHG will be useful in drug delivery due to its nontoxicity, nonimmunogenicity, and has the added advantage of a narrow molecular weight distribution and defined properties to form nanoparticles with a narrow size distribution [113, 114]. This study aimed to provide the framework for the future development of lymphocyte-targeted rHG nanocarrier delivery system with the potential to reach human clinical studies in the future.

As a nucleic acid molecule, siRNA is sensitive to nucleases (DNase or RNAse), possesses a negative charge due to their sugar phosphate backbones under physiological conditions, is hydrophilic, and has a relatively low molecular weight, which makes it a poor candidate for an active pharmaceutical agent and highlights the need of effective non-viral delivery systems for siRNA therapy [118]. This is evidenced by the rapid excretion of naked siRNA with an average diameter of less than 10 nm from the blood compartment through renal filtration clearance [119]. A desirable siRNA delivery system should increase the circulatory system retention time. It should also be capable of passing through the blood vessel endothelial wall to reach the target organ [120].

Non-viral siRNA vectors are internalized in cells by endocytic pathways, which can be subdivided into four different group, (1) clathrin-mediated endocytosis, (2) caveolae-mediated endocytosis, (3) clathrin- and caveolae-independent endocytosis, and (4) phagocytosis and macropinocytosis [121, 122]. A targeting ligand on the surface of a polymeric gelatin nanoparticle may recognize a specific antigen on the surface of target cells and enhance the cellular uptake by binding to cell surface receptors. Upon intracellular uptake, the endocytic vesicles containing the nanocarrier system travel along microtubes and fuse with the early endosomes. Subsequently, these mature into late endosomes (pH 5-6) and into lysosomes (pH ~4.5) that contain many
nucleases [122]. The siRNA loaded nanocarrier must escape from the endosomes before lysosomal degradation can occur. A major barrier for efficient siRNA delivery is endosomal escape [123]. The protein-sponge effect involves cationic polymers with strong buffering capacity between pH 5-7 and the acidic late endosome and lysosomal environment causes protonation of the amino groups on the polymer. The protonation of the polymer amino groups then causes osmotic swelling and vacuole disruption that results in the endosomal release of the polymer and siRNA cargo [123].

1.1.4. Nanocarriers for STAT6 siRNA Delivery and Asthma Therapy

An ideal delivery system must be biocompatible, biodegradable, and site-specific, while both avoiding siRNA degradation and uptake by normal cells [124]. Gelatin is a biodegradable and biocompatible polymer approved by FDA for intravenous administration. The following attributes highlight Gelatin’s usefulness within a nanotherapeutic delivery system: biodegradability, biocompatibility, non-toxicity, ease of chemical modifications, and cross-linking capacity, thus making GNC a promising carrier system for siRNA delivery. An established, simple and reproducible two-step desolvation technique was used to prepare GNC using Type “A” gelatin with 175 g gel Bloom strength [125, 126]. Bloom is a test to measure the strength of a gel or gelatin by determining the weight needed by a probe to deflect the surface of a gel composed of 6.67% gelatin solution kept for 17-18 hr at 10°C by 4 mm without breaking it, and is expressed between 30-300 Bloom [127]. Type B gelatin based NCs have been utilized for siRNA delivery [128], however reports comparing Type A and Type B gelatin [129] show Type A gelatin to be superior for bio-therapeutic delivery and has the advantage of being positively charged that leads to cytosolic delivery due to endosomal escape through a proton-sponge effect mechanism. A literature search revealed that to date gelatin has not been employed for targeting of S6S to the T immune cells and this will be the first report of its type.

The availability of modifiable amine groups on the periphery of the gelatin nanocarriers makes them an appropriate carrier system for targeting the therapeutic agent to the site of action by conjugation them with a targeting ligand, and for providing stealth properties by conjugating PEG. The act of PEGylating the gelatin nanocarriers should not increase their antigenicity because both polymers are non-antigenic. Further, PEGylation has been shown to reduce immunogenicity and antigenicity of various polymeric delivery systems [130, 131]. However, certain targeting peptides may increase the antigenicity of nanocarriers based on their sequence [132]. The targeting peptide used in this study does not show properties that will lead to increased antigenicity of the
gelatin [133]. Type A gelatin-based architectures are capable of selectively delivering the loaded nano-particles into the cytoplasm (an event known as endosomal escape) owing to protonation of amine groups of gelatin under sub-physiological endosomal pH (pH ~ 5.5). Cationic GNC allows for additional method of escaping the harsh and destructive lysosomal environment by triggering endosomal escape by eliciting the proton-sponge effect [123, 134]. In a murine model, the main binding site of IL-4 to IL4Rα was found to be between residues 79 to 62, with the sequence of QRLFRAFR. [133]. Further, to mediate intracellular S6S delivery, the PEGylated [polyethylene glycol (PEG) - MW 5000] mouse IL4Rα binding peptide (P) having sequence of QRLFRAFR (identical to 79–86 amino acid residues in IL4), will be coupled to the S6S loaded GNC (S6S-GNC) to facilitate the targeted delivery of S6S to the site of action, i.e T cells (T naïve, Th0 cells and Th2 cells) [133]. Therefore, the formulation will incorporate gelatin based NC system conjugated with IL4Rα binding peptide to attain site-specific targeted delivery of S6S. Naïve T, Th0 and Th2 cells abundantly express IL4R and its expression is up-regulated in asthmatic persons [135, 136]. Hence, S6S-GNC conjugated with PEGylated IL4Rα binding peptide (S6S-GNC-P) is hypothesized to be able to impart selective and targeted delivery of S6S to the site of action i.e T naïve, Th0 and Th2 cells to efficiently silence the STAT6. Off-target effects in B cells and bronchial airway epithelial cells are suspected to occur in vivo due to their inherently high IL4Rα expression, but these effects are expected to augment the anti-asthmatic effects of Th2 STAT6 downregulation [137, 138]. STAT6 silencing in dendritic cells, which also highly express IL4Rα, has been shown to lead to normal development, function, and inflammatory cytokine production [139]. Asthma is a multifaceted allergic disease, which mandates approaches that target multiple cells involved in asthma pathogenesis. Targeting the Th2 cells, which are in larger population in the lungs and play prominent roles in asthma, along with B and airway epithelial cells will be of great benefit for asthma therapy.

1.1.5. IL4Rα Targeting

IL4 is a pleiotropic cytokine derived from different cells of hematopoietic origin, such as T cells, mast cells and basophils and is required for the development of Th2 cells [140-145]. Transgenic mice overexpressing IL-4 showed induction of allergy-like diseases and autoimmunity [146]. In addition, IL4 leads to Th1 differentiation and CD8+ T-cell activation [147-149]. Type I IL4R is a heterodimer of IL4Rα and γc and is expressed mostly on hematopoietic cells, whereas type II IL4R is a heterodimer of IL4Rα and IL13Rα1 and is preferentially expressed on non-
hematopoietic cells [150-153]. The expression of IL4Rα on naïve T, Th0, and Th2 cells makes them an ideal target to deliver S6S to silence STAT6 [135, 136]. In contrast to the methods used to prevent the effects of IL4, blocking IL4Rα does inhibit AHR [154]. Consequently, IL4Rα mediated S6S delivery to attain STAT6 silencing is considered a superior target than IL4 alone. Since the IL13R shares a common α-chain with the IL4-Rα, to activate a STAT6-dependent signal transduction pathway, our STAT6 silencing approach will simultaneously block both IL4 as well as IL13-mediated asthma progression [155]. The specific IL-4Rα binding peptide has a sequence similar to that of IL4 proteins IL4Rα binding region [133, 156], which will help to achieve the site specific delivery of S6S-GNC-P to T cells via IL-4Rα receptor mediated internalization. The practice of organized conjugation of PEG chains on the surface of nano architecture is referred to as PEGylation [130, 157]. The terminal amine group on gelatin easily allows PEGylation, via amide to carboxy carbodiimide conjugation, which has been well reported to overcome the uptake and clearance of delivery systems by the reticuloendothelial system (RES) [158]. In addition, the use of PEGylated delivery systems has demonstrated increased salt and serum stability [130, 159-164]. Nanoparticles in the size range of 50-100 nm are most often coated with PEG 3400-10,000 to attenuate a half-life shortening from enhanced RES due to increased hydrodynamic diameter [165]. PEG with a MW of 5000 Dalton was found optimal for decreasing the clearance, increasing biocompatibility, stability and performance [166]. Therefore, a PEG-5000 as a spacer chain between the IL4Rα targeting peptide and the S6S-GNC was selected. Because of the direct delivery of S6S to the T cells, we expect to greatly improve the overall effectiveness of S6S to silencing the STAT6, reduce adverse side effects to other tissues, and overcome current limitations associated with S6S delivery.

Nanocarriers have generated potential interest for asthma therapy. However, the outcomes with passively delivered nanocarriers are poor due to their inefficient cell internalization, non-specific distribution and uptake in non-target cells. This project utilized a new and unique concept that involves the active, targeted delivery of S6S to T cells (T naïve, Th0 and Th2) by using a novel PEGylated S6S loaded gelatin (FDA approved polymer) nanocarrier (GNC) system coupled with an IL4 receptor alpha (IL4Rα) specific binding peptide having sequence of ‘QRLFRAFR’ that selectively recognizes the IL4Rα expressing T cells. To overcome the problems associated with the intracellular S6S delivery, the S6S loaded GNC (S6S-GNC) will be conjugated with PEGylated IL4Rα specific binding peptide (S6S-GNC-P) will be coupled to the S6S loaded GN (S6S-GNC)
to attain a selective and targeted delivery of S6S to T cells. Furthermore, the high expression of IL4Rα on T cells makes them an ideal target to deliver S6S. The delivery of S6S using novel S6S-GNC-P has several advantages such as targeted delivery of S6S to T cells (T naïve, Th0 and Th2), effective STAT6 silencing, high therapeutic benefit, and reduction in side effects.

IL4Rα was highly expressed on Th2 cells compared to other cells or tissues [137, 167-170]. Th2 cells are greater in population in lungs of asthmatic patients [171, 172]. STAT6 is promptly phosphorylated by the Jak family of tyrosine kinases following the interaction of IL4 and IL13 with the IL4Rα and IL13 receptor alpha (IL13Rα) chains, respectively [50, 173, 174]. Additionally, IL4Rα is expressed on B cells and airway epithelial cells involved in asthma [170, 175]. The expression of IL4Rα on Th2 cells, as well as in B cells and epithelial cells, and the impact of STAT6 activation in asthma make them ideal targets to deliver STAT6 siRNA using an IL4Rα-targeting peptide. The mouse IL4Rα targeting peptide having sequence of QRLFRAFR [176, 177] was selected to conjugate on PEGylated nanocarriers based on its reported efficacy [133]. The terminal amine group on gelatin easily allows PEGylation, which has been used to overcome the uptake and clearance of delivery systems by the reticuloendothelial system [178]. In addition, the use of PEGylated delivery systems has demonstrated increased salt and serum stability [130, 179]. Polyethylene glycol (PEG) of MW 5000 Da was found to be optimal towards decreasing the clearance, increasing biocompatibility and serum stability [180-182].

The i.v. administration of nanocarriers conjugated to IL4Rα binding peptide is expected to deliver the STAT6 siRNA via IL4Rα targeting peptide to asthma contributing cell types in lungs (pulmonary circulation, which has a low-pressure with high capacitance, and 98% cardiac output [183]) and spleen. We expect to significantly improve the overall effectiveness of STAT6 siRNA, reduce off-target effects to other tissues, and overcome current limitations associated with siRNA delivery. Since the IL-4R type I and II share a common IL4Rα-chain to activate a STAT6 pathway, our STAT6 transcriptional gene silencing approach will target both the IL4 and IL13 mediated asthma progression [51, 173, 184]. The IL4Rα-guided, active delivery of STAT6 siRNA using nanocarriers for asthma therapy is innovative and has not been published previously.

New asthma therapeutic approaches must take into consideration the key elements of the asthmatic cascade and be both effective and safe. Currently, there are no FDA approved STAT6 inhibitors and the use of small STAT6 inhibitor compounds under preclinical studies has been limited due to the poor efficacy and associated adverse side effects observed [52, 53, 63].
Therefore, there is a strong argument and potentially large market for alternate STAT6 silencing strategies. It is proposed to formulate S6S as nanotherapeutic by encapsulation inside gelatin polymer based S6S-GNC-P. The proposed S6S-GNC-P will specifically target T cells by means of surface-anchored, PEGylated IL4Rα targeting peptide and is hypothesized to be efficacious in alleviating asthma. This project will significantly improve the scientific knowledge by creating new tools for efficient targeted siRNA delivery and help in overcoming the limitations associated with current siRNA delivery approach. The in vitro studies will elucidate the role of the S6S-GNC-P for asthma therapy. This nanotherapeutic based targeted approach is also intended to result in a reduced dose of S6S, overcoming the degradation/off-site silencing limitations and hence potentially reduce the risk of adverse effects associated with passive delivery. In addition, the positively charged GNC will escape from endosomes after being internalized due to pH buffering effects [123]. Therefore, it is expected that the S6S-GNC-P dosage form will deliver significant S6S into the cytosol of T cells to silence the STAT6 and exert anti-asthmatic activity.

**Figure 1.2.** IL4Rα mediated targeted delivery of S6S-GNC-P. Schematic representing selective targeting of S6S-GNC-P to IL-4R α expressing T immune cells, Interaction of S6S-GNC-P with IL-4Rα followed by endosomal uptake of S6S-GNC-P inside the T cells, endosomal escape of positively charged GNC via proton-sponge effect and release of free S6S inside cytoplasm to exert the silencing effect by formation of S6S complex. This results in the silencing of STAT6 by S6S-RISC on STAT6 followed by cleavage of STAT6, which leads to blockade of phosphorylation and activation of STAT6.
CHAPTER 2. HYPOTHESIS, OBJECTIVES, AND RATIONALE

The central hypothesis of this work is that targeted STAT6 siRNA loaded gelatin nanocarriers will selectively deliver STAT6 siRNA to T cells and silence STAT6 mRNA and protein expression thereby inhibiting the transcription of master gene GATA3 involved in the initiation and progression of asthma and attenuating allergic asthma. The overall objective of this work was to formulate and characterize gelatin nanocarriers for the targeted delivery of STAT6 siRNA to T cells and to evaluate their effects in vitro. This development and evaluation of STAT6 siRNA loaded gelatin nanocarriers, which are targeted to an interleukin receptor asthma biomarker, represents the first critical step in the pre-clinical development of a novel nanotherapeutic for asthma therapy.

Objective 1. To develop and evaluate S6S-GNC for their particle size, zeta potential, loading efficiency, release kinetics, stability, cytotoxicity and gene silencing within a human adenocarcinoma cell line.

Working Hypothesis: The working hypothesis of this project was that the development of gelatin-based nanotherapeutics of S6S will provide biostability and will deliver the S6S to the tumor cells and thereby will exert a significant anticancer activity.

Rationale: RNAi is a naturally occurring gene silencing process that has the advantages of a high degree of specificity and the potential to silence genes of interest [185]. Small-interfering RNAs (siRNA) are synthetic double-stranded RNA (dsRNA) of 21-23 base pairs that can be designed to suppress target sequences, in a process known as post-transcriptional gene silencing. In order to exert the therapeutic effect the siRNA must be incorporated into the multi-protein RNA-induced silencing complex (RISC)[186]. The siRNAs, as a class of therapeutic agents, are capable of efficient knockdown of targeted genes and may have a more rapid bench-to-bedside development compared to other conventional anti-cancer therapies and have potential in the treatment of other gene related disease states[75].

The signal transducer and activator of transcription 6 (STAT6) is one of the most prominent transcription factors that regulate gene expression in response to extracellular polypeptides that lead to cellular proliferation, differentiation, and apoptosis [50]. STAT6 is a member of a transcription factor family that is present in the latent form within the cytoplasm cells and is promptly phosphorylated by the Janus Kinase (Jak) family of tyrosine kinases following the
interaction of IL4 and IL13 with the IL4 receptor alpha (IL4Rα) and IL13 receptor alpha (IL13Rα) respectively [50]. When activated by Janus kinases, STAT6 translocates to the nucleus where it may regulate cytokine-induced gene expression. Phosphorylated STAT6 is required for responsiveness to IL-4 and IL-13 [151]. Inhibition of Protein phosphatase 2A is also involved in the regulation of IL-4 mediated STAT6 signaling by inducing serine phosphorylation of STAT6 and inhibits the DNA binding of STAT6 [187]. STAT6 has often been associated with asthma and allergic inflammation, however it has been shown to have a role in other disease states [188, 189]. It has also been reported that STAT6 is a survival factor in human prostate cancer [190] and STAT6 increases the expression of COX-2, thereby protecting non-small cell lung cancer (NSCLC) against apoptosis [191, 192]. Dubey et al. found that STAT6 silencing in NCI-H460 lung cancer epithelial cells lead to an increase in cholesterol production and confirmed the anti-apoptotic effects of STAT6 [193]. STAT6 knockdown has been shown to inhibit proliferation and allow for apoptosis in COX-2 positive HT-29 colon cancer cells [194]. The STAT6 transcription activator is activated by an IL4-dependent pathway and is found to up-regulate the expression of BCL2L1/BCL-X(L), which is directly responsible for the anti-apoptotic effect of IL4 [193, 195]. Unfortunately, currently no STAT6 inhibitors have been approved by the FDA, and those within experimental studies (like leflunomide, pitrakinra and salicylates) have poor clinical efficacy, pharmacokinetic properties and adverse effects [196, 197], and this highlights the need to develop alternative STAT6 targeting approach.

The clinical utility of siRNA has been limited to their inherent properties, such as, naked siRNA is prone to degradation, have a shorter plasma half-life, rapid renal clearance, and limited permeability across cell membranes [198]. A variety of nanocarrier (NC) systems in early cancer therapeutic clinical results showed enhanced efficacy and reduced side effects[199-208]. Cationic lipid based systems have emerged as the most attractive for siRNA delivery, however the use is limited due to poor transfection efficiency and toxicity in vivo [209]. Natural polymer-based delivery systems are biocompatible and biodegradable with high physiological tolerance and low immunogenicity [210-216]. To circumvent this difficulty with siRNA delivery, we have formulated S6S as a nanotherapeutic agent by entrapping siRNA within a gelatin nanocarrier (GNC). Gelatin is a FDA approved natural biopolymer, which is inexpensive, biodegradable, nontoxic, has low antigenicity and is easily modified [217]. The working hypothesis of this project was that the development of FDA-approved gelatin based nanotherapeutics of S6S will provide
biostability and will deliver the S6S to the tumor cells and thereby will exert a significant anticancer activity. The mechanism of S6S cellular delivery will be achieved via electrostatic passive cellular uptake as illustrated in Figure 2.1. The core objective of this investigation was to develop and evaluate the biocompatible S6S nanoformulation that will enable effective intracellular delivery in an adenocarcinoma cell line.

**Figure 2.1.** Cellular uptake and intracellular mechanism of action (MOA) of targeted S6S-GNC.

**Objective 2.** To prepare, optimize, and characterize the IL4 receptor targeting peptide conjugated STAT6 siRNA loaded gelatin nanocarriers (S6S-GNC-P) and to evaluate the *in vitro* efficacy of S6S-GNC-P for cell binding, cell internalization, antiproliferative activity, STAT6 protein and mRNA downregulation in a mouse Th2 cell line.

**Working Hypothesis:** The working hypothesis of this study was that the S6S-GNC-P will effectively deliver STAT6 siRNA to Th2 cells to downregulate STAT6 expression. The proposed S6S-GNC-P will specifically target T immune cells by means of surface anchored PEGylated IL4Rα targeting peptide and was hypothesized to play a significant role in alleviating asthma.

**Rationale:** More Americans than ever before are suffering from asthma. Asthma is the leading chronic disease among children and accounts for approximately 13 million missed school days per year [3]. Furthermore, asthma is the third leading cause of hospitalization among children under the age of 15. Although asthma is more common in children than in adults, the 250,000
deaths annually occur mostly in adults [4]. Asthma costs the U.S. $56 billion each year and impacts of this disease on human health are devastating. Asthma disproportionately affects certain groups in the U.S., with higher rates for African Americans, American Indians, and Native Hawaiians. Despite the overwhelming prevalence of the disease and the suffering it causes, relatively little progress has been made to date in improving treatment and prevention of asthma. The current therapies include a combination of long-term control medications and fast-acting relief medications. These therapies mainly relieve symptoms rather than targeting underlying pathogenic elements of the disease [9, 10, 26]. A recent national asthma survey revealed that asthma healthcare is suboptimal and the disease still remains poorly controlled. Eighty-five percentage of asthma patients who were on asthma controller medications showed uncontrolled persistent asthma [27]. Allergic asthma symptoms are not controllable in most of the cases and some patients develop resistance to all available treatments. Therefore, newer effective and safer therapies targeting the pathogenic elements of asthma are essential to improve the clinical outcome.

Allergic (atopic) asthma is caused by a Th1/Th2 imbalance favoring Th2 cells, which facilitates production of inflammatory mediators, IgE, airway hyper-responsiveness (AHR), and mucus hyperplasia [218, 219]. STAT6 plays a key role in Th2 imbalance and asthma progression, thus it is a very promising target for asthma therapy [138, 220-222]. The lack of FDA approved STAT6 inhibitors have demanded the urgent need for alternate STAT6 targeting strategies. In addition to the important role of STAT6 in Th2 cells, the roles of B cells and airway epithelial cells are dependent on STAT6 activation and Th2 cell differentiation. Asthma is a multifaceted allergic disease, which mandates approaches that target multiple cells involved in asthma pathogenesis. Targeting the Th2 cells, which are in larger population in the lungs and play prominent roles in asthma, will be of great benefit for asthma therapy.

Despite an emerging understanding of the pathophysiology of asthma, there is still an urgent need for reliable and successful asthma therapies. Current asthma treatment is mainly aimed at relieving symptoms rather than targeting underlying pathogenetic elements of the disease [7-11]. New approaches must take into consideration the key elements of the asthmatic cascade and be both effective and safe. STAT6 is a crucial transcription factor that plays a key role in Th2 differentiation and gene transcription in response to IL4 and IL13 cytokines that promote asthma symptoms. Currently, there are no FDA approved STAT6 inhibitors and the use of small STAT6 inhibitor compounds under preclinical studies has been limited due to the poor efficacy and
associated adverse side effects observed [52, 53, 63]. Therefore, there is a strong argument and potentially large market for alternate STAT6 silencing strategies.

In this study, the STAT6 pathway was targeted using PEGylated STAT6 siRNA loaded gelatin nanocarriers conjugated with an IL4Rα targeting peptide (S6S-GNC-P) for significant improvement in asthma therapy (Figure 2.2.). A clinically validated biodegradable gelatin polymer was selected to develop the proposed nanocarriers. Gelatin has been previously approved for parenteral administration of other formulations according to the FDA Inactive Ingredients Database [94]. I hypothesized that the S6S-GNC-P will effectively deliver STAT6 siRNA to Th2 cells to downregulate STAT6 expression. The proposed S6S-GNC-P will specifically target T immune cells by means of surface-anchored, PEGylated IL4Rα targeting peptide and is hypothesized to play a significant role in alleviating asthma. Although the delivery of therapeutic agents using NC is widely reported, the proposed S6S-GNC-P that specifically and actively deliver S6S to the T cells via IL4Rα-mediated endocytosis is a novel approach. The in vitro studies will elucidate the role of the S6S-GNC-P for asthma therapy. This nanotherapeutic based targeted approach is also intended to result in a reduced dose of S6S, overcoming the degradation/off-site silencing limitations and hence potentially reduce the risk of adverse effects associated with passive delivery.

**Figure 2.2.** IL4Rα targeted S6S-GNC-P, (A) S6S-GNC-P internalization via IL4R endocytosis, (B) STAT6 siRNA release and formation of STAT6 siRNA-RISC complex, and (C) STAT6 silencing in Th2 cells.
Objective 3. To prepare a gelatin nanocarrier formulation designed to be administered to the lungs via nebulized nanosuspension, preserved in the lyophilized powder state, characterized for physical and aerodynamic properties, and evaluated under *in vitro* cell-based assays.

**Working Hypothesis:** The working hypothesis of this study was that the formulation of Gemcitabine-loaded gelatin nanocarriers (Gem-GNC) with particle size in the range of 150 nm to 200 nm will allow for a controlled release of Gem, have an aerodynamic particle size distribution within the appropriate limits, and outperform free Gem in metabolic activity MTT assays.

**Rationale:** Despite the recent advances in diagnosis and treatment, lung cancer is the second most common cancer only trailing prostate cancer in men and breast cancer in women [223]. In the United States, lung cancer is the leading cause of cancer deaths at an estimated 158,000 deaths in 2015 [224, 225]. An estimated 221,200 new cases of lung cancer were diagnosed in 2015, accounting for approximately 13% of all cancer diagnoses [225]. The lung cancer 5-year survival rate for cases where the disease is still localized within the lungs is 54%, however only 15% of lung cancer cases are diagnosed at an early stage [226]. For metastasized tumors, the 5-year survival rate is 4% [226]. The overall lung cancer survival rate is much lower than other leading cancer causes at 17.8% [226]. Non-small cell lung cancer (NSCLC) occurs when malignant cells form in the tissues of the lung and can be classified as squamous cell carcinoma, large cell carcinoma, adenocarcinoma, pleomorphic, carcinoid tumor, salivary gland carcinoma, and unclassified carcinoma [223]. Approximately 85% of all lung cancers are identified as NSCLC, where 75% of these cases are metastatic at diagnosis [227]. Standard treatment involves combinations of surgery, chemotherapy, and radiation therapy. Although early detection and treatment makes a significant difference in life expectancy, the majority of patients diagnosed with lung cancer present with locally advanced or metastatic disease [228]. Current NSCLC anticancer drugs have poor tumor tissue selectivity and toxicity issues that contribute to their overall low efficacy and detrimental effects to normal tissues. A well-designed drug-delivery system that can deliver anticancer therapeutics to cancerous cells should be developed to avoid adverse effects and to increase efficacy.

Gemcitabine (Gem), 4-amino-1-(2-deoxy-2,2-difluoro-β-D-erythropentofuranosyl)pyrimidin-2(1H)-one hydrochloride (2’,2’-difluorodeoxycytidine), is a deoxycytidine analogue that has been identified as a first-line treatment for NSCLC in combination
with cisplatin [229-231]. The therapeutic potential of Gem in the treatment of cancer is hindered by its short plasma half-life (short infusions have a half-life ranging from 30-90 min, long infusions have a half-life ranging from 4-11 hr), poor metabolic stability, and fast elimination rate [232]. Gem is metabolized by cytidine deaminase following systemic administration to the inactive 2’-deoxy-2,2’-difluorouridine, which is then cleared by urinary excretion by the kidneys. This leads to a half-life of 30-90 min, making frequent administration necessary to induce a pharmacological effect. The tolerability of Gem is more favorable than other anti-cancer drugs such as cisplatin plus etoposide in NSCLC patients, however serious side effects including myelosuppression, neutropenia, thrombocytopenia and anemia may occur [233-235]. The anti-cancer effects of Gem are dependent on frequent administration, making daily administration or prolonged infusion necessary to achieve therapeutic responses [236]. Therefore, a more efficient strategy to deliver Gem at the site of action for prolonged duration of time is necessary to improve the therapeutic potential and to reduce complications due to side effects.

Since Gem is a prodrug, it must first be transported into the cell via a nucleoside transporter and then it must be phosphorylated by deoxycytidine kinase to become pharmacologically active. Gem cellular uptake requires active transporters due to its lack of passive diffusion [237]. Since the uptake of Gem is mediated by both types of transporters, sodium independent and dependent, activity of these nucleoside transporters are fundamental to the inhibition of cell growth and essential for the clinical efficacy of the nucleoside analog [238]. This is supported through studies that have shown that nucleoside transporter deficiency or inhibition causes considerable resistance of tumor cells to Gem [237, 239]. A new drug delivery system capable of efficient Gem delivery to its site of action may avoid the development of resistance and should overcome the limitations associated with transporter deficiency resistance.

Drug delivery via the inhalation route of administration has the ability of allowing a high extent of local absorption by taking advantage of the large surface area, thin alveolar epithelium, permeable membrane and extensive vasculature [240]. Consequently, the use of the local passive administration of an ideal inhalable lung cancer therapy to the tumor site allows for maximum therapeutic concentration of drug while maintaining lower adverse side effects associated with systemic administration [241]. Administration by inhalation offers a non-invasive mean to circumvent first-pass metabolism and systemic toxicity, to reduce the therapeutic dose and frequency of administration, and delivery of drugs directly to their site of action with increased local
drug concentrations [242]. However, if a substance that has pulmonary toxicity properties is delivered by the inhalation route, careful dosing should be performed to avoid pulmonary toxicity. Gemcitabine has been found to cause dyspnea, and in rare but fatal cases, pneumonitis occurs [243, 244]. Also, due to the nature of inhalation delivery, a portion of the emitted dose will be swallowed and delivered to the GI tract. Oral gemcitabine delivery was generally well tolerated in a study completed by Veltkamp et al., where it was discovered that dFdC was converted to dFdU, via first pass metabolism [245]. A safe and effective drug delivery system that releases drug in a sustained manner is desirable in order to limit exposure to normal tissues while delivering the active chemotherapeutic to the cancer cells [246].

Nanocarriers represent a class of drug delivery system that should minimize degradation of therapeutic agent, prevent adverse side effects, and increase the availability of the drug at its intended site of action for therapeutic benefit. Administering a drug to the site of therapeutic action allows for generally lower doses to achieve clinically effective results. Therefore, nanocarriers should be engineered to slowly degrade, react to stimuli and to be site specific [247]. Nanocarrier formulations like nanoliposomes, nanostructured lipid carriers, dendrimers, and polymeric nanoparticles have previously been used for targeting lung cancer [248-251]. Polymeric nanoparticles represent an interesting lung cancer targeting platform because they may entrap drug and imaging agents, and may also exhibit lung cancer tissue targeting surface characteristics [252, 253]. The most commonly used polymers for lung cancer targeting nanoparticles are polylactic acid (PLA), poly(ε-caprolactone) (PCL), poly(lactide-co-glycolide (PLGA), alginic acid, chitosan, and gelatin. Entrapping chemo agents within a polymeric nanoparticle allows for the chemotherapeutic agent to maintain the biodistribution pattern of the polymer, as opposed to conventional therapeutics. This allows for sustained release of drug and protects from degradation of the active pharmaceutical ingredient [254].

Gelatin is a biocompatible, biodegradable, and naturally derived polymer that elicits a high physiological tolerance and low immunogenicity [255]. It has been previously used in other formulations for oral, intravenous, and respiratory inhalation administration [256]. Gelatin possesses carboxyl and amine functional groups that allow for surface modification with targeting molecules, or may be modified by the addition of varying levels of crosslinking agent to modify release characteristics [255, 257]. There have been few studies on the development of gelatin nanoparticles containing chemotherapeutics for the local treatment of lung cancer by the inhalation
route of administration [258-260]. Importantly, there have not been any previous studies that examine the formulation of a Gem-loaded gelatin nanocarrier for aerosol inhalation delivery. In this study, we have successfully prepared gelatin nanocarriers containing Gem and have characterized their physical and aerodynamic properties.

Nanocarriers with particle size of <200 nm may have increased uptake and action compared to larger particles with particle size of >200, possibly due to their ability to evade detection and removal by alveolar macrophages [261]. In addition, it was previously demonstrated nanoparticles with particle size of >100 nm and ≤200 nm were capable of penetrating the respiratory mucus [262]. Nanocarriers are difficult to deliver to the deep lung due to their inherent aerodynamic property of remaining suspended in the air. To achieve nanocarrier deposition to the distal lung tissue an appropriate pulmonary delivery device must be used such as a dry powder inhaler, metered dose inhaler, or nebulizer. A stable gelatin nanocarrier hydrocolloid suspension was designed for nebulization since previous studies have demonstrated the stability of aerosolized gelatin particles [263, 264].

The working hypothesis of this study was that the formulation of Gem-loaded gelatin nanocarriers (Gem-GNC) with particle size in the range of 150 nm to 200 nm will show a controlled release and have an aerodynamic particle size distribution within the appropriate limits. The objective of this study was to use Taguchi design of experiments to analyze how particle size is affected by the desolvating agent, the concentration of crosslinking agent, and the gelatin concentration. In this investigation, a Gem loaded gelatin nanocarrier formulation was designed to potentially be administered to the lungs via a nebulized nanosuspension, preserved in the lyophilized powder state, and characterized for physical and aerodynamic properties. Additionally, the GNCs were characterized for physicochemical properties and evaluated under cell based assays.
CHAPTER 3. LITERATURE REVIEW

3.1. Introduction to Asthma Nanotherapeutics and Aerosolized siRNA Nanoparticle Delivery Systems

Asthma is a multifaceted immune disease driven and characterized by varying degrees of airway hyperresponsiveness, bronchoconstriction, mucus hypersecretion, airway remodeling, and chronic inflammation. As understanding of asthma immunology grows, novel therapies could provide the opportunity to personalize asthma management. Drug or gene delivery systems which could selectively deliver immunomodulatory therapeutics directly to their site of action would be a superior treatment modality rather than use of conventional methods. Nanotechnology has a great potential in revolutionizing the drug and gene delivery approaches. A considerable amount of literature has been reviewed on the use of nanoparticles in the diagnosis, treatment, vaccination, and disease prevention. However, there has been no systematic review on the use of nanoparticles as therapeutic carriers for asthma therapy. This review article discusses asthma pathogenesis, mechanistic pathways for therapeutic asthma intervention, and the natural or bioengineered nanotechnology based targeted delivery of drugs or genes for the treatment of asthma. The various aspects of formulation, bioengineering, characterization, biocompatibility, preclinical evaluation and clinical application of nanomedicine, and regulatory considerations will also be discussed.

In the next section, the pulmonary route of administration, aerosol delivery devices, characterization of pulmonary drug delivery systems, and the rationale for inhaled delivery of siRNA will be discussed. The final section of this chapter reviews the applications of engineered nanoparticles containing siRNA for inhalation delivery. Diseases with known protein malfunctions may be mitigated through the use of siRNA therapeutics. The inhalation route of administration provides local delivery of siRNA therapeutics for the treatment of various pulmonary diseases, however barriers to pulmonary delivery and intracellular delivery of siRNA exists. siRNA loaded nanocarriers can be used to overcome the barriers associated with the pulmonary route, such as anatomical barriers, mucociliary clearance, and alveolar macrophage clearance. Apart from naked siRNA aerosol delivery, previously studied siRNA carrier systems comprise of lipidic, polymeric, peptide, or inorganic origin. Such siRNA delivery systems formulated as aerosols can be successfully delivered via an inhaler or nebulizer to the pulmonary region. Preclinical animal investigations of inhaled siRNA therapeutics rely on intratracheal and intranasal siRNA and siRNA nanocarrier delivery. Aerosolized siRNA delivery systems may be characterized using in
vitro techniques, such as dissolution test, inertial cascade impaction, delivered dose uniformity assay, laser diffraction, and laser Doppler velocimetry. The ex vivo techniques used to characterize pulmonary administered formulations include the isolated perfused lung model. In vivo techniques like gamma scintigraphy, 3D SPECT, PET, MRI, fluorescence imaging and pharmacokinetic/pharmacodynamics analysis may be used for evaluation of aerosolized siRNA delivery systems. The use of inhalable siRNA delivery systems encounters barriers to their delivery, however overcoming the barriers while formulating a safe and effective delivery system will offer unique advances to the field of inhaled medicine.

3.2. Recent Progress and Advances in Nanoparticle Drug Delivery Systems for Asthma Therapy

3.2.1. Rationale for Nanoparticulate Drug Delivery Systems for Asthma Therapy

Asthma is a chronic inflammatory respiratory disease with an allergic component characterized by varying degrees of airway hyperresponsiveness, bronchoconstriction, mucus hypersecretion, airway remodeling, intermittent airway, and chronic inflammation [265]. It is characterized by infiltration of the bronchial mucosa by leukocytes, sub-epithelial fibrosis, mucus hyperproduction, goblet cell metaplasia, airway hyper-responsiveness (AHR), and elevated serum IgE. Asthma affects approximately 300 million people, causes a quarter of a million deaths a year, and results in billions of dollars in healthcare costs [266, 267]. Although regular use of glucocorticoids and other currently available asthma management medications have decreased asthma-related deaths, the prevalence of asthma continues to increase [266, 268-270]. Novel, effective asthma treatments are required to reduce the burden of this disease. Nanotherapeutics represents a treatment modality that may have a high impact in this area by sufficiently delivering novel drugs to asthma immunological pathogenesis and progression targets.

As understanding of asthma immunology grows, novel drugs could provide the opportunity to personalize asthma management and directly target mechanisms responsible for the underlying disease. Drug delivery systems which could selectively delivery these immune system modulating therapeutics directly to their site of action would be a superior treatment modality rather than treating systemically. A considerable amount of literature has been published on the use of nanoparticles in the diagnosis, treatment, and disease prevention. However, there has been relatively little literature published on the use of nanoparticles as therapeutic carriers for asthma therapy. This review discusses asthma pathogenesis, host responses in asthma and related
pathways for therapeutic asthma intervention, current asthma therapeutics and investigational drugs, and the nanoparticle-based therapeutics agents under preclinical investigation for the treatment of asthma.

3.2.2. Asthma Pathogenesis and Pathophysiology

Description of the complete pathogenesis and pathophysiology of asthma is outside the scope of this review, however plenty of publications discuss this in detail [265, 271]. Asthma initially develops with bronchial inflammation and airway swelling (lamina reticularis). Apparent mucus viscoelasticity is increased, producing a thick and different to clear mucus when compared to normal mucus. This is due to amplified mucus cell growth and the resultant mucus hypersecretion. Together, this leads to enhanced predisposition to lung hyperinflation, smooth muscle hypertrophy, cilia cell disruption, and edema [272]. Although the symptoms of asthma are mostly reversible, the associated chronic inflammation of the pulmonary tract may lead to permanent structural changes, known as airway remodeling [273, 274]. The important host responses in the pathogenesis of asthma are shown in Figure 3.1.

**Figure 3.1.** Host responses in the pathogenesis of asthma. Several asthma drugs in pre-clinical and clinical trials target the cytokines or receptors that are central to these pathways, including IL-4, IL-5, IL-13, IL-17. TSLP=thymic stromal lymphopoietin. Reprinted with permission from Ref. [275]. Copyright 2014 BMJ Publishing Group Ltd.
Asthmatic phenotypes have been reviewed previously [276]. Asthmatic patients can be subdivided into one of five clusters governed by their clinical physiological, and pathological variables, as discussed in previously published research and review articles [276, 277]. These groups have been theoretically grouped based on the distinction between high Th2 and non-Th2 asthma [278]. Early onset allergic disease with a prominent T helper type 2 (Th2) signatures represents one group of severe asthmatics. Hallmarks of this phenotype are high levels of airway eosinophils, mast cells, IgE, and exhaled nitric oxide (ENO). Candidate gene analysis in this cohort have indicated that Th2 inflammatory pathways are active in these patients [276]. Another group of asthmatics have adult onset asthma with notable eosinophilia generally in the absence of other important allergic disease. Th2 pathways are important in this group with notable patterns of interleukins such as IL4, IL5, and IL13 in the blood. The third group has asthma symptoms that are exercise induced. The fourth group show minimal Th2 response but notable obesity. The fifth group shows a minimal Th2 response and notable sputum neutrophilia with a Th type 17 cell response. Children may also fall into clusters, although the determinants of pediatric clusters do not mirror those of adults [279, 280]. Although efforts to identify phenotypic clusters help distinguish different subtypes of patients, clustering has not yet led to differential treatment strategies. However as new treatments emerge, it is hopeful that endotyping, or defining disease subtypes by predominant molecular mechanisms or treatment responses, will lead to more approaches that focus on targeted fundamental asthma factors [275].

Although the majority of asthma is associated with atopy and has its onset in early childhood across all ages and especially after the age of 40 years, there are forms of asthma that appear to be independent of atopy [281, 282]. These non-allergic forms of the disease have been subject to careful comparative investigation but no clear pathogenic pathways have been identified [283]. There is some evidence to suggest that local IgE mechanisms may be involved with the detection of IgE isotype switching in airway biopsies [284, 285]. The immunopathology of late-onset non-allergic asthma appears to be similar to that of allergic asthma, although there have been some differences reported in the relative proportion of the various inflammatory cells present. It is important to recognize that some forms of late-onset asthma have an occupational cause due to non-IgE dependent sensitization to chemicals in the workplace. In addition to immunologic sensitization to chemicals, both intrinsic asthma and asthma caused by diisocyanate exposure are associated with antibodies directed to epithelial components. Other autoantibodies in asthma
include those directed to heat-shock protein (HSP)-70, CD2, and α-enolase. Whether such antibodies are truly pathogenetic or related more to tissue damage and inflammation remains to be established.

Pathological changes associated with the varying degrees of inflammation, bronchial constriction, mucus hypersecretion, and airway remodeling. These pathological changes results in different asthma endotypes. Complex interactions between genetic, epigenetic, and environmental factors predispose patients to develop a limited number of dysfunctional immunologic regulatory patterns, which in turn dictate the presentation of clinical endotypes [275]. It has been suggested that no single drug will be effective for all asthma patients, but some drugs might be very effective in selected patients who are identified on the basis of their underlying immunological processes [286].

The airways of the lungs consist of cartilaginous bronchi, membranous bronchi, and gas-exchanging bronchi termed the respiratory bronchioles and the alveolar ducts. While the first 2 types function mostly as anatomical dead space, they also contribute to airway resistance. The smallest non-gas-exchanging airway, the terminal bronchioles, are approximately 0.5 mm in diameter; airways are considered small if there are less than 2 mm in diameter. Airway structure consists of mucosa, which is composed of epithelial cells that are capable of specialized mucus production and a transport apparatus known as mucociliary clearance, basement membrane, smooth-muscle matrix extending to the alveolar entrances, and predominantly fibrocartilaginous or fibroelastic-supporting connective tissue. Cellular elements include mast cells, which are involved in the complex control of releasing histamine and other mediators. Basophils, eosinophils, neutrophils, and macrophages also are responsible for extensive mediator release in the early and late stages of bronchial asthma. Stretch and irritant receptors reside in the airways, as do cholinergic motor nerves, which innervate the smooth muscle and glandular units. In bronchial asthma, smooth muscle contraction in an airway is greater than that expected for its size if it were functioning normally, and this contraction varies in its distribution.

Airway remodeling refers to the lung structural changes that occur due to asthma which includes epithelial injury, increased thickness of the basement membrane, increased volume of airway smooth muscle, goblet cell metaplasia, and increased airway angiogenesis and lymphangiogenesis [287]. Several biopsy studies have shown that the airway epithelial injury, including disruption of tight junctions and cell denudation, occurs in asthma [288-290]. Epithelial
cells undergo rapid repair mechanisms and initiate signal cascades central to asthma in response to several stimuli, including epithelial growth factor [291-293]. Abnormal repair processes and decreased barrier function have also been shown to play a role in airway epithelial injury to cause airway remodeling [290].

Biopsy studies have also shown increases in reticular basement membrane thickness, thought to be mediated by myofibroblasts, in patients with asthma [294]. In children, these changes did not correlate with Th2 cytokine profile or with future lung function [295, 296]. The role of connective tissue outside of the basement membrane is also unclear. It has also been reported that certain patients with asthma have notable hyperinflation and decreased elastic recoil, possibly because of changes in connective tissue [297].

Increased airway smooth muscle mass has been a recognized feature of asthma for decades. These increases are mediated in part by the release of cystinyl leukotriene from eosinophils. Smooth muscle has a role in bronchoconstriction, which is triggered by several direct and indirect stimuli, and contributes to symptoms, exacerbations, and the remodeling process [298, 299]. The increase in smooth muscle mass is associated with increases in growth factors including TGF-β1 and platelet derived growth factor [300, 301]. The muscle itself may also act as a secretory organ in asthma, promoting maladaptive growth and immunologic responses. A recent review of these properties highlighted IL5, IL13, TGF-β1, IL1β, and tumor necrosis factor α (TNF-α) as important mediators in this process [299].

Goblet cell metaplasia is another important structural change that occurs in asthma. It has been observed in models of Th2-driven asthma, but is not a feature of TH1 models of asthma [302, 303]. The process seems to be dependent on the actions of the epidermal growth factor receptor as well as IL13 and may be inhibited by IFN-γ [304]. Calcium activated chloride channel proteins may mediate mucus hypersecretion at a downstream level [305].

3.2.2.1. Cells Mitigating Asthma

The pathology of asthma is mitigated through several airway cells and immune cells. Susceptibility genes for asthma along with important signaling molecules expressed by and directed to these cells are important therapeutic targets as shown in Figure 3.2.
Figure 3.2. Susceptibility of genes within asthma-associated cells. Representation of the most robust asthma candidate genes identified through association studies or positional cloning in a cell-based framework. Reprinted with permission from Ref. [306]. Copyright 2008 Nature Publishing Group.

3.2.2.1.1. Airway Epithelial Cells

Airway epithelial cells are the main cells that form the barrier against mechanical stress, oxidant stress, allergens, pollutants, infectious agents, and leakage of endogenous solutes. These cells also have important roles in mucociliary clearance and signaling. Various types of pattern recognition receptors, including Toll-like receptor 1 (TLR1), are expressed on epithelial cells, enabling responses to allergic and infectious stimuli [307]. In asthma, epithelial cell derived cytokines and chemokines (including IL25, IL33, thymic stromal lymphopoietin (TSLP), and granulocyte macrophage colony stimulating factor (GM-CSF)) signal effector cells (including basophils, eosinophils, mast cells, and lymphocytes) and dendritic cells to shape characteristic asthmatic immune response patterns to allergens, pollutants, and infectious agents [308].
3.2.2.1.2. Dendritic Cells

Dendritic cells are directly exposed to the external environment, like airway epithelial cells. Pulmonary dendritic cells act as antigen presenting cells and express a variety of pattern recognition receptors on their cell surface. Dendritic cells can also be recruited to the airway in response to allergens and pathogens [309, 310]. They can be directly stimulated by surface binding of allergens or infectious agents or indirectly stimulated by airway epithelial cells (by mediators such as IL25, IL33, TSLP, and GM-CSF) [311, 312]. Locally, dendritic cells can recruit eosinophils [307]. Migration of dendritic cells through the lymphatics to regional lymph nodes are mediated by multiple factors including C-C chemokine receptor type 7 (CCR7), CCR8, and CCRL2 [313, 314]. Dendritic cells affect T cell differentiation and under certain circumstances generate the Th2 response commonly seen in atopic asthma [315].

3.2.2.1.3. T Lymphocyte Subsets

Several T cell subsets are important in asthma. Traditionally, Th2 cells have been thought to predominate, with characteristic raised levels of IL4, IL5, and IL13 [172]. IL4 and IL13 promote inflammation (through signaling to eosinophils and B cells) and remodeling (through signaling to fibroblasts, airway smooth muscle, dendritic cells, and epithelial cells) [173]. IL5 is crucial for B cell survival and maturation and for stimulating eosinophils. Some patients with asthma show a predominance of Th2 cells. This pattern can develop under the influence of IL-18 and interferon γ (IFN-γ) and is characterized by further production of IFN-γ [316]. Th17 cells, which are CD4 positive T cells that express IL-17, also play a role in a subset of patients with asthma. This pattern is unusual, in that the resulting Th17 pathways result in neutrophils being the primary effector cells [317, 318]. Th9 cells are CD4 positive T cells that secrete IL9. Numbers of Th9 lymphocytes are raised in people with atopy, and these cells promote allergic responses, probably through activation of mast cells [319]. T regulatory cells, characterized by secretion of transforming growth factor β (TGF-β) and IL10, are thought to be important because of their role in reducing atopic responses [320].

3.2.2.1.4. B Lymphocytes

B cells are important in atopic asthma because they produce IgE [321]. IL5 and B cell activating factor promote B cell survival. Under the influence of IL4 or IL13, B cells need to bind to T cells (through CD40 and the CD40 ligand, respectively) to be activated to produce IgE,
generally within regional lymph nodes. Secreted IgE is primarily bound through the high affinity Fc epsilon receptors on mast cells and basophils, and when crosslinked by aeroallergen causes these cells to degranulate and release their mediators [322].

3.2.2.1.5. Innate Lymphoid Cells

The innate lymphoid cell is a recently described effector leukocyte that is stimulated by IL25 and IL33 (seen in response to viral illness) and requires the transcription factor RORα for signaling. These cells have the potential to differentiate into macrophages and granulocytes while producing notable quantities of Th2 cytokines and stimulating eosinophils in the process [323].

3.2.2.1.6. Eosinophils

Eosinophils are bone marrow derived granulocytes that play a central role in asthma. The biology of the eosinophil is complex, and the effects of its secreted products are diverse. Cellular differentiation in the bone marrow is mediated by IL3, IL5, and GM-CSF [324]. Recruitment of eosinophils is mediated by IL13, histamine, prostaglandin type 2, and eotaxins (through the CCR3 receptor) [325]. The survival of eosinophils is promoted by IL5 and apoptosis is signaled through binding of the siglec-8 and siglec-F receptors [326, 327]. In addition to releasing toxic granular proteins, such as eosinophilic cationic protein and eosinophil derived neurotoxin, eosinophils secrete dozens of cytokines and chemokines, which promote inflammation through the Th2 pathway and airway epithelial damage [328, 329].

3.2.2.1.7. Mast Cells

Mast cells are also important in the pathogenesis of asthma. Maintained near mucosal surfaces by IL-9, these cells can be activated by binding of stem cell factor to the surface receptor c-kit, IgE crosslinking, or binding of tyrosine kinase [330]. Activated mast cells are an important source of histamine, cysteinyi leukotrienes, and prostaglandins [331]. These mediators are central to bronchoconstriction, vasodilation, and the allergic inflammatory cascade.

3.2.2.1.8. Neutrophils

Neutrophils probably play a role in specific asthma endotypes. Recruited through TH17 pathways, neutrophil numbers are raised in patients with asthma, especially those who are relatively unresponsive to inhaled steroids [332]. It has been difficult to prove that neutrophils are involved in the pathogenesis of severe asthma because the use of inhaled steroids may suppress eosinophilia and result in airway neutrophilia [276, 333-335].
3.2.2.1.9. Macrophages and monocytes

Monocytes differentiate into macrophages in the presence of GM-CSF and dendritic cells in the presence of IL4 [336, 337]. In chronic asthma both monocytes and macrophages are prominent cells in the airway mucosa play important roles in asthma pathogenesis. While these cells are an important source of cysteinyi leukotrienes, reactive oxygen species, and a variety of lysosomal enzymes, their precise role in mediated tissue damage and contributing to the overall airway pathology of asthma remains unclear. In corticosteroid-refractory asthma, monocytes and macrophages are thought to play an increasingly important role and may well account for the ongoing chronic inflammation associated with their preferential infiltration into the airway wall in patients with longstanding corticosteroid-resistant disease. The role of how monocytes and macrophages fit into the overall Th2 paradigm and whether they represent a separate component of the immune and inflammatory response is an interesting area of research [271, 338].

3.2.2.1.10. Basophils

Basophils are mostly circulating IgE-triggered inflammatory cells, however in certain immune responses, they accumulate in tissues. The discovery of unique basophil-specific markers such as basogranulin has enabled their identification in the airways of subjects with asthma. It is not clear what their precise roles are in either acute or chronic disease, although it is known that they share many of their recruitment mechanisms with eosinophils and are likely to be companions of eosinophil infiltration.

3.2.3. Current FDA Approved Therapeutic Agents and Investigational Drugs

Asthma therapy generally aims to reduce symptoms, maintain pulmonary function, prevent recurrent exacerbations, and minimize hospitalization [339]. For all but the most severely affected patients, the ultimate goal is to prevent symptoms, minimize morbidity from acute episodes, and prevent functional and psychological morbidity to improve the patient’s quality of life [340]. Although allergen avoidance and the management of comorbidities such as smoking and obesity should be addressed first, drugs remain the cornerstone of treatment [341, 342]. Several drugs have been approved for asthma and the role of many of them have been defined in a comprehensive systematic review from the Global Initiative for Asthma [341, 342]. A summary of the stepwise asthma therapy selection is shown in Figure 3.3.
3.2.3.1. Currently Approved Therapeutic Agents

The standard treatment of asthmatic patients consists of trigger avoidance, bronchodilation, and anti-inflammatory therapy using long-term control and fast-acting relief medications. Environmental exposures and irritants can play a strong role in symptom exacerbations. The use of skin testing or in vitro testing to assess sensitivity to perennial indoor allergens is important. Once the offending allergens are identified, the physician should counsel their patient on how to avoid them. These allergens commonly exist in the home, where specific triggers induce dust mites, animals, cockroaches, mold, and pollen. Control agents include inhaled corticosteroids, inhaled cromolyn (Intal) or nedocromil (Tilade), long-acting bronchodilators, theophylline (Theo-
24, Theochron, Uniphyl), leukotriene modifiers, and anti-IgE antibodies [275, 343]. Relief medications include short-acting bronchodilators, systemic corticosteroids, and ipratropium (Atrovent) [344]. The pharmacologic treatment of asthma is based on stepwise therapy in which the asthma medications are added or removed from the regimen as the frequency or severity of the symptoms change.

Short acting β agonists (SABAs) counteract bronchoconstriction regardless of the trigger for contraction. In most patients with asthma, inhaled corticosteroids are a highly effective controller therapy (defined as a daily treatment designed to decrease the frequency of baseline symptoms and exacerbations that require short acting β agonists). Consistent use of inhaled corticosteroids (such as beclometasone, budesonide, ciclesonide, fluocinolide, fluticasone, and mometasone) can improve asthma symptoms, quality of life, measures of airway function, hyper-responsiveness, and the frequency and severity of exacerbations [341]. Long acting β agonists (LABAs), such as formoterol and salmeterol, are effective when used in combination with inhaled corticosteroids in patients with symptoms or exacerbations [345]. In addition, since LABAs provide sustained bronchodilation but have no significant anti-inflammatory effects themselves, they should only be used in combination with inhaled corticosteroids. Prospective studies of both salmeterol and formoterol (both LABAs) have identified an increased risk of death or near death fatal outcomes when used alone [346]. However, studies have not identified an increased risk of death of near death fatal outcomes in patients using both LABAs and ICS at the same time. Leukotriene antagonists, like montelukast also show efficacy in asthma, alone or combined with other controller therapy, especially in patients with prominent allergic disease or exercise symptoms [347]. Long acting muscarinic antagonists (such tiotropium and aclidinium) result in bronchodilation and show modest efficacy as adjuncts to inhaled corticosteroids and long acting β agonists [348, 349].

Glucocorticoids exert their effects by binding to the glucocorticoid receptor, which once inside the nucleus, modulates several DNA transcription factors [350, 351]. This leads to the upregulation of anti-inflammatory protein production and to a concomitant downregulation of pro-inflammatory protein production. In addition to such relatively slow genomic effects, glucocorticoids also display more rapid non-genomic effects, including inhibition of arachidonic acid release and alterations in cation transport across the plasma membrane [351]. Because of their broad pharmacologic activity, glucocorticoids are notorious for their side effects [351]. These
include immunosuppression, mucoskeletal complications, growth suppressive effects in children, hypertension, rapid weight gain, diabetes, hypertriglyceridemia, hypercholesterolemia, dermatological effects, glaucoma, peptic ulcer disease, decelerated wound healing, and electrolyte imbalance [20, 21, 351].

Glucocorticoids are a class of steroid hormones that are strong immunosuppressive and anti-inflammatory agents. Inhaled glucocorticoids are prescribed frequently in asthma therapy, because of their anti-inflammatory properties [351, 352]. However, inhaled glucocorticoids have some limitations due to long-term side effects, most often seen in elderly patients [353]. Additionally, with the need of daily dosing, these effects may lead to patient noncompliance [274]. Since severe asthmatics require higher doses of inhaled corticosteroids, it would be highly advantageous to develop inhaled corticosteroids with lower side effects even at high doses. Ciclesonide is an inhaled corticosteroid that has the least systemic effects and local side effects [354]. It has lower systemic effects because it is a prodrug which is only activated in the lungs to the active principle des-ciclesonide by esterases, whereas there is little activation is the oropharynx and GI [354]. This suggests that high doses of ciclesonide may be useful in severe asthma treatment. Inhaled corticosteroids in pressurized metered-dose inhalers are now administered with hydrofluoroalkane 134a because it results in lower particle sizes when compared to chlorofluorocarbon propellants, making it more likely to be deposited within the lower airways [355].

Cytokines are, in part, responsible for chronic airway inflammation and remodeling. Therefore, in severe asthmatics who do not respond to ICSs, cytokines have become important targets [356]. Several cytokines have been implicated in asthma and have already been targeted in clinical studies [357]. It is difficult to inhibit inflammatory processes because there is redundancy of cytokines. Another problem is the high cost of blocking mAbs, and therefore these drugs are more likely to be cost-effective in patients with severe disease. This problem may be resolved by the manufacturing of cheaper alternatives, like higher affinity antibodies or antibody fragments, or through the advancement of antibody preparation methodologies [358].

Omalizumab, a monoclonal antibody (mAb) directed against IgE, is currently recommended by US National Heart, Lung, and Blood Institute’s commissioned national asthma and education prevention program guidelines for use in severe treatment refractory atopic asthma [341]. Omalizumab is a mAb that binds the Fc portion of IgE and thus prevents activating its high-
affinity IgE receptor (FcεRI) on mast cells, basophils, and dendritic cells, as well as a low-affinity receptor (FcεRII) expressed on several immune and inflammatory cells, including macrophages, eosinophils, and T and B lymphocytes [359]. Omalizumab decreases the number of exacerbations in adults and children [359]. However, its use is limited by its high cost [360]. Clinical trials have demonstrated the clinical efficacy of omalizumab in reducing maintenance doses of oral corticosteroids and ICSs [361-363]. Patients with non-atopic (intrinsic) asthma were excluded, but because of the evidence for airway IgE production in some non-atopic patients, further study in these patients is needed [358, 364]. Patients with severe uncontrolled asthma with total IgE levels of > 300 IU/mL were excluded because it is not possible to give enough antibody to effectively block IgE [358]. Antibodies with a higher affinity for IgE might be developed so that it could be possible to treat patients with very high total IgE levels.

Inhaled corticosteroid treatment is effective for some asthmatic patients, but corticosteroids may not work for individuals with eosinophilic asthma. This type of asthma is characterized by a high level of eosinophils (a type of granulocyte), and people with this condition are at an increased risk for severe asthma attacks. The new drug reslizumab has been created to treat eosinophilic asthma. As an interleukin-5 (IL5) inhibitor, reslizumab blocks the action of IL5, a cytokine that is associated with the growth and movement of eosinophils. The recent FDA-approval for GlaxoSmithKline PLC’s Nucala® (mepolizumab) has applications for use as an add-on maintenance therapy for severe eosinophilic asthma patients aged 12 years and older. Mepolizumab is an approved IL5 targeting biological therapy. IL5 plays a major role in eosinophils, which contribute to asthma pathophysiology. This biologic is administered as fixed 100 mg dose by subcutaneous injection every four weeks. Several clinical trials have involved manipulation of IL5 signaling in patients with asthma. Early trials of mepolizumab, a monoclonal antibody that targets IL5, decreased eosinophil counts in blood and sputum, but had no noticeable effects on airway function [365, 366]. However, in trials of patients with sputum eosinophilia despite the use of high dose inhaled corticosteroids or prednisone, mepolizumab significantly decreased the number of exacerbations when compared with placebo [367-369].

Cromones (cromolyn sodium and nedocromil sodium) inhibit the activation of human mucosal mast cells and are very effective against allergen and other indirect challenges that involve mast cell activation [370]. The effects of cromones are closely mimicked by the diuretic furosemide, suggesting that they might act through ion channels. However, the molecular target
for cromones was never identified, although recent studies suggest that an orphan G protein–coupled receptor called GPR35 might be a target [358, 371].

Bronchial thermoplasty is an endoscopic procedure available at specialized centers that uses thermal energy to disrupt bronchial smooth muscle [372]. Recent large open label studies have shown a sustained decrease in the frequency of exacerbations for as long as five years in patients with severe asthma who undergo this procedure, suggesting that it has benefit in exacerbation prone populations [373, 374]. This treatment performed in 3 week intervals of 3 procedures.

Currently available combination products are administered twice daily, and consist of fluticasone propionate/salmeterol (Seretide), budesonide/formoterol (Symbicort and DuoResp SpiroMax), extra-fine beclomethasone/formoterol (Fostair) and fluticasone proionate/formoterol (Flutiform). A new combination multidose dry powder inhaler device (Relvar Ellipta) containing the ICS fluticasone furoate and the LABA vilanterol trifenatate (92/22 and 184/22 µg) is licensed as the first once-daily maintenance treatment for asthma. Once-daily treatment with fluticasone/vilanterol has the potential to improve adherence in asthma, although to date there are no published data to support improved adherence or effectiveness compared to other ICS/LABA combinations. The Single Inhaler Maintenance And Reliever Therapy (SMART) regiment, using both budesonide and formoterol (Symbicort SMART), or the combination of inhaled extra-fine particle size beclomethasone and formoterol pressurized metered dose inhaler as Maintenance and Reliever Therapy (Fostair MART) regiment for maintenance treatment and relief of symptoms, reduce the frequency of severe exacerbations compared to a fixed-dose combination plus a short-acting beta2-agonist (SABA) as a reliever. The SMART strategy cannot be used with salmeterol or the once-daily β2-agonists, such as indacaterol and vilanterol because of their cumulative side effects [358]. The scientific rationale for SMART is now well understood because the ICS used as rescue therapy has a rapid onset of its anti-inflammatory effect and prevents the build-up of inflammation that precedes an acute exacerbation [375, 376]. The SMART strategy is effective in patients with moderate and severe asthma, and also may be effective even when patients forget doses of their maintenance therapy and therefore addresses the key issue of poor adherence with controller therapy [358].
3.2.3.2. Investigational Drugs in Clinical trials and Preclinical Studies

Currently available asthma drugs have provided asthma symptom and exacerbation control for millions of people. However, despite the general success of currently available asthma drugs, there are several reasons to pursue new ones. The prevalence of asthma is increasing and its burden on society remains high. To date, there are no known cures or effective preventive strategies for asthma [377, 378]. Asthma remains uncontrolled in about 10% of patients who are adherent to their prescribed drugs [379]. Unfortunately, the effects of inhaled corticosteroids on asthma rapidly disappear when the drug is discontinued [378]. Moreover, current asthma drugs generally do not reverse or slow down most of the long term remodeling changes that occur in various cell types in the airway [380]. Despite prescription of drugs to control asthma, many patients still experience ongoing symptoms [381, 382]. Ongoing research is attempting to identify more effective and less toxic agents to control asthma. Investigational approaches to asthma management are presented here.

3.2.3.2.1. Small Molecules

3.2.3.2.1.1. Battling Corticosteroid Resistance

Since inhaled corticosteroids are absorbed in the lungs and may cause systemic side effects, safer inhaled corticosteroids with reduced oral bioavailability, reduced systemic absorption in the lungs, or inactivation in the bloodstream. To separate the side-effect mechanisms from the anti-inflammatory mechanisms, “dissociated” steroids have been designed. ICS side effects are mediated through transactivation and binding of glucocorticoid receptors to DNA, whereas anti-inflammatory effects are largely mediated through transrepression of transcription factors through a non-genomic effect [350, 383, 384]. The dissociated corticosteroids are still in preclinical development despite promising data published in animal models of asthma [385-387]. Nonsteroidal selective glucocorticoid receptor activators, such as AL-438 and mapracorat have been under clinical development [388-391]. Some of the anti-inflammatory effects of corticosteroids may be due to transactivation of anti-inflammatory genes, and therefore selective glucocorticoid receptor activators might not be as efficacious as existing inhaled corticosteroids [392]. Corticosteroids switch off inflammatory genes by recruiting the nuclear enzyme histone deacetylase-2 (HDAC2) to the activated inflammatory gene initiation site so that activators of this enzyme might also have anti-inflammatory effects or might enhance the anti-inflammatory effects of corticosteroids [383].
Several molecular mechanisms have now been described to account for corticosteroid resistance in asthmatic patients, including activation of p38 MAPK activity, increased expression of an alternatively spliced variant of the glucocorticoid receptor GRβ, increased production of macrophage migratory inhibitory factor (MIF), and reduced expression of HDAC2 due to oxidative and nitrative stress [350, 386, 393-395]. This results in increased acetylation of the glucocorticoid receptor, which prevents it from inhibiting NF-κB–driven inflammation [396]. Taken together, this suggests that there might be therapies that could potentially reverse corticosteroid resistance and that there might be different phenotypes of corticosteroid resistance in asthma that could require different therapeutic approaches. A novel therapeutic strategy is reversal of this corticosteroid resistance by increasing the expression and activity of HDAC2. p38 MAPK inhibitors have been shown to increase the anti-inflammatory responses to corticosteroids in PBMCs from patients with severe asthma, and as discussed above, p38 MAPK inhibitors are in clinical development for the treatment of severe asthma [397]. MIF is reported to be increased in patients with severe asthma and block the anti-inflammatory effects of corticosteroids, but the molecular mechanisms are poorly understood, and it has been difficult to find drugs that block its actions [398, 399]. MIF can signal and cause corticosteroid resistance through the activation of p38 MAPK [400].

Theophylline has been shown to increase alveolar macrophage expression of HDAC2, causing restoration of ICS response [401, 402]. The addition of low-dose theophylline to moderate doses of ICSs is more effective in patients with severe asthma than increasing the dose of ICS to the maximum tolerated dose and that withdrawal of low-dose theophylline causes a loss of asthma control in patients with severe asthma [403, 404]. In asthmatic smokers who are refractory to corticosteroids, low-dose theophylline added to a dose of ICS was effective of increasing corticosteroid sensitivity even when the ICS alone was ineffective[405]. Theophylline’s molecular mechanism of action in increasing HDAC2 levels is independent of PDE inhibition and is mediated by oxidant-activated PI3Kδ inhibition [406, 407]. The antidepressant nortriptyline reverses corticosteroid resistance by inhibiting PI3Kδ [408]. Nortriptyline may have potential as an add on therapy, however the potential clinical benefits have not been evaluated in human clinical trials [408]. Selective PI3Kδ inhibitors have underwent clinical development for the treatment of B-cell leukemia but might also be useful as add-on therapies with ICSs, especially if administered by inhalation to avoid any hematologic side effects [358].
During asthma exacerbations, reactive oxygen species are likely to amplify inflammation and contribute to the pathophysiology of asthma [409]. Oxidative stress reduces steroid responsiveness through a reduction in HDAC2 activity and expression. This suggests that antioxidants might reverse corticosteroid resistance and also reduce inflammation. Glutathione-based antioxidants are inactivated by oxidative stress, and therefore more potent and stable antioxidants are needed for asthma therapy, such as superoxide dismutase mimics and NADPH oxidase inhibitors [410]. In severe asthmatic patients, there is dysfunction of the transcription factor nuclear factor erythroid 2–related factor 2 (Nrf2), which plays key roles in endogenous antioxidant gene regulation [358]. Nrf2 activators, such as sulforaphane (Food source: broccoli) and the synthetic triterpenoid 1-(2-cyano-3,12-dioxooleana-1,9-dien-28-oyl)imidazole-methyl ester, have now been identified, and Nrf2 activators are in pre-clinical and clinical development [358, 411, 412].

3.2.3.2.1.2. Bronchodilators

Long acting muscarinic antagonists (LAMAs) have shown efficacy in randomized controlled trials in patients with uncontrolled asthma on low dose inhaled corticosteroids (primary outcome of morning peak flow) and those whose disease remains uncontrolled receiving combined inhaled corticosteroids and long acting β agonists (primary outcome of time to first exacerbation) [348, 349]. β2-Agonists act as functional antagonists of airway smooth muscle contraction, irrespective of the stimulus to constriction. Due to the safety concerns of LABAs, such as salmeterol and formoterol, which cause increased severe exacerbations and mortality when used alone, LABAs should be used only in combination inhalers containing a corticosteroid [413-415]. Indacaterol, a 24 hour LABA, has demonstrated safety and efficacy in terms of airway function within clinical trials [416, 417]. Fluticasone furoate-vilanterol, a ICS and LABA combination inhaler, had shown efficacy equivalent to fluticasone propionate-salmeterol in phase III clinical trials [418].

β2-Agonists and theophylline relax human airway smooth muscle by activating large conductance Ca2+-activated potassium channel activators, but other activators of these channels have not yet been tested for asthma therapy in clinical trials. Rho kinase inhibitors have potential as bronchodilators because of the role Rho kinase plays in airway smooth muscle contraction [419-421]. In addition, agonists of bitter taste receptors (TAS2Rs), such as quinine, chloroquine, and saccharine, relax human airways in vitro by increasing local Ca2+ release, resulting in the opening
and hyperpolarization of airway smooth muscle cells [422]. In mouse models, inhaled bitter tastants appear to be more effective than a β-agonist, although it is possible that since this model species is not very responsive to β2-agonists that the results are biased [358]. Theophylline relaxes human airway smooth muscle by inhibiting phosphodiesterase (PDE) 3 in airway smooth muscle cells, so it is reasonable that that selective PDE3 inhibitors, such as cilostazol and milrinone, are potential bronchodilators. PDE3/4 inhibitors are also of interest because PDE3 and PDE4 are both found in airway smooth muscle and are both likely to mediate some degree of cAMP degradation. Combined PDE3/4 inhibitors are currently in development as inhaled therapy for asthma and COPD, although there is some concern over increased cardiovascular mortality in past clinical trials [423]. Although the potential therapeutic utility of PDE inhibitors has been demonstrated in various animal models of asthma, their clinical efficacy has been restricted by the dose-limiting side effects; apart from Methylxanthines (e.g. theophylline), no other PDE inhibitors have been approved for the treatment of patients with asthma [358]. Although new PDE inhibitors have been synthesized, most data are from cellular and tissue-level studies [424]. CHF 6001, an inhaled PDE4 inhibitor, and RPL554, a dual PDE3/4 inhibitor, are still under clinical development [424]. Prostaglandin E2 (PGE2) relaxes human airway smooth muscle through E prostanoid subtype 4 (EP4) receptors, suggesting that EP4 selective agonists might be useful bronchodilators and might avoid coughing induced by PGE2 through E prostanoid subtype 3 (EP3) receptors on sensory nerve endings [425].

The most advanced of the anti-inflammatory therapies are PDE4 inhibitors, which have a wide spectrum of anti-inflammatory effects that are relevant to severe asthma, inhibiting T cells, eosinophils, neutrophils, mast cells, airway smooth muscle, epithelial cells, and nerves and are highly effective in animal models of asthma [426]. PDE4 inhibitors are effective against neutrophilic inflammation, making them an attractive potential therapy for severe asthma when there is neutrophilic inflammation. An oral PDE4 inhibitor, roflumilast, has an inhibitory effect on allergen-induced responses in patients with mild asthma and also reduces symptoms and lung function similar to a low dose of ICS [427]. Roflumilast is currently licensed for use in patients with severe COPD, and therefore there has been increased interest in its potential for the treatment of severe asthma. However, a major limitation to this class of drug is the mechanism-based side-effect profile, including nausea, headaches, and diarrhea, which is dose limiting. On the basis of animal models, the anti-inflammatory effects appear to be mediated by inhibition of PDE4B,
whereas nausea and vomiting are mediated through PDE4D inhibition, suggesting that PDE4B-selective inhibitors might be better tolerated [428]. Another approach is to deliver PDE4 inhibitors by means of inhalation, but thus far, these drugs have had no efficacy. Inhaled PDE3/4 inhibitors are also in development and might have the advantage of bronchodilatation through PDE3 inhibition [423].

Antimuscarinic (anticholinergic) bronchodilators are the preferred first-line therapy in patients with COPD, but in patients with asthma, they are less effective than β2-agonists. Antimuscarinic bronchodilators block only the cholinergic component of bronchoconstriction, whereas β2 agonists reverse all bronchoconstrictors, including the direct effects of inflammatory mediators, such as histamine, leukotriene (LT) D4, and prostaglandin (PG) D2 [429]. The long acting muscarinic antagonist (LAMA) tiotropium bromide completely blocked the late response to inhaled allergen in a sensitized guinea pig asthma model, suggesting an important role for cholinergic mechanisms [430]. Tiotropium inhibits eosinophilic inflammation in the airways and airway hyperresponsiveness, even in vagotomized animals, suggesting that tiotropium is blocking the effects of non-neuronally released acetylcholine on muscarinic M3 receptors and that tiotropium also blocks eosinophilic inflammatory mechanisms [431]. This response is also blocked by drugs that block TRPA1, an activating ion channel on airway sensory nerves, suggesting that allergens release a mediator that activates TRPA1, leading to cholinergic reflex bronchoconstriction. There is evidence that muscarinic receptors can be activated by acetylcholine released from airway epithelial and inflammatory cells [432, 433]. Choline acetyltransferase can be induced in epithelial cells by inflammatory mediators, such as TNF-a, suggesting that acetylcholine synthesis might be increased in the airways of asthmatic patients. Tiotropium inhibits Th2 cytokine release in allergen-exposed sensitized mice and that from human PBMCs [434]. It reduces eosinophilic inflammation, mucin gene expression, and airway remodeling in a murine model of asthma, possibly through a direct effect on fibroblasts [435]. Tiotropium inhibits neutrophilic inflammation and airway fibrosis after repeated LPS challenge in guinea pigs [436]. These experimental studies suggest that tiotropium might have anti-inflammatory effects through antagonism of neuronally and extraneuronally released acetylcholine on M3 receptors on inflammatory cells. There is also evidence that blocking M3 receptors with tiotropium inhibits acetylcholine-induced release of neutrophil chemotactic factors (mainly LTB4) from human macrophages [437]. These in vitro and experimental studies have paved the way to recent clinical
studies of tiotropium in patients with asthma, particularly in those with severe disease. Recent studies have shown that once-daily tiotropium provides useful additional bronchodilatation when added to a LABA in some patients with severe asthma. In one study approximately 30% of patients with severe asthma showed a good additional response when tiotropium was added [438]. Addition of tiotropium significantly improves lung function in patients whose symptoms are not controlled by high doses of ICSs and LABAs, although there is no improvement in symptoms or health status [349, 439]. Another study showed that tiotropium was comparable with salmeterol in terms of bronchodilator response when added to an ICS in patients who show a good response to a short-acting anticholinergic [440]. In asthmatic patients with the Arg16/Arg16 genotype of the b2-receptor, who have previously been reported to be less responsive to b2-agonists, once-daily tiotropium was no less effective than twice daily salmeterol in patients whose symptoms are not controlled with ICSs alone [441]. These studies suggest that addition of a LAMA to existing therapy in patients with severe asthma not controlled with ICSs and LABAs is beneficial and might be indicated in certain cases [358].

There are several other LAMAs in clinical development for COPD, including once-daily glycopyrrolate and GSK573719 and twice-daily aclidinium bromide [442]. There appears to be additivity between LABAs and LAMAs, suggesting that a triple combination of a LABA plus a LAMA plus an ICS might be useful in some patients with severe asthma [443]. Several bifunctional molecules that have LABA and LAMA activity are also in development, but it might prove difficult to balance the β-agonist and anticholinergic activities [444]. It would be logistically easier to combine a bifunctional molecule that has LABA and LAMA activity and an ICS in a combination inhaler to provide triple activity [358].

Statins, widely used cholesterol lowering agent, have found a new niche in the area of asthma therapy. Simvastatin, in an addition therapy to inhaled corticosteroids and bronchodilators can reduce systemic and airway inflammation, improve lung function and symptoms, and reduce acute exacerbations in patients with severe asthma who are already on controller inhaler therapy. A recent investigator-initiated, single-center, early Phase II, cross-over, randomized clinical trial, titled “Randomized Trial of Simvastatin for the Treatment of Severe Asthma” was conducted at the University of California Davis Medical Center, in Sacramento California. The purpose of this trial was to evaluate Simvastatin for treatment of asthma in subjects with severe asthma who are already taking inhaler controller therapy. The investigators hypothesized that treatment with
Simvastatin 40 mg administered once daily will improve indicators of airway and systemic allergic/Th2 inflammation and will also reduce acute exacerbations and improve lung function. All patients were on standard controller therapy including appropriate doses of inhaled corticosteroids and long-acting bronchodilators. The primary outcome measures were Th2 gene expression in nasal epithelial cells including IL-13, eosin and IL-4, 5, and 6, and STAT6 by RT-PCR and Exhaled nitric oxide (ENO) measure with a NIOX-Mino portable NO analyzer. Secondary outcome measures include acute exacerbations, lung function in terms of FEV1, FVC, and FEV, FVC ratio.

3.2.3.2.1.3. Novel Receptor Antagonists and Agonists for Asthma Therapy

Antileukotrienes, which are cysteinyi leukotriene receptor antagonists are less effective than ICSs [445]. Leukotriene B4 (LTB4) levels are increased in severe asthmatics and it serves as a chemoattractant of neutrophils, mast cells, and T cells, including effector CD81 memory T cells [446]. An LTB4 receptor (BLT1) antagonist had no effect in patients with mild asthma but has not been tested in patients with more severe disease, in whom it would be more likely to be effective [447]. A low-affinity BLT2 receptor is expressed on several cell types, including T cells and mast cells, and when inhibited by oligonucleotides, there is a reduction in allergic inflammation in a murine model [448]. BLT2 receptors are upregulated on mast cells after allergen challenge and mediate the synthesis of Th2 cytokines [448, 449]. To target both BLT1 and BLT2, LTB4 synthesis can be reduced by an inhibitor of LTA4 hydrolase, and such an approach is effective in a murine model of asthma [450-452].

Inflammation in some patients with severe asthma is predominantly neutrophilic so that inhibitors of neutrophilic inflammation are needed, and corticosteroids are poorly effective against neutrophilic inflammation [453]. Several approaches to treating neutrophilic inflammation might be applicable to the treatment of severe asthma. Although several classes of broad-spectrum non-corticosteroid anti-inflammatory treatments have been in development, there have usually been problems with side effects when the drugs are administered orally, and this has had limited clinical development. Peroxisome proliferator–activated receptor (PPAR) γ agonists have a wide spectrum of anti-inflammatory effects, including inhibitory effects on macrophages and T cells and neutrophilic inflammation, and polymorphisms of the PPARγ gene have been linked to increased risk of asthma [454]. The PPARγ agonist rosiglitazone produced a small improvement in lung function in smoking asthmatic patients in whom ICSs were ineffective and a modest (15%)
reduction in late response to inhaled allergen in patients with mild asthma [455, 456]. This suggests that PPARγ agonists, such as thiazolidinediones, have little therapeutic potential in asthma therapy.

Prostaglandin D2 (PGD2) is released from mast cells, Th2 cells, and dendritic cells and activates DP2 receptors, also called the chemoattractant homologous receptor expressed on Th2 cells (CRTH2), which mediate Th2 cell and eosinophil chemotaxis [16, 457]. There is increased expression of PGD2 in patients with severe asthma [458]. Several CRTH2 antagonists have shown clinical efficacy as oral treatments for asthma and allergic rhinitis, including AMG-853, Setipiprant, OC000459, and MK-7246 [459, 460]. A study of OC00049 in steroid-naïve asthmatic patients showed no improvement in lung function but a reduction in symptoms and no significant reduction in sputum eosinophil numbers compared with those values after placebo [461]. PGD2 also activates DP1 receptors, which mediate vasodilatation and enhance Th2 cell polarization by dendritic cells, so that a dual DP1/DP2 antagonist might be more effective, whereas an inhibitor of PGD synthase would block PGD2 synthesis and also prevent the bronchoconstrictor effects of PGD2 that are mediated through thromboxane receptors on airway smooth muscle [358].

### 3.2.3.2.1.4. Chemokine Receptor Inhibitors

Chemokines are small cytokines that attract inflammatory cells, including mast cells, eosinophils, and Th2 cells, into the airways and are therefore appropriate targets for therapy, particularly because they signal through G protein-coupled receptors for which small-molecule antagonists can be developed [462]. The major focus of interest in asthmatic patients has been the chemokine receptor CCR3, which is predominantly expressed on eosinophils and mediates the chemotactic response to CXCL11 (eotaxin), which is secreted in asthma. CCR3 is also expressed on mast cells and some Th2 cells. Several small-molecule inhibitors of CCR3 have been in clinical development, but their effects in asthmatic patients have not yet been reported because they have usually been discontinued because of toxicology problems. An inhaled antisense oligonucleotide that targets CCR3 has some effect in reducing sputum eosinophils, but results are difficult to interpret because IL5 and GM-CSF b chain antisense were coadministered [463]. Other chemokine receptors that are targeted for asthma therapy are CCR2 on monocytes and T cells and CCR4, CCR8, and CXCR4 on Th2 cells. CXC chemokine receptor 2 antagonists, which may help in the management of neutrophilic disease by decreasing IL8 activity, have shown some promise in early human trials by decreasing sputum neutrophilia in an ozone challenge model [464]. An afucosylated antibody to CCR4 (mogamulizumab, also known as KW-0761 and AMG- 761)
results in prolonged cytotoxic effects on Th2 cells, marked and prolonged depletion of Th2 cells, and reduced lung inflammation in animal models. Afucosylated antibodies are designed so that the Fc region does not contain fucose sugars within the oligosaccharides. This antibody is now in clinical trials for asthma and adult T-cell leukemia-lymphoma [465]. CXCR2 is expressed on neutrophils and monocytes and might be involved in the recruitment of neutrophils into the airways of patients with severe (neutrophilic) asthma. Several small-molecule inhibitors of CXCR2 are now in clinical development [466]. An oral CXCR1/CXCR2 antagonist, navarixin (SCH-527123), is effective in blocking ozone-induced sputum neutrophilia in healthy subjects and is currently in clinical trials for the treatment of severe asthma.

3.2.3.2.1.5. Kinase Inhibitors

Kinases play a key role in regulating the expression of inflammatory genes in asthmatic patients and might amplify inflammation in patients with severe asthma [467]. There are now several kinase inhibitors that might be useful in the treatment of severe asthma. The transcription factor nuclear factor κB (NF-κB) regulates many of the inflammatory genes that are abnormally expressed in asthmatic patients and is activated in asthmatic airways. Small-molecule inhibitors of the key enzyme IKK2/IκKb (inhibitor of κB kinase) block inflammation induced by NF-κB activation and are now in preclinical testing [468].

The p38 mitogen-activated protein kinase (MAPK) activates similar inflammatory genes to NF-κB, is activated in cells from patients with severe asthma, and has been linked to corticosteroid resistance [469, 470]. A p38 MAPK inhibitor appears to improve corticosteroid responsiveness in cells from patients with severe asthma [397]. p38 MAPK also plays a key role in activation of GATA3, a transcription factor that regulates Th2 cell differentiation and expression of Th2 cytokines [471]. Corticosteroids block GATA3 activation and are mimicked by p38 MAPK inhibitors [472]. An antisense that blocks p38 MAPK demonstrated efficacy in a murine asthma model [473]. Several small-molecule p38 inhibitors are now in clinical development for the treatment of inflammatory diseases, but side effects after systemic administration have proved to be a major problem [474].

Phosphoinositide 3-kinase (PI3K) also regulates inflammation and has several isoforms, but nonselective inhibitors are likely to be toxic [475]. The isoenzyme PI3Kγ is important in chemotactic responses, and selective inhibitors are in development, whereas PI3Kδ activation results in reduced corticosteroid responsiveness through reduced HDAC2 activity, so that PI3Kδ
inhibitors might potentially reverse corticosteroid resistance in patients with severe asthma [406]. In addition to its other pharmacology, theophylline inhibits PI3Kδ, preferentially over other PI3K isoforms. Therefore, theophylline derivatives that lack PDE inhibition or lead to preferential PI3Kδ inhibition might be of therapeutic value. A general concern about novel kinase inhibitors is that they might have side effects because they target mechanisms that are found in many cell types. Consequently, it might be necessary to develop inhaled formulations for use in asthmatic patients in the future, as proved necessary for corticosteroids.

Spleen tyrosine kinase (Syk) is involved in activation of mast cells and other immune cells, and several small-molecule Syk kinase inhibitors are in development, particularly for patients with severe asthma [476]. An antisense inhibitor of Syk kinase is effective in an animal model of asthma, and the small-molecule inhibitor R112 administered nasally reduces nasal symptoms in patients with hay fever [477, 478]. More potent inhibitors, such as R343 and Bay 61-3606, are in development for inhalation in asthmatic patients. Because Syk is widely distributed in immune and neuronal cells, there are concerns about side effects. As with other kinase inhibitors, there can be side effects with systemic administration, and therefore inhalation might be preferred.

Masitinib is a potent tyrosine kinase inhibitor that blocks c-Kit (as well as platelet-derived growth factor receptors) and provides some symptomatic benefit in patients with severe asthma [479]. Mast cell activation is important as a driving mechanism in some patients with severe asthma [458]. There are several approaches to inhibiting mast cell activation, and anti-IgE has already been shown to be of value in the treatment of some patients with severe asthma [480]. Stem cell factor is a key regulator of mast cell survival in the airways and acts through the receptor c-Kit on mast cells [330]. Plasma concentrations of stem cell factor are increased in patients with severe asthma [481]. Blockade of stem cell factor or c-Kit is effective in animal models of asthma, suggesting that this pathway might be a good target for new asthma therapies.

Janus kinase (JaK) inhibitors function by inhibiting the activity of one or more of the Janus kinase family enzymes (Jak1, Jak2, Jak3, Tyk2), thereby interfering with the JAK-STAT signaling pathway. These inhibitors have therapeutic action in the treatment of cancer and inflammatory diseases [482]. Cytokines that bind type I and type II cytokine receptors include interferons, interleukins, colony stimulating factor, and classic hormones such as erythropoietin, prolactin, and growth hormone [483]. A family of structurally distinct kinases are responsible for the receptor signaling of these cytokine receptors. The Janus family of kinases (Jaks) consists of four members:
Tyk2, Jak1, Jak2, and Jak3. These Jaks associate with the membrane proximal domains of type I and type II receptors in different combinations. When ligand is bound, Jaks phosphorylate cytokine receptors. Therefore, Jaks induce the recruitment of various signaling intermediates, including the STAT family of transcription factors, which directly modulates gene transcription[484, 485]. Th2 dominant immune responses are responsible for the chronic inflammatory respiratory distress that the disease asthma causes. Asthma prevalence remains high in industrial countries, and there are significant socioeconomic burdens of exacerbations of the disease or steroid-refractory asthma. Since JAK/STAT pathways have a role in the differentiation of T cells, including Th2 cells, Jak inhibitors have been studied in animal models of asthma.

Dual inhibition of Jak1 and Jak3 leads to superior cellular potency than selective inhibition of only Jak3 [486]. This is consistent with the model in which Jak1 has a dominant role over Jak3 in γC-dependent signal transduction and indicate that selective Jak3 inhibition is not enough to achieve sufficient immunosuppression [486]. Jak3 has been of particular interest because unlike other Jak family members, its expression is restricted to cells of the hematopoietic lineage. Jak3 (together with Jak1) is involved in the signaling initiated with IL2, IL4, IL7, IL9, IL15, IL21, which are important in immune homeostasis and, when deregulated, in pathologies of the immune system [487]. These receptors share the common gamma chain (CD132, γC) which is paired with an alpha chain (for IL4, IL7, IL9, and IL21) or beta chain (IL2 and IL15) as signaling partners and, in the case of IL2 and IL15, a signaling alpha chain. The pan-JAK inhibitor, Tofacitinib, was shown to reduce the allergen-induced airway eosinophilia and IL-13 levels in mice [488]. Pyridone 6, another pan-JAK inhibitor, was shown to reduce airway hyperresponsiveness (AHR), eosinophilia, and mucus hypersecretion when mice were treated during the challenge phase [489] including the alteration of regulatory T (Treg) cell activity or interference with other important pathways of JAK2.

3.2.3.2.1.6. Macrolides

Some severe asthmatics are chronically infected with atypical bacteria, such as Mycoplasma pneumoniae and Chlamydia pneumoniae [358, 490]. In patients with confirmed infections, there was a significant improvement in FEV1 after a 6-week course of clarithromycin [491]. Conversely, in a larger trial of patients with poorly controlled asthma, treatment with clarithromycin over a 16-week period did not produce any clinically meaningful improvement in asthma control, even in the patients who had positive PCR results for atypical bacteria, although
there was a significant reduction in airway hyperresponsiveness [492]. It has long been recognized that macrolides have anti-inflammatory effects that might be independent of their antibiotic effects [493]. Macrolides appear to inhibit inflammation by inhibiting NF-κB and other transcription factors, but the precise molecular mechanisms have not yet been determined. In patients with severe neutrophilic asthma, a course of azithromycin significantly reduced sputum neutrophil numbers and CXCL8 concentrations, with some improvement in symptoms [494]. This suggests that it might be worth using a therapeutic trial of macrolide antibiotics in patients with severe asthma who have predominantly neutrophilic inflammation. A nonantibiotic macrolide (EM-703) reverses corticosteroid resistance caused by oxidative stress by increasing HDAC2 activity [495]. Several non-antibiotic macrolides are now in development as anti-inflammatory therapies [496].

3.2.3.2.2. Biologics

3.2.3.2.2.1. Targeting IL-4/IL-13

Inhibition of IL4 by using inhaled soluble receptors proved to be disappointing, but there is continued interest in blocking IL13, a related cytokine that regulates IgE formation, particularly in patients with severe asthma. IL13 can also induce corticosteroid resistance and therefore appears to be an appropriate target for patients with severe asthma [497, 498]. Pitrakinra is a mutated form of IL4 that blocks IL4 receptor α, the common receptor for IL4 and IL13, and significantly reduces the late response to inhaled allergen in patients with mild asthma when administered subcutaneously or by means of nebulization, and larger clinical trials are currently in progress with this protein [499]. Several IL13 and IL4 receptor blocking antibodies are also in clinical development, but thus far, clinical studies in patients with severe asthma have been disappointing. A blocking mAb to IL13, lebrikizumab, has been studied in asthmatic patients whose symptoms are not controlled with high doses of ICSs and showed a small increase in FEV1 (approximately 5%) compared with placebo after 12 weeks but no significant effect at 24 weeks [500]. There are no significant improvements in symptoms or asthma-related health status and no reduction in exacerbations. Interestingly, increased concentrations of the plasma biomarker periostin, which was discovered by means of proteomic analysis of IL13–stimulated epithelial cells, showed a slightly better response (approximately 8%) than low concentrations, suggesting that it can be used as a biomarker to predict greater responses. An mAb targeting IL4 receptor α (AMG317), which therefore blocks the effects of IL4 and IL13, has been ineffective in controlling asthma symptoms or lung function in 3 different doses over a 12-week period in patients with mild asthma [501].
IL4 and IL13 are also central to the allergic response and are found in increased levels in the airways and sputum of asthmatics [502, 503]. IL4 is produced mainly by Th2 cells and mast cells, whereas IL13 is produced by a variety of cells including Th2 cells, mast cells, eosinophils, and basophils [172, 504-508]. Although these two cytokines do not show a high degree of sequence homology, they share a common receptor, IL4Rα [153]. Both IL4 and IL13 affect transcription through the STAT6 signaling pathway [509]. IL4 promotes Th2 cell development and B cell isotype switching, and it affects the production of chemokines by the airway epithelium [510]. IL13 promotes the allergic phenotype through effects on hematopoetic cells as well as airway epithelium, smooth muscle, fibroblasts, and the endothelium [155].

Several compounds in various phases of development aim to modulate IL4 and IL13 responses [173]. Nebulized IL4R has been shown to be safe in a clinical trial setting and efficacious in terms of symptoms and airway function when compared in a randomized controlled trial with placebo in the context of inhaled steroid withdrawal [511, 512]. In a placebo controlled randomized clinical trial of AMG 317, an IL4Rα blocker, the intervention did not show statistical and clinical benefit in symptoms as measured by the asthma control questionnaire or lung function as measured by pre-bronchodilator forced expiratory volume in one second [513]. A randomized controls trial found that dupilumab, a monoclonal antibody that inhibits IL4Rα, was superior to placebo in preventing asthma exacerbations in the context of withdrawal of long acting β agonists and inhaled corticosteroid in patients with blood or sputum eosinophilia despite the use of these two treatments [514]. Lebrikizumab, a monoclonal antibody that targets IL13, was superior to placebo with respect to airway function in patients whose asthma was uncontrolled despite the use of inhaled corticosteroids. This effect was most prominent in those with a high level of periostin, a stable blood marker of IL13 activity [500]. Another IL13 antibody, tralokinumab, was found to be effective in improving lung function when compared to placebo in a phase II clinical trial in moderate-to-severe asthma [515].

To discover whether the doses of anti-IL13 strategies are sufficient to block endogenous IL13 in the airways, a blocking anti–IL13 mAb was studied. It was found to profoundly suppress IL13 in nasal secretions after local allergen challenge, yet there was no significant reduction in eosinophil numbers or nasal symptoms [516]. IL4 and IL13 signal through the transcription factor signal transducer and activator of transcription 6, and small-molecule inhibitors, such as
AS1517499, have now been developed that are active in a murine model of asthma but have yet to be developed clinically [62].

3.2.3.2.2.2. Targeting IL5

There is increased production of IL5 in the peripheral circulation and in the airways of asthmatics [517, 518]. IL5 is produced by Th2 cells, natural killer cells, eosinophils, basophils, and CD34 positive cells [519-523]. The IL5 receptor has a unique subunit as well as a subunit shared by the IL3 and GM-CSF receptors, and it signals through multiple pathways including the JAK-STAT, Ras-MAPK, PI3K-ERK, and p38-NF-κB pathways [524]. IL-5 enhances eosinophil growth, maturation, and migration, while inhibiting eosinophil apoptosis [525-527]. IL-5 also enhances basophil development [528].

IL5 is of critical importance for eosinophilic inflammation, and a blocking antibody to IL5 (mepolizumab) depletes eosinophils from the circulation and sputum of asthmatic patients but disappointingly has no effect on the response to inhaled allergen, airway hyperresponsiveness, symptoms, lung function, or exacerbation frequency in asthmatic patients [529, 530]. However, more recent studies show that mepolizumab reduces exacerbations in highly selected patients who have persistent sputum eosinophilia despite high doses of ICSs, although there is no improvement in symptoms, lung function, or airway hyperresponsiveness [368, 531].

Reslizumab, another monoclonal antibody to IL5, improved airway function in patients with persistent sputum eosinophilia but had no significant effect on asthma symptoms or exacerbations when compared with placebo [532]. Reslizumab failed to improve asthma control over 12 weeks in patients with sputum eosinophilia despite high doses of ICSs, but there was some reduction in symptoms and sputum eosinophils [532].

An antibody against the IL5 receptor a (benralizumab, MEDI-563) might more effectively deplete airway eosinophils than blocking IL5 itself through antibody-dependent cytotoxicity of eosinophils and is currently being studied in clinical trials [533]. Results of phase IIb and III trials have not yet been reported for benralizumab, but this agent has been shown to decrease eosinophils in blood, sputum, and airways. A phase III trial (Clinical-Trials.gov identifier NCT01914757) of benralizumab is currently in progress [534, 535].
Overall, blocking IL5, although effective in reducing eosinophilic inflammation, has been disappointing, although it might be effective in highly selected patients, as well as in patients with other hypereosinophilic diseases, such as Churg-Strauss syndrome and eosinophilic esophagitis [536, 537].

3.2.3.2.2.3. Targeting IL9

Another Th2 cytokine that is currently being targeted is IL9, which plays a role in mast cell proliferation, although there is recent evidence that it is produced particularly by a subset of CD41 T cells designated Th9 cells [538]. Clinical studies have been encouraged by animal studies showing that inhibition of IL9 leads to reduced allergic inflammation and mucus hypersecretion, and a blocking IL9 antibody (MEDI-528) has been shown to be safe after weekly subcutaneous injections, with a trend toward reduction in exercise-induced asthma, which is mediated through mast cell activation [539]. Larger clinical trials are now in progress. There has been considerable interest in thymic stromal lymphopoietin (TSLP), an IL7–related cytokine that is secreted by airway epithelial cells, because it instructs dendritic cells to secrete chemokines that attract Th2 cells into the airways and potentiates the activation of these cells [540]. TSLP expression by airway epithelial cells is increased in a subset of patients with severe asthma treated with high doses of ICSs, suggesting that this might be a good target, especially because it acts as an upstream cytokine [541]. Several pharmaceutical companies are developing antibodies to TSLP and its receptor, as well as to OX40 and OX40 ligand, which act as costimulatory molecules to TSLP, although bronchial OX40/OX40 ligand expression is not increased in patients with severe asthma compared with that seen in patients with mild asthma [542].

3.2.3.2.2.4. Targeting IL2

Activation of Th2 cells by allergen leads to production of interleukin (IL) 2 and its receptor IL2R [543]. Binding of IL2 to Th2 cells expressing IL2R leads to proliferation of that clone of specifically sensitized Th2 cells. The humanized monoclonal antibody to the CD25 subunit of IL2R, daclizumab, inhibits various T cell functions, including T cell proliferation and cytokine production [544]. The potential role of daclizumab in the treatment of moderate to severe asthma was examined in a study of 115 patients randomly assigned (3:1) to intravenous daclizumab every two weeks or placebo for 12 weeks [544]. Daclizumab treatment was associated with small improvements in pulmonary function and asthma control [544]. The potential risks of suppression of the IL2 pathway may limit the utility of this type of immunosuppression in asthma.
3.2.3.2.2.5. Targeting IL17

IL17, a cytokine produced by Th17 cells, plays an important role in the immunologic responses seen in asthma [545]. There are multiple IL17 receptors, the functions of which may differ slightly [545]. Receptor activation leads to the secretion of several inflammatory mediators including IL1β, IL6, TNF-α, and GM-CSF, which ultimately leads to neutrophil recruitment [545, 546]. Higher levels of IL17 than normal have been found in the blood, sputum, and human airway cells of patients with asthma [546-548]. Corticosteroids are very effective in suppressing Th2 cell–driven inflammation, at least in part because they are very potent inhibitors of the Th2 regulating transcription factor GATA3 [331, 472]. Animal models of asthma have also been developed that highlight Th2 cell–driven inflammation, and these models have been suggest to be inappropriate for the development of treatments for severe asthma [358]. Because different immune mechanisms are likely operate in many patients with severe asthma, it might be necessary to target different sets of cytokines, such as those involving Th1 and Th17 cells. Th17 cells have been implicated in patients with severe asthma, particularly those patients with a predominantly neutrophilic pattern of inflammation [549]. Interestingly, Th17 cells appear to be corticosteroid resistant and might therefore contribute to the corticosteroid resistance seen in patients with severe asthma [550].

Brodalumab is a human monoclonal antibody that binds the IL17Rα, effectively inhibiting signaling of several members of the IL17 family, including IL25. Three doses of brodalumab were compared with placebo in a phase IIa trial of adults with moderate to severe asthma. In this trial, safety was demonstrated, but efficacy (defined by the primary outcome of change in the asthma control questionnaire) was not apparent in the group as a whole. A specified subgroup with high bronchodilator reversibility reported improved scores on the asthma control questionnaire (although the results were not adjusted for multiple comparisons). No other clinically meaningful differences were found between the brodalumab groups and the placebo group [551]. Of note, groups in this study were not stratified by predetermined inflammatory profiles.

3.2.3.2.2.6. Targeting Other Asthma Pathways

Another cytokine targeted in asthmatic patients is TNF-α, which might play a significant role in those with severe asthma. Several uncontrolled or small studies suggested that anti-TNF therapies (TNF blocking antibodies infliximab or soluble receptor etanercept) might be useful in reducing symptoms, exacerbations, and airway hyperresponsiveness in patients with severe asthma, but a recent large multicenter trial with the humanized antibody golimumab showed no
beneficial effect on lung function, symptoms, or exacerbations, and there were increased reports of pneumonia and cancer [552-554]. A study of etanercept over 4 weeks in patients with moderate-to-severe asthma showed no clinical efficacy, but there were no safety problems [555]. Another biologic, the vasoactive intestinal peptide analog (Ro 25-1553) has bronchodilator activity in asthmatic patients but does not show efficacy improvements over inhaled formoterol [556]. Vasoactive intestinal peptide is a neuropeptide that is expressed by lymphoid and neural cells, which has effects on cellular mediators of inflammation and has potential use for asthma therapeutics [557].

3.2.3.2.3. Oligonucleotide-based Asthma Therapy

The siRNA designed for asthma therapies have been recently reviewed by Xie and Merkel [558]. The siRNAs directed towards cytokines, chemokines, transcription factors, tyrosine kinases, and costimulatory factors for asthma treatment are summarized in Table 3.1.

Table 3.1. Summary of biological molecular targets for siRNA-mediated asthma therapies. Used with permission from Ref. [558]. Copyright John Wiley and Sons 2015.

<table>
<thead>
<tr>
<th>Route of Administration</th>
<th>Formulation/vector</th>
<th>Target Molecule</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intratracheal</td>
<td>Naked/modified siRNA</td>
<td>CD86</td>
<td>[559]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rip2</td>
<td>[560]</td>
</tr>
<tr>
<td></td>
<td>Lentivirus</td>
<td>IL-5</td>
<td>[561]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nerve growth Factor</td>
<td>[562]</td>
</tr>
<tr>
<td></td>
<td>Atelocollagen</td>
<td>Syntaxin4</td>
<td>[563]</td>
</tr>
<tr>
<td>Intranasal</td>
<td>Lentivirus</td>
<td>GATA3</td>
<td>[564]</td>
</tr>
<tr>
<td></td>
<td>Naked/modified siRNA</td>
<td>GATA3</td>
<td>[565]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>STAT6</td>
<td>[51]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SYK</td>
<td>[566]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c-Kit</td>
<td>[567]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SOCS3</td>
<td>[568]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IL-4</td>
<td>[569]</td>
</tr>
<tr>
<td>Intravenous</td>
<td>Plasmid</td>
<td>CD40</td>
<td>[570]</td>
</tr>
</tbody>
</table>
Small interfering RNA (siRNA) are naturally occurring intermediates in the RNA interference (RNAi) pathway, but can also be synthesized and delivered to cells. Short hairpin RNA (shRNA) are produced within the target cell from DNA constructs that have been delivered to the nucleus. siRNA can be synthesized within the laboratory and delivered to cells either naked or via synthetic or natural polymers and lipids within the cytoplasm of the target cells. shRNA, on the other hand, are prepared using nuclear expression and delivered to the cells via viral or other gene therapy vectors to the nucleus [571]. Because siRNA has high degradation of 99% within 48 hr, they require a high dose relative to shRNA [571]. Antisense oligonucleotides are single-stranded polymers of approximately 15-20 synthetically modified deoxynucleotides and are complementary to the sense sequence of a mRNA molecule [572]. A review comparing laboratory and clinical gene silencing of siRNAs and antisense oligonucleotides has been published by Watts et al. [572].

Antigen presenting cells, such as dendritic cells, can initiate and maintain inflammatory responses in the airways in the pathogenesis of asthma. A CD86 siRNA delivered to bone marrow derived dendritic cells in vitro lost their ability to activate Th2 cells through the costimulatory molecule CD86 [559]. The same siRNA, labeled with Texas Red, was delivered in vivo to an OVA asthmatic mouse model via intranasal administration and was found to efficiently overcome the epithelial barrier and co-localize with the dendritic cells. For three days, the CD86 siRNA was delivered intranasally at a dose of 12.5 µg/day [559]. The effects following these doses were reduction of eosinophilia, AHR, levels of Th2 associated interleukins, while goblet cell metaplasia did not change. It is possible that a higher dose, or more prolonged doing regime may be required to reduce all of the asthma symptoms [558].

Transcription factors of the production of cytokines or chemokines that regulate the growth and differentiation of inflammatory cells, such as GATA3, STAT6, and receptor interacting protein 2 (Rip2), have been reported as potential asthma therapeutic targets because of their upstream locations in the inflammatory process [558]. The GATA3 transcription factor determines the differentiation of Th2 cells and production of associated interleukins. Both GATA3 siRNA and antisense oligonucleotides have been created and evaluated. The 200 µg GATA3 anti-sense oligonucleotides delivered intranasally for 4 days led to local anti-inflammatory effects when compared to healthy control and a dexamethasone treated control in an OVA asthmatic mouse model [573]. Another group delivered a GATA3 small hairpin RNA (shRNA) using a lentiviral
delivery vector to OVA asthmatic mice and also found similar results [564]. Sel et al. evaluated a GATA3 DNAzyme efficacy compared to GATA3 siRNA and anti-sense oligonucleotides delivered intranasally (200 µg) [565]. A DNAzyme are DNA oligonucleotides that catalyze specific chemical reactions, which degrade the target protein. The GATA3 DNAzyme was found to be superior to the GATA3 oligonucleotides or siRNA in terms of reduction of inflammatory mediators including the number of eosinophils in the BAL and the IL-5 to IFN-γ ratios [565]. The GATA3 siRNA effectively reduced the amount of goblet cells and AHF at levels comparable to the GATA3 DNAzyme [558, 565]. This study did not evaluate the siRNA or oligonucleotides within a delivery vector, such as a nanocarrier. Therefore, it may be possible that the effects of the gene silencing were reduced due to degradation or poor cellular uptake, which could be resolved through the use of an oligonucleotide delivery system. GATA3 downregulation using a DNAzyme was evaluated as a therapeutic strategy recently in a human phase 3 clinical trials (ClinicalTrials.gov number: NCT01743768) [574]. The clinical study concluded that the GATA3 specific DNAzyme significantly reduced both late and early asthmatic responses, including Th2 regulated inflammatory responses, following allergen challenge in patients with allergic asthma [574].

Intranasally delivered STAT6 siRNA (100 µg) to asthmatic mice reduced the leukocyte infiltration in the BALF and reduced AHR compared to the GFP siRNA control [51]. The levels of IL-4, IL-5, IL-13, T cells infiltration, and histological changes were reduced and suggested that the STAT6 siRNA was effective at reducing airway inflammatory events associated with asthma [51]. However, STAT6 siRNA deposition and cellular internalization was more localized to the airway epithelial cells instead of the T and B lymphocytes, suggesting that even more reduction in asthmatic symptoms may be achieved if the siRNA could be preferentially delivered to these cells types [558].

The Syk and the NF-κB are other potential targets that are upstream of the inflammatory process involved in asthma [575]. Syk is expressed in immune cells and are responsible for initiating and mediating cellular signaling pathways, including the development and maturation of B cells, proliferation of T cells, and the production of inflammatory mediators from macrophages and mast cells [558, 576]. Syk anti-sense oligodeoxynucleotide loaded liposomes that were delivered to OVA asthmatic rats via aerosol inhalation successfully reduced leukocyte infiltration in the BALF and antigen induced upregulation of adhesion molecules in eosinophils, neutrophils,
and macrophages [477]. There was no significant change in pulmonary inflammation in the lungs of the Syk anti-sense oligo treated- and the saline- treated rats, suggesting that possibly increased dose, more frequent doing intervals, or possible a different oligonucleotide may be necessary [477]. From this partial success, another group studied the effect of a Syk siRNA (0.1, 1, 10 µg) delivered intranasally for 3 days before inducing lung inflammation [566]. Less inflammatory cell infiltration was observed in the animals treated with 1 and 10 µg siRNA, which indicated that the Syk siRNA had a dose-dependent effect at reducing inflammatory cell recruitment during times of lung inflammation [558, 566]. A Syk siRNA, Excellair™ by ZaBeCor has underwent Phase II clinical trials, although results of this study are not yet avaiable [558].

Another potential target for asthma therapy is the protein, stem cell factor (SCF), which is a ligand for the tyrosine kinase receptor c-Kit. Intranasal administration of methylated c-Kit siRNA at 35 µg/day for 3 days to allergic asthmatic mice led to significant reduction in IL-4, IL-5, and SCF in the BALF [567]. The treatment also inhibited inflammatory cell infiltration, eosinophilia, and reduced mucus secretion [567].

The blockade cytokine or chemokine receptors using siRNA has been previously reported using suppressor of cytokine signaling 3 (SOCS3), IL-4, and IL-5 [568, 569]. An IL-5 targeting siRNA loaded onto a lentiviral vector that was delivered intratracheally to asthmatic mice for 3 days was found to reduce the IL-5 expression by half and reduced AHR [561]. Expectedly, the lung eosinophilia was reduced in response to the lowered IL-5 expression [561].

Other siRNAs that have been proposed for possible asthma therapy include those siRNA targeted against the transient receptor potential cation channel (TRPV1), nerve growth factor, and the soluble membrane N-ethylmaleimide-sensitive factor attachment protein receptor protein (syntaxin4) [562, 577, 578].

Other reported oligonucleotide delivery for asthma therapy examples include the antisense oligonucleotide CCR3 antagonist (co-administered with an antisense oligonucleotide targeting the βc subunit of the IL-3, IL-5, and GMCSF receptors) which had shown some early efficacy in phase II trials, decreasing sputum eosinophils in response to allergen challenge [463]. Inhaled antisense oligonucleotides that block the common b chain of IL-5 and GM-CSF receptors together with the chemokine receptor CCR3 (TPI ASM8) have a small effect in reducing allergen responses and airway inflammation [463].
3.2.4. Nanoparticle-Based Therapeutic Delivery Systems Currently Under Pre-Clinical Investigation for the Treatment of Asthma

Nanotechnology, or nanoscience, refers to the study of specialized architectures at a scale of 100 nm or less, however nanomedicine has been suggested to include structures up to 500 nm [579, 580]. A part of nanotechnology, the field of nanomedicine refers to highly specific medical intervention at the molecular scale for the diagnosis, prevention, and treatment of various diseases [579]. Active pharmaceutical ingredients (APIs) may be nanocrystals, integrated into the matrix, reside within an inner space, or be attached to the inner or outer surfaces of the nanoparticle. Nanoparticles for use as therapeutic drug delivery systems are generally greater than 100 nm in one direction and may consist of biodegradable materials such as natural and synthetic polymers, dendrimers, liposomes, or metals [580]. If appropriately designed, nanocarriers can theoretically deliver a wide variety of drugs, target them to sites of interest, and protect them from degradation and inactivation by the body to improve drug efficacy and decrease potential adverse side effects [241, 580].

3.2.4.1. Polymeric Nanoparticles

Polymers should be biocompatible in that they have properties which include non-toxicity, non-antigenicity, biodegradability and biocompatibility. The natural polymers that have commonly been used for preparation of polymeric nanoparticles are chitosan, gelatin, sodium alginate, and albumin [99, 581, 582]. The well-known synthetic polymers include polyactides (PLA), polyglycolides (PGA), Polylactide co-glucolides (PLGA), polyanhydrides, polyorthoesters, polycyanoacrylates, polycaprolactone, polyglutamic acid, polycrylic acid, poly(N-vinyl pyrrolidone), poly(methyl methacrylate), poly(vinyl alcohol), poly(acrylic acid), polyacrylamide, polyethylene glycol, and polymethacrylic acid. Advantages of polymers are that they are biocompatible, capable of having a high payload, easily modified, and capable of being surface modified. Disadvantages of polymer delivery systems stem from the use of organic solvents in production processes that leave residues, polymer cytotoxicity, and scalability of the production process.

3.2.4.1.1. Gelatin

Gelatin is a natural biopolymer, which has the advantages of being inexpensive, biodegradable, non-toxic, not antigenic, and easily modifiable [217]. Natural polymer based delivery systems are biocompatible and biodegradable with high physiological tolerance and low
immunogenicity[210-216]. Gelatin is the hydrolysis product of collagen isolated from animal skin and bones, and treated with either acid, which is known as Type A gelatin, or treated with base to form Type B gelatin. Gelatin contains about 33% glycine and is also rich in proline and 4-hydroxyproline. An example of a typical structure of gelatin is -Ala-Gly-Pro-Arg-Gly-Glu-4Hyp-Gly-Pro, similar to what is shown in Figure 3.4.

Figure 3.4. Representative image of the gelatin polymer structure.

Gelatin is composed of single-or multi-stranded polypeptides with left-handed proline helix conformation containing 50-1000 amino acids. The triple helix of type I collagen extracted from animal skin and bones, is composed of three chains each with a molecular mass of approximately 95 kDa. The gelatin polymer that results from the partial hydrolysis of collagen is composed of mixtures of the α1(I) and α2(I) chains. Gelatin solutions undergo coil-helix transition followed by aggregation of the helices by the formation of collagen-like right-handed triple-helical proline/hydroxyproline rich areas. Gelatin crosslinking agents include transglutaminase, glutaraldehyde, and genipin to link reactive side chains like primary amines on lysines.

Recurrent airway obstruction (RAO), a common allergic disease in horses, is an asthma-like disease. In horse RAO, hypersensitivity reactions to environmental antigens cause an allergic inflammatory response within the airways. Cytosine triphosphate deoxynucleotide connected to a quinine triphosphate deoxynucleotide by a phosphodiester linkage oligonucleotides (CpG-ODN) are known to direct the immune system toward a Th1-pathway, and away from the pro-allergic Th2-line (Th2/Th1-shift). Cytosine-phosphate-guanosine-oligodeoxynucleotides (CpG-ODN) loaded gelatin nanoparticles effectively protected the oligonucleotides from DNAase degradation and were designed to elicit a Th2 to Th1 shift [583]. Preliminary studies on the inhalation of gelatin nanoparticle-bound CpG-ODN in RAO-affected horses showed promising results so the group performed follow up clinical studies in horses [584]. In the follow up study by Klier et al., the aim was to evaluate the clinical and immunological effects of gelatin nanoparticle-bound CpG-
ODN in a double-blinded, placebo-controlled, prospective, randomized clinical trial in horses and to verify a sustained effect post-treatment [585]. Twenty-four RAO-affected horses received a single inhalation every 2 days for 5 consecutive inhalation doses. Horses were examined for clinical, endoscopic, cytological, and blood biochemical variables before the inhalation regimen, immediately afterwards, and 4 weeks post-treatment. Before and immediately after the inhalation regimen, the gelatin nanoparticle-bound CpG-ODN treatment produced a statistically significant decrease in respiratory effort, amount and viscosity of nasal and tracheal secretions, alveolar-arterial oxygen gradient, and neutrophil percentage, and an increase in arterial oxygen pressure when compared to the placebo. Administration of a gelatin nanoparticle-bound CpG-ODN formulation caused a potent and persistent effect on allergic and inflammatory-induced clinical variables in RAO-affected horses. This treatment could serve as a model for future asthma therapy in humans.

3.2.4.1.2. Albumin

Albumin carriers are an attractive delivery vehicle due to their non-toxicity, non-immunogenicity, biocompatibility, and biodegradability. Albumin nanoparticles have a high binding capacity of many drugs and are well tolerated in vivo [586].

Wang et al. reported a method employing drug-loaded albumin nanoparticles, which efficiently deliver drugs into neutrophils adherent to the surface of the inflamed endothelium [587]. These nanoparticles have been shown to prevent neutrophil activation and subsequent tissue damage from inflammation. Inflammatory diseases such as acute lung injury and ischaemic tissue injury are caused by the adhesion of polymorphonuclear neutrophils to the lining of the vascular endothelium and neutrophil transmigration. Nanoparticle-mediated targeting of activated neutrophils on vascular endothelial cells at the site of injury may be a useful means of directly inactivating neutrophil transmigration and hence mitigating vascular inflammation. Using intravital microscopy of TNF-α-challenged mouse cremaster post-capillary venules, the authors demonstrated that fluorescently tagged albumin nanoparticles are largely internalized by neutrophils adherent to the activated endothelium via cell surface Fcγ receptors [587]. Administration of albumin nanoparticles loaded with the Syk inhibitor, piceatannol, which blocks ‘outside-in’ β2 integrin signalling in leukocytes, detached the adherent neutrophils and elicited their release into the circulation. This is an approach which addresses the abhorrent neutrophil sequestration and activation associated with inflammatory diseases, like certain types of asthma,
by inactivating the pro-inflammatory function of neutrophils by delivering a drug-loaded albumin nanoparticle. This may be of benefit in neutrophilic asthma, a non-atopic asthma phenotype which is not easily controlled with conventional ICSs or omalizumab anti-IgE therapies.

In mice with vascular inflammation, bovine serum albumin nanoparticles were preferentially internalized by neutrophils adhering to the inflammation site. In these mice, albumin nanoparticles loaded with piceatannol, a Syk inhibitor, decreased Syk pathway–induced vascular adhesion of neutrophils compared with unloaded nanoparticles via a Syk mediated pathway. In mouse models of acute lung injury, piceatannol-loaded nanoparticles decreased neutrophil infiltration and lung inflammation compared with free piceatannol. with the authors planned to study these model albumin nanoparticles loaded with other available Syk inhibitors or other classes of neutrophil adhesion inhibitors in models of acute lung injury, sepsis and/or ischemia/reperfusion injury. Potentially, they may decide to use GS-9973 (Gilead Sciences Inc.), an oral Syk inhibitor, in Phase II trials to treat hematological malignancies, fostamatinib disodium (FosD; Rigel Pharmaceuticals Inc.), an oral Syk inhibitor, in Phase II testing to treat idiopathic thrombocytopenic purpura (ITP), or Excellair (ZaBeCor Pharmaceutical Co.), an siRNA targeting Syk, in Phase II testing to treat asthma.

3.2.4.1.3. Poly lactic-co-glycolic acid (PLGA)

Poly lactic-co-glycolic acid (PLGA) is an FDA-approved biodegradable polymer that has high biocompatibility while being resilient carriers for many active pharmaceutical ingredients including small molecules, proteins, and oligonucleotides [588]. In addition to its drug delivery roles, PLGA functions more appropriately as a specific immunotherapy adjuvant for allergen vaccines than does conventional Al(OH)3 due to its superior efficacy, longer potency, and markedly fewer side effects [589].

Salem et al. recently reported the development of a nanoparticle-based vaccine for protection and treatment against dust mite allergies which has potential for preventing asthma pathogenesis[590]. The delivery system that they developed was composed of PLGA particles carrying Dermatophagoides pteronyssinus-2 (Der p2) antigen and cytosine-phosphate-guanine motif (CpG) adjuvant which serves as a vaccine system to stimulate systemic antigen-specific immune response. The development of such a vaccine is important because in the USA, 84% of homes have measurable levels of house dust mite (HDM) allergens and a quarter of these houses have greater levels of allergens than the proposed limit for asthma [591]. Continuous exposure to
HDM allergens can lead to lung inflammation as evidenced by lung eosinophilia and airway obstructions. This can lead to asthma attacks in sensitized individuals. Persistent exposure to HDM allergens stimulates dendritic cells (DCs) in lungs to initiate Th2 immune responses [308]. These Th2 responses promote eosinophil recruitment to lungs, inflammatory cytokines; and B cell production of IgE antibodies [592].

Mazzarella et al. have shown that different sizes of PLGA particles encapsulating CpG, prepared using a double emulsion solvent evaporation method, had a size-dependent decrease in the proportion of eosinophils found in BAL fluids of mice vaccinated with Der p2 and different sizes of empty or CpG-loaded PLGA particles. Mice vaccinated with the Der p2 coated on 9-μm-sized empty PLGA particles showed increased levels of IgE and IgG1 antibodies as well as increased airway hyperresponsiveness, while all sizes of CpG loaded PLGA particles prevented airway hyperresponsiveness following exposure to Der p2. Inflammatory responses to Der p2 exposure were significantly reduced when smaller PLGA particles were used for vaccination. In addition, encapsulating CpG in PLGA particles increased IgG2a secretion. This study shows that the size of PLGA particles used for vaccination plays a major role in the prevention of house dust mite-induced allergy and that incorporation of CpG into the PLGA particles preferentially develops a Th1-type immune response. The authors suggest that their developed vaccine should ideally induce Th1 immune responses that result in production of IFN-γ and IgG2a antibodies, as well as reduce Th2-stimulated airway inflammation and initiate protective immunity by Th1 cells that would reduce symptoms associated with the allergy [593].

Pollen allergies are common in certain atopic asthmatics and are typically caused by pollens from hyperallergenic birch, olive, ragweed, and also from the tropical Palmae. The immunotherapeutic effects of recombinant Caryota mitis profilin (rCmP)-loaded PLGA nanoparticles for the vaccine adjuvant to prevent and treat allergic disease were studied by Xiao et al. [589]. An allergic asthma mouse model was established for specific immunotherapy using rCmP-loaded PLGA nanoparticles as the adjuvant. The model was evaluated by determining airway hyperresponsiveness and levels of serum-specific antibodies (IgE, IgG, and IgG2a) and cytokines, and observing histologic sections of lung tissue. The rCmP-loaded PLGA nanoparticles inhibited specific IgE production and secretion of the Th2 cytokine IL4, while facilitating the generation of specific IgG2a and secretion of the Th1 cytokine interferon-gamma, converted the Th2 response to Th1, and evidently alleviated allergic symptoms. These rCmP-loaded PLGA
nanoparticles are another example of PLGA adjuvant vaccine delivery for specific immunotherapy for allergic asthma.

Th2 cytokines and their downstream JAK-STAT pathways play a critical role in allergic asthma [138, 489, 594]. The effects of pyridone 6, pan-JAK inhibitor, on asthmatic responses and the resultant mechanisms of biological effects in a ovalbumin (OVA) induced asthmatic mouse model was investigated by Matsunaga et al. [489]. Pyridone 6 treatment during the challenge phase suppressed eosinophilia in bronchoalveolar lavage (BAL) fluids but did not affect airway hyperresponsiveness (AHR). To improve the efficacy of the JAK inhibitor, pyridone 6 was encapsulated in polylactic-coglycolic acid nanoparticles (pyridone 6-PLGA) [489]. Pyridone 6-PLGA treatment just before OVA challenge suppressed both airway eosinophilia and AHR. Although the IL-13 levels in BAL fluids and the OVA-specific IgE levels in serum after the challenge phase treatment with pyridone 6-PLGA were similar to those after a sham treatment, the eotaxin levels in BAL fluids and lung mCLCA3/Gob-5 expression were decreased in pyridone 6-PLGA-treated mice. Interestingly, the local IL-13 levels and serum OVA-specific IgE were decreased, while IL-17-producing T cells were increased by pyridone 6-PLGA treatment during the sensitization plus challenge phases. In vitro, pyridone 6 strongly suppressed the differentiation of Th2 from naive CD4 T cells, but it partly enhanced Th17 differentiation. Pyridone 6 potently suppressed IL-13-mediated STAT6 activation and mCLCA3/Gob-5 expression in mouse tracheal epithelial cells. These findings suggest that the JAK inhibitor pyridone 6 suppresses asthmatic responses by inhibiting Th2 inflammation and that application of PLGA nanoparticles improves the therapeutic potency of pyridone 6.

A study by Onoue et al. was aimed to develop a respirable sustained-release powder (RP) formulation of long-acting vasoactive intestinal peptide derivative, [Arg(15, 20, 21), Leu(17)]-VIP-GRR (IK312532), using PLGA nanospheres (NS) with the aim of improving the duration of action as a bronchodilator[595]. NS formulation of IK312532 (IK312532/NS) was prepared by an emulsion solvent diffusion method in oil, and a mixture of the IK312532/NS and erythritol was jet-milled and mixed with lactose carrier to obtain the IK312532/NS-RP. Physicochemical properties were characterized focusing on appearance, particle size, and drug release, and in vivo pharmacological effects were assessed in antigen-sensitized rats. The IK312532/NS with a diameter of 140 nm showed a biphasic release pattern in distilled water with ca. 20% initial burst for 30 min and a sustained slow release up to ca. 55% for 24h. Laser diffraction analysis
demonstrated that IK312532/NS-RP had fine dispersibility and suitable particle size for inhalation. In antigen-sensitized rats, insufflated IK312532/NS-RP (10 μg of IK312532/rat) could suppress increases of granulocyte recruitment and myeloperoxidase in pulmonary tissue for up to 24h after antigen challenge, although IK312532-RP at the same dose was less effective with limited duration of action. From these findings, newly prepared IK312532/NS-RP might be of clinical importance in improving duration of action and medication compliance for treatment of airway inflammatory diseases including asthma.

3.2.4.1.4. Dendrimers

Dendrimers, are monodisperse highly branched macromolecules which are discovered in early 1980’s by Donald Tomalia and coworkers [168,169]. Dendrimers are monodisperse, nanoscale sizes that matches with the size of biomolecules [170]. Several polyamine polymers have been explored as carriers for drug delivery including poly(amido amine) (PAMAM) dendrimers. The PAMAM dendrimers, also known as starburst dendrimers are the first one to be investigated which included ammonia as the core [174]. Their size and molecular mass is easily controllable and their solubility characteristics can be varied based upon the nature of surface groups [171]. Dendrimer surfaces may be functionally designed to enhance or resist trans-cellular, epithelial or vascular permeability [172]. Mathematically defined numbers of terminal surface groups (Z) present on dendrimers are suitable for conjugation of drugs, signaling groups and targeting moieties [173]. Dendrimers can also be employed to attain pH reliant release with a slower release under normal physiological conditions and a burst release of loaded bioactive at the acidic tumor environment [173]. Dendrimers are routinely synthesized as tuneable nanostructure that may be designed and regulated as function of their shape, size, surface chemistry and interior void space for active ingredient loading [203].

Researchers at the University of California Davis have previously developed a well-defined non-toxic PEG-dendritic block telodendrimer for successful delivery of chemotherapeutics agents and, in these studies, they applied this technology for therapeutic development in asthma [81]. Their findings from early-stage drug development studies suggest that the encapsulation and protection of anti-inflammatory agents such as corticosteroids in nanoparticle formulations can improve efficacy. Further development of novel drugs in nanoparticles is warranted to explore potential treatments for chronic asthma. In these proof-of-concept experiments, the authors hypothesized that dexamethasone loaded self-assembling nanoparticles (Dex-NP) and delivered
systemically would target the lung and decrease allergic lung inflammation and airways hyper-
responsiveness to a greater degree than equivalent doses of dexamethasone (Dex) alone. The
formulation was evaluated in a OVA induced asthmatic mouse model. Mice treated with Dex-NP
were found to have significantly fewer total cells and eosinophils in the lung lavage than Ova-
exposed mice alone. Also, lower levels of the inflammatory cytokines IL-4 and MCP-1 were found
in lungs of the Dex-NP compared to control, and they were not lower in the Dex alone group [81].
In addition, respiratory system resistance was lower in the Dex-NP compared to the other Ova-
exposed groups suggesting a better therapeutic effect on airways hyperresponsiveness [81]. It
would be interesting to evaluate the effectiveness of other anti-inflammatory agents in these types
of nanoparticles delivery systems for the treatment of asthma.

The first work to report the formulation of inhalable siRNA with aerosol properties suitable
to deep lung deposition using pMDIs devices that maintain characteristics like being inexpensive
and portable for improved patient compliance. This study, conducted by Conti et al, had shown
that siRNA-G4NH2 dendriplexes can efficiently target lung alveolar epithelial A549 cells and
silence genes even after siRNA has been exposed to the propellant environment [250]. Small
interfering RNA (siRNA)-based therapies have utilities in the treatment of asthma. However,
progress in this area has been hindered due to the lack of carriers that can efficiently deliver siRNA
to lung epithelial cells, and also due to challenges in developing oral inhalation (OI) formulations
for the regional administration of siRNA and their carriers to the lungs. Conti et al. report the
ability of generation four, amine-terminated poly(amidoamine) (PAMAM) dendrimer (G4NH2)-
siRNA complexes (dendriplexes) to silence the enhanced green fluorescent protein (eGFP) gene
on A549 lung alveolar epithelial cells stably expressing eGFP [250]. They also report the
formulation of the dendriplexes and their aerosol characteristics in propellant-based portable OI
devices. The size and gene silencing ability of the dendriplexes was seen not to be a strong function
of the N/P ratio. Silencing efficiencies of up to 40% are reported. Stable dispersions of the
dendriplexes encapsulated in mannitol and also in a biodegradable and water-soluble co-oligomer
were prepared in hydrofluoroalkane (HFA)-based pressurized metered-dose inhalers (pMDIs).
Their aerosol characteristics were very favorable, and conducive to deep lung deposition, with
respirable fractions of up to 77%. Importantly, siRNA formulated as dendriplexes in pMDIs was
shown to keep its integrity after the particle preparation processes, and also after long-term
exposures to HFA.
Methylprednisolone is an important corticosteroid used in the treatment (through inhalation) of lung inflammation associated with asthma. The ability of methylprednisolone-polyamidoamine (PAMAM) dendrimer conjugate to improve the airway delivery was evaluated in a pulmonary inflammatory murine model that was based on an 11-fold enhancement of eosinophil lung accumulation following five daily inhalation exposures of sensitized mice to the experimental allergen, ovalbumin. Methylprednisolone was successfully conjugated to PAMAM-G4-OH dendrimer yielding 12 methylprednisolone molecules per dendrimer, and further solubilized in lysine carrier [84]. Five daily trans-nasal treatments with the carrier alone, free methylprednisolone, and methylprednisolone-dendrimer at 5 mg kg(-1) (on a drug basis) did not induce additional lung inflammation, although free methylprednisolone decreased baseline phagocytic cell recoveries by airway lavage and tissue collagenase dispersion. Methylprednisolone treatments alone decreased ovalbumin-associated airway and tissue eosinophil recoveries by 71 and 47%, respectively. Equivalent daily methylprednisolone dosing with methylprednisolone-dendrimer conjugate further diminished these values, with decreases of 87% and 67%, respectively. These findings demonstrated that conjugation of methylprednisolone with a dendrimer enhances the ability of methylprednisolone to decrease allergen-induced inflammation, perhaps by improving drug residence time in the lung. This is supported by the fact that only 24% of a single dose of dendrimer delivered to the peripheral lung is lost over a 3-day period. Therefore, conjugation of drugs to a dendrimer may provide an improved method for retaining drugs within the lung when treating such inflammatory disorders as asthma.

Previously, G protein-coupled receptor (GPCR) agonists were tethered from polyamidoamine (PAMAM) dendrimers to provide high receptor affinity and selectivity. Kecskes et al. prepared GPCR ligand--dendrimer (GLiDe) conjugates from a potent adenosine receptor (AR) antagonist; such agents are of interest for treating asthma and other conditions [596]. Xanthine amine congener (XAC) was appended with an alkyne group on an extended C8 substituent for coupling by Cu(I)-catalyzed click chemistry to azide-derivatized G4 (fourth-generation) PAMAM dendrimers to form triazoles. These conjugates also contained triazole-linked PEG groups (8 or 22 moieties per 64 terminal positions) for increasing water-solubility and optionally prosthetic groups for spectroscopic characterization and affinity labeling. Human AR binding affinity increased progressively with the degree of xanthine substitution to reach K(i) values in the nanomolar range. The order of affinity of each conjugate was hA(2A)AR > hA(3)AR
> hA(1)AR, while the corresponding monomer was ranked hA(2A)AR > hA(1)AR ≥ hA(3)AR. The antagonist activity of the most potent conjugate 14 (34 xanthines per dendrimer) was examined at the G(i)-coupled A(1)AR. Conjugate 14 at 100 nM right-shifted the AR agonist concentration--response curve in a cyclic AMP functional assay in a parallel manner, but at 10 nM (lower than its K(i) value), it significantly suppressed the maximal agonist effect in calcium mobilization. This is the first systematic probing of a potent AR antagonist tethered on a dendrimer and its activity as a function of variable loading.

3.2.4.1.5. Chitosan

Chitosan, derived from the naturally occurring chitin, is a fully biodegradable and biocompatible natural polymer, which can be used to prepare nanoparticles [597]. Chitosan has been used for nasal drug delivery because of its biocompatibility, biodegradability and bioadhesiveness [598].

Allergic asthmatics produce low amounts of IFN-γ, a pleiotropic Th1 cytokine that downregulates Th2-associated airway inflammation and hyperresponsiveness (AHR) of allergic asthma. Adenovirus-mediated IFN-γ gene transfer reduces AHR, Th2 cytokine levels and lung inflammation in mice, but its use would be limited by the frequency of gene delivery required. Therefore Kumar et al. tested chitosan/IFN-γ pDNA nanoparticles (CIN) for in situ production of IFN-γ and its in vivo effects and found that mucosal CIN therapy can reduce established allergen-induced airway inflammation and AHR [599]. CIN were administered to OVA-sensitized mice to investigate the possibility of using gene transfer to modulate ovalbumin (OVA)-induced inflammation and AHR. Mice treated with CIN exhibit significantly lower AHR to methacholine challenge and less lung histopathology. Production of IFN-γ is increased after CIN treatment while the Th2-cytokines, IL-4 and IL-5, and OVA-specific serum IgE are therapeutically in mice with established asthma. CIN was found to inhibit epithelial inflammation within 6 hours of delivery by inducing apoptosis of goblet cells. Experiments performed on STAT4-defective mice do not show reduction in AHR with CIN treatment, thus implicating STAT4 signaling in the mechanism of CIN action.

Kong et al. demonstrated that treatment with chitosan interferon-gamma (IFN-gamma) plasmid deoxyribonucleic acid (DNA) nanoparticles (chitosan interferon-gamma nanogene [CIN]) led to in situ production of IFN-gamma and a reduction in inflammation and airway reactivity in mice, but the mechanism underlying the immunomodulatory effects of CIN remains unclear [600].
In this report, the effect of CIN treatment on the immune responses of CD8+ T cells and dendritic cells was examined in a BALB/c mouse model of ovalbumin (OVA)-induced allergic asthma. OT1 mice (OVA-T cell receptor [TCR] transgenic) were also used to test the effects of CIN on OVA-specific CD8+ T cells. CIN treatment caused a reduction in IFN-gamma production in a subpopulation of OVA-specific CD8+ T cells cultured in vitro in the presence of OVA. CIN also reduced apoptosis of the CD8+ T cells. Examination of dendritic cells from lung and lymph nodes indicated that CIN treatment decreased their antigen-presenting activity, as evident from the reduction in CD80 and CD86 expression. Furthermore, CIN treatment significantly decreased the number of CD11c+b+ dendritic cells in lymph nodes, suggesting that endogenous IFN-gamma expression may immunomodulate dendritic cell migration and activation. CIN therapy results in a reduction in proinflammatory CD8+ T cells and decreases the number and antigen-presenting activity of dendritic cells.

Chen et al. developed oral dispersible tablets containing prednisolone (PDS)-loaded chitosan nanoparticles using microcrystalline cellulose (MCC 101), lactose, and croscarmellose sodium (CCS) [601]. An oral disintegrating tablet was formulated in order to increase compliance in pediatric patients who have difficulty swallowing tablets or capsules. The PDS-loaded chitosan nanoparticles were formulated by ionotropic external gelation technique in order to enhance the solubility of PDS in salivary pH. Prepared nanoparticles were used for the development of oral fast disintegrating tablets by direct compression method. Other BCS class II active pharmaceutical ingredients could potentially be formulated using similar methodology. The prepared tablets were evaluated for disintegration time, in vitro drug release, thickness, weight variation, drug content uniformity, friability, and hardness. The effect of concentrations of the dependent variables (MCC, lactose, CCS) on DT and in vitro DR was studied. Fast disintegrating tablets of PDS can be prepared by using MCC, CCS, and lactose with enhanced solubility of PDS. The minimum disintegration time was found to be 15 seconds, and the maximum drug release within 30 minutes was 98.50% [601]. Oral fast disintegrating tablets containing PDS nanoparticles could be the better choice for the pediatric patients that would result in better patient compliance. From this study, it can be concluded that fast disintegrating tablets could be a potential drug delivery technology for the management of asthma in pediatrics.

Ou et al. created a hypoallergenic vaccine by forced ubiquitination by intranasal immunization with a DNA vaccine coexpressing Der p1 and ubiquitin in an allergic rhinitis mouse
Allergic rhinitis has the same Th2 imbalance like atopic asthma, making this study relevant for asthma therapy or prevention. In this study, the authors constructed a DNA vaccine coexpressing Der p 1 allergen and murine ubiquitin, using chitosan nanoparticles as a carrier. This DNA vaccine pVAX1-Ub-Derp1/CS nanoparticle transfected 293T cells \textit{in vitro} but also transform cells \textit{in vivo} effectively \cite{602}. The inflammation of nasal mucosa in an allergic rhinitis murine model via immunization with pVAX1-Ub-Derp1/CS was less severe than those without treatments. Furthermore, it found that mice immunized with pVAX1-Ub-Derp1/CS generated a high level of specific IgG but a low level of specific IgE (P < .01). The significantly increased levels of interferon-\(\gamma\) and the significantly decreased levels of interleukins 4, 10, and 17 indicated that a TH1-type response was elicited by immunization with pVAX1-Ub-Derp1/CS and this effect was augmented following intranasal immunization. The authors concluded that the nasal mucosal immunization and ubiquitination using nanoparticle pVAX1-Ub-Derp1/CS is expected to be a new kind of effective vaccine for allergic rhinitis and possibly for asthma because of their efficacy and safety.

To optimize the properties of chitosan as siRNA carrier, Luo \textit{et al.} synthesized guanidinylated chitosan (GCS). To target their polyplexes to the smooth muscle cells in the lung for potential applications in asthma or COPD, they also synthesized salbutamol-modified chitosan that was subsequently guanidinylated. To test the efficacy \textit{in vivo}, the authors nebulized their formulations with an Aeroneb Pro device first and then administered the collected condensate i.t. with a PennCentury microsprayer to EGFP expressing mice. The animals were treated with 5 \(\mu\)g of siRNA each day for 3 consecutive days, and \textit{in vivo} knockdown was quantified by confocal laser scanning microscopy of tissue sections and by Western Blot to confirm 40\% gene knockdown with the targeted formulation \cite{603}. Unfortunately, the authors did not include negative control siRNA in the \textit{in vivo} study and did only check effects of negative control siRNA \textit{in vitro} with the guanidinylated chitosan but not with the salbutamol-modified polymer. These additional controls would be very helpful for the interpretation of the results, however. Another modified chitosan that mediated nucleic acid delivery to the lung was described by Park \textit{et al.} who prepared O-carboxymethyl chitosan-graft-branched polyethylenimine (OCMPEI). Their copolymer was tested for \textit{in vitro} gene silencing with siRNA and \textit{in vivo} gene delivery with plasmid DNA. The polyplexes were injected i.v. and mediated excellent GFP expression in the lung \cite{604}. Thus, it is possible
that this delivery system may as well be successful for siRNA delivery to the lung after i.v. or even
local administration.

Isatin, an endogenous indole compound, prevents atrial natriuretic peptide (ANP) from
signaling through its cell-surface receptor, NPRA. Allergic airway inflammation has been linked
to natriuretic peptide signaling and blocking this signaling axis in the lung prevents allergen-
induced pathology. Kandasamy et al. encapsulated isatin in chitosan nanoparticles and tested them
in a mouse model of allergic asthma by intranasal delivery to the lung [605]. Isatin nanocapsules
reduced lung pathology by blocking ANP signaling, but surprisingly also by reducing the
expression of NPRA. Ovalbumin-allergic mice were treated intranasally with isatin-containing
chitosan nanocapsules either before or after allergen challenge, and lung function, cytokine levels,
histopathology and cellular infiltration were determined. ANP activity was quantitated by
measuring changes in intracellular cyclic GMP and changes in NPRA levels were determined. For
comparison with isatin's effects, the expression of the receptor was inhibited with small interfering
RNA against NPRA mRNA. Isatin nanocapsules administered locally to the lung reduced cGMP
production and NPRA expression and protected allergic mice from airway hyperreactivity and
lung inflammation when given either before or after allergen challenge. Leukocyte infiltration was
reduced and lung cytokine profiles showed a repolarization from a Th2-like to a Th1-like
phenotype. Isatin nanocapsules administered locally to the lung inhibit NPRA signaling but also
are capable of lowering the expression of NPRA, thus effectively reducing inflammation in a
mouse model of allergic asthma. Pharmacological intervention to reduce NPRA activity through
the inflammatory natriuretic peptide axis in the lung may be a useful adjunct therapy for treating
lung disease.

Atrial natriuretic peptide (ANP) and its receptor, NPRA, have been extensively studied in
terms of cardiovascular effects and it was shown that ANP-NPRA signaling pathway is also
involved in airway allergic inflammation and asthma. ANP, a C-terminal peptide (amino acid 99-
126) of pro-atrial natriuretic factor (proANF) and a recombinant peptide, NP73-102 (amino acid
73-102 of proANF) have been reported to induce bronchoprotective effects in a mouse model of
allergic asthma. The effects of vessel dilator (VD), another N-terminal natriuretic peptide covering
amino acids 31-67 of proANF, on acute lung inflammation in a mouse model of allergic asthma
was reported by Wang et al. [606]. Here, A549 cells were transfected with pVD or the pVAX1
control plasmid and cells were collected 24 hrs after transfection to analyze the effect of VD on
inactivation of the extracellular-signal regulated receptor kinase (ERK1/2) through western blot [606]. For determination of VD's attenuation of lung inflammation, BALB/c mice were sensitized and challenged with ovalbumin and then treated intranasally with chitosan nanoparticles containing pVD. pVD nanoparticles inactivated ERK1/2 and downregulated NPRA expression in vitro, and intranasal treatment with pVD nanoparticles protected mice from airway inflammation. VD's modulation of airway inflammation may result from its inactivation of ERK1/2 and downregulation of NPRA expression. The authors concluded that chitosan nanoparticles containing pVD may be therapeutically effective in preventing allergic airway inflammation.

The same group determined whether siRNA for natriuretic peptide receptor A (siNPRA) protected against asthma when administered transdermally [607]. Imiquimod cream mixed with chitosan nanoparticles containing either siRNA green indicator (siGLO) or siNPRA was applied to the skin of mice. Delivery of siGLO was confirmed by fluorescence microscopy. The anti-inflammatory activity of transdermal siNPRA was tested in OVA-sensitized mice by measuring airway hyperresponsiveness, eosinophilia, lung histopathology and pro-inflammatory cytokines. SiGLO appearing in the lung proved the feasibility of transdermal delivery. In a mouse asthma model, BALB/c mice treated with imiquimod cream containing siNPRA chitosan nanoparticles showed significantly reduced airway hyperresponsiveness, eosinophilia, lung histopathology and pro-inflammatory cytokines IL-4 and IL-5 in lung homogenates compared to controls. These results demonstrate that topical cream containing imiquimod and siNPRA nanoparticles exerts an anti-inflammatory effect and may provide a new and simple therapy for asthma.

Theophylline is a drug that reduces the inflammatory effects of allergic asthma but, due to its narrow therapeutic window, is difficult to administer at an appropriate dosage without causing adverse side effects [608, 609]. Adverse side effects of theophylline include nausea, headache, and cardiac arrhythmias [609]. Theophylline suppresses the activation of inflammatory cells including neutrophils and eosinophils at concentrations lower than 10-20 mg/L, which is required to achieve bronchodilation [609-611]. It was hypothesized that adsorption of theophylline to chitosan nanoparticles modified by the addition of thiol groups would improve theophylline absorption by the bronchial epithelium and enhance its anti-inflammatory effects [612]. Lee et al. sought to develop an improved drug-delivery matrix for theophylline based on thiolated chitosan, and to investigate whether thiolated chitosan nanoparticles (TCNs) can enhance theophylline's capacity to alleviate allergic asthma [612]. A mouse model of allergic asthma was used to test the effects
of theophylline in vivo. BALB/c mice were sensitized to ovalbumin (OVA) and OVA-challenged to produce an inflammatory allergic condition. They were then treated intranasally with theophylline alone, chitosan nanoparticles alone or theophylline adsorbed to TCNs. The effects of theophylline on cellular infiltration in bronchoalveolar lavage (BAL) fluid, histopathology of lung sections, and apoptosis of lung cells were investigated to determine the effectiveness of TCNs as a drug-delivery vehicle for theophylline. Theophylline alone exerts a moderate anti-inflammatory effect, as evidenced by the decrease in eosinophils in BAL fluid, the reduction of bronchial damage, inhibition of mucus hypersecretion and increased apoptosis of lung cells. The effects of theophylline were significantly enhanced when the drug was delivered by TCNs. Intranasal delivery of theophylline complexed with TCNs augmented the anti-inflammatory effects of the drug compared to theophylline administered alone in a mouse model of allergic asthma. The beneficial effects of theophylline in treating asthma may be enhanced through the use of this novel drug delivery system.

In a study by de Lima et al., the interaction between sodium cromoglycate, a drug used in asthma treatment, and chitosan [613]. Cromoglicic acid, also known as cromolyn, is traditionally described as a mast cell stabilizer, and is commonly marketed in the sodium salt forms sodium cromoglicate or cromolyn sodium. Sodium cromoglycate prevents the release of inflammatory chemicals including histamine from mast cells. Equilibrium experiments showed that Sips (or Freundlich-Langmuir) isotherm described well the resultant data and adsorption possibly occurred as in multilayers. A model based on ordinary reaction-rate theory, compounded of two processes, each one with a correlated velocity constant, described the kinetics of sorption. Kinetic and equilibrium data suggested the possibility of surface rearrangement, favored by the increase of temperature. This delivery system may prolong the release of sodium cromoglycate, thus decreasing the amount of necessary doses. Because of the convenience and perceived safety of leukotriene receptor antagonists, they have largely replaced sodium cromoglycate as the non-corticosteroid asthma treatment of choice. Cromoglicic acid requires administration four times a day, and does not provide additive benefit in combination with inhaled corticosteroid [8].

Oyarzyn-Ampuero et al. produced mucoadhesive nanocarriers made from chitosan (CS) and hyaluronic acid (HA), and containing the macromolecular drug heparin, suitable for pulmonary delivery [614]. For the first time, this drug was tested in ex vivo experiments performed in mast cells, in order to investigate the potential of the heparin-loaded nanocarriers in
antiasthmatic therapy. CS and mixtures of HA with unfractionated or low-molecular-weight heparin (UFH and LMWH, respectively) were combined to form nanoparticles by the ionotropic gelation technique. The resulting nanoparticles loaded with UFH were between 162 and 217 nm in size, and those prepared with LMWH were 152 nm. The zeta potential of the nanoparticle formulations ranged from +28.1 to +34.6 mV, and in selected nanosystems both types of heparin were associated with a high degree of efficiency, which was approximately 70%. The nanosystems were stable in phosphate buffered saline (PBS), pH 7.4, for at least 24h, and released 10.8% of UFH and 79.7% of LMWH within 12h of incubation. Confocal microscopy experiments showed that fluorescent heparin-loaded CS-HA nanoparticles were effectively internalized by rat mast cells. *Ex vivo* experiments aimed at evaluating the capacity of heparin to prevent histamine release in rat mast cells indicated that the free or encapsulated drug exhibited the same dose-response behavior.

Oyarzun-Ampuero *et al.* further studied a nanomedicine consisting of heparin-loaded polysaccharide nanocarriers for the treatment of asthma [614, 615]. This nanomedicine consisted of chitosan/carboxymethyl-β-cyclodextrin loaded with unfractioned or low-molecular-weight heparin is described and its potential in asthma treatment is evaluated. nanoparticles are prepared by ionotropic gelation showing a size that between 221 and 729 nm with a positive zeta potential. The drug association efficiency is higher than 70%. Developed nanosystems are stable in Hank's balanced salt solution at pH = 6.4, releasing the drug slowly. *Ex vivo* assays show that nanocarriers lead to an improvement of heparin preventing mast cell degranulation. These results agree with the effective cellular internalization of the fluorescently labeled nanocarriers, and suggest these nanomedicines as promising formulations for asthma treatment.

In 2008, a study of the effect of chitosan microparticles loaded with major epitope peptide of mite group 2 allergen Der f 2 from Dermatophagoides farinae (Der f 2-47-67) in alleviating asthma in mice was published [616]. Li *et al.* injected Derf2-47-67 entrapped chitosan nanoparticles intraperitoneally into BALB/c mice prior to an intranasal challenge with a Der f extract allergen [616]. Airway hyperreactivity was measured via whole-body plethysmography, and bronchoalveolar lavage (BAL) fluid was collected to calculate total cell and eosinophil counts. Changes in lung histology were assessed after hematoxylin-eosin staining, and serum levels of Der f-specific immunoglobulin (Ig) G2a and IgE were determined by enzyme-linked immunoabsorbent assay. Mice immunized with Der f 2-47-67-loaded chitosan microparticles displayed decreased
airway hyperreactivity, reduced numbers of eosinophils in BAL fluid, alleviated lung inflammation and mucus production, a reduced serum level of Der f-specific IgE and an increased serum level of Der f-specific IgG2a. This data showed that Der f 2-47-67-loaded chitosan microparticles inhibited airway allergic inflammation. Liu et al. tested immunotherapeutic efficiency by intranasal administration of Der f entrapped in CS microparticles to sensitized mice [617]. BALB/c mice were sensitized intraperitoneally with Der f extract absorbed to alum, followed by intranasal treatment with PBS, CS, Der f or Der f-CS nano-vaccine for 6 weeks. The mice were subsequently challenged intranasally with Der f extract for 1 week, and we analyzed their clinical symptoms, antibody expression levels, cytokine levels, T cell proliferation and regulatory T cell numbers. Mice treated with intranasal Der f-CS nano-vaccine prior to challenge displayed an alleviated spectrum of symptoms including airway hyper-reactivity, lung inflammation and mucus production and had fewer eosinophilic cells in bronchoalveolar lavage fluid (BALF). Interestingly, the cytokine levels in Der f-specific IgE were reduced, but IgA in serum and BALF was increased. The authors also observed that IL-4 was reduced and IFN-gamma and IL-10 were increased among splenocytes and in BALF, which inhibits Der f-specific T-cell proliferation in splenocytes and increases regulatory T cells in the spleen. However, the mice challenged without intranasal Der f or Der f-CS vaccine treatment developed allergic asthma. These results illustrated that intranasal administration of Der f-CS nano-vaccine plays roles in immunologic protection in murine allergic asthma by inducing regulatory T cells and Th1-type reaction.

The therapeutic effect of sublingual use of Dermatophagoides farinae/chitosan nanoparticle vaccine on murine asthma was studied by Yu et al. [618]. The objective of this study was to prepare Dermatophagoides farinae (Der f)/chitosan nanoparticle vaccine (DCN), and to investigate the effect of sublingual administration with DCN in asthma mice model. DCN were prepared by ionotropic gelation. 30 BALB/c mice were randomly divided into 5 groups: normal control group (A), PBS control group (B), Chitosan group (C), Der f group (D), DCN group (E). Group A were treated with normal saline (100 microl) all the time. Mice in other groups were sensitized intraperitoneally with 50 microg dust mite extracts plus 2 mg Al(OH)3, and on day 28 given a sublingual vaccination of PBS (group B), or empty CS nanoparticles (group C), or Der f (group D, 1 mg Der f) or DCN (group E, loaded with 1 mg Der f). All the mice received 18 doses at 1-day intervals. One week after the last immunization, mice in group B, C, D, and E were intranasally
challenged with 50 microg Der f extract daily for seven days. Twenty-four hours after the last challenge, AHR was assessed by using whole-body plethysmography. Two days post challenge, mice were sacrificed and BALF was collected. Number of the total cells and eosinophils was determined. Level of cytokines in the supernatant of splenocyte culture was assayed by ELISA. Level of Der f specific IgE, IgG2a and IgA in the sera was determined by ELISA. Airway inflammation was analyzed by HE staining. Spleen lymphocyte proliferation responses were analyzed by MTT colorimetry. Compared with group B, AHR and the lung inflammation in groups D and E were greatly reduced. Numbers of total cells and eosinophils in BALF of groups D and E were significantly lower than that of group B. The level of specific IgE was significantly lower in groups D and E, and that of IgA in groups D and E was significantly higher than that in group B. The level of IL-4 in BALF and cultured splenocytes of groups D and E was significantly lower than that of group B. While IFN-gamma and IL-10 in BALF in groups D and E were significantly higher than that of group B, and same with IFN-y and IL-10 in spleen cultured supernatants of groups D and E. The allergen-specific splenocyte proliferation was inhibited in groups D and E, and there was no significant difference between group C and group B. Dermatophagoides farinae (Der f)/chitosan nanoparticle vaccine has therapeutic effect on murine asthma.

Respiratory syncytial virus (RSV) causes severe bronchiolitis and is a risk factor for asthma. Since there is no commercially available vaccine against RSV, a short interfering RNA against the RSV-NS1gene (siNS1) was developed and its potential for decreasing RSV infection and infection-associated inflammation in rats was tested by Kong et al. 2007 [619]. Plasmids encoding siNS1 or an unrelated siRNA were complexed with a chitosan nanoparticle delivery agent and administered intranasally. Control animals received a plasmid for a non-specific siRNA. After expression of the plasmid in lung cells for 24 hours, the rats were intranasally infected with RSV. Prophylaxis with siNS1 significantly reduced lung RSV titers and airway hyperreactivity to methacholine challenge compared to the control group. Lung sections from siNS1-treated rats showed a sizable reduction in goblet cell hyperplasia and in lung infiltration by inflammatory cells, both characteristics of asthma. Also, bronchoalveolar lavage samples from siNS1-treated animals had fewer eosinophils. Treatment of rats with siNS1 prior to RSV exposure was effective in reducing virus titers in the lung and in preventing the inflammation and airway hyperresponsiveness associated with the infection that has been linked to development of asthma.
The use of siNS1 prophylaxis may be an effective method for preventing RSV bronchiolitis and potentially reducing the later development of asthma associated with severe respiratory infections.

### 3.2.4.1.6. Other Polymers

A study by Ballester et al. found that pluronic-stabilized poly(propylene sulfide) nanoparticle conjugation enhances the immunomodulatory effects of intranasally delivered CpG in house dust mite-allergic mice [620]. The emerging strategy in preventing and treating airway allergy consists of modulating the immune response induced against allergens in the lungs. CpG oligodeoxynucleotides have been investigated in airway allergy studies, but even if promising, efficacy requires further substantiation. The effect of pulmonary delivery of nanoparticle (NP)-conjugated CpG on lung immunity it was found that NP-CpG led to enhanced recruitment of activated dendritic cells and to Th1 immunity compared to free CpG. We then evaluated if pulmonary delivery of NP-CpG could prevent and treat house dust mite-induced allergy by modulating immunity directly in lungs. When CpG was administered as immunomodulatory therapy prior to allergen sensitization, we found that NP-CpG significantly reduced eosinophilia, IgE levels, mucus production and Th2 cytokines, while free CpG had only a moderate effect on these parameters. The NP-CpG pre-treatment before HDM sensitization and challenge reduced the mucus production in the lungs of allergic mice, as shown in Figure 3.5. In a therapeutic setting where CpG was administered after allergen sensitization, although both free CpG and NP-CpG reduced eosinophilia and IgE levels to the same extent, NP conjugation of CpG significantly enhanced reduction of Th2 cytokines in lungs of allergic mice. Taken together, these data highlight benefits of NP conjugation and the relevance of NP-CpG as allergen-free therapy to modulate lung immunity and treat airway allergy.
Figure 3.5. Pre-treatment with NP-CpG reduces mucus production in lungs of allergic mice. Mice were pre-treated with CpG and then sensitized and challenged with HDM. (A) Representative periodic acid-Schiff (PAS)-stained lung sections showing mucus-producing goblet cells (dark magenta) within bronchi (scale bar, 100 µm). (B) Quantification of mucus-producing goblet cells per unit length. (C) Representative PAS-stained lung sections showing leukocyte accumulation (blue) around blood vessels (Scale bar, 100 µm). (D) Inflammation score, defined as area of leukocyte infiltration and normalized to the average values in naïve mice. Data shows mean ± SEM from three independent experiments, 14 mice per group (5 mice in naïve group). *P < 0.05, **P < 0.01; ##P < 0.01 compared to naïve mice. Reprinted with permission from Ballester et al. 2015 under Creative Commons Attribution 4.0 International License (CC BY 4.0) [620].

The most promising treatments for asthma are based on specific immunotherapies, but they lack efficiency and can induce deleterious side effects. Among new modalities of immunotherapy currently in development, DNA vaccination presents a promising approach, as it enables targeted immunotherapy in association with reduced allergenicity. Beilvert et al. have developed an innovative, DNA-based vaccine against Dermatophagoides farinae 1 allergen (Der f 1), one of the allergens most commonly encountered by asthma patients in Europe [621]. Intramuscular administration of a Der f 1-encoding plasmid formulated with the block copolymer 704 in healthy mice induced a strong humoral and cellular response with a pro-helper T cell type 1 bias. Administration of the same formulation in asthmatic mice, according to an early vaccination protocol, led to a reduction of airway hyperresponsiveness and a significant decrease in the level of inflammatory cytokines in the bronchoalveolar lavage of Der f 1-vaccinated mice.
Yoo et al. reported a new family of fully biodegradable hydroxybenzyl alcohol (HBA)-incorporated polyoxalate (HPOX) as a novel therapeutics of airway inflammatory diseases [622]. HPOX was designed to incorporate antioxidant and anti-inflammatory HBA and peroxalate ester linkages capable of reacting with hydrogen peroxide (H2O2) in its backbone. HPOX nanoparticles exhibited highly potent antioxidant and anti-inflammatory effects by scavenging H2O2, reducing the generation of intracellular oxidative stress and suppressing the expression of pro-inflammatory mediators such as inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and interleukin (IL)-1β in stimulated macrophages. The potential of HPOX nanoparticles as an anti-asthmatic agent was evaluated using a murine model of asthma. Intratracheal administration of HPOX nanoparticles remarkably reduced the recruitment of inflammatory cells and expression of pro-inflammatory mediators such as IL-4 and iNOS. Based on their excellent antioxidant, anti-inflammatory and anti-asthmatic activities, HPOX nanoparticles have great potential as therapeutics and drug carriers for the treatment of airway inflammatory diseases such as asthma.

Although inhaled steroids are the treatment of first choice to control asthma, administration of systemic steroids is required for treatment of asthmatic exacerbation and intractable asthma. To improve efficacy and reduce side effects, Matsuo et al. examined the effects of betamethasone disodium phosphate encapsulated in biocompatible, biodegradable blended nanoparticles (stealth nanosteroids) on a murine model of asthma [623]. These stealth nanosteroids were found to accumulate at the site of airway inflammation and exhibit anti-inflammatory activity. Significant decreases in BALF eosinophil number were maintained for 7 days with a single injection of nanosteroids containing 40 microg betamethasone disodium phosphate. Airway responsiveness was also attenuated by the injection of stealth nanosteroids. A single injection of 40 microg of free betamethasone disodium phosphate and 8 µg of free betamethasone disodium phosphate once daily for 5 days did not show any significant effects. The authors concluded that stealth nanosteroids achieve prolonged and higher benefits at the site of airway inflammation compared to free steroids.

Despite wide exploitation of corticosteroid drugs for the treatment of asthma, the poor therapeutic effect on a neutrophilic subtype of asthma prohibits the full recovery of asthma patients. In work conducted by Kim et al., dexamethasone (Dexa) was loaded in Flt1 peptide-hyaluronic acid (HA) conjugate nanoparticles to overcome the limitation of corticosteroid resistance for the treatment of neutrophilic pulmonary inflammation [624]. Flt1 peptide-HA conjugates are self-assembled to nanoparticles because of hydrophobic Flt1 peptide conjugated to
HA by benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) chemistry. HA receptors include CD44, LYVE-1 and HARE are capable of binding specifically to HA [625, 626]. CD44 positive epithelial cells have been confirmed to be located within asthmatic lung epithelium [627]. *In vitro* bioimaging showed efficient internalization of Flt1 peptide-HA conjugate nanoparticles into lung epithelial cells by CD44-receptor mediated endocytosis. Also, *ex vivo* imaging for the biodistribution in ICR mice revealed long-term retention of Flt1 peptide-HA conjugate nanoparticles in deep lung tissues possibly due to mucoadhesive property of HA. On the basis of bioimaging results for pulmonary drug delivery applications, we prepared Dexa-loaded Flt1 peptide-HA conjugate nanoparticles. Transmission electron microscopy (TEM) and dynamic light scattering (DLS) confirmed the formation of nanoparticles, which reduced cytokine levels of lipopolysaccharide (LPS)-stimulated cells more efficiently than free Dexa. Furthermore, according to the bronchoalveolar lavage (BAL) cellularity and histological analysis, Dexa loaded Flt1 peptide-HA conjugate nanoparticles showed remarkable therapeutic effects in both eosinophilic and neutrophilic asthma model mice.

A different biopolymer that is used for nucleic acid delivery and that has received attraction for pulmonary delivery is atelocollagen, a highly purified and pepsin-treated type I collagen obtained from calf dermis. Liu *et al.* formed polyplexes between the protein and siRNA by mixing and incubating for 16 h before they were administered i.t. to Wistar or EGFP expressing rats [563]. As a therapeutic gene, syntaxin4, one of the soluble membrane N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins was chosen as a novel target that regulates the function of mucosal-type mast cells. After it was shown that syntaxin4 knockdown with siRNA significantly decreases the release of granule contents from rat mucosal mast cells, rats were sensitized and challenged with ovalbumin (OVA) to establish inflammation-associated symptoms of asthma. The rats were then treated i.t. with siRNA/atelocollagen complexes once a day for 5 days which decreased the levels of rat mast cell protease-II (RMCPII) in the BALF, and asthmatic airway constriction was prevented. The authors therefore concluded that syntaxin4 knockdown stabilizes mucosal mast cells [563].

Protein-cage nanoparticles are promising multifunctional platforms for targeted delivery of imaging and therapeutic agents owing to their biocompatibility, biodegradability, and low toxicity. The major advantage of protein-cage nanoparticles is the ability to decorate their surfaces with multiple functionalities through genetic and chemical modification to achieve desired
properties for therapeutic and/or diagnostic purposes. Specific peptides identified by phage display can be genetically fused onto the surface of cage proteins to promote the association of nanoparticles with a particular cell type or tissue. Upon symmetrical assembly of the cage, peptides are clustered on the surface of the cage protein in bunches. The resulting PBNC (peptide bunches on nanocage) offers the potential of synergistically increasing the avidity of the peptide ligands, thereby enhancing their blocking ability for therapeutic purposes. Jeon et al. demonstrated a proof-of-principle of PBNCs, fusing the interleukin-4 receptor (IL-4R)-targeting peptide, AP-1, identified previously by phage display, with ferritin-L-chain (FTL), which undergoes 24-subunit assembly to form highly stable AP-1-containing nanocage proteins (AP1-PBNCs). AP1-PBNCs bound specifically to the IL-4R-expressing cell line, A549, and their binding and internalization were specifically blocked by anti-IL-4R antibody. AP1-PBNCs exhibited dramatically enhanced binding avidity to IL-4R compared with AP-1 peptide, measured by surface plasmon resonance spectroscopy. Furthermore, treatment with AP1-PBNCs in a murine model of experimental asthma diminished airway hyper-responsiveness and eosinophilic airway inflammation along with decreased mucus hyperproduction. These findings hold great promise for the application of various PBNCs with ligand-specific peptides in therapeutics for different diseases, such as cancer.

A novel stabilized aggregated nanogel particle (SANP) drug delivery system was prepared by Deshmukh et al. for injectable passive lung targeting. Gel nanoparticles (GNPs) were synthesized by irreversibly cross-linking 8 Arm PEG thiol with 1,6-hexane-bis-vinylsulfone (HBVS) in phosphate buffer (PB, pH 7.4) containing 0.1% v/v Tween™ 80. Aggregated nanogel particles (ANPs) were generated by aggregating GNPs to micron-size, which were then stabilized (i.e., SANPs) using a PEG thiol polymer to prevent further growth-aggregation. The size of SANPs, ANPs and GNPs was analyzed using a Coulter counter and transmission electron microscopy (TEM). Stability studies of SANPs were performed at 37°C in rat plasma, phosphate buffered saline (PBS, pH 7.4) and PB (pH 7.4). SANPs were stable in rat plasma, PBS and PB over 7 days. SANPs were covalently labeled with HiLyte Fluor™ 750 (DYE-SANPs) to facilitate ex vivo imaging. Biodistribution of intravenous DYE-SANPs (30 μm, 4 mg in 500 μL PBS) in male Sprague-Dawley rats was compared to free HiLyte Fluor™ 750 DYE alone (1mg in 500 μL PBS) and determined using a Xenogen IVIS® 100 Imaging System. Biodistribution studies demonstrated that free DYE was rapidly eliminated from the body by renal filtration, whereas
DYE-SANPs accumulated in the lung within 30 min and persisted for 48 h. DYE-SANPs were enzymatically degraded to their original principle components (i.e., DYE-PEG-thiol and PEG-VS polymer) and were then eliminated from the body by renal filtration. Histological evaluation using H & E staining and broncho alveolar lavage (BAL) confirmed that these flexible SANPs were not toxic. This suggests that because of their flexible and non-toxic nature, SANPs may be a useful alternative for treating pulmonary diseases such as asthma, pneumonia, tuberculosis and disseminated lung cancer.

A reduction of oxidative stress by p-hydroxybenzyl alcohol-containing biodegradable polyoxalate nanoparticulate antioxidant was observed by Kim et al. [629]. The large production of reactive oxygen species (ROS) leads to the oxidative stress and the subsequent functional decline of organ systems. p-Hydroxybenzyl alcohol (HBA) is known to inhibit oxidative stress. We have developed biodegradable antioxidant copolyoxalate, in which HBA is chemically incorporated into its backbone for the treatment of oxidative stress-related diseases. HBA-incorporated copolyoxalate (HPOX) was designed to possess aromatic peroxalate ester linkages in its backbone and release HBA during its hydrolytic degradation. Peroxalate ester linkages in the backbone reacted with and scavenged hydrogen peroxide, leading the release of HBA in vitro. HBA released from HPOX exerted excellent antioxidant activity, such as inhibition of nitric oxide (NO) production by suppressing iNOS (inducible nitric oxide synthases) expression in lipopolysaccharide (LPS)-activated RAW 264.7 cells. HPOX nanoparticles delivered intranasally significantly reduced pulmonary inflammation and suppressed the iNOS expression. Given their excellent antioxidant and anti-inflammatory activities, HPOX nanoparticles maybe highly effective in the treatment of asthma, or other oxidative damage-related diseases.

In an effort to selectively deliver siRNA to activated T cells, Xie et al. had formulated transferrin-polyethylenimine (Tf-PEI) as a pulmonary delivery system of siRNA [630]. The authors utilized siRNA gene downregulation capabilities to silence inflammation related genes in activated T cells, such as Th2 cells, to lessen their effect on airway inflammation and inflammatory cell chemoattraction. The polyplexes which had optimal physicochemical properties, had shown significantly increased cell internalization and gene downregulation compared to non-targeted controls. The polyplexes delivered in a murine asthmatic model displayed adequate biodistribution for delivery to activated T cells involved in asthma [630].
3.2.4.2. Lipid-Based Nanoparticles

Lipid-based delivery systems are commonly used to deliver siRNA \textit{in vitro} or \textit{in vivo} [631]. Most often, cationic lipids or liposomes are used to form complexes, termed lipoplexes with anionic siRNA through spontaneous electrostatic interaction. Commercial siRNA transfection agents are commonly lipid-based systems, including Oligofectamin™, TransIT-TKO, Lipofectamine® RNAiMAX, and DharmaFECT [632-635]. A major challenge of using lipid-based siRNA delivery systems is their toxicity and non-specific activation of inflammatory cytokines and interferon responses [636]. Since aerosolization is a high shear stress process, the stability of the liposomes should be monitored since this process may cause physical and chemical changes that can lead to early siRNA release and degradation of the siRNA [637]. Lipid based delivery systems for siRNA include liposomes, solid lipid nanoparticles and nanostructured lipid carriers.

A study conducted by Lisitsa \textit{et al.} found that a significant positive effects of a phospholipid nanoparticle therapy on the dynamics of the main parameters of oxidative stress including reduced concentration of nitric oxide metabolites and increased total antioxidative activity of plasma without any clinically significant reactions [638]. This study consisted of a simple blind randomized placebo-controlled study for the evaluation of dynamics of biomarkers of oxidative stress, which was the total concentration of nitrate- and nitrite-anions in condensed exhaled breath and plasma, pH of exhaled breath, total antioxidative activity of plasma in patients with bronchial asthma inhaling phospholipid nanoparticles. The same group conducted a single-blind placebo-controlled study of the efficacy and safety of inhalation of phospholipid nanoparticles in patients with bronchial asthma [639]. They found a statistically significant positive effect of the proposed therapeutic modality on the clinical status of the patients and lung function tests, without clinical significant adverse events [639].

3.2.4.2.1. Liposomes

Liposomes have been used extensively for drug delivery since Alec Bandham first described liposomes more than 50 years ago [640]. Due to their relatively straightforward preparation and their excellent biodegradability and biocompatibility, liposomal systems have progressed into one of the most extensively used and most clinically advanced drug delivery platform. Liposomes are composed of phospholipids, which due to their amphipathic nature; spontaneously self-assemble into vesicular structures when dispersed in aqueous media. In these
lipid vesicles, the hydrophilic head groups line up and face the outer aqueous environment, while another layer of polar heads face the aqueous interior, segregating the hydrophobic tail groups of both layers from the aqueous environment.

Several studies have demonstrated that the employment of a drug delivery platform for inhaled glucocorticoid therapy in asthma may have a direct and distinct pulmonary effect with reduced side effects [641, 642]. By delivering liposomes locally to the lungs for asthma therapy, an optimized pulmonary residence time of the glucocorticoid is achieved by increasing lung deposition and decreasing upper respiratory tract retention, while drug redistribution to non-target tissues are reduced [643, 644]. One of the first clinical strides involving liposomal glucocorticoids in asthma evaluated the use of nebulizers to administer dilauroyl phosphatidylcholine (DLPC) liposomes containing beclomethasone dipropionate (Bec-DP) [645]. Using 18 different types of nebulizers, the local lung deposition efficiency of liposomes in the diameter of 1-3 µm was evaluated. While the majority of nebulizers were able to provide acceptable performance for delivering Bec-DP liposomes, only two of them, i.e. Aerotech II and Spira, achieved high localization in alveolar airways, and relatively low deposition in mouth and throat. The lung deposition and clearance of 99mTechnetium-labeled Bec-DLPC liposomes was visualized and quantified in a follow-up study [646]. These experiments showed that~75% of the inhaled liposomes were in the pulmonary tract, ~12% in the nasopharynx, and ~13% in the stomach and intestine. Although free 99mTc was cleared within minutes, ~50% of the liposome-associated radioactivity was still found to be present in the lungs 24h after inhalation, indicating a substantially prolonged retention of radio labeled liposomes in the lungs. Also in healthy human volunteers, a strong deposition in the lungs and oropharynx was observed upon using the Aerotech II and Spira nebulizer. Once deposited, a large proportion of inhaled radiolabeled liposomes remained in the lung for >3 hr [646]. The clearance levels differed between the two nebulizers, likely because of aerosol particle size (much larger in the case of the Spira, resulting in less deep and homogenous deposition, and faster mucociliary clearance. No significant side effects, either local or systemic, were observed upon assessing the tolerability of Bec-DLPC liposomal aerosol formulation in healthy volunteers, in spite of efficient deposition and distribution [647]. Also in patients, the inhalation of Bec-DLPC liposomes resulted in a beneficial distributional pattern of high overall localization in the lungs, but moderate to low deposition in the upper respiratory tract (i.e. the oropharynx, mouth, and throat) and in the gastrointestinal (GI) tract [648]. In the case of
severe asthma, as compared to mild asthma, there was some increased clearance and heterogeneous deposition, but in both groups, more than half of the dose was still present in the lung 1 day after administration [649]. A similar study using DPPC liposomes showed 88% lung deposition at 6 h after aerosol inhalation, suggesting that a single daily dose of inhaled liposomal glucocorticoids might be enough for proper therapeutic efficacy [650].

In some cases, when moderate inhaled glucocorticoid administration is ineffective, its combination with a long-lasting β2-agonist, such as formoterol, provides better therapeutic outcome than just using higher inhaled glucocorticoid doses [651]. Similarly, the co-administration of formoterol with 99mTc-labelled Bec-DLPC liposomes significantly improved the liposomal localization and therapeutic activity, as measured by spirometry, of pulmonary administered glucocorticoid liposomes in asthma. Although formoterol could potentially stimulate liposomal clearance (since β2 agonists are known to improve mucociliary clearance both in vitro and in vivo; particularly in patients with bronchitis, the pulmonary retention of the 99mTc-labelled Bec-DLPC liposomes remained unchanged by formoterol therapy [652, 653].

PEGylated liposomal aerosols (e.g. containing budesonide) have also been evaluated. Weekly administration of budesonide loaded PEGylated liposomes resulted in a similar efficacy as equal daily doses of free budesonide [654]. Interestingly, the therapeutic efficacy of weekly administered budesonide-encapsulating conventional liposomes was much lower. Moreover, the budesonide-loaded PEG-liposomes induced an effective decrease in serum eosinophil peroxidase activity (EPO, an eosinophilic activation marker in asthma), while the other treatments tests, including non-PEGylated liposomal budesonide, failed to demonstrate an effect. This positive contribution of PEGylation on the efficacy of pulmonary administered liposomal aerosol formulation seems to be related to their improved physicochemical stability, such as less aggregation or less opsonization, as compared to non-PEGylated liposomes [274].

Youngren et al. developed and evaluated freeze-dried manniosylated liposomes for the m-cell targeted oral delivery of selenium to the immune system for antioxidant effects [655]. Dipalmitoylphosphatidylcholine, distearoylphosphatidylglycerol, and cholesterol were dissolved in a chloroform and methanol mixture and allowed to form a thin film within a rotatory evaporator. This thin film was hydrated with a sodium selenite (5.8 μM) solution to form multilamellar vesicles and homogenized under high pressure to yield unilamellar nanoliposomes. Se-loaded nanoliposomes were manniosylated by 0.1% w/v mannosamine (Man-Lip-Se) prior to being
lyophilized. Mannosamine concentration was optimized with cellular uptake studies in M receptor expressing cells. Non-lyophilized and lyophilized Man-Lip-Se were characterized for size, zeta potential, and entrapment efficiency. The influence of liposomal composition on the characteristics of Man-Lip-Se were evaluated using acidic and basic medium for 24 h. Thermal analysis and powder X-ray diffraction were used to determine the interaction of components within the Man-Lip-Se. The size, zeta potential and entrapment efficiency of the optimum Man-Lip-Se were observed to be 158 ± 28.9 nm, 33.21 ± 0.89 mV, and 77.27 ± 2.34%, respectively. An in vitro Se release of 70-75% up to 24 h in PBS pH 6.8 and <8% Se release in acidic media (0.1 N HCl) in 1 h was observed. The Man-Lip-Se were found to withstand gastric-like environments and showed sustained release. Stable freeze-dried Man-Lip-Se were successfully formulated with a size of <200 nm, ≈ 75% entrapment, and achieved controlled release of Se with stability under acidic media, which may be of importance in the targeted delivery of Se to the immune system for asthma selenium supplementation.

The severity of allergic asthma is dependent, in part, on the intensity of peribronchial inflammation. P-selectin is known to play a role in the development of allergen-induced peribronchial inflammation and airway hyperreactivity. John et al. demonstrated that nanoparticles displaying P-selectin blocking arrays were functionally active in vivo, significantly reducing allergen-induced airway hyperreactivity and peribronchial eosinophilic inflammation in a murine model of asthma [656]. Selective inhibitors of P-selectin-mediated leukocyte endothelial-cell interactions may therefore attenuate the inflammatory processes associated with allergic airway disease. In a study by John et al., novel P-selectin inhibitors were created using a polyvalent polymer nanoparticle capable of displaying multiple synthetic, low molecular weight ligands [656]. By assembling a particle that presents an array of groups, which as monomers interact with only low affinity, we created a construct that binds extremely efficiently to P-selectin. The ligands acted as mimetics of the key binding elements responsible for the high-avidity adhesion of P-selectin to the physiologic ligand, PSGL-1. The inhibitors were initially evaluated using an in vitro shear assay system in which interactions between circulating cells and P-selectin-coated capillary tubes were measured. The nanoparticles were shown to preferentially bind to selectins expressed on activated endothelial cells.
3.2.4.2.2. Solid Lipid Nanoparticles and Nanostructured Lipid Carriers

Solid Lipid Nanoparticles (SLN) are prepared by replacing the oil of the fat emulsion by a solid lipid or a blend of solid lipids, which makes the lipid matrix of the SLN solid at room and body temperature [657-660]. SLN are composed of 0.1-30% w/w lipid dispersed in an aqueous solution of 0.5-5% w/w surfactant as a stabilizing agent [661, 662]. SLN provide physical stability, chemical stability, controlled release, and low cytotoxicity if appropriate excipients are utilized [663]. Production of SLN can be carried out without the use of organic solvents and can be scaled up. Disadvantages of SLN are low loading capacity and premature release during storage [664]. These events occur because the low ordered lipid modification of the particle matrix after production transforms to the highly ordered β-modification during storage. The β-modification is characterized by perfect crystal lattice with few imperfections and therefore, little room is left for siRNA storage. In order to overcome these issues, the second generation of lipid nanoparticles, known as Nanostructured Lipid Carriers (NLC) were developed. NLC have a solid lipid matrix at room and body temperature and consists of a blend of solid lipid and oil, preferably at a ratio of 70:30 up to a ratio of 99.9:0.1 [664]. Through mixing different kinds of lipids, a less ordered matrix with more room for active compounds is achieved [664].

Curcumin has shown considerable pharmacological activity, including anti-inflammatory properties, but its poor bioavailability and rapid metabolization have limited its application. Wang et al. formulated curcumin-solid lipid nanoparticles (curcumin-SLNs) to improve its therapeutic efficacy in an ovalbumin (OVA)-induced allergic rat model of asthma [665]. This studies observations implied that curcumin-SLNs could be a promising candidate for asthma therapy. A solvent injection method was used to prepare the curcumin-SLNs. Physiochemical properties of curcumin-SLNs were characterized, and release experiments were performed in vitro. The pharmacokinetics in tissue distribution was studied in mice, and the therapeutic effect of the formulation was evaluated in the model. The prepared formulation showed an average size of 190 nm with a zeta potential value of -20.7 mV and 75% drug entrapment efficiency. X-ray diffraction analysis revealed the amorphous nature of the encapsulated curcumin. The release profile of curcumin-SLNs was an initial burst followed by sustained release. The curcumin concentrations in plasma suspension were significantly higher than those obtained with curcumin alone. Following administration of the curcumin-SLNs, all the tissue concentrations of curcumin increased, especially in lung and liver. In the animal model of asthma, curcumin-SLNs effectively
suppressed airway hyperresponsiveness and inflammatory cell infiltration and also significantly inhibited the expression of Th2 cytokines, such as IL4 and IL13, in bronchoalveolar lavage fluid compared to the asthma group and curcumin-treated group.

Patil-Gadhe and Pokharkar developed a montelukast-loaded nanostructured lipid carrier formulation that improved the systemic bioavailability, avoided hepatic metabolism, and to reduced hepatic cellular toxicity due to metabolites of the conventional montelukast delivery [666]. Montelukast loaded NLC was prepared using melt-emulsification-homogenization method. Preformulation study was carried out to evaluate drug-excipient compatibility. MNLCs were prepared using spatially different solid and liquid lipid triglycerides. CAE (DL-Pyrrolidonecarboxylic acid salt of L-cocyl arginine ethyl ester), a cationic, biodegradable, biocompatible surfactant was used to stabilize the system. MNLCs were characterized by FTIR, XRPD and DSC to evaluate physicochemical properties. MNLCs having a particle size of 181.4 ± 6.5 nm with encapsulation efficiency of 96.13 ± 0.98% were prepared. FTIR findings demonstrated no interaction between the drug and excipients of the formulation which could lead to asymmetric vibrations. DSC and XRPD study confirmed stable amorphous form of the montelukast in lipid matrix. In vitro release study revealed sustained release over a period of 24 h. In vivo single dose oral pharmacokinetic study demonstrated 143-fold improvement in bioavailability as compared to montelukast-aqueous solution.

In a follow-up evaluation by Patil-Gadhe et al., montelukast loaded nanostructured lipid carrier (MNLC) for pulmonary application demonstrated the potential of montelukast lipidic nanoparticulate formulation to improve the efficacy with reduced toxicity leading to better performance of drug as MNLC-DPI for inhalation use [667]. The formulated nanoparticles were evaluated in vitro for aerodynamic characterization and in vivo for pulmonary pharmacokinetics in Wistar rats. The in vitro cytotoxicity was performed on A549 cell line and compared with montelukast-aqueous solution. MNLC was prepared with montelukast (0.2%), Precirol ATO5 (solid lipid), and Capryol-90 (liquid lipid) in the ratio of 7:3 using melt-emulsification-homogenization method. dl-Pyrrolidonecarboxylic acid salt of l-cocyl arginine ethyl ester (CAE), a biodegradable surfactant in the concentration of 1% was used to stabilize the nanoparticles. The particle size and encapsulation efficiency (EE) were 184.6 ± 2.7 nm and >95%, respectively. MNLC-Dry powder for inhalation (DPI) was prepared by lyophilization using 3% mannitol as cryoprotectant and carrier. MNLC-DPI was evaluated for flow, crystallographic and thermal
properties. Mass median diameters (MMD) and density for MNLC-DPI were found to be $15.1 \pm 1.4 \, \mu m$ and $0.051 \pm 0.002 \, g/cc$, respectively. *In vitro* aerosol performance study indicated more than 95% of the emitted dose (ED) at both the flow rates studied. Mass median aerodynamic diameters (MMAD) of $3.24 \pm 0.67 \, \mu m$ with $69.98 \pm 1.9\%$ fine particle fraction (FPF) were obtained at 30 L/min flow rate, whereas at 60 L/min MMAD and FPF were found to be $2.83 \pm 0.46 \, \mu m$ and $90.22 \pm 2.6\%$, respectively. *In vitro* cytotoxicity study on A549 cells revealed higher safety of MNLC than pure drug. The pulmonary pharmacokinetic study demonstrated improved bioavailability, longer residence of drug in the lung and targeting factor of 11.76 for MNLC as compared to montelukast-aqueous solution.

### 3.2.4.2.2. Lipid Nanomicelles

Rehydrated, sterically stabilized, phospholipid nanomicelles of budesonide for nebulization was characterized for their physicochemical, *in vitro*, and *in vivo* characteristics [668]. Inhaled corticosteroids provide unique systems for local treatment of asthma or chronic obstructive pulmonary disease. However, the use of poorly soluble drugs for nebulization has been inadequate, and many patients rely on large doses to achieve optimal control of their disease. Theoretically, nanotechnology with a sustained-release formulation may provide a favorable therapeutic index. The aim of this study was to determine the feasibility of using sterically stabilized phospholipid nanomicelles of budesonide for pulmonary delivery via nebulization. PEG(5000)-DSPE phospholipid micelles containing budesonide (BUD-SSMs) were prepared by the co-precipitation and reconstitution method, and the physicochemical and pharmacodynamic characteristics of BUD-SSMs were investigated. The optimal concentration of solubilized budesonide at 5 mM PEG(5000)-DSPE was $605.71 \pm 6.38 \, \mu g/mL$, with a single-sized peak population determined by photon correlation spectroscopy and a particle size distribution of $21.51 \pm 1.5 \, nm$. The zeta potential of BUD-SSMs was $-28.43 \pm 1.98 \, mV$. The percent entrapment efficiency, percent yield, and percent drug loading of the lyophilized formulations were $100.13\% \pm 1.09\%$, $97.98\% \pm 1.95\%$, and $2.01\% \pm 0.02\%$, respectively. Budesonide was found to be amorphous by differential scanning calorimetry, and had no chemical interaction with PEGylated polymer according to Fourier transform infrared spectroscopy. Transmission electron microscopic images of BUD-SSMs revealed spherical nanoparticles. BUD-SSMs exhibited prolonged dissolution behavior compared with Pulmicort Respules ($P < 0.05$). Aerodynamic characteristics indicated significantly higher deposition in the lungs compared with Pulmicort Respules. The mass median aerodynamic,
geometric standard deviation, percent emitted dose, and the fine particle fraction were 2.83 ± 0.08 μm, 2.33 ± 0.04 μm, 59.13% ± 0.19%, and 52.31% ± 0.25%, respectively. Intratracheal administration of BUD-SSMs 23 hours before challenge (1 mg/kg) in an asthmatic/chronic obstructive pulmonary disease rat model led to a significant reduction in inflammatory cell counts (76.94 ± 5.11) in bronchoalveolar lavage fluid compared with administration of Pulmicort Respules (25.06 ± 6.91). The BUD-SSMs system might be advantageous for asthma or chronic obstructive pulmonary disease and other inflammatory airway diseases.

The local treatment of lung disorders such as asthma and chronic obstructive pulmonary disease via pulmonary drug delivery offers many advantages over oral or intravenous routes of administration. This is because direct deposition of a drug at the diseased site increases local drug concentrations, which improves the pulmonary receptor occupancy and reduces the overall dose required, therefore reducing the side effects that result from high drug doses. From a clinical point of view, although jet nebulizers have been used for aerosol delivery of water-soluble compounds and micronized suspensions, their use with hydrophobic drugs has been inadequate. Sahib et al, set forth to evaluate the feasibility of sterically stabilized phospholipid nanomicelles (SSMs) loaded with beclomethasone dipropionate (BDP) as a carrier for pulmonary delivery [669]. The SSM system might be an effective way of improving the therapeutic index of nebulized, poorly soluble corticosteroids.1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-methoxy-poly(ethylene glycol 5000) polymeric micelles containing BDP (BDP-SSMs) were prepared by the co-precipitation and reconstitution method, and the physicochemical and in vitro characteristics of BDP-SSMs were investigated. BDP-SSMs were successfully prepared with a content uniformity and reproducibility suitable for pulmonary administration. The maximum solubility of BDP in SSMs was approximately 1300 times its actual solubility. The particle size and zeta potential of BDP-SSMs were 19.89 ± 0.67 nm and -28.03 ± 2.05 mV, respectively. The SSMs system slowed down the release of BDP and all of the aerodynamic values of the aerosolized rehydrated BDP-SSMs were not only acceptable but indicated a significant level of deposition in the lungs.

3.2.4.2.3. Lipid Nanocapsules

Liposomes have some important drawbacks: they have a low capacity to encapsulate lipophilic drugs, they are manufactured through processes involving organic solvents, and they are leaky, unstable in biological fluids and more generally in aqueous solutions for being
commercialized as such. Huynh et al. have developed new nano-cargos, the lipid nanocapsules, with sizes below the endothelium fenestration (ϕ < 100 nm), that solve these disadvantages [670]. They are prepared according to a solvent-free process and they are stable for at least one year in suspension ready for injection, which should reduce considerably the cost and convenience for treatment. Moreover, these new nano-cargos have the ability to encapsulate efficiently lipophilic drugs, offering a pharmaceutical solution for their intravenous administration. The LNCs were prepared according to an original method based on a phase-inversion temperature process recently developed and patented. Their structure is a hybrid between polymeric nanocapsules and liposomes because of their oily core which is surrounded by a tensioactive rigid membrane. They have a lipoprotein-like structure. Their size can be adjusted below 100 nm with a narrow distribution. Importantly, these properties confer great stability to the structure (physical stability > 18 months). Blank or drug-loaded LNCs can be prepared, with or without PEGylation, that is a key parameter that affects the vascular residence time of the nano-cargos. Other hydrophilic tails can also be grafted. Different anticancer drugs (paclitaxel, docetaxel, etoposide, hydroxytamoxifen, doxorubicin, etc.) have been encapsulated. They all are released according to a sustained pattern. Preclinical studies on cell cultures and animal models of tumors have been performed, showing promising results.

Inhaled fluticasone propionate is often prescribed as a first-line therapy for the effective management of pulmonary diseases such as asthma. As nanocarriers offer many advantages over other drug delivery systems, a study by Umerska et al. investigated the suitability of lipid nanocapsules (LNCs) as a carrier for fluticasone propionate, examining the drug-related factors that should be considered in the formulation design and the behavior of LNCs with different compositions and properties suspended within aerosol droplets under the relatively hostile conditions of nebulization [671]. By adjusting the formulation conditions, particularly the nanocarrier composition, fluticasone propionate was efficiently encapsulated within the LNCs with a yield of up to 97%, and a concentration comparable to commercially available preparations was achieved. Moreover, testing the solubility of the drug in oil and water and determining the oil/water partition coefficient proved to be useful when assessing the encapsulation of the fluticasone propionate in the LNC formulation. Nebulization did not cause the fluticasone propionate to leak from the formulation, and no phase separation was observed after nebulization. LNCs with a diameter of 100 nm containing a smaller amount of surfactant and a larger amount
of oil provided a better fluticasone propionate loading capacity and better stability during nebulization than 30 or 60 nm LNCs.

3.2.4.3. Carbon-based Nanoparticles

An attractive property of carbon nanotubes (CNTs) is that the tube- or fiber-like structure allows for extensive functionalization and loading of cargo [672]. However, CNTs have been found to exacerbate asthma and inflammation in the lungs following inhalation, thus making them unlikely delivery systems for asthma therapy to the lungs [673]. Major factors that cause these toxic effects are the high aspect ratio, durability and residual metal content that generate reactive oxygen species [672]. On the other hand, fullerenes, another carbon-based nanoparticle delivery system, has shown promise in reducing oxidative stress within the asthmatic airways while delivering therapeutic cargo.

3.2.4.3.1. Fullerenes

Human mast cells (MC) and peripheral blood basophils are critical cells involved in the initiation and propagation of several inflammatory conditions, mainly type I hypersensitivity. Ryan et al. report an unanticipated role of fullerenes as a negative regulator of allergic mediator release that suppresses Ag-driven type I hypersensitivity [674]. Their findings identify a new biological function for fullerenes and may represent a novel way to control MC-dependent diseases including asthma. Human MC and peripheral blood basophils exhibited a significant inhibition of IgE dependent mediator release when pre-incubated with C(60) fullerenes. Protein microarray demonstrated that inhibition of mediator release involves profound reductions in the activation of signaling molecules involved in mediator release and oxidative stress. Follow-up studies demonstrated that the tyrosine phosphorylation of Syk was dramatically inhibited in Ag-challenged cells first incubated with fullerenes. In addition, fullerene pre-incubation significantly inhibited IgE-induced elevation in cytoplasmic reactive oxygen species levels. Furthermore, fullerenes prevented the in vivo release of histamine and drop in core body temperature in vivo using a MC-dependent model of anaphylaxis.

Riedl and Nel reviewed the evidence demonstrating the importance of oxidative stress in asthma pathogenesis and presented the therapeutic implications of these findings [675]. Mechanistically, the effect of oxidative stress on dendritic cells has been demonstrated to have a potent effect on Th1/Th2 skewing of the immune response. Investigations of gene-environment interactions have identified genetic polymorphisms associated with individual susceptibility to
pollutant-induced respiratory oxidative stress. The effects of current asthma therapy on oxidative stress are currently unclear, but previous trials using conventional antioxidant therapy in asthma have been largely ineffective. Recent investigations have identified two promising broad-based therapeutic approaches: Nrf2 pathway activation and the use of thiol precursors. Preliminary data suggest that fullerene nanomaterials and dietary interventions may also have potential benefits in asthma. Our current understanding of the role of oxidative stress in asthma suggests that antioxidant therapy may be important in optimizing asthma treatment and prevention. The future success of antioxidant asthma therapy will require strategies with broad effects on airway redox equilibrium and the selection of appropriate target populations.

Certain fullerene derivatives inhibit mast cell function in vitro, and therefore Norton et al. examined the in vivo therapeutic effect of fullerene derivatives on asthma, a disease in which mast cells play a predominant role [676]. The authors wanted to determine whether an efficient mast cell-stabilizing fullerene derivative (C(70)-tetragalloylate [TGA]) can inhibit asthma pathogenesis in vivo and to examine its in vivo mechanism of action. Asthma was induced in mice, and animals were treated intranasally with TGA either simultaneously with treatment or after induction of pathogenesis. The efficacy of TGA was determined through the measurement of airway inflammation, bronchoconstriction, serum IgE levels, and bronchoalveolar lavage fluid cytokine and eicosanoid levels. TGA-treated mice had significantly reduced airway inflammation, eosinophilia, and bronchoconstriction. The TGA treatments are effective, even when given after disease is established. Moreover, we report a novel inhibitory mechanism because TGA stimulates the production of an anti-inflammatory P-450 eicosanoid metabolites (cis-epoxyeicosatrienoic acids [EETs]) in the lung. Inhibitors of these anti-inflammatory EETs reversed TGA inhibition. In human lung mast cells incubated with TGA, there was a significant upregulation of CYP1B gene expression, and TGA also reduced IgE production from B cells. Lastly, mast cells incubated with EET and challenged through FcεRI had a significant blunting of mediator release compared with nontreated cells. The inhibitory capabilities of TGA reported here suggest that fullerene derivatives might be used a platform for developing treatments for asthma. Epoxyeicosatrienoic acids are involved in the C(70) fullerene derivative-induced control of allergic asthma [676].

A study by Dellinger et al. investigated the role and mechanism of action of fullerenes in deactivating mast cell-base inflammation, paving the way to the development of novel, non-steroid therapy in reactive airway disease [677]. Water-soluble fullerenes can be engineered to regulate
activation of mast cells and control mast cell-driven diseases in vivo. To further understand their anti-inflammatory mechanisms a C70-based fullerene conjugated to four myo-inositol molecules (C70-I) was examined in vitro for its effects on the signaling pathways leading to mediator release from human lung mast cells [677]. The C70-I fullerene stabilizes mast cells and acts synergistically with long-acting β2-adrenergic receptor agonists (LABA) to enhance inhibition of mast cell mediator release through FcεRI-simulation. The inhibition was paralleled by the upregulation of dual-specificity phosphatase one (DUSP1) gene and protein levels. Concomitantly, increases in MAPK were blunted in C70-I treated cells. The increase in DUSP1 expression was due to the ability of C70-I to prevent the ubiquitination and degradation of DUSP1. These findings identify a mechanism of how fullerenes inhibit inflammatory mediator release from mast cells and suggest they could potentially be an alternative therapy for steroid resistant asthmatics.

Lipids peroxidative oxidation as well as antioxidative enzymes superoxidisedismutase and catalase activity in OVA sensitized mice, and their inactivation by fullerene FC60 (FC60-OVA, mFC60 mFC60-OVA) modified and conjugated with ovalbumines have been studied. It has been demonstrated that the mice sensibilization with ovalbumin leads to the tissues peroxidative lipid oxidation processes enforcement as well as lowering antioxidative enzymes activity in lungs and spleen. Brova et al. used different fullerene forms expressed antioxidative effect and modifying effect to antioxidative protection enzymes at a given pathology [678]. The influence of fullerene FC 60 and its modified form (1,2-methanofullerene-C60)61-carbolacid was the mostly effective. The data warrants further investigation of fullerenes as the potential medicines.

3.2.4.4. Metal-based Inorganic Nanoparticles

Inorganic nanoparticles represent an efficient alternative due to the lower toxicity [123] and also can be modeled to possess the controlled release properties [124]. In perspective of drug delivery, bioactives can be incorporated inside inorganic nanoparticulate systems without any chemical modifications of bioactives [125]. The inorganic nanoparticles that have been used for delivery of drugs comprise of silica, calcium, gold, magnesium, strontium, quantum dots, etc. [126]. Inorganic nanoparticles possess several versatile properties suitable for the cellular delivery including biocompatibility, controlled release of therapeutics agents, and capability of targeted drug delivery. The inorganic nanoparticles can be used for various routes of administration including nasal, parenteral, and inhalation. The inorganic nanoparticles possess ability to accumulate in cells without being recognized by P-gp, one of the main mediators of MDR,
resulting in the increased intracellular concentration of drugs [127]. However, metallic nanoparticles and metallic oxide nanoparticles have been reported to exert adverse effects on the lung due to poor biocompatibility [673]. Upon inhalation, clearance of metallic particles occurs through mucociliary clearance, translocation to interstitial sites and to regional lymph nodes, and macrophage pinocytosis [673]. If the metallic particle deposition rate is more than the clearance rate, then inhaled nanoparticles in the lung may prolong alveolar macrophage clearance continuously and cause airway epithelial cell injury [673, 679].

3.2.4.4.1. Super-paramagnetic Iron Oxide (SPIO) Nanoparticles

The inhalation of medical aerosols is widely used for the treatment of lung disorders such as asthma, chronic obstructive pulmonary disease, cystic fibrosis, respiratory infection and, more recently, lung cancer. Dames et al. suggest that nanomagnet aerosols may be useful for treating localized lung disease, by targeting foci of bacterial infection or tumor nodules [680]. Targeted aerosol delivery to the affected lung tissue may improve therapeutic efficiency and minimize unwanted side effects. Despite enormous progress in optimizing aerosol delivery to the lung, targeted aerosol delivery to specific lung regions other than the airways or the lung periphery has not been adequately achieved to date. Targeted aerosol delivery to the lung can be achieved with aerosol droplets comprising superparamagnetic iron oxide nanoparticles, termed nanomagnetosols, in combination with a target-directed magnetic gradient field [680].

Simultaneous inhibition of IL4 and IL13 via the common receptor chain IL4Rα to adequately block their biological effects presents a promising therapeutic approach to give the addition relief required for asthma patients. In a study by Al Faraj et al., superparamagnetic iron oxide nanoparticles were conjugated with anti-IL4Rα blocking antibodies via polyethylene glycol (PEG) polymers [681]. The delivery of these blocking antibodies to the inflammatory sites in the lung via the developed nanocarriers was assessed using noninvasive free-breathing pulmonary MRI. Biocompatibility assays confirmed the safety of the developed nanocarriers for pre-clinical investigations. For all the investigated formulations, nanocarriers were found to be very stable at neutral pH. However, the stability noticeable decreased with the PEG length in acidic environment and thus the loaded antibodies were preferentially released. Immunofluorescence and fluorimetry assays confirmed the binding of the nanocarriers to the IL4Rα asthma biomarker. Pulmonary MRI performed using an ultra-short echo time sequence allowed simultaneous noninvasive monitoring of inflammatory responses induced by ovalbumin challenge and tracking of the developed
nanocarriers, which were found to co-localize with the inflammatory sites in the lung, as shown in Figure 3.6. Targeting of the developed nanocarriers to areas rich in IL4Rα positive inflammatory cells was confirmed using histological and flow cytometry analyses. The anti-IL4Rα-conjugated nanocarriers developed here have been confirmed to be efficient in targeting key inflammatory cells during chronic lung inflammation following intrapulmonary administration. Targeting efficiency was monitored using noninvasive MRI, allowing detection of the nanocarriers’ co-localization with the inflammatory sites in the lung of ovalbumin-challenged asthmatic mice.

Figure 3.6. Noninvasive lung MRI. (a) Representative MR images of mouse lung for control mice, ovalbumin-challenged mice (i.e. OVA), and OVA mice following intrapulmonary administration of either SPIONs (upper row) or SPIONs–IL4Rα (lower row) acquired at 2 h, 48 h, and 7 days post administration. White arrows highlight the presence of void signal dots related to SPIONs (with or without IL4Rα conjugation). SPIONs–IL4Rα were found to preferentially co-localize with the inflammatory (hyper-intensity) regions in the asthmatic lung (blue circles) following ovalbumin challenge. (b) Quantitative assessments of inflamed lung volume (ILV) for control versus OVA mice at 2 h, 24 h, 48 h, and 7 days post administration of either SPIONs or SPIONs–IL4Rα. Error bars are standard deviations of triplicates. Reprinted with permission from Ref. Al Faraj et al. 2015 [681]. Copyright © 2015 John Wiley & Sons, Ltd.
3.2.4.4.2. Gold

Although gold nanoparticles have been shown to exhibit a range of beneficial biological properties, including anti-inflammatory and anti-oxidant effects, their putative impact on allergic asthma has not been addressed. Barreto et al. evaluated the potential of nasal-instilled gold nanoparticles to prevent allergen-induced asthma in distinct murine models of this disease. Swiss-Webster (outbred) and A/J (inbred) mice were sensitized with ovalbumin and then treated with intranasal injections of gold nanoparticles (6 and 60 µg/kg), 1 h before ovalbumin challenges [682]. Lung function, leukocyte infiltration, mucus exacerbation, extracellular matrix deposition, cytokine generation, and oxidative stress were evaluated 24 h after the last challenge. In both mice strains, gold nanoparticles clearly inhibited (70-100%) allergen-induced accumulation of inflammatory cells as well as the production of both pro-inflammatory cytokines and reactive oxygen species. In A/J mice, recognized as genetic asthma prone animals, instilled gold nanoparticles clearly prevented mucus production, peribronchiolar fibrosis and airway hyperreactivity triggered by allergen provocation. The findings of this study lead to a conclusion that gold nanoparticles prevented pivotal features of asthma, including airway hyperreactivity, inflammation and lung remodeling. Such protective effects are accounted for by reduction in lung tissue generation of proinflammatory cytokines and chemokines, in a mechanism probably related to downregulation in the levels of oxidative stress.

In healthy lungs, deposited micrometer-sized particles are efficiently phagocytosed by macrophages present on airway surfaces; however, uptake of nanoparticles (NP) by macrophages appears less effective and is largely unstudied in lung disease. Using mouse models of allergic asthma and chronic obstructive pulmonary disease (COPD), Geiser et al. investigated NP uptake after challenge with common biogenic ambient air microparticles [683]. Bronchoalveolar lavage (BAL) cells from diseased mice (allergic asthma: ovalbumin [OVA] sensitized and COPD: Scnn1b-transgenic [Tg]) and their respective healthy controls were exposed ex vivo first to 3-µm fungal spores of Calvatia excipuliformis and then to 20-nm gold (Au) NP. Electron microscopic imaging was performed and NP uptake was assessed by quantitative morphometry. Macrophages from diseased mice were significantly larger compared to controls in OVA-allergic versus sham controls and in Scnn1b-Tg versus wild type (WT) mice. The percentage of macrophages containing AuNP tended to be lower in Scnn1b-Tg than in WT mice. In all animal groups, fungal spores were localized in macrophage phagosomes, the membrane tightly surrounding the spore,
whilst AuNP were found in vesicles largely exceeding NP size, co-localized in spore phagosomes and occasionally, in the cytoplasm. AuNP in vesicles were located close to the membrane. In BAL from OVA-allergic mice, 13.9 ± 8.3% of all eosinophils contained AuNP in vesicles exceeding NP size and close to the membrane. Overall, AuNP uptake by BAL macrophages occurred mainly by co-uptake together with other material, including micrometer-sized ambient air particles like fungal spores. The lower percentage of NP containing macrophages in BAL from Scnn1b-Tg mice points to a change in the macrophage population from a highly to a less phagocytic phenotype. This likely contributes to inefficient macrophage clearance of NP in lung disease. Finally, the AuNP containing eosinophils in OVA-allergic mice show that other inflammatory cells present on airway surfaces may substantially contribute to NP uptake.

In contrast, low, intrapulmonary doses of Au NPs can aggravate pulmonary inflammation and AHR in a mouse model of diisocyanate-induced asthma [684]. Hussain et al. conducted a study to investigate the modulation of an asthmatic response by gold (Au) nanoparticles in a murine model of diisocyanate-induced asthma [684]. On days 1 and 8, BALB/c mice received 0.3% toluene diisocyanate (TDI) or the vehicle acetone-olive oil (AOO) on the dorsum of both ears (20 μL). On day 14, the mice were oropharyngeally dosed with 40 μL of a NP suspension (0.4 mg·mL⁻¹ (∼0.8 mg·kg⁻¹) Au). 1 day later (day 15), the mice received an oropharyngeal challenge with 0.01% TDI (20 μL). On day 16, airway hyperreactivity (AHR), bronchoalveolar lavage (BAL) cell and cytokine analysis, lung histology, and total serum immunoglobulin E were assessed. NP exposure in sensitized mice led to a three-fold increase in AHR, and a five-fold increase in BAL total cell counts, mainly comprising neutrophils and macrophages. The NPs taken up by BAL macrophages were identified by energy dispersive X-ray spectroscopy. Histological analysis revealed increased edema, epithelial damage and inflammation.

**3.2.4.4.3. Silver**

The use of silver in the past demonstrated the certain antimicrobial activity, though this has been replaced by other treatments. However, nanotechnology has provided a way of producing pure silver nanoparticles, and it shows cytoprotective activities and possible pro-healing properties. Antioxidant effects of silver nanoparticles could be one of the molecular bases in the murine model of asthma. These findings may provide a potential molecular mechanism of silver nanoparticles in preventing or treating asthma. Park et al. used ovalbumin (OVA)-inhaled female C57BL/6 mice to evaluate the roles of silver nanoparticles and the related molecular mechanisms in allergic
airway disease. In this study with an OVA-induced murine model of allergic airway disease, and they found that the increased inflammatory cells, airway hyperresponsiveness, increased levels of IL-4, IL-5, and IL-13, and the increased NF-κB levels in lungs after OVA inhalation were significantly reduced by the administration of silver nanoparticles [685]. In addition, we have also found that the increased intracellular reactive oxygen species (ROS) levels in bronchoalveolar lavage fluid after OVA inhalation were decreased by the administration of silver nanoparticles. These results indicate that silver nanoparticles may attenuate antigen-induced airway inflammation and hyperresponsiveness.

The anti-inflammatory action of silver nanoparticles (NPs) has been reported in a murine model of asthma in a previous study. Jang et al. performed more specific mechanisms of silver NPs in an attenuation of allergic airway inflammation have not yet been established [686]. Silver NPs substantially suppressed mucus hypersecretion and PI3K/HIF-1α/VEGF signaling pathway in an allergic airway inflammation. Vascular and mucous changes are believed to contribute largely in pathophysiology in asthma. Among various factors related to vascular changes, vascular endothelial growth factor (VEGF) plays a pivotal role in vascular changes in asthma. Mucin proteins MUC5AC and MUC5B have been implicated as markers of goblet cell metaplasia in lung pathologies. The aim of this study was to investigate the effects of silver NPs on VEGF signaling pathways and mucus hypersecretion. Ovalbumin (OVA)-inhaled female BALB/c mice were used to evaluate the role of silver NPs and the related molecular mechanisms in allergic airway disease. In this study, with an OVA-induced murine model of allergic airway disease, it was found that the increased levels of hypoxia-inducible factor (HIF)-1α, VEGF, phosphatidylinositol-3 kinase (PI3K) and phosphorylated-Akt levels, and mucous glycoprotein expression (Muc5ac) in lung tissues were substantially decreased by the administration of silver NPs.

3.2.4.5. Nanocrystals

Tranilast (TL), an antiallergic agent, has been clinically used in the treatment of bronchial asthma, although the clinical use of TL is limited because of its poor solubility and systemic side effects. To overcome these drawbacks, a novel respirable powder (RP) of TL for inhalation therapy was developed using nanocrystal solid dispersion of TL (CSD/TL) [687]. In the CSD/TL, wet-milled crystalline TL particles with a mean diameter of 122 nm were dispersed, and there was a marked improvement in dissolution behavior of the CSD/TL-RP compared with that of a physical mixture of TL and carrier. Laser diffraction and cascade impactor analyses on the CSD/TL-RP
demonstrated high disperability and deposition in the respiratory organs with emitted dose and fine particle fraction of ca. 98 and 60%, respectively. Inhaled CSD/TL-RP could attenuate antigen-induced inflammatory events in rats, as evidenced by histochemical analyses and inflammatory biomarkers such as lactate dehydrogenase, eosinophil peroxidase, and myeloperoxidase. The CSD/TL-RP seemed to be more potent than the physical mixture in inhibiting inflammatory responses, possibly due to the improved dissolution behavior. Systemic exposure of TL after intratracheal administration of CSD/TL-RP at a pharmacologically effective dose (100 μg of TL/rat) was found to be fivefold less than that of the oral TL dosage form at clinical dose (1.67 mg/kg). Given the improved pharmacodynamics and lower systemic TL concentration, the inhalable TL formulation might provide an interesting alternative to oral therapy with a better safety margin for the treatment of asthma and other airway inflammatory diseases.

Tranilast (TL) has been clinically used for the treatment of airway inflammatory diseases, although the clinical use of TL is limited because of its poor solubility and systemic side effects. To overcome these drawbacks, a novel respirable powder of TL (CSD/TL-RP) for inhalation therapy was developed by Kawabata et al. using nanocrystal solid dispersion of TL (CSD/TL) [688]. Inhalable TL formulation might be an interesting alternative to oral therapy for the treatment of asthma and other airway inflammatory diseases with sufficient dispersing stability. Stability study on CSD/TL-RP was carried out with a focus on inhalation performance. Even after 6 months of storage at room temperature, there were no significant morphological changes in micronized particles on the surface of carrier particles as compared with that before storage. Cascade impactor analyses on CSD/TL-RP demonstrated high inhalation performance with emitted dose and fine particle fraction (FPF) of ca. 98% and 60%, respectively. Long-term storage of CSD/TL-RP resulted in only a slight decrease in FPF value (ca. 54%). Inhaled CSD/TL-RP could attenuate antigen-induced inflammatory events in rats, as evidenced by marked reduction of granulocytes in bronchoalveolar lavage fluid and inflammatory biomarkers such as eosinophil peroxidase, myeloperoxidase, and lactate dehydrogenase. These findings were consistent with decreased expression levels of mRNAs for nuclear factor-kappa B and cyclooxygenase-2, typical inflammatory mediators.

A study by Jacobs and Muller described the production of a budesonide nanosuspension by high-pressure homogenization for local pulmonary delivery from 40 mL up to 300 mL [689]. The aim was to obtain a nanosuspension that can be nebulized and is also long-term stable. The
Nanosuspension was produced by high-pressure homogenization. The formulation was stabilized by a combination of the electrostatic stabilizer lecithin and a steric stabilizer tyloxapol. Particle size analysis was performed by laser diffraction and photon correlation spectroscopy. For further particle characterization, zeta potential was determined. To investigate the aerosolization properties, the nanosuspension was nebulized and afterward analyzed on particle size. It was possible to obtain a long-term stable budesonide nanosuspension. Mean particle size of this nanosuspension was about 500-600nm, analyzed by photon correlation spectroscopy. Analysis by laser diffraction showed that the diameters 95% and 99% were below 3 μm. Budesonide nanosuspension showed a long-term stability; no aggregates and particle growth occurred over the examined period of 1 year. The PCS diameter before and after aerosolization did not change, and the LD diameters increased negligibly, showing the suitability for pulmonary delivery. The scale-up from 40 mL up to 300 mL was performed successfully. High-pressure homogenization is a production method to obtain nanosuspensions with budesonide for pulmonary application. The budesonide nanosuspension was stable during one year of storage at room temperature and proved to be safe and effective in healthy volunteers [690].

Nanocrystal budesonide (nanobudesonide) is a suspension for nebulization in patients with steroid-responsive pulmonary diseases such as asthma [689]. The pharmacokinetics and safety of the product were compared to those of Pulmicort Respules by Kraft et al. [690]. Sixteen healthy volunteers were administered nanobudesonide 0.5 and 1.0 mg, Pulmicort Respules 0.5 mg, and placebo in a four-way, randomized crossover design. All nebulized formulations were well tolerated, with no evidence of bronchospasm. Nebulization times were significantly shorter for nanobudesonide compared to Pulmicort Respules. Because of a low oral bioavailability, plasma concentration of budesonide is a good marker of lung-delivered dose. The pharmacokinetics of nanobudesonide 0.5 and 1.0 mg were approximately dose proportional with respect to Cmax, AUC(0-t), and AUC(0-infinity). Nanobudesonide 0.5 mg and Pulmicort Respules 0.5 mg exhibited similar AUCs, suggesting a similar extent of pulmonary absorption. A higher Cmax was noted with nanobudesonide 0.5 mg, and the tmax was significantly different, suggesting a more rapid rate of drug delivery of nanobudesonide 0.5 mg than Pulmicort Respules. In conclusion, nebulized nanobudesonide 0.5 mg was safe in healthy volunteers, with a similar extent of absorption as Pulmicort Respules.
Pranlukast, one of the potential therapeutic tools in the treatment of asthma, has limited clinical applications due to its poor water solubility. The study is aimed to provide a platform for better utilizing pranlukast with enhancement of the dissolution rate and, thus, the oral bioavailability of pranlukast by preparing nanosuspensions through high-pressure homogenization method. In a study by Wang et al., Poloxamer407 and PEG200 were chosen as stabilizer and surfactant [691]. The formulation was investigated systematically with the dissolution tests as predominant method. Nanosuspensions were prepared by programmed high-pressure homogenization method. The product was characterized by particle size analysis, TEM and XRD are evaluated by in vitro dissolution tests and in vivo absorption examination. In addition, nanosuspensions with only pranlukast were prepared and compared with formulated nanosuspensions. The optimal values of formulation were 0.5% (w/v) pranlukast with 0.375% (w/v) Poloxamer407, 0.375% (w/v) PEG200 and the screened programming homogenizing procedure parameters were 680 bar for the first 15 circles, 1048 bar for the next 9 circles and 1500 bar for the last 9 circles. Nanosuspensions of 318.2 ± 7.3 nm, -29.3 ± 0.8 mV were obtained. The XRD analysis indicated no change of crystalline occurred in the process of homogenization. The in vitro dissolution behavior of nanosuspensions exhibited complete release in 30 min with a remarkable fast dissolution rate. The in vivo bioavailability of formulated pranlukast nanosuspensions demonstrated its enhancement of fast onset of therapeutic drug effects with 4.38-fold improved compared to that of raw crystals. The study provides a feasible, practical thinking of industry development in the clinical use of pranlukast.

Nanoparticle agglomerates of fluticasone propionate in combination with albuterol sulfate as dry powder inhalers were developed by El-Gendy et al. [692]. Particle engineering strategies remain at the forefront of aerosol research for localized treatment of lung diseases and represent an alternative for systemic drug therapy. With the hastily growing popularity and complexity of inhalation therapy, there is a rising demand for tailor-made inhalable drug particles capable of affording the most proficient delivery to the lungs and the most advantageous therapeutic outcomes. To address this formulation demand, nanoparticle agglomeration was used to develop aerosols of the asthma therapeutics, fluticasone or albuterol. In addition, a combination aerosol was formed by drying agglomerates of fluticasone nanoparticles in the presence of albuterol in solution. Powders of the single drug nanoparticle agglomerates or of the combined therapeutics possessed desirable aerodynamic properties for inhalation. Powders were efficiently aerosolized
(~75% deposition determined by cascade impaction) with high fine particle fraction and rapid dissolution. Nanoparticle agglomeration offers a unique approach to obtain high performance aerosols from combinations of asthma therapeutics.

Charpentier et al. conducted a study of the rapid expansion of supercritical solution (RESS) process for producing beclomethasone-17,21-dipropionate particles suitable for pulmonary delivery [693]. The purpose of this research was to micronize beclomethasone-17,21-dipropionate (BDP), an anti-inflammatory inhaled corticosteroid commonly used to treat asthma, using the RESS technique. The RESS technique was chosen for its ability to produce both micron particles of high purity for inhalation, and submicron/nano particles as a powder handling aid for use in next generation dry powder inhalers (DPIs). Particle formation experiments were carried out with a capillary RESS system to determine the effect of experimental conditions on the particle size distribution (PSD). The results indicated that the RESS process conditions strongly influenced the particle size and morphology; with the BDP mean particle size decreasing to sub-micron and nanometer dimensions. An increase in the following parameters, i.e. nozzle diameter, BDP mol fraction, system pressure, and system temperature; led to larger particle sizes. Aerodynamic diameters were estimated from the SEM data using three separate relations, which showed that the RESS technique is promising to produce particles suitable for pulmonary delivery.

An aerosol flow reactor method, a one-step continuous process to produce nanometer-sized drug particles with unimodal size distribution, was developed by Eerikainen, et al. [694]. This method involves first dissolving the drug material in question into a suitable solvent, which is then followed by atomizing the solution as fine droplets into carrier gas. A heated laminar flow reactor tube is used to evaporate the solvent, and solid drug nanoparticles are formed. In this study, the effect of drying temperature on the particle size and morphology was examined. A glucocorticosteroid used for asthma therapy, beclomethasone dipropionate, was selected as an experimental model drug. The geometric number mean particle diameter increases significantly with increasing reactor temperatures due to formation of hollow nanoparticles. Above 160 degrees C, however, further increase in temperature results in decreasing particle size. The produced nanoparticles are spherical and show smooth surfaces at all studied experimental conditions.
3.2.4.5. DNA and RNA Nanoparticles

Thymulin has been shown to present anti-inflammatory and anti-fibrotic properties in experimental lung diseases. Da Silva et al. hypothesized that a biologically active thymulin analog gene, methionine serum thymus factor, delivered by highly compacted DNA nanoparticles may prevent lung inflammation and remodeling in a mouse model of allergic asthma [695]. The DNA nanoparticles are composed of a single molecule of plasmid DNA compacted with block copolymers of poly-L-lysine and polyethylene glycol (CK30PEG), which have been found safe in a human phase I/II clinical trial. Thymulin plasmids were detected in the lungs of ovalbumin-challenged asthmatic mice up to 27 days after administration of DNA nanoparticles carrying thymulin plasmids. A single dose of DNA nanoparticles carrying thymulin plasmids prevented lung inflammation, collagen deposition and smooth muscle hypertrophy in the lungs of a murine model of ovalbumin challenged allergic asthma, leading to improved lung mechanics. In this study, highly compacted DNA nanoparticles using thymulin analog gene modulated the inflammation and remodeling processes which together improved lung mechanics.

Activation of the transcription factor signal transducer and activator of transcription 5b (STAT5b) is a key event in the development of asthma. The potent ability of small interfering RNA (siRNA) to inhibit the expression of STAT5b mRNA has provided a new class of therapeutics for asthma. However, efficient delivery of siRNAs remains a key obstacle to their successful application. A targeted intracellular delivery approach for siRNA to specific cell types would be highly desirable. Qiu et al. used packaging RNA (pRNA), a component of the bacteriophage phi29-packaging motor, to deliver STAT5b siRNA to asthmatic spleen lymphocytes [696]. This pRNA was able to spontaneously carry siRNA/STAT5b and aptamer/CD4, which is a ligand to CD4 molecule. Based on RT-PCR data, the pRNA dimer effectively inhibited STAT5b gene mRNA expression of asthmatic spleen lymphocytes, without the need for additional transfections. We conclude that the pRNA dimer carrying both siRNA and aptamer can deliver functional siRNA to cells; possibly, the aptamer acts as a ligand to interact with specific receptors. The pRNAs were evaluated with a CCK-8 kit and were found to have little cytotoxicity. We conclude that pRNA as a novel nanovehicle for RNA worth further study.

Allergic asthma is characterized by airway inflammation caused by infiltration and activation of inflammatory cells that produce cytokines. Many studies have revealed that c-kit, a
proto-oncogene, and its ligand, stem cell factor (SCF), play an important role in the development of asthmatic inflammation. Intranasal small interference RNA (siRNA) nanoparticles targeting specific viral gene could inhibit airway inflammation. Wu et al. assessed whether silencing of c-kit with intranasal small interference RNA could reduce inflammation in allergic asthma [567]. A mouse model of experimental asthma was treated with intranasal administration of anti-c-kit siRNA to inhibit the expression of the c-kit gene. We assessed the inflammatory response in both anti-c-kit siRNA-treated and control mice. Local administration of siRNA effectively inhibited the expression of the c-kit gene and reduced airway mucus secretion and the infiltration of eosinophils in bronchoalveolar lavage fluid. Moreover, c-kit siRNA reduced the production of SCF, interleukin-4 (IL-4), and IL-5, but had no effect on interferon-γ (IFN-γ) generation. These results show that intranasal siRNA nanoparticles targeting c-kit can decrease the inflammatory response in experimental allergic asthma.

3.2.4.6. Microparticle-based Carriers for Nanoparticle Delivery

A lectin-anchored chitosan nanoparticles within microparticles system showed a triggering effect of N-acetylglucosamine on controlling drug release. Li et al. investigated the use of N-acetylglucosamine (NAG) to accelerate drug release from a lectin-modified carrier [697]. A wheat germ agglutinin (WGA)-anchored salmeterol xinafoate (SalX)-loaded nanoparticles-in-microparticles system (NiMS) was prepared with an ionotropic gelation technique combined with a spray drying method. The formulated microparticles were spherical, with diameters ranging mainly from 2 to 8 μm; the drug entrapment efficiency was >70% (w/w), and the loading capacity was approximately 8% (w/w). Drug release from WGA-SalX-NiMS, within the first 4h, was approximately 30% less than that from SalX-NiMS, indicating an effect of lectin-modification to retard drug release from the NiMS. Due to "sugar-lectin" interactions, drug release from WGA-SalX-NiMS was substantially increased after the addition of NAG to the release medium. However, no significant influence of NAG was observed on the drug release profile of SalX-NiMS without WGA anchorage. The characteristics of NAG-WGA interaction may provide valuable insights into the "triggering-effects" of specific sugars on drug release from lectin-anchored carriers. These results suggest that it is possible to control drug release from a lectin-anchored drug delivery system using a specific sugar, and that the designed novel WGA-SalX-NiMS may be a suitable formulation for chronotherapy of asthma.
Although not used directly for mitigating asthma, a study by Ruge et al. described the mechanistic study of disintegration of nano-embedded microparticles after deposition in mucus [698]. The conversion of colloidal drug carriers/polymeric nanoparticles into dry microparticulate powders (e.g., by spray-drying) is a prominent approach to overcome the aerodynamic limitations of these formulations for delivery via inhalation. However, to what extent such nano-embedded microparticles disintegrate into individual/intact nanoparticles after contacting relevant physiological media has so far not been addressed. Polymeric nanoparticles were spray-dried into nano-embedded microparticles (NEMs) using different amounts of trehalose as embedding matrix excipient. Formulations were characterized and then evaluated for their disintegration behavior after aerosolization onto model mucus. Although a rapid and complete aqueous re-dispersion was observed for specific excipient/nanoparticle weight ratios (i.e., greater than 1/1), the same formulations revealed no disintegration after deposition onto a static mucus layer. Double-labeled NEMs powders (i.e., dual color staining of polymeric nanoparticles and trehalose) demonstrated rapid matrix dissolution, while the nanoparticle aggregates persisted. When deposited onto agitated mucus, however, sufficient disintegration of NEMs into individual polymeric nanoparticles was observed. These findings indicate that mechanical forces are necessary to overcome the attraction between individual nanoparticles found within the NEMs. Thus, it remains questionable whether the lung mechanics (e.g., breathing, mucociliary clearance) acting on these formulations will contribute to the overall disintegration process.

### 3.2.5. Summary and Future Directions of Asthma Nanotherapeutics

Asthma is a multifaceted immune disease driven and characterized by varying degrees of airway hyperresponsiveness, bronchoconstriction, mucus hypersecretion, airway remodeling, and chronic inflammation. As understanding of asthma immunology grows, novel therapies could provide the opportunity to personalize asthma management along with the targeted delivery mediated by nanoparticles. Drug or gene delivery systems which could selectively deliver immunomodulatory therapeutics directly to their site of action would be a superior treatment modality rather than use of conventional methods. Nanotechnology has a great potential in revolutionizing the drug and gene delivery approaches.
3.3. Aerosol Delivery of siRNA to the Lungs - Rationale for Gene Delivery Systems

3.3.1. Rationale for Aerosol Delivery of siRNA to the Lungs

RNA interference (RNAi) is a process in which RNA molecules inhibit gene expression by causing the destruction of specific messenger RNA (mRNA). Small interfering RNA (siRNA) are double stranded RNA molecules containing 20-25 nucleotides that are involved in the RNAi pathway and interfere with the expression of a specific gene with complementary nucleotide sequences [699]. As shown in Figure 3.7, siRNA degrades mRNA after transcription, thereby preventing translation, or plays a role in RNAi-related pathways [699]. siRNA has potential therapeutic applications in treating ‘undruggable’ diseases by downregulating the expression of a target gene in a post-transcriptional manner. Since the discovery of siRNA in 1998 by Fire and Mello et al., the mechanisms of RNAi have been extensively reviewed [700-703]. The siRNA possesses a specific sequence that is complementary with its target mRNA that induces site-specific cleavage and subsequent inhibition of intracellular protein synthesis. The siRNA, once in the cytoplasm of the cell, can incorporate within the RNA-induced silencing complex (RISC) and activates this complex. The sense strand is then removed from the duplex and degraded by nucleases in the activated RISC complex. At the same time, the antisense strand directs the RISC to the base-complementary sequence of the target mRNA located within the cell cytoplasm. Binding of mRNA to the antisense strand in the activated RISC eventually induces cleavage by the endonuclease Argonaute and post-transcriptional silencing of the target gene expression. Major advantages of siRNA over small molecule drugs or protein therapeutics are that the sequences can be rapidly designed for highly specific inhibition of the target of interest and that the synthesis of siRNAs is relatively simple because it does not require a cellular expression system, complex protein purification, or refolding schemes [704].

Pulmonary diseases such as lung cancer, cystic fibrosis, pulmonary hypertension, asthma, and chronic obstructive pulmonary disorder (COPD) have potential siRNA therapeutic targets [704-706]. Nevertheless, siRNA delivery systems are likely to have instability issues that cause premature release of the nucleic acids, especially with systems that incorporate their cargo through electrostatic interactions. Due to the high negative charge density and relatively large size of the siRNA molecules, naked siRNA molecules are not able to enter cells efficiently [707]. Pulmonary delivery of siRNA faces major challenges that involve a lack of correlation between in vitro and
in vivo experiments, difficulty in translation from animal models to humans, and non-applicable administration routes used in animal studies for human use [708].

Figure 3.7. siRNA pathway schematic. Reprinted with permission from Ref. [709, 710]. Copyright: (2007) Nature Publishing Group.

In Section 3.3., the modes of pulmonary delivery of siRNA, the evaluation of aerosol drug delivery systems, and the rationale for the use of nanocarriers to overcome the barriers of pulmonary delivery and cellular uptake of siRNA are reviewed. The following section, Section 3.4., focuses on the siRNA loaded non-viral particulates for aerosolized delivery systems, and preparation and characterization techniques for siRNA loaded nanoparticles.

3.3.2. Pulmonary Route of Administration

To achieve pulmonary delivery, inhalable aerosols generated by an inhaler or nebulizer are the preferred option. Before entering clinical trials, new therapeutic agents must demonstrate
preclinical efficacy in appropriate animal models that are translatable to humans [711]. Pulmonary aerosols are usually administered via the inhalation, intratracheal, or intranasal routes. Intratracheal and intranasal routes of administration are commonly used to deliver therapeutic siRNA or other therapeutic agents to the lungs of animals due to ease of experimental setup and control [712]. In preclinical studies, the very different lung anatomy of mice and humans needs to be considered while selecting the route of administration to assess delivery and efficacy. Formulations administered via the pulmonary route of administration are required to be nonirritating to reduce risk of pharyngeal edema, bronchial spasm, anaphylaxis, peracute death, and chronic pulmonary fibrosis [713]. These factors are vital to the successful development of an orally or intranasally inhaled siRNA delivery system.

3.3.2.1 Inhalation Route

The most non-invasive way to locally deliver therapeutics to the lungs is through inhalation. Four types of inhalation devices are currently available including pressurized metered dose inhalers (pMDIs), dry powder inhalers (DPIs), nebulizers, and soft mist inhalers (SMIs). With appropriate developmental optimization, these devices may deliver siRNA to the lungs. During development, key parameters should be considered for an optimum inhaler system, as shown in Table 3.2.

Table 3.2. Parameters to consider in siRNA formulation design for inhalation.

<table>
<thead>
<tr>
<th>Property</th>
<th>Parameters</th>
</tr>
</thead>
</table>
| Aerosol properties  | Mass median aerodynamic diameter  
|                     | Geometric standard deviation  
|                     | Fine particle fraction  
|                     | Air/particle velocity  |
| Particle properties | Volume diameter  
|                     | Bulk density  
|                     | Tap density  
|                     | Shape  
|                     | Charge  |
| Physiochemical properties | Solubility  
|                     | Hygroscopicity  |
| Lung properties     | Disease state on airway structure and breathing pattern  
|                     | Disease state on surfactant production  
|                     | Disease state on mucus production and mucociliary clearance  
|                     | Age of patient, pulmonary or nasal breathing  
|                     | Airway structure and diameter  |
3.3.2.1.1 Inhalation Aerosol Delivery Devices

pMDIs are currently the most commonly used inhalers. The therapeutic agents within a pMDI are in either a suspended particulate state or dissolved within propellants, such as chlorofluorocarbons (CFCs) and hydrofluoroalkanes (HFAs) [708]. The propellants are an indispensable part of pMDIs as they supply the energy required to aerosolize the drug for inhalation. siRNA or siRNA loaded nanocarriers may not be compatible with propellant vehicles which limit the formulation of siRNA into the pMDIs [708]. However, crosslinked chitosan-PEG1000 based nanocarriers with particle size of less than 230 nm were found to be physically stable within HFA-227, highly dispersible, and successfully delivered to the deep lung airways using pMDIs [714]. Selecting a stable and appropriate formulation for use with the pMDI devices will allow for the development of siRNA containing pMDIs.

DPIs and their formulations allow for the inhalation of aerosol clouds of dry particles. DPI device design has a major impact on their performance. Advantages of DPIs are that they have improved sterility and chemical stability of biomolecules compared to liquid aerosols [708]. Drug deposition from inhaled DPIs is dependent on the inspiration flow rate of the patient, and often times the illness that warrants their use of the inhalation therapy causes their inspiration flow rates to be abnormally low [715, 716]. The device must be designed to minimize these variation with respect to patient disease state [717]. Spray drying techniques are often used to prepare protein and peptide inhalable DPI formulations that have demonstrated successful in vivo delivery of therapeutic macromolecules [718-721]. The DPIs formulation can be used for pulmonary delivery of siRNA. The issues and challenges that have risen from the formulation of biological macromolecules, such as flowability, dispersibility, and biochemical stability needs to be also considered for development of siRNA based DPIs [722]. Considerations for formulation of proteins as powder aerosols have been reviewed previously [722]. Maintaining biochemical stability of siRNA during formulation limits the processes that may be used to prepare siRNA based dry powder formulations.

Nebulizers generate liquid aerosols that can deliver saline-based solutions or suspensions of drug product at large volumes via inhalation [723]. Nebulizer treatments usually last over several minutes, rather than single inhalation. Generated droplets have aerodynamic diameters between 1-10 µm, depending on the formulation and the type of nebulizer device. The four major
categories of nebulizers are jet (pneumatic), vibrating mesh/membrane, smart, and ultrasonic nebulizers [724, 725].

A jet, or pneumatic nebulizer, can be used to deliver suspension formulation which makes them suitable for delivering siRNA or siRNA carrier delivery systems. Breath-enhanced jet nebulizers release more aerosol during inhalation, whereas breath-actuated jet nebulizers sense the patient’s inspiratory flow and deliver aerosol only during inhalation [726-728]. In an air jet nebulizer, compressed gas draws up bulk liquid to a jet by the Bernoulli effect and atomization takes place where the liquid emerging from the jet interacts with the shear force set up by the gas flow. Rayleigh dispersion of bulk liquid into droplets occurs and baffles remove the coarse droplets, which fall back into the reservoir, whereas droplets with aerodynamic diameters of < 10 µm are available for inhalation. It has been estimated that 99% of the generated aerosols are recycled back to the reservoir, thus the shear stress is exerted multiple times on the formulation and hence, the possible degradation of naked siRNA during this process needs to be evaluated [729].

Vibrating mesh nebulizers use micropumps to force liquid through a mesh or aperture plate for aerosol generation. Suspensions, nanocarriers, and nucleic acids have been successfully delivered via mesh nebulizers [603, 730-735]. Mesh nebulizers have consistent and improved aerosol generation efficiency, large fine-particle fraction, low residual volume, and the ability to nebulize low volumes [725, 736-738]. Vibrating mesh/membrane nebulizers are more expensive than jet nebulizers because of the electronics involved with controlling the vibration process. In addition, mesh nebulizers are difficult to clean. However, mesh nebulizers are more efficient than jet nebulizers at providing higher drug doses to patients [725]. The limitations of mesh nebulizers include the blocking of the mesh or apertures during the nebulization of viscous suspensions or the precipitation and crystallization of drug or excipients [725, 739]. This should be a consideration when formulating a siRNA nanocarrier suspension for nebulization.

There are two types of vibrating mesh nebulizers, termed active and passive. Active mesh nebulizers utilize a piezoelectrical element that expands and contracts upon application of electrical currents which then vibrates a mesh in contact with the medication to generate the aerosol. Passive mesh nebulizers use a transducer horn that induces passive vibrations on the mesh plate for aerosol generation [725].
Smart nebulizers incorporate adaptive aerosol delivery (AAD®) technology that analyzes the patient’s breathing pattern in order to determine the timing of aerosol drug delivery during inhalation [740]. The device is able to adapt to the patients breathing pattern, therefore it is able to reduce the aerosol losses and the variation in drug delivery. Smart nebulizers can provide effectiveness and end of dose feedback to the patient during therapy, which can increase patient compliance [740, 741].

Ultrasonic nebulizers utilize a piezoelectric crystal vibrating at high frequencies of 1-3 MHz to produce aerosols. These types of nebulizers have the limitations of large residual volumes, inability to aerosolize viscous solutions, and degradation of heat sensitive materials [725, 742, 743]. Therefore, ultrasonic nebulizers may not be useful for suspensions of naked siRNA or siRNA nanocarriers [744, 745].

Chemical and physical stability of the naked siRNA during the nebulization process is of high concern. Therefore, delivery of siRNA using nebulizers should involve the development of a formulation which protects the siRNA from physical degradation, shear stress, and chemical degradation mediated via hydrolysis and endogenous enzymes [708].

SMIs are a recently introduced category of inhaler delivery system marketed as Respimat® produced by Boehringer-Ingelhem [746]. This technology provides a metered dose to the user who activates the inhaler and energy from a spring imposes pressure on the liquid container. The SMI generates the aerosol by impinging opposing droplet streams emerging from a uniblock nozzle created using silicon wafer technology [746]. The advantages of the SMI are that it has high lung deposition due to the low velocity of the aerosol cloud and thus minimizing the deposition in the mouth and throat [746, 747]. In addition, the patient is not required to exert excessive inspiratory force in generating the aerosol clouds associated with DPIs. The effect on physical and chemical stability of siRNA must be considered while delivering a formulation via SMIs [723, 748]. The feasibility of SMIs as delivery devices for non-viral siRNA delivery has not been explored, but it may be a viable option.

### 3.3.2.2 Intratracheal Route

The intratracheal route of administration is used most often in animal studies for assessing the inhalation delivery of various drugs. However, the clinical application of this route is limited due to its invasive setup and uncomfortable delivery technique [712, 749]. The intratracheal route is not feasible for human clinical studies due to method invasiveness that involves inserting a tube
to dispense the formulation within a surgical incision made within the tracheal rings [708]. The traditional method involves the animal to be anesthetized, undergo surgical tracheotomy, and after the trachea is exposed, an endotracheal tube or needle is inserted into the incision between the tracheal cartilaginous rings, with the tip projected at a defined position before the tracheal bifurcation, as shown in Figure 3.8. The drug solution or suspension formulation is administered through the tube using a micro syringe and instilled into the airways. Bivas-Benita et al. described a non-invasive intratracheal instillation that did not involve surgical resection of the trachea that is also known as the oro-tracheal route [750]. Aerosol is delivered to the lungs by placing a microsprayer over the anesthetized animals tongue and down to the trachea or the animal is intubated and the drug is instilled in solution or suspension form. This method has the drawbacks of difficulty in accurate placement of the microsprayer catheter and reduced mucociliary clearance due to anesthesia.

![Figure 3.8](image)

**Figure 3.8.** Intratracheal instillation and bronchoalveolar lavage on rat.

There have been a number of studies that utilized the intratracheal route for siRNA delivery to the lungs [634, 751-756]. Drug deposition by this route is less uniform than by the inhalation route of administration [749]. Since this route avoids oropharynx deposition, the amount of drug loss is lower compared to the inhalation route. However, this route of delivery does not allow for determination of the effect of aerodynamic particle size on the lung deposition, therefore the *in vivo* intratracheal instillation studies in the animals do not reflect the intricacies of lung deposition. The intratracheal instillation route of delivery is suitable for providing proof-of-concept for local lung delivery studies in animal models.
3.3.2.1 Intratracheal Aerosol Delivery Devices

Intratracheal aerosolization devices consist of an atomizer located in the distal tip of a long, narrow, stainless steel or plastic tube used to deliver an aerosol directly to the lungs when inserted down the trachea, above the carina or further down, in an anesthetized animal. Devices for preclinical studies include commercially available technologies for administration of solution and for dry powder aerosol formulations. Intratracheal delivery can be achieved through devices, such as the Penn-Century microsprayer [751, 757], the Micro-Mist nebulizer [758], or the Aeroprobe nebulizing catheter [759]. The Penn-Century, Inc. (Wyndmoor, PA, USA) offers an air-free liquid device and an air-driven dry powder device for preclinical use [750, 760]. The miniaturized nebulizing catheter system has shown success for targeted gene delivery to the lungs [761, 762]. The nebulizing catheter device (NCD) was first adapted for the delivery of peptide therapeutics to the rat lungs (Aeroprobe™, Trudell Medical International, London, Ontario, Canada) [762]. This NCD delivers a liquid, which is transported down a tube through a central lumen and pressurized gas is pushed through the peripheral lumens from a compressed airway source at 50 psi. The close proximity of the liquid and gas lumens at the distal tip produces an aerosol in the particle size range of 20-40 µm [762].

3.3.2.3 Intranasal Route

The intranasal route provides a straightforward animal model experimental setup for delivery of siRNA to the lungs, therefore it is another common siRNA delivery route [68, 632, 633, 757, 763-769]. The animals must be anesthetized and siRNA formulations are then instilled into the nasal cavity to be breathed in. Although this route has shown success in siRNA delivery to the lungs in mouse animal models, the translation of these studies to humans has been limited. Humans are not obligate nose breathers and have a nasal cavity that filters out the majority of particulates, therefore human intranasal studies do not see such a high lung deposition [770]. Additionally, anesthetics cause the animals to have reduced mucociliary action, therefore the reduced mucociliary clearance overestimates the transfection efficiency of the formulations [771]. A study by Heyder et al. found that only 3% of monodisperse powder particles with aerodynamic diameters of 1-5 µm were deposited within human bronchial airways after intranasal administration [772]. Since approximately 97% of the monodisperse particles were deposited within the nasopharynx area, the intranasal route of delivery is ideal for targeting this site in humans. The ALN-RSV01 siRNA, which targets the mRNA encoding the N-protein of respiratory
syncytial virus (RSV), developed by Alnylam Pharmaceuticals (Cambridge, MA, USA), has completed phase II clinical trials for the treatment of human RSB infections using the intranasal route of administration [773-775]. This naked siRNA was delivered via a nasal spray to the upper respiratory tract.

The intranasal route may also be used to allow for systemic or central nervous system (CNS) delivery of siRNA. Protein and peptide biological macromolecules have been previously administered via the intranasal route to access the systemic circulation [771]. The nasal cavity has a relatively large surface area and vascularization for facilitating rapid absorption. The intranasal route can access the CNS by bypassing the blood-brain barrier [776, 777].

3.3.2.3.1 Intranasal Aerosol Delivery Devices

Intranasal delivery devices for liquid solution or suspension include the rhynyle catheter and instillation tube. This simple method, often used on anesthetized or sedated animals, involves the intranasal insertion of a fine catheter or micropipette followed by instilling the liquid into the desired area to be aspirated into the airways during breathing [778]. The use of small volumes of 5 µL per nostril or 10 µL total limited drug deposition to the nasal cavity, whereas larger volumes of 25 µL per nostril or 50 µL total allowed for deposition with the lung upper airways [779]. Intranasal instillation is limited due to its hindered ability to reach the deep lung.

For the intranasal delivery of dry powders to the pulmonary route of administration, the Dry Powder Insufflator™ for mice can be used to aerosolize the powder dose [780]. This device is actuated using either an air syringe or a Penn-Century air pump, which generates 200 µL puffs of air that generates the powder dose and carries it into the deep lung.

3.3.2.4 Passive Inhalation Exposure Chambers for Animal Studies

Aerosol exposure chambers may be classified as nose-only, head-only, and whole-body exposure chambers. These systems are commonly used to deliver nebulized formulations [781]. An example of a nose-only exposure chamber is the InExpose™ (SCIREQ®) model as shown in Figure 3.9. This device has been evaluated for use with inhalable drug carrier systems containing antisense oligonucleotides and siRNA for the treatment of lung cancer [782]. Liposomes with particle size and mass median diameter of 130 nm and 270 nm respectively, aerosolized by a jet Collison nebulizer were found to be stable over the continuous aerosolization and a higher lung dose and retention time compared to intravenous route was observed [782]. Other examples of nose-only exposure chambers include the Oro-Nasal and Respiratory Exposure Systems (CH
Technologies, Westwood, NJ) and the In-Tox Products Small Animal Exposure System (Intox Products, Edgewood, NM). Nose-only and head-only exposure chambers avoid exposure of the aerosol to other parts of body. These set-ups can be stressful for the animal due to the restraint and exposure facemasks placed on the animals face and neck [781].

The whole-body exposure setup represents a less invasive exposure chamber because rodents are free to move during the passive aerosol delivery [781]. Whole-body exposure can also simulate environmental exposures to aerosols [783]. These exposure chambers are usually composed of a chamber where the animal resides during the aerosol therapy, and a port where aerosol can be delivered to the inside of the chamber [784-786]. When delivering a therapeutic aerosol via a whole-body exposure chamber, there is possible administration of significant amount of formulation to other routes, such as the oral route [781]. Parameters that impact the variation of the study may include the size of the chamber, the animals size, lung capacity, the inspiratory flow rate, and the formulation characteristics [781].

![Figure 3.9. InExpose™ (SCIREQ®) nose-only exposure system.](image)

3.3.3. Evaluation of Pulmonary Drug Delivery Systems

Aerosolized siRNA formulations are characterized using various \textit{in vitro}, \textit{ex vivo}, and \textit{in vivo} techniques that are shown in \textbf{Table 3.3.} [787, 788]. The purpose of these performance tests is primarily to establish the efficacy of the drug product and device delivery platform have been addressed. These tests were elaborated in Chapter <601> of the USP [789].
Table 3.3. Techniques for evaluating pulmonary drug delivery systems.

<table>
<thead>
<tr>
<th>Type</th>
<th>Technique</th>
<th>Measurement determined</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vitro</td>
<td>Dissolution test</td>
<td>Dissolution rate</td>
</tr>
<tr>
<td></td>
<td>Inertial impaction using cascade impactor</td>
<td>Aerodynamic particle size distribution (APSD)</td>
</tr>
<tr>
<td></td>
<td>Delivered dose assay</td>
<td>Total delivered dose uniformity</td>
</tr>
<tr>
<td></td>
<td>Laser diffraction</td>
<td>Particle size and particle size distribution</td>
</tr>
<tr>
<td></td>
<td>Laser Doppler velocimetry</td>
<td>Aerosol velocity</td>
</tr>
<tr>
<td>Ex vivo</td>
<td>Isolated perfused lung</td>
<td>Mechanisms of drug transport and deposition</td>
</tr>
<tr>
<td></td>
<td>Scintigraphy</td>
<td>Visualization and quantification of aerosol deposition in respiratory tract</td>
</tr>
<tr>
<td>In vivo</td>
<td>Pharmacokinetic (PK)-animal model/clinical trial</td>
<td>PK parameters</td>
</tr>
<tr>
<td></td>
<td>Pharmacodynamics (PD)</td>
<td>Biochemical and physiological effects of a drug</td>
</tr>
</tbody>
</table>

3.3.3.1 In vitro Characterization

Aerosolized siRNA formulation dissolution tests may be performed by adding the formulation within dissolution medium vessel under constant agitation [790, 791]. The solution is then sampled at pre-determined time intervals to measure the amount of siRNA released from the nanoparticles. Since there is not a standard method developed for determining siRNA dissolution rate, various media, such as distilled water [792], phosphate buffer [793], simulated lung fluid
(SLF) and modified SLF have been used. In addition, various apparatus, such as the flow-through cell [794], standard USP dissolution apparatus, and stirring vessels have been used. Test conditions, such as sink, and particle introduction into the medium being either direct or by aerosolization have been used to characterize the dissolution rate of inhaled drugs.

Inertial impaction is a standard *in vitro* technique used to predict aerosol deposition in the lungs [795]. The cascade impactor is the equipment of choice for particle size analysis of most inhaled aerosols because it allows for assay of mass of drug, determined aerodynamic size directly, and provide size information that be indicative of lung deposition. The inertial size-separation and fractionation in a cascade or sequence of single stages within the cascade impactor apparatus measures aerodynamic particle size distribution (APSD) [789, 796]. The jet stream is aimed towards a flat impaction plate, upon which the flow of particles having high inertia is disrupted and impact on the plate. Meanwhile, particles with lower inertia remain within the streamline airflow and do not impact onto the plate [789, 796, 797]. Several impactor plates separate aerosol particles into size ranges according to their aerodynamic size. The larger particles will impact on the plate, whereas the smaller ones will remain airborne. The Stokes number, which is defined as the ratio of the stopping distance to the orifice diameter, can be used to estimate the impaction efficiency [798].

The available cascade impactors accepted by the United States Pharmacopeia (USP) and the European Pharmacopeia (Ph.Eur.) are listed in Table 3.4 [799, 800]. The Andersen Cascade Impactor (ACI) is a pharmacopeia method for pMDIs and DPIs and is the most commonly used inertial impactor instrument (Figure 3.10 A). Other cascade impactors include the Marple-Miller model 160 (Figure 3.10 B) and the multistage liquid impinger (Figure 3.10 C) for DPIs. Nebulizers are suggested to be characterized by the USP <601>/Ph.Eur. 2.9.44 using the Next-Generation Impactor (NGI) at 15 L/min or with a suitable validated cascade impactor that operates at ≤15 L/min [799].
### Table 3.4. USP and Ph.Eur. Cascade Impactors for orally inhaled dosage forms.

<table>
<thead>
<tr>
<th>Dosage Form</th>
<th>USP</th>
<th>Ph.Eur.</th>
</tr>
</thead>
</table>
| pMDI        | - Andersen 8-stage non-viable cascade impactor configuration at 28.3 L/min flow rate | - Single Stage Twin Impinger  
- Andersen 8-stage non-viable cascade impactor configuration at 28.3 L/min flow rate  
- NGI |
| DPI         | - Marple-Miller impactor (model 160, MMI)  
- Andersen 8-stage non-viable cascade impactor configuration at flow rates other than 28.3 L/min  
- Multi-stage liquid impinger  
- NGI | - Multi-stage liquid impinger  
- Andersen 8-stage non-viable cascade impactor configuration at 28.3 L/min flow rate  
- NGI |
| Nebulizer*  | - Harmonized with Ph.Eur. | - Single-stage twin impinger  
- NGI |

*operated at a flow rate of 15 L/min. Ph.Eur. 2.9.44 includes the NGI and is harmonized with <1601> of the USP.
Cascade impactors are apparatuses for measuring APSD of inhaled aerosols and are not lung simulators or models [798]. Since cascade impactors operate at a constant flow rate, they do not simulate the environment of the respiratory tract, such as temperature, relative humidity, and the size-selectivity of the various processes that govern the deposition in the human respiratory
tract. The constant particle velocity within a cascade impactor contrasts with the decreasing air velocity with increasing airway generation number in the lung. The cascade impactor stage selectivity is much steeper than selectivity for regional deposition [802].

Non-aerodynamic droplet or particle size distribution was determined using laser diffraction [795, 803, 804]. This technique measures the low angle scattering of monochromatic coherent light from every particle in the measurement zone. These simultaneous measurements are combined to contribute to the angular scattered light intensity profile. Laser diffraction has the advantages of that it is non-invasive, rapid, has high resolution, wide-dynamic range, can measure volume weighted particle size distribution directly, and is an absolute technique that does not require calibration or verification using optical reticles [805]. During a laser diffraction particle size determination measurement, particles of the same size scatter light to the same position in the detector array, where smaller particles scatter light to wider angles [803]. Efficient data inversion algorithms ensure rapid convergence to a valid solution. Laser diffraction method utilizes the interpretation of the relationship between scattered light intensity and scattering angle, which is the distance from the central axis of the photodetector. The Fraunhofer approximation is a simplified description assuming particles absorb light completely [806]. In this case, only scattering at contour is considered and the particle refractive index (Np) is not needed. The Fraunhofer model works best when particle size is much larger than the wavelength of light, and when the particles are opaque, spherical, and when Np is different to the refractive index of the surrounding medium (e.g. air) [807]. The Lorenz-Mie theory is a complete description of angular light scattering process that requires a full description of complex particle refractive index, which at times is unknown [806]. The Lorenz-Mie model applies to all sizes of particles, but assumes that particles are homogeneous, isotropic, spherical, and has a known complex Np that incorporates both refraction and absorption components. A time-averaged data presentation mode is useful to capture overall performance of a pulmonary inhaled product. The size distribution can be presented in both differential and cumulative formats and the key metrics like particle size of the 10th, 50th, and 90th volume percentiles can be obtained [803]. Laser diffraction equipment can either be an open bench or a close cell. Open bench is simple to perform and is widely used where the aerosol can be directed across the measurement zone. Closed sampling is necessary for DPI testing and may be needed to control evaporation for some measurements, especially with continuous nebulizers. The limitations for laser diffraction are that the method has no assay for
drug mass, particles are assumed to be spherical, constant droplet density versus size, vignetting, beam splitting, and droplet evaporation [803].

The delivered dose uniformity assay is used to quantitatively determine the delivered dose and dose uniformity using a dose uniformity sampling apparatus (DUSA) [789]. The apparatus is connected to an aerosol delivery system through a mouthpiece adapter and a vacuum pump is connected at the other end to provide the desired air flow. The aerosol released into the sampling apparatus is captured onto a filter that is then assayed to quantify the delivered dose. The dose uniformity is determined from variability of the measured delivered dose by the collection of the total mass of contents for the actuation of the clinical dose on the label, excluding volatile propellant and low-volatile species.

3.3.3.2 Ex vivo Characterization

Ex vivo studies are used to identify lung-specific PK without interference from systemic absorption, distribution, metabolism, and excretion (ADME) that occur during in vivo studies [749]. The isolated perfused lung method includes the isolation of a lung from the body and keeping it within an artificial system under certain experimental conditions. These tests utilize isolated perfused lung models that maintain lung tissue functionality and architecture [749, 781]. The isolated perfused lung model is often used to establish the mechanisms of drug absorption and deposition in the lungs. The shortcoming of ex vivo methods include relatively shortened timeframes for data collections due to the viability of the perfused lungs, complex experimental set up, and the absence of tracheo-bronchial circulation and mucociliary clearance [781].

The isolated perfused lung preparation has been used in rats, guinea pigs, rabbits, dogs, and monkeys [749]. The preparation, as shown in Figure 3.11 A-B, consists of peristaltic pumps and a tubing assembly to carry the perfusate to and from the lung, and a double-jacketed artificial thorax to house the isolated perfused lung at 37°C. Perfusate flow, which is normally at 12-15 mL/min in rat lungs, is pumped from the reservoir to the isolated perfused lung through a central porthole of the thorax lid. The bottom of the thorax has an opening, where the perfusate can return to the reservoir or be collected. The thorax is sealed, which enables negative or positive pressure ventilation or the maintenance of pressure [749]. While the animal is under anesthesia, the pulmonary circulation in cannulated via the pulmonary artery and the lung is perfused with autologous whole blood or a buffered artificial medium at pH 7.4 [749]. The surgical procedures involved in preparing the isolated perfused lung model was discussed previously [749]. Following
perfusion, the formulations are administered via the tracheal port and the perfusate samples may be collected at predetermined time points to establish absorption profiles [749].

**Figure 3.11.** Isolated perfused rat lung preparations: (a) horizontally positioned isolated perfused rat lung with a scheme of forced solution instillation, (b) vertically positioned isolated perfused rat lung with a scheme of nebulization catheter dosing. Reprinted with permission from Ref. [749]. Copyright 2003.
3.3.3.3 In vivo Characterization

Gamma scintigraphy is a non-invasive imaging technique that allows for visualization of drug deposition following pulmonary delivery in the respiratory tract, predicts in vivo efficacy, and estimates mucociliary clearance [808-810]. Prior to pulmonary delivery, the drug formulation is radio-labelled with gamma-ray-emitting radiotracer, such as Technetium 99m (99mTc). Alternatively, the radiolabel can be incorporated or attached to the microparticle or nanocarrier instead of the drug [801]. Radiolabeling could be simply achieved by mixing a radiotracer solution with the formulation [811]. An appropriate in vitro validation is required to ensure distribution of the radiotracer across particle size range and that the radiotracer is not affecting the particle size distribution [810]. A gamma camera is used to visualize and quantify the drug deposition in regions of interest within the respiratory tract following inhalation of the radiolabeled formulation. Scintigraphy data are considered an equivalency assessment tool since the data have been demonstrated to correlate well with clinical efficacy data [810, 812]. After pulmonary delivery of the formulation, a gamma camera scans the thorax and radioactive counts can be digitalized to get a 2D image of the lungs, oropharynx, stomach, the inhalation device, and the exhalation filter [801, 813]. The periphery of the lungs is imaged by $^{80m}$Kr ventilation or transmission scans, and is superimposed to quantify the gamma-labelled particles to establish distribution and permeation [801, 814]. However, the two dimensional (2D) nature of gamma scintigraphy images make it difficult to differentiate the overlaid anatomical structures. The ratio of peripheral to central deposition, known as the penetration index (PI), measures the extent to which an aerosol reached the lower airways in the lung periphery [801, 814].

Three dimensional (3D) imaging methods, such as single photon emission computed tomography (SPECT) overcome 2D imaging issues in distinguishing physiological landmarks [815]. Radiolabeled formulations are tracked by a gamma camera that rotates completely around the subject to form a topographical image in all planes [801, 814, 816]. 3D images can be formed using computer software [808]. A limitation of SPECT is the possible occurrence of regional deposition, redistribution by mucociliary clearance, coughing, and absorption to the bloodstream during the 30 min method timeframe [801, 817]. Other limitations are the requirement of a high dose and the use of radiation. Other in vivo imaging methods have been applied to overcome these issues, nevertheless the relatively lower cost and technical skills required are much lower for planar
gamma scintigraphy or SPECT imaging [801, 818]. SPECT 3D imaging is suitable for correlating in vitro and in vivo data [808, 819, 820].

Positron emission tomography (PET) utilizes positron labeled drugs that emits two high-energy photons through electron annihilation [801, 821-823]. These photons are concurrently emitted at 180° from one another, then detected and counted as a single event [801]. Accurate delineation regions of interest are obtained by combining the 3D PET image with MRI or spiral CT scan of the thorax [824, 825]. This allows for the calculation of the percentage of the inhaled dose deposited in these regions [801]. The advantage of PET is that the drug can act as the radioactive tracer by incorporating a positron emitting isotope (\( ^{11}C, ^{15}O, ^{13}N, ^{18}F, ^{64}Cu, \) and \( ^{124}I \)) by isotopic substitution at trace amounts [814, 826-828]. PET has been employed to analyze lung pathology, biochemistry, inflammation, transgene expression, and cellular responses in vivo [826]. The utility of the method is limited by the short half-life (\( ^{11}C\ t_{1/2} \sim 20 \text{ min} \)) of radionuclides that necessitates the need to be close to a cyclotron during the study [829]. PET has been suggested to have no advantage over planar gamma scintigraphy and PK studies for the calculation of total lung deposition of pulmonary inhaled formulations [801, 808].

Magnetic resonance imaging (MRI) does not require the radiolabeling of an aerosol to determine qualitative and quantitative deposition of particles in the lungs, but rather is based on the Nuclear Magnetic Resonance phenomenon. This method utilizes non-ionizable radiation to generate images that can be augmented through the use of soft tissue contrast mechanisms and orientations of 2D and 3D images [801]. The interaction of nuclear magnetic moments of the experimental sample tissue with an external magnetic and electromagnetic fields form spin density, \( T_1 \) and \( T_2 \) relaxation times, and motion which ranges from diffusion to rapid produce the signals the MRI equipment utilized to form images. \( T_1 \) and \( T_2 \) depend on the local nuclei environment which is subject to disease state of the tissues, where \( T_1 \) is the longitudinal relaxation time constant and \( T_2 \) is the transverse relaxation time constant [801, 830]. Signals measured from the nuclei are either full or half integer values of spin in proportion to the angular momentum that results from an odd number of protons or neutrons. The majority of MRIs measure the signal from the ubiquitous hydrogen (\(^1H\)) atom, however lung imaging performed using helium-based (\(^3\)He) methods is now well established because the non-lipid soluble properties of helium allow it to stay within the airspace [801, 831, 832]. Hyperpolarized \(^3\)He or \(^{129}\)Xe gas MRI produce more
polarization, which leads to better imaging [830, 833, 834]. MRI has been employed within in vivo animal studies and within in vitro human lung airway replicas [830, 835-838].

Fluorescence imaging tracks fluorescently tagged particles within an aerosol to monitor their deposition in the lungs [801, 839]. This method is limited due to the issues of the excitation and emission fluorescence signals are scattered, reflected, and absorbed by the tissues [801, 840]. This affects the light captured by the detector and the actual number of molecules. In addition, a heterogeneous background signal exists because biological tissue has the capability to autofluoresce [840]. The autofluorescence background can be measured using untreated tissue and subtracted from the experimental background or the use of near-infrared wavelengths can be used to overcome this issue [841-844]. Fluorescent dyes are aerosolized, delivered to the lungs via inhalation, intracheal, or intranasal routes, and imaged using an in vivo imaging system (IVIS). The particle deposition in the lobes of the lungs can be visualized and quantified using image analyzing software [801]. Fluorescence imaging eliminates the need for radioactive tags, therefore it may be used to image deposition patterns in live animals [839].

PK studies investigate ADME of drugs by collecting lung deposition and plasma concentration data from either animal or human test subjects to determine the PK parameters. These parameters include drug absorption parameters such as the peak drug concentration (C\text{max}), the time C\text{max} occurs (t\text{max}), and the absorption rate constant (k\text{a}) to determine the absorption rate and the area under curve (AUC) to estimate bioavailability. Also, drug distribution and elimination parameters such as clearance (CL), volume of distribution (Vd), and half-life of the drug (t_{1/2}) can be determined. Mice and rat animal models are commonly used in animal PK studies because of their development in modeling various respiratory diseases, smaller drug requirement, and low cost [749, 781]. The drug can be administered through intranasal or intratracheal routes or by using passive inhalation [749, 781, 845]. Intratracheal or intranasal direct administration avoids oropharyngeal deposition, enabling accurate and reproducible dosing [846].

3.3.4. siRNA Delivery to the Lungs

For clinical use, siRNA needs to be delivered to the target region of the lung and be released within the cytoplasm after entering the target cells as shown in Figure 3.12. Successful gene therapy requires the effective levels of transgene expression in specific cell types to enhance the treatment efficacy and to avoid adverse side effects caused by the expression in inappropriate cell types.
siRNA is a highly negatively charged, hydrophilic, and large sized (approximately 13.3 kDa) macromolecule that cannot cross biological membranes to reach their target sites. Viral vectors have previously demonstrated cell uptake and siRNA efficacy, however, major limitations to human therapeutic delivery exist, such as uncontrolled viral replication, immunogenicity, tumorigenicity, and toxicity [847]. Due to these concerns, non-viral delivery systems have been developed and successfully used to deliver siRNA. An ideal siRNA delivery system should (1) condense siRNA into nano-sized particles, (2) protect siRNA from enzymatic degradation, (3) facilitate cellular uptake, (4) promote endosomal escape to release siRNA to the cytoplasm where the RISC is located, (5) have negligible effects on gene silencing activity or specificity and (6) have negligible toxicity [708, 848]. Non-viral delivery systems include naked siRNA delivery, and delivery vectors such as lipids, polymers, peptides, and inorganic materials.

**Figure 3.12.** Aerosol siRNA delivery to the lungs. (A) pulmonary delivery of naked or siRNA nanocarriers, (B) Carrier-based delivery of siRNA cellular uptake. The aerosolized formulation of siRNA and use of appropriate device delivers siRNA by inhalation, intratracheal, or intranasal delivery to the human or animal lung. Once the siRNA carriers reach the lower respiratory tract, they may reach target cells and become internalized through endocytosis. Carriers capable of endosomal escape avoid degradation in the lysosome and release free siRNA within the cytoplasm. The RNAi pathway may then be initiated, ultimately leading to mRNA degradation and gene silencing.
3.3.4.1 Barriers of Pulmonary Delivery of siRNA

Delivering siRNA efficiently to the lungs via the pulmonary route requires understanding of the anatomical and physiological characteristics of the respiratory tract. The human respiratory tract has a highly efficient gas exchange capacity and to keep particulates out (Table 3.5) as discussed previously [748]. Therefore, the development of siRNA aerosol delivery systems should consider careful control of particle or droplet size to bypass the lungs natural defense mechanisms.

**Table 3.5.** Pulmonary barriers to lung siRNA delivery [748].

<table>
<thead>
<tr>
<th>Airway Defenses</th>
<th>Alveoli Defenses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper airway (nose, throat, and trachea)</td>
<td>Degradative Enzymes e.g. proteases, RNase</td>
</tr>
<tr>
<td>Bifurcations</td>
<td>Neutrophils</td>
</tr>
<tr>
<td>Mucus</td>
<td>Opsonins</td>
</tr>
<tr>
<td>Mucociliary Clearance</td>
<td>Surfactant</td>
</tr>
<tr>
<td>Cough Clearance</td>
<td>Complement</td>
</tr>
<tr>
<td>Airway epithelium</td>
<td>Alveolar Macrophages</td>
</tr>
</tbody>
</table>

The respiratory tract is divided into two regions, (1) the conducting airways and (2) the respiratory region. The conducting airways encompass the nasal cavity, pharynx, trachea, bronchi, and bronchioles and the respiratory region consists of the respiratory bronchioles and alveoli [708]. The human respiratory tract has evolved to be a size selective fractionator that prevents particulates from reaching the deep lung. For example, the largest particles are removed at the naso- or oropharynx and progressively finer particles deposit in the proximal, intermediate, and distal airways. The main feature of the respiratory tract is the high degree of bifurcations that have been described according to Weibel’s lung model to have 24 generations in total. These gradually narrowing airways from the carina to the alveolar sacs cause increased particle velocity, which causes particles to impact the walls of airways instead of reaching the lower regions of the lungs. Therefore, for a therapeutic agent to reach the target site of action, it must possess aerodynamic properties to allow it to pass through the branched airways to the deep lung.
Aerodynamic particle size distribution is an important influence on the location of lung deposition of a formulation [849]. Three forces dominate particle motion within an aerosol cloud: gravitational sedimentation, inertial impaction, and Brownian diffusion. Gravitational sedimentation and inertial impaction are dominant when the aerodynamic diameter is > 2 µm. Large particles with aerodynamic particle size of > 6 µm deposit mostly on the airway walls at bifurcations in the upper airways due to their high momentum. Smaller particles of aerodynamic diameters of < 1 µm deposit due to Brownian diffusion [850]. If submicron sized particles are delivered without a larger carrier particle or droplet, then they would be mostly exhaled in the breath. A breath hold method may be used to increase deposition within the lower respiratory tract, however, it has lower efficiency. The most optimum aerodynamic particle sizes for deposition within the lower airways are between 1 to 5 µm [729, 749]. As particle size decreases below 1 µm, lung deposition increases due to increased diffusional mobility [851]. For particles less than 100 nm in size, approximately 50% of the nanoparticles will deposit within the alveolar region of the lungs. These nanoparticles often enter the lungs as large agglomerates that break apart upon deposition.

The elimination pathways for nanoparticles within the lungs involve coughing, dissolution, clearance via the mucociliary escalator, translocation from the airways, phagocytosis by macrophages and neuronal uptake [845, 852, 853]. Nanoparticle degradation is dependent on the composition of the delivery system and mediated through hydrolysis, oxidation, and reduction pathways [853, 854]. After deposition in the lower airways following inhalation, free siRNA will be susceptible to degradation from RNase, whose enzymatic activity is very high in the lungs [855-857].

A major pulmonary delivery barrier is the mucociliary clearance driven by the physical action of the ciliated epithelial cells, which move the mucus and alveolar fluid towards the upper airways, and eventually, the throat. Mucociliary clearance also includes the alveolar macrophages along the airways, which phagocytose foreign particles and deposits their remains to be removed by the mucociliary escalator. Mucus is present on the respiratory epithelium from the nasal cavity to the terminal bronchioles [858]. Types of glycosylated protein, known as mucins, are the main component of mucus [859]. Mucus is a physical barrier as it increases in viscosity of the moist surface of the lung epithelial cells, thereby reducing drug penetration and diffusion rate [860, 861]. Particles that are deposited on the mucosal layer above the ciliated epithelial cells are rapidly
removed by mucociliary clearance and are eventually either coughed out or swallowed into the gastrointestinal tract [858, 862]. The alveolar fluid is found on the surface of alveoli epithelium as a thin layer of pulmonary surfactant which is composed of phospholipids and surfactant-associated proteins known as SP-A, SP-B, SP-C and SP-D [863, 864]. Following delivery of foreign antigen or nanocarrier, pulmonary surfactant proteins SP-A and SP-D have been shown to play a role in macrophage uptake by acting as opsonins and scavenger molecules [865]. These pulmonary surfactants are located at the air-liquid interface of the alveoli, therefore they can interact with particulate aerosols that reach the lower airways [866]. Other major opsonins of the airways and alveoli of the lungs include immunoglobulins (IgG or IgM), complement fragments (C3, C4, C5), fibronectin, and laminin [867, 868]. The alveolar macrophages located within the alveoli rapidly engulf foreign particles by phagocytosis as a defense mechanism [869-871].

In addition to macrophages, the other major phagocytes are the neutrophils, or polymorphonuclear neutrophilic leukocytes (PMNs or polys), that are abundant in the blood but are not present in normal healthy tissue [872]. Like macrophages, neutrophils are phagocytic cells that have a role in the innate immunity because they recognize, ingest, and destroy many pathogens without the adaptive immune response [872]. When macrophages first encounter a pathogen within the lung, their action is augmented by the recruitment of large number of neutrophils to the infection site [872, 873]. Pulmonary administered naked siRNA is susceptible to alveolar macrophage or neutrophil uptake and are subsequently degraded within these cells, thus reducing their therapeutic efficacy.

Considering disease state is an important aspect of pulmonary deposition and clearance since the physiological conditions of the airways are altered and pose a threat on the efficiency of an aerosolized siRNA delivery system. During infection and inflammation, the airways become congested because there is increased mucus secretion and decreased mucociliary clearance [874, 875]. The thickness, viscosity, viscoelasticity, and the composition of the mucus layer depend on the pathological condition and have inter-patient variability [852]. Impaired lung function can greatly reduce the inspiratory force and coordination of the patient, making effective inhalation difficult, and therefore drug deposition can be suboptimal. For different therapeutic applications, disease state of the patient should be considered in order to design an effective aerosolized delivery system.
In addition to using particles with small aerodynamic diameter suitable for deposition in the lower airways, it has been reported that the use of large porous particles can prolong retention time in the lungs by effectively avoiding alveolar macrophage phagocytosis [876-878]. Porous particles over 10 µm in geometric diameter have a smaller aerodynamic diameter due to their mass-inertia relationship. Their smaller aerodynamic diameters of 1-3 µm fall within the ideal aerodynamic size range for effective lung deposition, while their actual geometric size is larger than the size range of alveolar macrophage uptake and removal.

The mucus layer may be partially deteriorated using mucolytic agents that break down the three dimensional gel network of mucus. A mucus inhibitor, such as glucopyrrolate, may also be used to overcome the mucus barrier [879]. In patients with bronchiectasis or cystic fibrosis, mannitol has been clinically proven to improve the hydration and viscoelastic surface properties of the sputum, which increased mucus clearance [880]. The deposition site of nucleic acid delivery systems in the airways has been previously manipulated and directed through the use of ultrasound and magnetic field technologies [680, 881, 882]. Another less-invasive option to enhance the transport across the mucus layer is to coat the surface of nanoparticles with neutrally charged molecules, like polyethylene glycol (PEG) [262].

Lung disease involving chronic lung inflammation, like cystic fibrosis, emphysema, or asthma, are characterized by increased levels of pulmonary proteases [883]. These proteases may degrade peptide-conjugated siRNA or peptide-based-polymeric nanocarriers of siRNA before the carrier gets to target specific cells of interest.

3.3.4.2 Barriers of Intracellular siRNA Delivery

To exert their effects, the siRNA must cross the cellular membrane and be released within the cytoplasm of the target cells of the respiratory tract, and it must access the RISC, where their intracellular interaction takes place. Due to the size of 13 kDa and a negative charge, the siRNA does not readily cross biological membranes. Therefore, an appropriate delivery system should facilitate siRNA cellular uptake.

The major cellular uptake pathway for non-viral siRNA delivery systems that are under 150 nm in size is known as endocytosis [884]. Endocytosis is the mechanism by which cells engulf polar molecules or nanocarriers that cannot readily pass through the hydrophobic cellular membrane. The four endocytosis pathways are clathrin-mediated endocytosis, caveolae-mediated endocytosis, micropinocytosis, and phagocytosis [885]. For the inhalation route of administration,
a particle size of less than 150 nm also avoids macrophage uptake, thus slowing lung clearance [886]. Clathrin-mediated endocytosis is the major pathway that allows for particle uptake through enclosed clathrin-coated vesicles [884]. These vesicles then fuse with early endosomes to form late endosomes, which subsequently form into lysosomes. As this process progresses from early endosome to lysosome, the pH within the vesicles drop down to approximately pH 5.0 and degradative DNase and RNase are present within the lysosome [884, 887, 888]. For a siRNA to elicit therapeutic effects within the cell, it must escape from the endosome and be released within the cytoplasm to avoid degradation by degradative enzymes. To promote endosomal escape, the “proton sponge hypothesis” can be practiced by using a high buffering capacity polymer with a large pH range, such as polyethylenimine (PEI), which will become protonated as the pH of the endosome drops [889, 890]. This protonation of the polymer causes an influx of chloride ions, protons and water into the endosomes. The building osmotic pressure causes the endosomes to burst, thus releasing its contents into the cytoplasm [890]. However, the use of PEI has been associated with toxicity issues [891]. An alternative strategy to promote endosomal escape is through the use of pH sensitive fusogenic peptides. These peptides undergo pH-dependent conformation changes at low pH which cause a membrane disrupting conformational change that destabilizes endosomal membranes [892-895].

Another cellular uptake mechanism of siRNA delivery systems is the caveolae-mediated endocytosis pathway [884]. The delivery system is internalized through a caveolin-coated vesicle known as a caveosome. The caveosomes are void of nucleases, degradative enzymes, and have a non-acidic pH. The delivery systems avoid lysosomal degradation because the contents of the caveosomes are transported to the Golgi or endoplasmic reticulum. Caveolin is expressed within lung tissues, therefore it may be a more efficient route for inhalation siRNA delivery systems compared to clathrin-mediated endocytosis, especially if the delivery system doesn’t possess endosomal escape properties [708, 896, 897].

Another cellular uptake mechanism of cells of the lungs is phagocytosis [884]. This route can only be performed by alveolar macrophages and other specialized cells. Uptake of delivery systems by this pathway should be avoided since siRNA molecules taken up are eventually degraded by the phagolysosomes of the cells.
3.3.5. Summary of the Rationale for Gene Delivery Systems for Aerosol Delivery of siRNA to the Lungs

Lower airway pulmonary delivery can be achieved through the inhalation of an aerosol from delivery devices such as pMDIs, DPIs, nebulizers, and SMIs. Preclinical safety and efficacy studies using animal models often deliver pulmonary aerosols via the inhalation, intratracheal, and intranasal routes. The lung physiological and anatomical differences between human and animals are an integral consideration when selecting the route of administration to assess the efficacy of an aerosol. Intratracheal administration involves an invasive surgical setup and anesthesia. The intranasal route of administration can be used to reach the airways in mouse models by instilling and aspirating the liquid solution or suspension into the nasal cavity of the animal. Dry powder formulations can be delivered using a microsprayer that when actuated, generates the aerosol and propels it into the airways. The nose-only exposure chamber used in preclinical studies has provided a robust means of delivering aerosols to the deep lung. Aerosolized siRNA formulations are characterized using *in vitro*, *ex vivo*, and *in vivo* techniques. *In vitro* techniques measure the dissolution rate, aerodynamic particle size distribution, total delivery dose uniformity, particle size, and aerosol velocity. *Ex vivo* techniques analyze the mechanisms of drug transport, deposition, and absorption. *In vivo* techniques provide visualization and quantification of aerosol deposition in the respiratory tract, PK parameters, and biochemical and physiological effects of the pulmonary delivered drug. Barriers for pulmonary delivery of siRNA exist due to the natural defenses of the lungs to keep exogenous particulates and substances out. Delivering aerosolized siRNA effectively to the lungs and to their target site-of-action involves the understanding of the anatomical and physiological characteristics of the respiratory tract. The barriers for aerosolized delivery of siRNA include degradation by RNase and mucociliary clearance. Intracellular naked siRNA delivery is impeded due to their large size, negative charge, and susceptibility to degradation. These intracellular delivery constraints may be mitigated through the use of siRNA nanocarrier systems for aerosol delivery including those of lipidic, polymeric, peptide, or inorganic origin. Part II of this review article will discuss the preparation methods of various siRNA nanocarrier systems, accompanied by a series of examples.
3.4. Nanocarrier-based Delivery Systems for Aerosol Delivery of siRNA to the Lungs

3.4.1. Rationale for Nanocarrier-based Delivery Systems

siRNA has potential therapeutic applications in treating ‘undruggable’ diseases via post-transcriptional downregulation of target gene expression. The mechanisms of RNAi have been comprehensively reviewed [700-703]. The siRNA is composed of a specific sequence that is complementary with its target mRNA and induces site-specific cleavage and subsequent inhibition of intracellular protein synthesis. The synthesis of siRNAs is relatively simple compared to other therapeutic classes because it does not require a cellular expression system, complex protein purification, or refolding schemes [704]. A major advantage of siRNA over small molecule drugs or protein therapeutics is that the sequences can be rapidly designed for highly specific inhibition of the target of interest.

Pulmonary diseases such as asthma, lung cancer, cystic fibrosis, pulmonary hypertension, and chronic obstructive pulmonary disorder (COPD) have potential siRNA therapeutic targets [704-706]. However, due to the high negative charge density and the relatively large size of the siRNA molecules, naked siRNA molecules are not able to enter cells efficiently [707]. Thus, delivery systems for siRNA need to be developed to successfully protect and deliver these agents. Nevertheless, siRNA delivery systems are likely to have instability issues that cause premature release of the nucleic acids, especially with systems that incorporate their cargo through electrostatic interactions. Pulmonary delivery of siRNA faces major challenges that involve decreased correlation between in vitro and in vivo experiments, difficulty in translation from animal models to humans, and administration routes used in animal studies that are non-applicable for human use [708]. Another challenge in siRNA therapy is the possibility of off-target effects induction.

This Section 3.4 review chapter focuses on the pulmonary route of administration, siRNA loaded non-viral particulates for pulmonary or nasally inhaled delivery systems, and preparation techniques for siRNA loaded nanoparticles [898]. While the previous part (Section 3.3) covered the rationale for the use of various siRNA delivery systems, this part will focus on the preparation of siRNA loaded nanocarrier systems including examples of their pulmonary delivery.
3.4.2. Non-viral Delivery of siRNA to the Lung

siRNA is a highly negative charged, hydrophilic, and large-sized (approximately 13.3 kDa) macromolecule that cannot cross biological membranes to reach their target sites. Viral vectors have previously demonstrated cell uptake and siRNA efficacy, however, major limitations to human therapeutic delivery exist, such as uncontrolled viral replication, immunogenicity, tumorigenicity, and toxicity [847]. Due to these concerns, non-viral delivery systems have been developed and successfully used to deliver siRNA. An ideal siRNA delivery system should (1) condense siRNA into nano-sized particles, (2) protect siRNA from enzymatic degradation, (3) facilitate cellular uptake, (4) promote endosomal escape to release siRNA to the cytoplasm where the RNA-induced silencing complex (RISC) is located, (5) have negligible effects on gene silencing activity or specificity and (6) have negligible toxicity [708, 899]. Non-viral delivery systems include naked siRNA, and delivery vectors such as lipids, polymers, peptides, and inorganic materials, as shown in Figure 3.13.

Figure 3.13. Schematic of different non-viral siRNA delivery vectors.
3.4.2.1 siRNA Delivery

3.4.2.1.1 Introduction to siRNA Delivery

Unformulated or naked siRNA involves the delivery of siRNA without the use of a delivery vehicle or carrier. Advantages of this strategy include the ease of preparation and the facility of delivery by inhalation, intratracheal, or intranasal routes. A major disadvantage of this strategy is that the delivered siRNA is susceptible to poor cell targeting and uptake, and to degradation within the airways. As discussed in part 1 of the review article, the major barriers for the delivery of siRNA to the lung include the presence of mucus, alveolar fluid, alveolar macrophages, and mucociliary clearance. Unfavorable physicochemical properties of siRNAs (negative charge, large molecular weight) and instability in plasma (half-life 10 min) also poses major delivery challenges. A major disadvantage of this strategy is that the delivered siRNA is susceptible to poor cell targeting and uptake, and to degradation within the airways. As discussed in part 1 of the review article, the major barriers for the delivery of siRNA to the lung include the presence of mucus, alveolar fluid, alveolar macrophages, and mucociliary clearance. Unfavorable physicochemical properties of siRNAs (negative charge, large molecular weight) and instability in plasma (half-life 10 min) also poses major delivery challenges [900]. Furthermore, after being transported intracellularly into the lysosomes via endocytosis, siRNA gets degraded in the lysosomes which diminishes the activity of siRNA therapeutics [631]. Since unmodified siRNA is prone to enzymatic degradation, new methods such as chemical modification of siRNA have been developed to increase stability. The chemical modification also improves specificity and potency, and reduces the immune response and off-target effects [901].

3.4.2.1.2 RNA Modifications & Preparation Methods

The local delivery of siRNA is particularly well-suited for lung disease and infection therapy [902]. The direct instillation of siRNA into the lungs through intranasal or intratracheal routes results in direct contact with lung epithelial cells. Non-modified siRNA’s can also induce nonspecific activation of immune system through the Toll-like receptor 7 pathway [903]. However, chemical modifications can be introduced into the RNA duplex structure which can enhance biological stability without adversely affecting gene-silencing activity and prevent nonspecific immune activation. Some of the modifications include incorporation of 2′-O-methyl modifications into the sugar structure of selected nucleotides within both the sense and antisense strands. The therapeutic efficacy of delivered siRNA can also be improved using conjugation of small molecules or peptides to the sense strand of siRNA. Several other modifications have been reported to eliminate the off-target effects such as phosphorothioate or boranophosphate introduction [904].

In a recent study, Antagomir-122 was synthesized from a hydroxyproline-linked cholesterol solid support and 2′-O-methyl phosphoramidites. On administration a marked decrease in endogenous miR-122 levels in the liver was observed [905]. This and such other newer strategies
have shown promise in protecting the siRNA against degradation by endonucleases thus allowing them to reach their site of action.

There are 3 main methods used for the production of siRNA in vitro which includes 1) chemical synthesis, 2) in vitro transcription of small RNA’s and 3) in vitro transcription of long RNA’s produced by the digestion with Dicer enzyme [906]. The in vitro chemical synthesis of siRNAs involves in vitro transcription and digestion of long dsRNAs by an RNase III family enzyme (e.g. Dicer, RNAse III). These production methods require the design of siRNA sequences before siRNA preparation. Recently, Aalto et al. utilized an in vitro system using the combination of T7 RNA polymerase and RNA-dependent RNA polymerase (RdRP) of bacteriophage f6 to generate siRNA molecules. They further used an in vivo RNA replication system to produce siRNA. This system was based on carrier state bacterial cells containing the f6 polymerase complex which can result in unlimited amounts of siRNA up to 4.0 kb in size. Unmodified or modified siRNA can be prepared with simple reconstitution within normal saline or 5% dextrose solution for inhalation delivery. Aerosolization of the siRNA solution can be conducted using a nebulizer. A new class of naked siRNA has been developed by Hamasaki et al., termed ribophorin II (PnkRNA™) and nkRNA ® [907]. This novel class of RNAi agents was synthesized as single-stranded RNA on a solid phase that, following synthesis, self-anneal into a unique helical structure containing a central stem and two loops [748]. Furthermore, a naked and unmodified novel RNAi agent, such as RPN2-PnkRNA, which has been selected as a therapeutic target for lung cancer when delivered via inhalation resulted in efficient inhibition of tumor growth without any significant toxicity.

3.4.2.1.3 Examples of siRNA Delivery

Luciferase expressing mice were treated by intratracheal administration of 10 nmol siRNA duplex or 10 nmol phosphorothioate locked antisense oligonucleotide free nucleic acids [908]. Ex vivo luminometry elucidated organ associated luciferase knockdown. IVIS imaging of oligonucleotides labeled with Cy5 combined with confocal microscopy was used to determine their biodistribution [908]. Upon administration, the oligonucleotides underwent fast systemic distribution by transcytosis and renal clearance. The kidney and liver uptake of the phosphorothioate locked nucleic acid antisense oligonucleotides caused gene knockdown in these organs. However, protein expression was not downregulated in purified lung tissue cells [908]. The reduced therapeutic efficacy of free RNA supports observations by another group, who
concluded that intratracheally delivered free TNF-α siRNA post-hemorrhage was inefficient in reducing the symptoms in a septic shock model of acute lung injury [909]. After bleomycin triggered pulmonary fibrosis was induced in mice with transgenic expression of human TGF-β1, 5 mg/kg TGF-β1 siRNA or a scrambled control was delivered intratracheally. This study found that siRNAs sequences shared by human and rodents successfully knocked down TGF-β1 expression in human derived cell lines and significantly inhibited pulmonary fibrosis in vivo [910].

3.4.2.2 Lipid-based Delivery Systems
3.4.2.2.1 Introduction to Lipid-based Delivery Systems

Lipid-based delivery systems are commonly used to deliver siRNA in vitro or in vivo [631]. Most often, cationic lipids or liposomes are used to form complexes, termed lipoplexes with anionic siRNA through spontaneous electrostatic interaction. Commercial siRNA transfection agents are commonly lipid-based systems, including Oligofectamine™, TransIT-TKO, Lipofectamine® RNAiMAX, and DharmaFECT [632-635]. A major challenge of using lipid-based siRNA delivery systems is their toxicity and non-specific activation of inflammatory cytokines and interferon responses [636]. Since aerosolization is a high shear stress process, the stability of the liposomes should be monitored since this process may cause physical and chemical changes that can lead to early siRNA release and degradation of the siRNA [637]. Lipid based delivery systems for siRNA include liposomes, solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC).

SLN are prepared by replacing the oil of the fat emulsion by a solid lipid or a blend of solid lipids, which makes the lipid matrix of the SLN solid at room and body temperature [657-660]. SLN are composed of 0.1-30% w/w lipid dispersed in an aqueous solution of 0.5-5% w/w surfactant as a stabilizing agent [661, 662]. SLN provide physical stability, chemical stability, controlled release, and low cytotoxicity if appropriate excipients are utilized [663]. SLN can be produced without the use of organic solvents and can be scaled up. Disadvantages of SLN are low loading capacity and premature release during storage [664]. These events occur because the low ordered lipid modification of the particle matrix after production transforms to the highly ordered β-modification during storage. The β-modification is characterized by perfect crystal lattice with few imperfections and therefore, little room is left for siRNA storage. In order to overcome these issues, a second generation of lipid nanoparticles, the NLC were developed. NLC have a solid lipid matrix at room and body temperature and consist of a blend of solid lipid and oil, preferably at a
ratio of 70:30 up to a ratio of 99.9:0.1 [249, 664, 911]. Through mixing different kinds of lipids, a less ordered matrix with more room for active compounds is achieved [664].

3.4.2.2.2 Preparation Methods

3.4.2.2.2.1 Cationic Lipoplexes

Cationic lipids or liposomes can spontaneously form complexes with negatively charged siRNA through electrostatic interactions to form lipoplexes (Figure 3.14). Complexation of cationic lipids and siRNA occurs through the negatively charged siRNA interaction with the positively charged lipids. This interaction causes neutralization of the cationic lipids, causing them to aggregate and encapsulate the siRNA [912]. It is necessary to optimize the lipid composition, lipids to siRNA ratio and the lipoplexes preparation methods, in order to improve stability and delivery efficiency while reducing possible adverse effects. Lipoplexes often have good transfection efficiency due to their efficient interaction with the negatively charged cell membranes as shown in Fig. 3. However, lipoplexes generally display poor stability and reproducibility when compared to other lipid based siRNA delivery systems, such as liposomes or SLN [913]. Another disadvantage is that cationic lipids are typically more toxic than neutral lipids [914]. To shield the positive charge of the cationic lipids, hydrophilic polymers such as PEG have been used to reduce inflammatory response.

Figure 3.14. Cationic liposome and anionic siRNA interaction forming a lipoplex. The cationic lipoplex interacts with the negatively charged cellular membrane leading to enhanced delivery within the cells via endocytosis.
Lipoplexes are not stable in liquid suspension for long-term storage, as shown by several studies in which the lipoplexes aggregate [915, 916]. This leads to the requirement of preparation immediately prior to administration or to improve lipoplex stability through the use of high pH diluents, sucrose gradients, and conjugation of PEG to the surface of lipoplexes [916-920].

Lyophilization is another method for intervening this issue that has been shown to inhibit aggregation for long-term storage of nucleic acid formulations [915, 921, 922]. Lyophilization of siRNA lipoplexes allows for longer storage times compared to leaving the lipoplexes in suspension and/or freezing them. Lyophilization allows for storage at room temperature, and therefore is preferred over freezing due to cost reduction in transportation and storage, and improved stability of biomolecules resulting from the removal of non-freezable water associated with most biomolecules [923]. Lyophilization can cause liposome fusion and phase separation during the drying and rehydration steps [924]. To overcome these problems, cryoprotectants, such as carbohydrates, are used. Cryoprotectants limit mechanical damage and rupture of the lipid bilayer, caused by ice crystals, during the freeze-drying and the rehydration process by maintaining the membrane in a flexible state [925].

### 3.4.2.2.2 Liposomes

The basic steps of loaded liposome production include (1) lipid hydration, (2) regulation of liposome size, and (3) removal of non-encapsulated drug [926].

Lipid hydration can be achieved through mechanical methods, organic solvent methods, or detergent removal methods [655, 912]. Mechanical methods for lipid hydration are based on using a rotating evaporator to form a thin-layer of phospholipids onto the wall of the rotating vessel. The layer is hydrated with an aqueous buffer solution that contains the siRNA to load while the vessel rotates. The process forms multilamellar vesicles, which can further be sonicated, extruded, or homogenized to form small unilamellar vesicles. Glass beads may be used to optimize the process by allowing the creation of thinner lipid films by increasing surface area. These thinner lipid films allow for higher hydration efficiency and siRNA entrapment. Lipid hydration by organic solvent methods requires the melting of lipids within an organic solvent followed by exposure to an aqueous phase that is then separated by evaporation. If the starting point is an organic solvent immiscible with the aqueous phase, then it is possible to use the reverse-phase evaporation vesicles technique, where the intermediate state is represented by emulsions. The lipid solution in an organic solvent with the aqueous buffer is sonicated to obtain a water-in-oil (W/O) emulsion.
Controlled evaporation of the organic solvent leads to the formation of unilamellar vesicles. When most of the organic solvent is removed, a gel is formed, and it has to be agitated to transform into a viscous fluid that contains liposomes. The gel collapse coincides with the conversion of the W/O emulsion in the liposome form. If the starting point is an organic solvent miscible with water, then it is possible to continue with a precipitation stage. An aqueous buffer is added to the miscible organic solvent to dilute it, leading to the precipitation of lipids which then aggregate to form liposomes. The speed of the dilution passage can be adjusted to obtain desirable liposomal particle size, with faster dilution passages being associated with smaller particle size of formed vesicles.

Detergent removal methods for lipid hydration form micellar structures that engulf more lipids as the detergent is removed to form unilamellar vesicles. This method is less efficient for low molecular weight compounds than other lipid hydration methods [927]. Since this method has not been studied for large hydrophilic compounds like siRNA molecules, it is assumed that liposomal formulation of siRNA using this method is not suitable [912].

Size optimization and removal of free siRNA should be performed following the lipid hydration step. Size of liposomes should be adjusted based on the siRNA that needs to be incorporated. This may be achieved through (1) extrusion at low or medium pressure through dimensionally defined pores, (2) fractionation of a heterogeneous population by centrifugation or size-exclusion chromatography, and (3) homogenization to obtain smaller sized liposomes [912]. Examples of extrusion devices are the Avanti Mini-Extruder and Nano DeBEE high pressure homogenizer shown in Figure 3.15A, B and C. Other mechanical dispersion methods include sonication, freeze-thawed liposomes, lipid-film hydration by shaking or freeze drying, or dried reconstituted vesicles [928]. The non-encapsulated siRNA can be removed by dialysis, ultrafiltration via ultracentrifugation, gel chromatography, and ionic-exchange resins [912].
Methods of encapsulating siRNA in liposomes include simple mixes of siRNA with pre-assembled liposomes, pre-condensation of siRNAs before liposome encapsulation, direct hydration of a lipid thin-film layer with a siRNA solution, and the ethanol dilution method [912]. A pre-assembled liposome can be complexed with siRNA with a simple mixture method. This method is not suitable for PEGylated liposomes, since siRNA is unable to penetrate the PEGylated lipid bilayer efficiently [757]. In this case, the siRNA would bind to the liposomal surface, which causes premature release of the siRNA from the liposome in vivo. To achieve a siRNA PEGylated liposome loaded using the simple mixture method, the siRNA and the non-PEGylated liposomes can be incubated to form the siRNA liposome complex and subsequent incubation with PEG-lipids at elevated temperatures. The PEG-lipids would be inserted within the lipid bilayer, which can be verified by measuring zeta potential [70]. An alternative is to covalently bind PEG chains to the siRNA liposome complexes after simple mixture loading of the siRNA. An example of this surface modification is the binding of PEG to the amine functional groups of the cationic cholesterol
polyamine (CDAN) component of a liposomes using a pH-sensitive oxime linkage that decomposes at pH <5.5 [929]. This technique, known as the “ABCD paradigm”, overcomes drawbacks of other PEGylation methods, in that there is adequate siRNA encapsulation, PEGylation elicits a stealth behavior, and the PEG chain release of the liposomes within the endosomal compartments leads to a pH triggered endosomal escape [930].

Another technique involves pre-condensation of siRNA with protamine, a natural cationic polypeptide, along with high molecular weight polyanions, such as hyaluronic acid, at a ratio allowing for negatively charged nanoparticles [931]. The high molecular weight polyanion is used to provide resistance to reticuloendothelial clearance instead of PEG [932]. These complexes are then incubated with cationic liposomes to form the siRNA encapsulated liposomes. Alternatively, siRNAs can be pre-condensed with calcium phosphate nanoparticles followed by the cationic liposomal coating and PEG-lipids [933].

The direct hydration of a thin-film lipid layer with siRNA solution allows for encapsulation of siRNA within the aqueous core of a liposome. Addition of a concentrated solution of siRNA to a dry thin lipid film layer allows for the incorporation of the siRNAs into the internal cationic space of a lipidic bilayer [934]. Since siRNA is present during the formation of the liposomes, approximately 50% of the siRNA is encapsulated as it is uniformly distributed across cationic lipids, including the internal and external lipid bilayers. An alternative approach involves the formation of a monophasic solution of siRNAs and DOTAP in water/methanol/chloroform mixture that leads to a perfect complex between the negative charge of siRNA and the positive charge of the cationic lipids [935]. Once excess water and chloroform are added, a phase separation occurs. siRNA/DOTAP micelles stay in the organic phase and the aqueous/methanol phase can be discarded. The neutral PEGylated lipids are added along with water and then the organic solvent can be removed by evaporation. The liposomes can then be extruded for further size control [912].

The ethanol dilution method for directly hydrating the lipid layer is an alternative method of direct hydration. Cationic PEGylated liposomes are mixed with siRNA in the presence of a critical concentration of ethanol (approximately 40%) in the aqueous buffer [936]. At this ethanol concentration, liposomes are destabilized and their membrane structural integrity is compromised, allowing siRNA to uniformly penetrate the liposomes and associate with the positive charges on the lipids. Ethanol concentration is pivotal since if it is at an inadequate level, the siRNAs would not be able to efficiently penetrate the lipid bilayer. If the ethanol concentration is too high,
liposomal aggregation would occur. The ethanol is removed by dialysis or tangential filtration following siRNA loading, forming the PEGylated cationic liposomes containing approximately 50% of the originally added siRNA. Stabilized Nucleic Acid Lipid Particles, or SNALPs, is a widely recognized example of liposomal complexes prepared using the ethanol dilution method [937]. A cationic lipid is protonated at low pH of 4-5 within the ethanol of the complex formation buffer, but is neutral when the acidic buffer is exchanged with a physiological buffer at pH 7.4. The advantages of cationic lipids are that siRNAs bound to the external surface of the liposome are detached when neutralized. The released siRNAs can be washed away from the complexes, together with ethanol, during the buffer exchange procedure. The final complexes can have a final siRNA complexation and encapsulation efficiency of around 90%. Other complexes of siRNA/PEGylated liposomes are prepared similarly to SNALPs only that they contain other cationic lipids and termed as Lipidoid Nanoparticles [938, 939]. This process is carried out with a combination of siRNAs in aqueous buffer with an equal volume of lipids (cationic, neutral, and PEGylated lipids), dissolved in butanol, the mixture is lyophilized and the lyophilized matrix is rehydrated [636, 938].

3.4.2.2.3 Solid Lipid Nanoparticles and Nanostructured Lipid Carriers

SLN formulations are composed of solid lipids, emulsifiers, and water [664]. A schematic of SLN preparation detailing the types of components and feed solutions used is shown in Figure 3.16. NLC formulations are composed of solid lipids, oil, surfactant, and water [664]. Lipids are used in a broad sense to include triglycerides, partial glycerides, fatty acids, steroids, and waxes. All classes of emulsifiers have been used to stabilize the lipid dispersion. Particle agglomeration may be efficiently prevented through the combination of emulsifiers selected in respect to charge and molecular weight. The use of physiologic lipids within the lipid matrix decreases the possibility of acute or chronic toxicity. The choice of emulsifier heavily depends on the administration route.
Initially, high shear homogenization and ultrasound are dispersing techniques used for the production of solid lipid nanodispersions. Despite both methods being common due to their ease of use, dispersion quality is poor due to the presence of microparticles and metal contamination.

High pressure homogenization is a reliable and powerful technique for the preparation of SLN. High pressure homogenization has been used for the production of nanoemulsions for parenteral nutrition; therefore, these homogenizers are available from several manufacturers with varying sizes. The scalability of this technique is highly efficient. High pressure homogenizers force liquid with 100-2000 bar pressure through a narrow orifice with size of approximately a few micrometers. Due to the narrowed orifice, the fluid accelerates on a very short distance to a high velocity of over 1000 km/h. High shear stress and cavitation forces disrupt the particles down to the nanoparticle size range. Typically, the lipid contents are between 5-10% and even higher lipid concentrations up to 40% have been successfully homogenized to lipid nanodispersions. A preparation step that incorporates drug into the bulk lipid by dissolving or dispersing the drug in the lipid melt followed by hot or cold homogenization techniques are used to prepare SLN.
Hot homogenization, or the homogenization of an emulsion, is performed at temperatures above the melting point of the lipid. The pre-emulsion of siRNA loaded lipid melt and the aqueous emulsifier phase is obtained using a high shear mixing device, such as an Ultra-Turrax homogenizer. The quality of the pre-emulsion affects the quality of the final product to a high degree since it is desirable to obtain droplets within the size range of a few micrometers. The higher temperature leads to a lower particle size because of the decreased viscosity of the inner phase. However, high temperatures may increase the degradation of the siRNA or the SLN. The homogenization step may be repeated several times to achieve the desired particle size, keeping in mind that the high pressure homogenization equipment increases the temperature of the sample by approximately 10°C for every 500 bar increase in pressure. Increasing the homogenization pressure and number of cycles over the recommended 500-1500 bar 3-5 cycles, respectively causes a particle size increase of the lipid nanocarriers due to particle coalescence as a result of high kinetic energy of the particles. The primary product of hot homogenization is a nanoemulsion due to the liquid state of the lipid. Cooling the nanoemulsion sample to a lower temperature forms the solid particles. Lipid crystallization is slowed due to the small particle size and the incorporation of emulsifiers, therefore the sample may remain as a supercooled melt for several months.

Cold homogenization is a high pressure milling of a suspension of solid lipids. Effective temperature control and regulation is needed in order to ensure the unmolten state of the lipid due to the increase in temperature during homogenization. Cold homogenization has been developed to overcome temperature induced drug degradation, drug distribution into the aqueous phase during homogenization, and the complexity of the crystallization step of the nanoemulsion leading to several modification or supercooled melts associated with hot pressure homogenization. The first preparatory step that includes the dispersing of the drug in the melt of bulk lipid is followed by a rapid cooling step within liquid nitrogen or dry ice. In this step, the high rate of cooling causes the formation of a homogenous distribution of the drug within the lipid matrix. The solid, siRNA containing lipid is then milled to microparticles. Ball or mortar milling typically results in particle sizes of 50-100 micrometers. Low temperatures increase the fragility of the lipid and causes particle comminution, or the reduction in particle size. The solid lipid microparticles are dispersed in a chilled emulsifier solution. Then the pre-suspension is subjected to high pressure homogenization at or below room temperature. Compared to hot homogenization, generally larger particle sizes and broader size distribution are obtained in cold homogenized samples. The method
of cold homogenization minimizes the thermal exposure of the sample, but it does not fully avoid it due to the melting of the lipid/siRNA mixture during the initial step.

SLN can be prepared by solvent emulsification or evaporation techniques by precipitation in o/w emulsions. Lipophilic material is dissolved in a water-immiscible organic solvent that is emulsified within an aqueous phase. Once the solvent evaporates, precipitation of the lipid in the aqueous phase forms a nanoparticle dispersion.

SLN can be prepared by the dilution of microemulsions. Since there are varying opinions on the structure and dynamics of a microemulsion, a detailed review on the subject has been published [941]. A microemulsion can be envisioned as a two-phase system composed of an inner and outer phase, such as o/w microemulsions. They are prepared by stirring an optically clear mixture at 65-70°C composed of a low melting fatty acid, an emulsifier, co-emulsifiers, and water. The hot microemulsion is dispersed in cold water (2-3°C) under stirring. Typical volume ratios of the hot microemulsion to cold water are between 1:25 to 1:50. The dilution process is determined by the composition of the microemulsion. The droplet structure is already contained in the microemulsion, therefore no energy is required to achieve nanoparticle sized particles. The temperature gradient, pH, and the composition of the micro emulsion determine the product quality. High-temperature gradients facilitate rapid lipid crystallization and prevent aggregation. The dilution step constrains the lipid to a considerably lower concentration level than high pressure homogenization based formulations.

Lyophilization increases the chemical and physical stability of SLN over an extended period of time. Transformation of the SLN into a solid form prevents crystal growth by Ostwald ripening and avoids hydrolysis reactions. Lyophilization is also possible for SLN formation of dry powders for inhalation or for reconstitution for nebulizer delivery. The solid state of the lyophilized SLN will have increased chemical and physical stability compared to the aqueous lipid dispersion. Two additional transformations between the formulations are necessary and may cause stability problems. The first transformation from aqueous dispersion to powder involves the freezing of the sample and the evaporation of water under vacuum. Freezing of the sample may cause stability problems due to the freezing out effect which results in changes in the osmolarity and the pH. The second transformation, resolubilization, involves low water and high particle content and high osmotic pressure that could facilitate particle aggregation.
The lipid content of the SLN dispersion should not exceed 5% to prevent an increase in particle size because direct content of lipid particles is decreased in more diluted samples. The protective effect of the surfactant can be compromised during lyophilization. Diluted SLN dispersions will also have higher sublimation velocities and a higher specific area. The addition of cryoprotectants will be necessary to decrease SLN aggregation and to obtain a better re-dispersion of the dry product. Typical cryoprotective agents are sorbitol, lactose, mannose, trehalose, glucose, and polyvinylpyrrolidone. They decrease the osmotic activity of water and crystallization and favor a glassy state of the frozen sample. Cryoprotectants are place holders that prevent contact between discrete lipid nanoparticles and they interact with polar head groups of the surfactants and serve as a pseudo hydration shell.

As an alternative to lyophilization, spray drying can be used to transform an aqueous SLN dispersion into a dry product [804, 942, 943]. Spray drying can cause particle aggregation due to high temperatures, shear forces, and partial melting of the particles, therefore it is recommended to use lipids with melting points > 70°C. The incorporation of carbohydrates and low lipid content improve the preservation of the colloidal particle size during spray drying. The melting of the lipid can be minimized by using ethanol-water mixtures as a dispersion medium instead of pure water due to the lower inlet temperatures.

**3.4.2.2.3 Examples of Lipid-based Delivery Systems**

Functional genomic studies to study novel targets for lung cancer or other diseases have commonly utilized commercially available cationic lipid transfection reagents, including Lipofectamine RNAiMAX [944], Lipofectamine2000 [945-954] and HiPerfect [954]. Other groups have made lipoplexes composed of phospholipids and cholesterol with particle size of approximately 100 nm that have shown higher cellular internalization in lung cancer cells than with Lipofectamine2000, but had higher cytotoxicity. Oxime ether lipid nanoparticles with particle size of 150-220 nm transfected cells more efficiently with serum present than conventional liposomal formulations [955].

A myeloid cell leukemia 1 (Mcl1)-specific siRNA administered in a lung metastases model at 0.21 mg/kg was delivered by a microsprayer intratracheally four times on alternating days starting at 5 days post intravenous (i.v.) injection of either B16 or Lewis Lung Carcinoma cells [956]. In this study cationic nanoliposomes with particle size of 200 nm were prepared using the thin-film hydration technique and were composed of the various lipid ratio of EDOPC, DOTAP,
DOTMA, DC-Cholesterol, DOPE, and Cholesterol [956]. The thin-films were hydrated with 1 ml of 20 mmol/l HEPES (pH 7.4), and the resulting nanoliposomes were extruded through 0.2 µm polycarbonate membrane filters three times using an extruder [956].

An intranasally delivered liposome formulation containing a novel siRNA sequence against the epithelial sodium channel (ENaC) alpha subunit was developed and evaluated by Clark et al. [957]. Liposomes administered intranasally caused significant ENaC downregulation without TLR3, 8, or 9 activation, thereby supporting that the effects were induced by RNAi [957].

An aerosolized liposomal formulation was developed by Mainelis et al., who prepared liposomes composed of egg phosphatidylcholine: 1,2-dipalmitoyl-sn-glycero-phosphatidylcholine:cholesterol containing doxorubicin and antisense oligonucleotides or siRNA targeted against multidrug resistance-associated protein 1 (MRP1). This group used these formulations to evaluate the delivery of a nose-only small animal exposure chamber [782]. Mainelis et al. characterized the aerosolized particles and found that the change of mean particle size was insignificant during the 1 hour of continuous aerosolization. It was shown that, of the nebulized formulation, 1.4% at each port was available for inhalation and the lung retention was higher than in the injected samples [782]. The lung tumor model was prepared by intratracheal administration of luciferase expressing A549-luc cells followed by detection of tumor growth by luminescence imaging. After inhalation treatment with antisense oligonucleotides and doxorubicin combination, the tumor volumes were 90% reduced, compared to 40% after doxorubicin i.v. injection [782].

3.4.2.3 Polymer-based Delivery Systems

3.4.2.3.1 Introduction to Polymer-based Delivery Systems

Polymers for use in nanoparticle drug delivery systems should be biocompatible in terms of non-toxicity, non-antigenicity, biodegradability, and biocompatibility. The natural polymers that have commonly been used for preparation of polymeric nanoparticles are albumin, chitosan, dextran, gelatin, lectins, and sodium alginate [99, 581, 582]. There are many synthetic polymers available, while some are still under development. The well-known synthetic polymers include Poly (e-caprolactone) (PECL), polylactide (PLA), polystyrene, polyglycolide (PGA), Poly(lactide co-glycosides (PLGA), polyanhydrides, polyorthoesters, polycyanoacrylates, polycaprolactone, polyglutamic acid, polymaleic acid, poly(N-vinyl pyrrolidone), poly(methyl methacrylate), poly(vinyl alcohol), poly(acrylic acid), polyacrylamide, polyethylene glycol, and polymethacrylic
acid. Polymeric nanoparticles include nanocapsules, which contain a reservoir core surrounded by a solid material shell, or nanospheres, which are solid matrix particles [958]. Polymeric siRNA carriers are capable of swelling due to hydration and release siRNA by diffusion. Another physicochemical mechanism of release is by enzymatic degradation of the polymer at the delivery site, thus releasing the siRNA from the core. The siRNA may also dissociate causing de-adsorption or release. An advantage of the use of polymers as a siRNA delivery systems is the possibility of chemical modifications, which include the synthesis of block polymers and comb polymers or attachment of targeting moieties [959]. Disadvantages of polymer delivery systems stem from the use of organic solvents in production processes that leave residues, polymer cytotoxicity, and obstruct scalability of the production process. Another disadvantage of peptide based polymer inhaled delivery systems is their ability to increase the elastic and viscous moduli of lung fluid and mucus, decreasing mucociliary clearance, which can complicate many diseases states [960-962].

3.4.2.3.2 Preparation Methods for Polymer-based Nanoparticle Delivery Systems

The preparation methods of polymeric nanoparticles from a polymeric dispersion include emulsification/solvent diffusion, solvent evaporation, nanoprecipitation, salting out, dialysis, and supercritical fluid (SCF) technology methods. The preparation methods of polymeric nanoparticles from polymerization of monomers are emulsion (including mini- and micro-), interfacial polymerization, and controlled/living radical polymerization (C/LRP). Ionic gelation and coacervation are methods for polymeric nanoparticle preparation from hydrophilic polymers.

3.4.2.3.2.1 Emulsion and Diffusion Techniques

Emulsions are typically formed by two immiscible phases and a surface active agent and some form of dispersive force. Polymer precipitation from emulsion droplets can be achieved by removing the polymer solvent through solvent evaporation, fast diffusion after dilution, or salting out [958]. These methods lead to formation of nanospheres when performed on simple oil-in-water emulsions. Oil containing nanocapsules can be obtained by adding oil in the polymer solution composing the emulsion droplets.

Emulsification and solvent diffusion is a modified version of the solvent evaporation method [963]. The encapsulating polymer is dissolved in a partially water soluble solvent such as propylene carbonate and saturated with water to ensure the initial thermodynamic equilibrium of both liquids. In order to produce the precipitation of the polymer and the consequent formation of nanoparticles, it is necessary to promote the diffusion of the solvent of the dispersed phase by
dilution with an excess of water when the organic solvent is partly miscible with water or with another organic solvent in the opposite case. The polymer-water saturated solvent phase is emulsified in an aqueous solution containing stabilizer, leading to solvent diffusion to the external phase. Resultant nanospheres or nanocapsules will be formed dependent on the oil-to-polymer ratio. The solvent is removed by evaporation or filtration.

This technique allows for high encapsulation efficiencies (>70%), high batch-to-batch reproducibility, eliminates the need for homogenization, ease of scale-up, and narrow size distribution. A disadvantage of this method is that it requires high volumes of water that has to be removed from the suspension and the leakage of water-soluble entrapped therapeutic agent drugs into the external saturated-aqueous phase during emulsification causes a reduction in entrapment efficiency [964]. This method has moderate efficiency in the entrapping hydrophilic molecules, therefore it may have limited applications for siRNA delivery systems.

Emulsion polymerization can be used to prepare nanoparticles by monomer polymerization. It is a fast and easily scalable nanoparticle preparation method. The continuous organic phase methodology involves the monomer dispersion into an emulsion, inverse microemulsion, or into a non-solvent of the monomer [964, 965]. Surfactants or protective soluble polymers were used in the initial stages of polymerization to prevent aggregation. Since this method utilizes toxic organic solvents, surfactants, monomers, and initiators that can be leached from the formed nanoparticles, this method has been used sparingly in recent years [965].

An alternate approach was developed because of the conventional method disadvantages of having non-biodegradable polymer and difficult procedure. Poly(methylmethacrylate) (PMMA), poly (ethylcyanoacrylate) (PECA), and poly (butylcyanoacrylate) nanoparticles were produced by a surfactant dispersion within an organic phase consisting of cyclohexane, n-pentane, and toluene [965]. The monomer is dissolved in the aqueous continuous phase and surfactants or emulsifiers are not required. Initiation occurs when collision happens between the monomer molecules and the initiator molecule, which could be an ion or a free radical, within the continuous phase [965]. Alternatively, the monomer molecule itself can be converted into an initiating radical by high-energy radiation, such as g-radiation, ultraviolet, or strong visible light. Polymer chains form, according to a mechanisms of anionic polymerization, when collision occurs between the initiated monomer ions or monomer radicals and other monomer molecules. Solid particle formation and phase separation may occur before or after the polymerization reactions [966, 967].
The mini-emulsion polymerization method has been developed using a wide range of polymer materials [968-970]. A typical mini-emulsion polymerization formulation contains water, monomer mixture, co-stabilizer, surfactant, and initiator. The key difference between emulsion polymerization and mini-emulsion polymerization is the use of a low molecular mass compound as the co-stabilizer and also the use of a high-shear device. Mini-emulsions are critically stabilized, require a high-shear to reach a steady state, and have an interfacial tension much greater than zero [971].

Micro-emulsion polymerization is used to prepare nano-sized polymeric particles that involves a kinetically different process than other emulsion methods [972]. Although these processes both produce high molar mass colloidal polymeric particles, the particle size achievable for micro-emulsion based techniques is much smaller than for other emulsion based techniques. For micro-emulsion polymerization, typically a water soluble initiator, is placed into the aqueous phase of a thermodynamically stable micro-emulsion containing swollen micelles [965]. These spontaneously formed thermodynamically stable micelles are where polymerization commences. This process relies on large quantities of surfactant systems, which have an interfacial tension at the oil/water interface close to zero. The particles formed are completely coated with surfactant because of this high concentration of surfactant. Polymer chains are first formed only in some droplets, as the initiation cannot be attained in all droplets simultaneously. Then, the osmotic and elastic influence of the chains destabilize the fragile micro-emulsions and typically lead to an increase in the particle size, the formation of empty micelles, and secondary nucleation. Very small lattices of particle size of 5-50 nm are formed with the presence of empty micelles. Critical factors of the kinetics of micro-emulsion polymerization and the resultant polymeric nanoparticle properties are the concentration of initiator, surfactant, monomer, and reaction temperature [965, 973].

3.4.2.3.2 Solvent Evaporation

The first step of the solvent evaporation technique involves the preparation of a polymer solution prepared in volatile solvents and emulsions. Ethyl acetate is the solvent of choice because it has a better toxicological profile when compared to the other widely used solvents, dichloromethane and chloroform [971]. The primary emulsion is transformed into a colloidal nanoparticle suspension following the evaporation of the solvent from the polymer, which is facilitated through the diffusion of the emulsion’s continuous phase [965]. The conventional
methods involve the formation of primary single emulsions, such as oil-in-water (o/w) or the formation of double-emulsions, such as (water-in-oil)-in-water (w/o)/w. Nanocarriers formed through this method utilize high-speed homogenization or ultra-sonication with subsequent solvent evaporation through continuous magnetic stirring or under vacuum. The resultant semi-solid particles may undergo ultracentrifugation and be washed with distilled water to collect and purify the nanocarriers. The resultant nanocarriers may then be freeze-dried with a suitable cryoprotectant and stabilizing agent. Particle sizes of these nanocarriers are dependent on the type and concentration of stabilizer, homogenizer speed, and polymer concentration. Reduction of particle size can be achieved through high-speed homogenization and ultra-sonication.

3.4.2.3.2.3 Nanoprecipitation

Nanoprecipitation, or the solvent displacement method, involves the precipitation of preformed polymer from an organic solution and the diffusion of the organic solvent in the aqueous medium with or without a surfactant [973-976]. The polymer is dissolved in a water miscible solvent of intermediate polarity, leading to the precipitation of spherical nanocarriers. This phase is injected into a stirred aqueous solution containing a stabilizer as a surfactant. Polymer deposition on the interface between the water and the organic solvent, caused by fast diffusion of the solvent, leads to instant formation of stable nanocarriers in suspension [977]. In order to facilitate the suspension formation, the first step is to perform phase separation with a completely miscible solvent that is also a non-solvent for the polymer [966]. This nanoprecipitation technique allows the preparation of nanocapsules when a small volume of nontoxic oil is incorporated in the organic phase. Considering the oil-based central cavities of the nanocapsules, high loading efficiencies of hydrophobic drugs are often reported. This technique spontaneously produces emulsification because of the high diffusion rates in result of limiting water-miscible solvents [965]. Spontaneous emulsification is not observed if the coalescence rate of the formed droplets is sufficiently high due to certain instability when mixed in water of some water-miscible solvents. Acetone/dichloromethane is used to dissolve drugs and increase their entrapment. However, dichloromethane increases particle size and has toxicological issues. This method is appropriate for lipophilic drugs; therefore, its usefulness for siRNA nanocarriers is limited by low entrapment efficiency.
3.4.2.3.2.4 Salting Out

The salting out effect causes the separation of a water miscible solvent from an aqueous solvent [964]. This procedure is considered a modified emulsification/solvent diffusion method. Drug and polymer are first dissolved in a solvent, like acetone, which is then emulsified with the salting-out agent into an aqueous gel. Salting-out agents include electrolytes, such as magnesium chloride or calcium chloride, or non-electrolytes, such as sucrose with a colloidal stabilizer such as polyvinylpyrrolidone or hydroxyethylcellulose [965]. Induction of nanoparticle formation occurs when the oil/water emulsion is diluted with enough water or aqueous solution to enhance the diffusion of acetone into the aqueous phase [964]. Salting out agent selection is highly influential in the drug encapsulation efficiency [965]. Formed polymeric nanoparticles are purified by cross-flow filtration to remove the solvent and salting out agent [965].

An advantage of the salting out method is that it minimizes stress to protein encapsulates [965, 978, 979]. The salting out procedure is useful for heat sensitive substances since it does not require increased temperature. The multiple washing steps pose a disadvantage when considering entrapment efficiency. The greatest disadvantage of this method is that it is used exclusively for lipophilic drugs, therefore its application to siRNA is low.

3.4.2.3.2.5 Dialysis

Nanocarriers with small particle size and narrow particle size distribution can be prepared by dialysis [971, 973, 980]. Polymer dissolved in an organic solvent is placed within a dialysis tube with desired molecular weight cut off [965]. Dialysis is then performed against a non-solvent miscible with the former miscible solvent. The use of the dialysis membrane or semi-permeable membranes allow the passive transport of solvents to slow down the mixing of the polymer solution with a non-solvent [965]. The displacement of the solvent inside the membrane is followed by the progressive aggregation of the polymer due to a loss of solubility and the formation of homogeneous suspensions of nanoparticles. The mechanism of nanocarrier formation by dialysis method is based on a mechanism similar to that of nanoprecipitation. The solvent used in the preparation of the polymer solution affects the morphology and particle size distribution of the nanoparticles.

3.4.2.3.2.6 Supercritical Fluid Technology (SCF)

Supercritical fluid technology has allowed for the use of more environmentally friendly solvents for the preparation of polymeric nanocarriers with high purity [981, 982]. Supercritical
fluid and dense gas technology are expected to offer an effective technique of nanoparticle preparation because it avoids most of the drawbacks of traditional techniques, such as presence of organic solvent [965].

The production of nanoparticles using supercritical fluids has two principles. The first is the rapid expansion of supercritical solution (RESS). The second principle is the rapid expansion of supercritical solution into liquid solvent (RESOLV). In traditional RESS, the solute is dissolved in a supercritical fluid, followed by the rapid expansion of the formed solution across a capillary nozzle or an orifice into ambient air. The resultant high degree of super saturation and rapid pressure reduction in the expansion results in homogenous nucleation and formation of dispersed particles [965]. Mechanistic studies of different model solutes for the RESS process indicate that within the expansion jet, both micrometer-sized and nanometer particles are present [983]. A few studies were carried out on the production of nanoparticles using RESS. Poly (perfluoropolyetherdiamide) droplets produced from the rapid expansion of CO$_2$ solutions. The RESS experimental apparatus consists of three major units: a high pressure stainless steel mixing cell, a syringe pump, and a pre-expansion unit. A solution of polymer in CO$_2$ is prepared at ambient temperature. The solution is heated isobarically to the pre-expansion temperature while pumped through pre-expansion unit before the solution reaches the nozzle. At this point, the supercritical solution is now allowed to expand through the nozzle, at ambient pressure. The concentration and degree of saturation of the polymer have a considerable effect on the particle size and morphology of the particles for RESS [984-987].

A RESS modification, known as RESOLV, involves the expansion of the supercritical solution into a liquid solvent instead of ambient air [971, 988]. The RESS technique results in microscaled sized particles rather than nanoparticles. In order to overcome this drawback, RESOLV was developed. In RESOLV, the liquid solvent suppresses the particle growth in the expansion jet, thus making it possible to obtain primarily nano-sized particles [965, 988, 989].

### 3.4.2.3.2.7 Interfacial Polymerization

Interfacial polymerization is one of the most established methods for the preparation of polymeric nanoparticles [990, 991]. In interfacial polymerization, there is stepwise polymerization of two reactive monomers or agents, which are dissolved respectively in two phases (continuous and dispersed), and the reaction takes place at the interface of the two liquids [992]. Hollow polymeric nanoparticles were synthesized with the use of interfacial cross-linking reactions as
polyaddition, polycondensation, or radial polymerization [993-996]. Polymerization of monomers at the oil/water interface of an oil-in-water micro-emulsion produce oil-containing nanocapsules [997]. The organic solvent, which is completely miscible with water, served as a monomer vehicle and the interfacial polymerization of the monomer was believed to occur at the surface of the oil droplets that formed during emulsification [958, 998, 999]. The use of acetone or acetonitrile, which are aprotic solvents, was recommended for nanocapsule preparation [965]. Alternatively, protic solvents, such as ethanol, m-butanol and isopropanol, were found to also induce the formation of nanospheres [965, 1000]. Nanocapsules containing aqueous water can be formed by the interfacial polymerization of monomers in water-in-oil interface and precipitated out forming the nanocapsule shell [1001, 1002]. Therefore, siRNA containing nanocapsules can be formed using the interfacial polymerization of monomers in water-in-oil microemulsions.

3.4.2.3.2.8 Controlled/Living Radical Polymerization (C/LRP)

The primary restrictions of radical polymerization are due to unavoidable radical to radical termination reactions and cause a lack of control over the molar mass, the end functionalities, and the overall macromolecular structure [965]. The C/LRP processes are based on previous polymerization techniques and opens new prospects in polymeric nanoparticle preparation [965, 1003-1005]. The growing public concern over environmental impacts and the growth of hydrophilic polymer applications in medicine have sparked interest in C/LRP process. These factors have given rise to green chemistry techniques and created a demand for environmentally and chemically benign solvents such as water and supercritical carbon dioxide [965]. Industrial radical polymerization in aqueous dispersed systems and specifically in emulsion polymerization is widely used. The primary goal was to control the characteristics of the polymer to modulate their function, molar mass, molar mass distribution, and structure. Implementation of C/LRP in aqueous dispersed systems, results in the formation of polymeric nanoparticles with precise particle size and size distribution control [965, 1006]. Among the available controlled/living radical polymerization methods successfully and extensively studied methods include nitroxide-mediated polymerization (NMP), atom transfer radical polymerization (ATRP), and reversible addition and fragmentation transfer chain polymerization (RAFT) [1007-1012]. The size of the resultant polymeric nanoparticles depends on the type of control agent, control agent concentration, monomer, initiator, and the emulsion type.
3.4.2.3.2.9 Hydrophilic Polymer Ionic Gelation/Coacervation

Polymeric nanoparticles are prepared by using biodegradable hydrophilic polymers such as chitosan or gelatin as shown in Figure 3.17. In this method, the positive charged amino groups of the polymer interact with the negatively charged triphosphate to form coacervates with a size in the nanometer range [1013-1015]. Coacervates are formed as a result of electrostatic interaction between two aqueous phases, whereas ionic gelation involves the material undergoing transition from liquid to gel due to ionic interaction conditions at room temperature [965].

![Diagram showing the preparation of polymeric nanoparticles using the coacervation (desolvation) technique.](image)

**Figure 3.17.** Diagram showing the preparation of polymeric nanoparticles using the coacervation (desolvation) technique.

3.4.2.3.3 Examples of Polymer-based Delivery Systems

Chitosan is a generally regarded as safe (GRAS) excipient and a natural polysaccharide [1016, 1017]. However, its applications in siRNA delivery systems have been hindered due to poor water solubility and low transfection efficiency [748]. An inhalable chitosan/siRNA dry powder composed of unmodified chitosan was prepared using the supercritical CO$_2$ technique followed by manual grinding in an investigation by Okuda et al. [1018]. Water, ethanol and CO$_2$ solvents were combined in a compressed column (35°C, 25 MPa). This supercritical CO$_2$ technique embodies tolerable conditions for the precipitation of powders capable of reconstitution without siRNA or nanoparticle degradation. The hydrodynamic particle sizes and zeta potentials of the developed chitosan particles were shown to be maintained throughout and after the powder formation process [1018]. Biodistribution of intratracheally administered Cy5.5 labeled siRNA formulations, including the nanoparticle suspension, the dry powder, and the free/naked siRNA were monitored.
The efficiency of the delivered siRNA was measured in a lung metastases model of colon26/Luc cells by luciferase downregulation. The dry powder particles had highest knockdown efficiency, however they had higher lung clearance than the nanoparticle suspension [1018]. This study is one of the few reports that describe the formulation of an inhalable dry powder containing siRNA.

Another dry powder formulation prepared using a double emulsion solvent evaporation method was designed by Jensen et al., where siRNA was entrapped within DOTAP-modified PLGA nanoparticles [1019, 1020]. The total concentration of DOTAP and PLGA in chloroform was kept constant and the optimal formulation contained 25% (w/w) DOTAP by varying the weight/weight (w/w) percentage of DOTAP. The particles were spray dried with mannitol and an aerodynamic size of 3.69 ± 0.18 μm obtained, which is within the optimal size range for deep lung deposition, and did not exhibit aggregation or coalescence [1019, 1020]. During spray drying, the sugar alcohol remained in its crystalline state, as determined by X-ray powder diffraction analysis, and therefore functioned as a stabilizer. The hydrodynamic diameters and cellular uptake remained unchanged before and after freeze-drying according to statistical analysis, which supported the conclusion that spray-drying is a powerful technique for engineering siRNA nanoparticle dry powder formulations [1020].

PLGA poly[vinyl-3-(dialkylamino) alkylcarbamate-co-vinyl acetate-co-vinyl alcohol]-graft-poly(D,L-lactide- co-glycolide), (DEAPA-PVA-g-PLGA), that contained covalently modified positively charged side groups for siRNA complexation was prepared by Benfer et al. [207]. The H1299 NSCLC cellular uptake of DEAPA-PVA-g-PLGA/siRNA nanoparticles increased after addition of surfactant and was internalized mostly by clathrin-mediated endocytosis [207]. Merkel et al. also showed that DEAPA-PVA-g-PLGA/siRNA nanoparticles remain in the lung for extended periods of time when compared to gold-standard PEI-formulated or free siRNA. Thus, making the formulation applicable for intratracheal administration [1021]. To prepare the inhalable dry DEAPA-PVA-g-PLGA/siRNA nanoparticle powder, the freeze-drying protocol was optimized. Cryoprotectants, such as dextrose, hydroxyproxypropyl-beta-cyclodextrin, lactosucrose, polyvinylpyrrolidone (PVP), sucrose, and trehalose can be used to stabilize the nanoparticles. Although particles freeze-dried in the cryoprotectant 10% glucose were about 150 nm in particle size after reconstitution, the particles freeze-dried in the cryoprotectant 10% sacharose were much larger with a particle size of 250 nm. However, the freeze-dried larger particles showed more similar and efficient cellular uptake when compared to freshly prepared
nanoparticles [1021]. It was demonstrated that gene downregulation efficacy was preserved through the freeze-drying process as assessed after reconstitution of the nanoparticles [1021]. These findings are similar to those of a study by Kasper et al. who showed that cryoprotectant concentrations of 12% and higher can prevent PEI/DNA polyplex aggregation which retains their transfection efficiency after re-dispersion [1022].

Luo et al. synthesized salbutamol-modified guanidylated chitosan and subsequently formed polyplexes targeted to lung smooth muscle cells for potential applications in asthma or COPD [603]. The formulations were nebulized with an Aeroneb ® Pro nebulizer (Aerogen, Galway, Ireland) and then the collected condensate was administered intratracheally with a PennCentury microsprayer to transgenic mice expressing enhanced green fluorescent protein (EGFP) [603]. The animals were treated daily with 5 μg of siRNA 3 days, and in vivo knockdown was quantified by confocal laser scanning microscopy (CLSM) of tissue sections and by Western blot. The targeted formulation achieved a 40% gene downregulation [603].

Atelocollagen, a highly purified and pepsin-treated type I collagen obtained from calf dermis also raised interest for its use in pulmonary delivery of siRNA [748]. Protein and siRNA polyplexes were prepared by mixing during a 16h incubation and were administrated intratracheally to Wistar or EGFP expressing rats [563]. The therapeutic gene target was syntaxin4, which regulates the function of mucosal-type mast cells and is one of the soluble membrane N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins. Following siRNA treatment, syntaxin4 knockdown significantly decreased granule contents release from rat mucosal mast cells. Rats were sensitized and challenged with ovalbumin (OVA) following the allergic mouse model protocols to elicit symptoms of asthma. The rats were then intratracheally instilled with atelocollagen siRNA complexes once a day for 5 days. These treatments decreased the rat mast cell protease-II (RMCPII) levels in the bronchoalveolar lavage fluid (BALF) and prevented asthmatic airway constriction. Therefore, the authors concluded that syntaxin 4 knockdown prevents degranulation and stabilizes mucosal mast cells [563].

Spermine, a naturally occurring polymer consisting of small linear tetra-amines with two primary and two secondary amines, is popular for the delivery of nucleic acids [748]. Endogenous spermines play a key role in the compact packaging of cellular DNA, this property is exploited artificially in nucleic acid delivery [1023, 1024]. Due to the short molecules and the resultant rigidity of spermine, unmodified spermine does not efficiently compact siRNA [748, 1025]. Jiang
et al. prepared glycerol propoxylate triacrylate-spermine (GPT-SPE) and shRNA polyplexes that had a particle size of 160 nm and had a zeta potential of +9 mV. Human NSCLC was mimicked using a KrasLA1 model and administered 8 mg of GPT–SPE and 0.8 mg of shRNA (scrambled or Akt1 shRNA) twice a week for 4 weeks as aerosol [1024]. The authors did not discuss whether the preparation and delivery processes had an effect on the polyplex physicochemical characteristics. In a later study, GPT-SPE delivered shRNA against the same target. The same nose-only exposure chamber was used but administered half of the previous dose (0.4 mg) DNA as an aerosol (scrambled or Akt1 shRNA) twice a week over a timeframe of 4 weeks [1026]. Aerosol delivery of Akt1 shRNA suppressed lung tumor growth and the authors elucidated the mechanism of action [1026]. The same group conducted another later study, which used the GTE-SPE polymer for sodium-dependent phosphate co-transporter 2b (NPT2b) siRNA delivery via nose-only inhalation [1027]. The animals were treated for four weeks with 0.5 mg of siRNA twice a week. Aerosolized NPT2b siRNA downregulated NPT2b expression levels as detected via Western blotting, densitometric analysis and qPCR, and significantly increased the pro-apoptotic protein levels. The activation of apoptosis was confirmed in TUNEL positive cells, and PCNA and VEGF levels were decreased after knockdown of Akt1 [1027].

Choi et al. synthesized a conjugate of dexamethasone and PEI (DEXA-PEI) polyplex to target the cellular nucleus via interaction with the glucocorticoid receptor [1028]. This concept was previously explored, but the novelty of this approach was induction of the model of acute lung inflammation by the instillation of baked SiO$_2$ nanoparticles [1028, 1029]. siRNA against macrophage migration inhibitory factor (MIF) was complexed with DEXA-PEI and particles obtained had a particle size of 355 nm, whereas PEI/siRNA complexes aggregated to a particle size of >600 nm. These particles were administered intratracheally with either 2.5 μg or 10 μg siRNA per animal and were shown to decrease pulmonary inflammation and the MIF mRNA and Muc5ac expression after treatment with conjugated and nonconjugated PEI complexes 72 h post-administration of the polyplexes [1028].

3.4.2.4 Peptide-based Delivery Vectors

3.4.2.4.1 Introduction to Peptide-based Delivery Vectors

Protein transduction domains (PTDs) are short amino acid chains that interact with the plasma membrane that allows for cellular uptake that can be used for siRNA delivery. Obtaining controlled siRNA cellular delivery through the use of peptides as carriers can overcome siRNA
delivery constraints, which include poor bioavailability and clinical efficacy. Chemical conjugation of cationic peptide cell penetrating peptides (CPP) to siRNA has facilitated enhanced siRNA tissue delivery and cellular internalization. The TAT protein from HIV-1 which was discovered to be responsible for the cellular uptake of the virus was of interest of drug delivery scientists. Since that time, a variety of CPPs have been developed to facilitate the cellular uptake transport of therapeutic molecules including siRNA [708]. Covalent peptide to siRNA disulfide bond formation attachment, or non-covalent electrostatic interactions between the siRNA and peptide to form complexes can be used to prepare siRNA peptide conjugates [708]. Their mechanism of action is also expected to vary due to the sequence diversity of different CPPs. These peptides function to improve cellular delivery by efficient transport across the cellular membrane or promote endosomal escape [1030]. The activities of different CPPs have been reviewed elsewhere [1031, 1032].

3.4.2.4.2 Preparation Methods

Double stranded siRNA, with or without modification, can form stable or cleavable peptide conjugates using a range of different conjugation chemistries [1033]. Designing conjugation schemes include selection of linkage chemistry, spacer molecule, and orientation of the siRNA and peptide components [1034]. There are two main strategies for synthesizing siRNA peptide conjugates: the total stepwise solid-phase synthesis and the solution-phase or solid-phase fragment coupling of peptides with oligonucleotides that have been prepared individually on solid supports [1035]. Stable linkages may be prepared from either method, but fragment coupling of peptides is necessary for cleavable disulfide linkages.

The most common conjugation chemistries have involved fragment coupling in aqueous solution. Peptide and siRNA fragments are prepared on their own solid supports by conventional automated solid-phase synthesis procedures. Each fragment requires a masked functional group to be incorporated during the assembly, which can be released chemo-selectively during a simple deprotonation step. A specific chemical reaction is then initiated in aqueous medium between the functional group on the oligonucleotide and that on the peptide to produce the desired conjugate. An advantage of the fragment-coupling method is that both components can be purified, for example by HPLC, before conjugation so that the conjugation product can be easily identifiable. This method’s disadvantage is that the aqueous conditions of conjugation sometimes need to be modified to maintain the solubility of both components and the conjugate product. A reaction of a
cysteine-substituted siRNA with a thioester-substituted peptide produces a stable amide linkage through the mechanism of native ligation. Another method involves the reaction of an aldehyde-containing siRNA with a cysteine-containing peptide, forming a thiazolidine linkage, or with an aminooxy peptide forming an oxime linkage, or with a hydrazinopeptide forming a hydrazine. An aldehyde oligonucleotide is unstable and must be generated by periodate treatment of a cis-diol substituted siRNA just before conjugation [1036].

The most straightforward method of fragment conjugation involves the synthesis of the siRNA component with an alkyl thiol linker on either of the 5’ or 3’-end forming a 3’-thiopropyl or a 5’-thiohexyl linker [1037]. Following activation with pyridylsulfide, the siRNA can be coupled to a peptide. These thiol-functionalized siRNAs can be reacted with a cysteine-containing peptide to form a cleavable disulfide linkage or with a bromoacetyl-substituted peptide to form a stable thioether linkage. A maleimide derivative of the peptide can also be prepared for the reaction with a thiol-substituted siRNA via a Michael-type addition reaction [1036].

3.4.2.4.3 Examples of Peptide-based Deliver Vectors

The PTDs and cell penetrating peptides are small (10-30 amino acid), positively charged molecules that usually contain arginine and lysine. Arginine and lysine amino acids provide primary and secondary amine functional groups in their side chains that can be protonated for electrostatic interaction with siRNA and cellular membranes to enhance the cellular permeability [748]. Delivery of siRNA using a short peptide conducted by Oh et al. who used R3V6 peptides, which were composed of 3 arginines and 6 valines [748, 1038]. Ternary complexes were formed with sphingosine-1-phosphate lyase (S1PLyase), a recombinant high mobility group box-1 box A peptide (HMGB1A), siRNA, and R3V6 (siS1PLyase/HMGB1A/R3V6). Premixture of siRNA and HMGB1A avoided aggregation and formed particles with sizes below 200 nm that elicited an anti-inflammatory effect [1038]. These ternary complexes were tested in vitro and delivered siRNA into non-phagocytosing LA-4 lung epithelial cells more efficiently than PEI and Lipofectamine, inferring that their uptake mechanisms were not based on phagocytosis [748, 1038]. Intratracheal administration of the ternary complexes reduced the S1PLyase level efficiently in an LPS-induced BALB/c ALI model. The cell penetrating peptides, TAT and double TAT (dTAT) was used for siRNA delivery by Baoum et al. [1039]. To form the complexes, siRNA was mixed with the polycations and then CaCl₂ was added to the mixture in order to compact the siRNA and to decrease the hydrodynamic diameters of the particles [1039]. Following i.v. injection of the
complexes, high gene knockdown was observed in the lung [1039]. These results suggest that formulating these siRNA complexes for pulmonary delivery by inhalation, would allow for high local concentration while keeping high accumulation in the lungs.

3.4.2.5 Inorganic-based Delivery Systems

3.4.2.5.1 Introduction to Inorganic-based Delivery Systems

Various promising inorganic delivery systems have been investigated for the delivery of siRNA devised for diagnostic and therapeutic purposes. They include carbon nanotubes and metals such as iron oxide, quantum dots, gold and silica [1040]. Mesoporous silica based nanoparticles (MSN) have been widely investigated as carriers for siRNA based targeted drug delivery systems [1041, 1042] (Table 1). The MSN are chemically stable, safe and biodegradable which makes it a promising gene delivery carrier. [1043, 1044]. MSN also possess several advantages over other inorganic carriers, such as capability to encapsulate higher amounts of drugs due to large pore volumes and improved stability due to their inorganic oxide framework [1045].

3.4.2.5.2 Examples of Inorganic-based Delivery Systems

Taratula et al. prepared MSNs with a 3-mercaptopropyl modification for labeling with Cy5.5 and with pyridylthiol for conjugation of a lutein hormone releasing hormone (LHRH) peptide via a PEG spacer and for attaching thiol modified siRNA [748, 1046]. The MSNs pores were loaded with either doxorubicin or cisplatin with two types of siRNA for a chemotherapeutic combination therapy, as shown in Figure 3.18. The MRP1 and BCL2 siRNA were selected based on their target mRNA’s role in suppression of pump and non-pump cellular chemoresistance. An orthotopic model of lung cancer was established by intratracheal installation of luciferase expressing A549 cells [1047]. Nanoparticle accumulation in the mouse lungs was detected by whole body fluorescence imaging and revealed that inhalation administration prevented the MSN systemic circulation delivery and thus limited their accumulation elsewhere in the body [1046].
Figure 3.18. Co-delivery of siRNA and anticancer agents using mesoporous silica nanoparticles (MSN) The surface engineered approach consists of surface bound siRNA and PEG-LHRH.

The same group further utilized the modified MSN’s to co-deliver anticancer drugs [doxorubicin (DOX) or cisplatin (CIS)], suppressor of pump resistance (siRNA targeting MRP-1 mRNA), and suppressor of non-pump cellular resistance (siRNA targeting BCL2 mRNA) using tumor targeting moiety LHRH peptide. The inhalation delivery of LHRH targeted MSN-drug complexes carrying both BCL2 and MRP1 siRNA (LHRH-PEG-siRNA-DOX-MSN) showed that 73.6% of MSN was retained in lung compared to 5% when i.v. injected [1048]. Also, after i.v. administration MSN-based DDS was found to be accumulated mainly in liver (73%), kidneys (15%) and spleen (7%) while inhalation delivery resulted in significant reduction in accumulation to only 17%, 9% and 1% in liver, kidneys and spleen respectively.

3.4.3. Ongoing clinical trials on aerosolized siRNA based medicines

Excellair™ from ZaBeCor Pharmaceuticals (Bala Cynwyd, Pennsylvania, USA) is an inhaled siRNA for the treatment of asthma. This siRNA functions as a spleen tyrosine kinase (Syk) inhibitor thereby inhibiting the transcription factors regulated by Syk. Syk is involved in the signaling from the B-cell receptor and regulates the downstream signaling cascades that ultimately leads to the activation of several pro-inflammatory transcription factors. Thus, the specific Syk inhibition by Excellair™ is designed to reduce the inflammation associated with asthma. In a phase
I study, patients with asthma received the inhaled siRNA therapeutic for 21 consecutive days [1049]. The drug was well tolerated in all asthma patients, with no serious adverse side effects. Moreover, 75\% of the patients treated reported improvement of breathing or reduced rescue inhaler use, while placebo patients reported no such improvement. These results helped propel Excellair™ into a phase II clinical trials in 2009, however these results are not available [1050].

Alnylam Pharmaceuticals (Cambridge, Massachusetts, USA) developed a nebulizer and nasal spray delivered siRNA therapeutic. The ALN-RSV01 siRNA was designed for the treatment of respiratory syncytial virus (RSV) targeting the nucleocapsid protein is indicated for prophylactic treatment against RSV infections in healthy patients (NCT00496821) and for treating RSV infection in lung transplant patients (NCT00658086 and NCT01065935). The ALN-RSV01 siRNA is composed of a double-stranded RNA duplex with 19 base pairs of complementary and 2-nt dT overhangs at both 3’ ends [773, 1051].

RSV is the leading cause of hospitalization of infants, infecting approximately 70\% of infants under a year old. A RSV vaccine is not available for pediatric patients due to the potential teratogenicity and reduced effectiveness [1052]. RSV may also produce severe respiratory diseases like pneumonia in immunocompromised adolescents and adults, and in the elderly [774]. RSV replicates in the outermost layer of the airway epithelium, including regions lining the nasal passages, trachea, and the bronchioles [1053]. The anti-viral efficacy of ALN-RSV01 was demonstrated by the reduced infection rate in healthy patients and by reducing the daily symptoms in lung transplantation patients [774, 775]. This example clearly shows the advancement that siRNA brings to clinical care by providing safe and efficacious treatment options.

3.4.4. Summary and Future Directions of Pulmonary siRNA Delivery

The therapeutic potential of siRNA in the treatment of lung diseases has yet to be fully explored. The use of inhalable nanoparticulate siRNA delivery systems have barriers to their effective delivery, such as degradation by RNase, mucociliary clearance, cough clearance, and alveolar macrophage clearance, but overcoming these constraints will offer unique advances to the field. Important parameters to consider in aerosolized siRNA formulation design and development are the particle, aerosol, physicochemical, stability, targeted site, and lung physiological properties.

siRNA carrier systems for aerosol delivery include those of naked siRNA formulation, lipidic, polymeric, peptide, or inorganic origin. Lipid-based delivery systems, such as liposomes
or solid lipid nanoparticles have received attention for inhaled pulmonary delivery by using endogenously present phospholipids and surfactant-like lipids. Polymer-based siRNA delivery systems can be composed of biocompatible polymers which may be chemically modified to improve cellular delivery, internalization, and intracellular release. Peptide-based siRNA delivery vectors improve the poor bioavailability and clinical efficacy associated with naked siRNA delivery. Inorganic-based delivery systems allow for the unique delivery of siRNA for diagnostic and therapeutic purposes at higher payloads compared to other delivery systems. Safety of these formulations must be proven through various laboratory and clinical studies. In vitro techniques for characterizing aerosolized pulmonary siRNA delivery systems include dissolution tests, inertial cascade impaction, delivered dose uniformity assays, laser diffraction, and laser Doppler velocimetry. Ex vivo techniques such as the isolated perfused lung model can be used to identify inhaled particulate distribution. In vivo techniques like scintigraphy and pharmacokinetic/pharmacodynamics analysis can give even more insight to the distribution, safety, and efficacy of a pulmonary inhaled siRNA nanocarrier. The vigorous work researchers have completed regarding the local pulmonary inhaled delivery of siRNA in various models of lung disease. Two inhalable therapeutic siRNA products have entered clinical trials. More clinically relevant preclinical studies utilizing biocompatible and safe siRNA delivery vehicles will provide inhalable aerosolized siRNA delivery vehicles for future clinical studies. Overall, recent work focused on inhalation delivery of siRNA for the treatment of pulmonary disorders has demonstrated the feasibility of this approach. The rapid progress in this area of research would facilitate the translation to the clinic.
CHAPTER 4. MATERIALS AND METHODS

This chapter describes the materials and methods used for the formulation of gelatin nanocarriers and their evaluation. The S6S-GNC were developed using Taguchi orthogonal array design of experiments of acetaminophen loaded gelatin nanoparticles, which underwent regression analysis to predict full factorial data. This full factorial data of the APAP-GNC formulations were compared using a desirability calculation to find the formulation with the smallest particle size, zeta potential close to +20 mV, and highest loading efficiencies. The formulation consisting of 2% w/v gelatin, 50% v/v acetone addition, and 0.02% w/v genipin crosslinker addition were selected for the formulation of STAT6 siRNA containing gelatin nanocarriers. The hypothesis was that the formulation of STAT6 siRNA loaded gelatin nanocarriers would exhibit stability in different physiologically relevant media, protect the siRNA from degradation, and promote STAT6 protein downregulation in Th2 through an IL4Rα mediated cellular uptake. In order to test this hypothesis, the S6S-GNC were characterized and evaluated using various methods such as dynamic light scattering, electrophoretic mobility, cellular binding and internalization assays, and evaluation in a lung cancer cell line, as well as protein downregulation analysis in a Th2 cell line. The inhalable GNC were prepared to provide evidence for the developed S6S-GNC-P as an asthma therapy delivered by the pulmonary route of administration. Gemcitabine loaded gelatin nanocarriers were prepared characterized for the aerodynamic and physical characteristics, as well as in vitro efficacy in lung cancer cell lines.

4.1. Materials

4.1.1. Cell Culture

The human NSCLC cell lines A549 (ATCC® CCL-185™) and NCI-H460 [H460] (ATCC® HTB-177™) were grown as monolayers in 75 cm² tissue culture flasks (Greiner Bio-One, Monroe, NC, USA) at 37°C under 5% CO₂ in F12-K and RPMI supplemented medium (Life technologies, Grand Island, NY, USA) with 10% fetal bovine serum (FBS) and an antibiotic antifungal solution of penicillin (5000 U/mL), streptomycin (0.1 mg/mL) and neomycin (0.2 mg/mL) (PSN), respectively. A549 cells are human lung adenocarcinoma epithelial cells line. H460 cells are human lung large cell carcinoma derived from pleural effusion of the lung.

The helper T lymphocytic (Th2) cell line, D10.G4.1 (ATCC® TIB-224™), isolated from the lymph node of adult male Mus musculus (mouse) strain AKR/J, was grown as suspension in suspension culture flasks (Greiner Bio-One, Monroe, NC, USA) at 37°C under 5% CO₂. The base
medium for this cell line was prepared according to ATCC® recommendations as follows: RPMI-1640 medium (ATCC Catalog No. 30-2001) containing 10% T-STIM with Con A (rat IL-2 culture supplement; Becton Dickinson, Catalog No. 354115), 10% fetal bovine serum, 0.05 mM 2-mercaptoethanol, 10 pg/ml mouse IL-1 alpha (R&D Systems, Catalog No. 400ML). When plating this Th2 cell line, cell density was adjusted to 2x10^5 viable cells/ml within complete growth medium. To help promote the expression of STAT6 protein, IL4 recombinant peptide (Shenandoah Biotechnology, Inc., Warwick, PA, USA) was added to the growth medium 5 days before plating for cell treatments at 100 ng/mL [1054, 1055].

Dulbecco’s Phosphate-buffered Saline (DPBS) was purchased from Mediatech, Inc., Manassas, VA, USA). Cell culture media and penicillin/streptomycin/neomycin stock solutions were purchased from Cellgro (Herndon, VA, USA). Heat inactivated FBS was purchased from Atlanta Biologicals (Lawrenceville, GA, USA).

4.1.2. Chemicals

Gelatin (type A; 175 g Bloom Strength; with isoelectric point of 8-9, and average molecular weight of 40-50 kDa; GELITA, USA) was graciously provided as a gift from the manufacturer. Low-endotoxin containing gelatin (GELITA® MedellaPro™, Lot # 7323-2C, Test date May 2011, Expiration date May 2016, GELITA, Sergeant bluff, IA, USA) was provided as a gift from GELITA®. Glutaraldehyde (GTA) was purchased from Alfa Aesar (Heysham, Lancaster) as a 25% aqueous solution. Genipin (GEN) was kindly provided as a gift sample from Wilshire Technologies, Inc. (Princeton, NJ, USA). Acetaminophen (APAP) was purchased from Sigma Aldrich. STAT6 siRNA (mouse) was purchased from Santa Cruz Biotechnology, Inc. (sc-36570; Santa Cruz, CA, USA). The custom STAT6 siRNA with a sense sequence of 5’-CGA CGA AGA ACU CAA GUU Utt-3’ and antisense sequence of 5’-AAA CUU GAG UUC UUC CUG Ctt-3’ containing 2’ O-Methyl modified Adenosine or Guanine, internal 2’ Fluoro modified Cytosine and Uracil, and phosphorothioate linkages was purchased from Sigma Aldrich custom siRNA Oligos services [1056, 1057]. Ethanol, dimethyl sulfoxide (DMSO), 3-[4,5-dimethylthizol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and lactose monohydrate was purchased from VWR International (Radnor, PA, USA). Spectra/Por Dialysis membranes (MWCO 25 kDa and 100 kDa) were obtained from Spectrum Laboratories Inc. (Rancho Dominguez, CA, USA).

Gelatin (type B; bloom strength of 225 g; isoelectric point of 4.7-5.2 [225 H 30 mesh Batch# 402101511] (Rousselot, Dubuque, IA, USA) was provided as a gift from the manufacturer.
Genipin \(\text{[Methyl } (1R,2R,6S)-2\text{-hydroxy}-9\text{-}(\text{hydroxymethyl})-3\text{-oxabicyclo}[4.3.0]nona-4,8\text{-dien}-5\text{-carboxylate}]\) was provided as a gift sample from Wilshire Technologies, Inc. (Princeton, NJ, USA). Gem \[\text{[4-amino-1-(2-deoxy-2,2-difluoro-}\beta\text{-D-erythro-pentofuranosyl]pyrimidin-2(1H)-one hydrochloride}]\) was purchased from Sigma Chemicals (St. Louis, MO, USA). Ethanol, dimethyl sulfoxide (DMSO), \(3\text{-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and lactose monohydrate were purchased from VWR International (Radnor, PA, USA). Spectra/Por Dialysis membrane (molecular weight cutoff of 25,000 Da) was obtained from Spectrum Laboratories In. (Rancho Dominguez, CA, USA). All chemicals used were either analytical or tissue culture grade.

Lactose monohydrate (Polystormor™, Lot # J340475) was purchased from Macron Chemicals (Avantor Performance Materials, Inc., Phillipsburg, NJ, USA). DEPC treated and nuclease free water for RNA work was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Acetone was purchased from BDH VWR International (Lot # 09301002, West Chester, PA, USA). N-hydroxysuccinimide (NHS) was purchased from Alfa Aesar (98+%., Lot # 5001V26X, Ward Hill, MA, USA). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) coupling agent for peptide conjugation was purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). The hydrophilic model drug Acetaminophen (APAP) was a gift from Dr. Rajesh N. Davé (Distinguished Professor of Chemical, Biological and Pharmaceutical Engineering, Site Director, NSF-ERC on Structured Organic Particulate Systems, New Jersey Institute of Technology, Newark, NJ, USA). Ultra-low range agarose was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Ultra-low range DNA ladder was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Chlorpromazine hydrochloride powder was purchased from MP Biomedicals (Santa Ana, CA, USA), Genestein was purchased from Acros Organics (Morris Plains, NJ, USA), and amiloride was purchased from TCI America (Portland, OR, USA). Sulfo Cyanine-5 (Cy5) was purchased from Cyandye, LLC (Sunny Isles Beach, FL, USA). Fluorescein isothiocyanate (FITC) was purchased from was purchased from AMRESCO® and supplied by VWR International (Radnor, PA, USA). Heat shock isolated, biotechnology-grade Bovine Serum Albumin (BSA) was purchased from AMRESCO ® (lot # 0912C494, Solon, OH, USA). Ribonuclease A (RNase A), isolated from bovine pancreas (Type XII-A), was purchased from Sigma Aldrich (St. Louis, MO, USA).
All chemicals and reagents were of analytical, HPLC, or biotechnology grade as appropriate for the intended experiments. Distilled deionized, and 0.22 μm filtered sterile water was used throughout the experiments. All chemicals were of reagent or tissue culture grade.

4.2. Methods

4.2.1. Selection of Gelatin Molecular Weight Fraction by Controlled Desolvation

The GNCs formed from the whole gelatin fraction were prepared by one step desolvation technique. A 1% w/v gelatin type “A” solution was prepared by dissolving gelatin in distilled de-ionized H₂O at 50°C under gentle stirring at 400 rpm. When the gelatin solution became homogeneous and transparent, the temperature of the solution was reduced to 35°C and 19.98 mg acetaminophen (model drug engaged to optimize formulation conditions) was added and dissolved. Then, the desolvation step was accomplished, wherein 80% (v/v) ethanol was added at a rate of 1 ml/min under constant stirring at 600 rpm. Following this, 150 μl 10% GTA was added at a rate of 0.2 ml/min to crosslink the nanocarriers. The formulation was stirred at a rate of 600 rpm for another 55 min and the stir rate was reduced to 200 rpm until ethanol completely evaporated (process required approximately 12 hr).

The high molecular weight (HMW) fraction was prepared by the classical 2-step desolvation technique, where 5% (w/v) gelatin type “A” was first desolvated with an equal volume of acetone for 12 minutes under gentle stirring. After 12 minutes, the supernatant that contained the low molecular weight (LMW) gelatin fraction, water, and acetone was decanted and discarded. The HMW fraction sediment was allowed to dry and underwent mass reconciliation. The HMW gelatin was re-dissolved in distilled de-ionized H₂O 1% (w/v) solution at 50°C under gentle stirring. When the gelatin solution became homogeneous and transparent, the temperature of the solution was reduced to 35°C and 19.80 mg acetaminophen was added and dissolved. Then, a second desolvation step commenced, where 80% v/v pure ethanol was added drop wise at a rate of 1 ml/min under a constant stirring rate of 600 rpm. Five minutes after the ethanol addition, 150 μl 10% GTA was added drop-wise at a rate of 0.2 ml/min to crosslink the gelatin and therefore harden the nanocarriers. The formulation was stirred at a rate of 600 rpm for another 55 min and then 5 ml distilled de-ionized H₂O was added and the stir rate was reduced to 200 rpm until ethanol completely evaporated.

The MMW fraction was prepared by a modified 2-step desolvation technique, where 5% w/v gelatin type “A” was first desolvated with an equal volume of acetone for 5 seconds, quickly
decanted into another beaker, and then allowed to desolvated for another 12 minutes where the LMW fraction was decanted and discarded. The first containing HMW fraction while the LMW gelatin in water and acetone supernatant was discarded. The MWW fraction sediment was allowed to dry and underwent mass reconciliation.

The MMW gelatin was re-dissolved in distilled de-ionized H₂O to make a 1% w/v solution at 50°C under gentle stirring at 400 rpm. When the gelatin solution became homogeneous and transparent, the temperature of the solution was reduced to 35°C and 22.92 mg acetaminophen was added and dissolved. Then, a second desolvation step commenced, where 80% pure ethanol was added drop wise at a rate of 1 ml/min under constant stirring at 600 rpm. Five minutes after the ethanol addition ended, 150 µl of 10% GTA was added drop wise at a rate of 0.2 ml/min to crosslink gelatin and therefore harden the nanocarriers. The formulation as stirred at a rate of 600 rpm for another 55 min and then 5 ml distilled de-ionized H₂O was added and the stir rate was reduced to 200 rpm until ethanol completely evaporated.

The whole, HMW, and MMW gelatin fractions were compared for their resultant nanocarrier particle size, polydispersity index, and entrapment efficiency (EE%).

4.2.2. Formulation and Optimization of gelatin nanocarriers using Design of Experiments (DoE)

4.2.2.1. Development of S6S-GNC using a Full Factorial DoE

Type A gelatin based nanocarriers were prepared using the 2-step desolvation technique with slight modifications (Figure 4.1.) [125, 1058].

Figure 4.1. Preparation of S6S-GNC. The 1% w/v aqueous gelatin solution was incubated with the STAT6 siRNA for 10 min at 35°C, then ethanol and crosslinker was added dropwise at a stirring rate of 600 rpm at 35°C for 1 hr, at which point the stirring rate was reduced to 200 rpm. After approximately 4 h, the ethanol was completely evaporated and STAT6 siRNA loaded gelatin nanocarriers remained in a colloidal suspension in water or PBS pH 7.4. The resultant nanoparticles were collected by centrifugation and re-suspended for subsequent characterization or lyophilization in the presence of 1% w/w lactose monohydrate.
The formulated GNC was cross-linked with more biocompatible cross linker, GEN as against to predominantly employed GTA cross-linker [1059, 1060]. GNC formulations were optimized using a full factorial design with the independent variables being stir rate, ethanol volume, and GEN concentration with particle size being the dependent variable. APAP was used as model drug to set formulation parameters. This optimized formula was used to prepare S6S loaded gelatin nanocarriers.

GNC formulations were optimized using a $3^3$ full factorial design with the independent variables being stirring rate, ethanol volume, and GEN concentration and the dependent variable of particle size (Table 4.1). Full factorial design has been used extensively in the literature to evaluate the critical factors and develop the optimal formulation by identifying all possible effects of combination of the factor levels, which is useful for studying the main effects of the design factors and the interactions between those factors [1061, 1062]. Full factorial design was employed to identify the relative significance of numerous variables and their interactions [1061-1063]. For the systematic optimization studies, APAP was employed as a model drug based on the hydrophilic nature which resembles the negatively charged siRNA [1064-1066]. At the formulation pH of 5.4, the acetaminophen will be protonated (pKa 9.5) and therefore be neutral. If the formulation pH was raised to above 9.5, the acetaminophen will become deprotonated and have a negative charge. Although having both hydrophilicity and a negative charge would more closely resemble siRNA, raising the pH of the formulation above 9.5 would deprotonate the amino groups (pKa= 9.2) and protonate the carboxylic acid groups (pKa=4) within the Type A gelatin (pKa=7-9) and charge on the gelatin will flip to negative. Any electric charge mediated loading of the APAP would become hindered at a pH higher than 9.5, therefore a pH of 5.4 was selected to maintain positively charged gelatin polymer and neutral acetaminophen.

Table 4.1. Full Factorial 3$^3$ design for the optimization of S6S-GNC using APAP as a model drug.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stirring Rate (rpm)</td>
<td>300, 600, 700</td>
</tr>
<tr>
<td>Ethanol proportions (% v/v)</td>
<td>41, 44, 47</td>
</tr>
<tr>
<td>10% GTA (μl/20 ml formulation)</td>
<td>100, 150, 300</td>
</tr>
</tbody>
</table>

S6S-GNC was formulated by employing the optimized 2-step desolvation methodology (Figure 4.1.) with slight modifications [125, 1060]. HMW gelatin fraction (Figure 4.2.), that
generated smallest sized nanocarriers with particle size approximately 80 nm, was selected for formulation development.

![Figure 4.2. Particle size analysis report for GNC formulated at 600 rpm stir rate (magnetic stir bar method). The bars and dots and error bars represent the mean ± standard deviation (n=3).](image)

One key amendment was made in relation to desolvating solvent, wherein, diluted ethanol was employed in our method as compared to 100% ethanol in reported methods of gelatin nanoparticle preparation [125, 1058]. It was anticipated that the use of a diluted ethanol solution (aqueous) will generate a milder environment for desolvation and hence lessen the chance to form larger, non-uniformly packed gelatin nanocarriers during the preparation stage. To conduct the second desolvation step, 9 ml of 9:1 (v/v) ethanol to water solution was added to 10 ml (total aqueous ethanol, 47% v/v) drop-wise at a temperature of 35°C at an injection speed of 1 ml/min). After ethanol addition, 200μl of 5 mg/ml GEN was added to drop wise to crosslink the formed nanocarriers. After 1 hr, the temperature and stir rate was reduced to 30°C and 200 rpm, respectively, in order to avoid thermal, as well as mechanical stress-induced aggregation or agglomeration. The resultant nanocarriers were purified by three cycles of centrifugation at 10,000 x g for 30 min followed by dispersion of the pellet in PBS pH 7.4 to the original volume.

**4.2.2.2. Development of Targeted STAT6 siRNA loaded Gelatin Nanocarriers**

Type A cationic gelatin nanocarriers were prepared using the 2-step desolvation technique with some modifications, followed by subsequent genipin crosslinking. For the Taguchi design of experiments, the independent factors were gelatin concentration, volume ratio of desolvating agent added to the gelatin solution batch volume, and APAP concentration and their levels were determined as shown in Table 4.2. Gelatin concentration was selected as the orthogonally distributed parameter. The orthogonal array (L₉, 3³) was utilized to determine the optimal parameters and to analyze the effect of these parameters. The formulation parameters were
assigned to each column and nine combinations were formed as shown batches F1-F9 in Table 4.2. The orthogonal array is configured in respect to the total degrees of freedom of the targeted function. The degrees of freedom (DF=9-1=8) for L9 orthogonal array can be equal to or more than the determined process parameters. The particle size error values were measured via the experimental design for each combination of control factors.

**Table 4.2.** Taguchi orthogonal array of APAP-GNC design of experiments parameters.

<table>
<thead>
<tr>
<th>Batch ID</th>
<th>[Gelatin]% w/v</th>
<th>[Acetone]% v/v</th>
<th>[APAP] mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>1</td>
<td>44.4</td>
<td>165</td>
</tr>
<tr>
<td>F2</td>
<td>1</td>
<td>47.4</td>
<td>331</td>
</tr>
<tr>
<td>F3</td>
<td>1</td>
<td>50</td>
<td>496</td>
</tr>
<tr>
<td>F4</td>
<td>2</td>
<td>44.4</td>
<td>331</td>
</tr>
<tr>
<td>F5</td>
<td>2</td>
<td>47.4</td>
<td>496</td>
</tr>
<tr>
<td>F6</td>
<td>2</td>
<td>50</td>
<td>165</td>
</tr>
<tr>
<td>F7</td>
<td>3</td>
<td>44.4</td>
<td>496</td>
</tr>
<tr>
<td>F8</td>
<td>3</td>
<td>47.4</td>
<td>165</td>
</tr>
<tr>
<td>F9</td>
<td>3</td>
<td>50</td>
<td>331</td>
</tr>
</tbody>
</table>

Acetaminophen (APAP) was used as a model drug to optimize the formulation parameters, as shown in Figure 4.3. The desolvating agent, acetone, was added dropwise at a rate of 1 mL per minute using a syringe pump (New Era Syringe Pumps, Inc, Farmingdale, NY, USA) and a 5 mL gas-tight glass syringe (Hamilton, Reno, NV, USA). The GNC colloidal suspension was corrected for volume with distilled deionized water at the process end stage to avoid aggregation. The
particle size and zeta potential of the resultant GNCs in suspension were obtained by dynamic light scattering and electrophoretic mobility, respectively.

Figure 4.3. APAP-GNC Preparation by the 2-step desolvation method and purification by centrifugation and dialysis.

Figure 4.4 shows the resultant data set obtained with the Taguchi orthogonal array included particle size, polydispersity index, zeta potential, and entrapment efficiency. The Taguchi results obtained from the experiments outlined in Table 4.2, is shown in Figure 4.4. These results show that for the prepared batches, the particle size ranged from approximately 60 nm to 200 nm, the Polydispersity indices were in the range of 0.4 to 0.6, the zeta potential (mV) was in the range of +11 to +18 mV, and the loading efficiency (LE%) was in the range of 0% to 8%. Batch 1 had a 0% loading efficiency, possibly due to the low APAP concentration within the acetone and the low gelatin concentration. Additionally, the low acetone addition may have produced more loosely associated polymeric matrix nanoparticles. Therefore, under the stress of filtration, the loosely associated APAP was released. Regression analysis of the Taguchi dataset was performed to predict full factorial data to better design a formulation with desired properties. The equations generated are shown below as Equations 4.1.-4.4.
Figure 4.4. Taguchi orthogonal array design of experiments for APAP-GNC preparation as a model for S6S-GNC. This Figure indicates the batch particle size, polydispersity index, zeta potential, and loading efficiency results. Bars represent mean and error bars are standard deviations (n=3).

\[
\text{Particle size (nm)} = 67.5 + 42.6 [\text{Gelatin}], \%_{w/v} - 0.29 [\text{Acetone}], \%_{v/v} - 0.0980 \ [\text{APAP}], \text{mM} \tag{4.1}
\]

\[
\text{Polydispersity index}= 0.184 - 0.0352 [\text{Gelatin}], \%_{w/v} + 0.00733 [\text{Acetone}], \%_{v/v} + 0.000012 [\text{APAP}], \text{mM} \tag{4.2}
\]

\[
\text{Zeta potential (mV)}= -2.99 + 1.28 [\text{Gelatin}], \%_{w/v} + 0.290 [\text{Acetone}], \%_{v/v} + 0.00471 [\text{APAP}], \text{mM} \tag{4.3}
\]

\[
\text{Loading Efficiency (\%)}= -211 + 11.1 [\text{Gelatin}], \%_{w/v} + 3.35 [\text{Acetone}], \%_{v/v} + 0.176 [\text{APAP}], \text{mM} \tag{4.4}
\]

These regression equations were used to generate data for a full factorial dataset as shown in batches F10-F36 within Table 4.3. The most desirable formulation would have the lowest
particle size, the lowest polydispersity index, a target zeta potential of +20 mV, and the highest entrapment efficiency. Therefore, a desirability equation can be generated to select the most desirable batch parameters as shown below in Equation 4.5. Where D is the desirability factor and PDI is the polydispersity index. The desirability function (Equation 4.5) was used to calculate the desirability of each formulation within the predicted full factorial design of experiments dataset. The lower the value of D, the more desirable the batch is as defined by the parameters above. From these predicted data, batch ID F27 with a gelatin concentration of 2% w/v, amount of acetone concentration in the desolvated gelatin of 50% v/v, and APAP concentration of 496 mM in the desolvating agent were the selected parameter levels for the preparation of S6S-GNC (Table 4.3.).

A validation experiment was performed and the prepared STAT6 siRNA loaded gelatin nanocarriers were characterized as discussed in the following sections.

\[
D = [(\text{Particle Size} - 0) + (\text{PDI} - 0) + (20 - \text{Zeta Potential}) + (100 - \text{LoadingEfficiency})] \\
\text{Equation 4. 5.}
\]
Table 4.3. Predicted full factorial data for APAP-GNC.

<table>
<thead>
<tr>
<th>Batch ID</th>
<th>[Gel], % w/v</th>
<th>[Acetone], % v/v</th>
<th>[APAP], mM</th>
<th>Mean particle size (nm)</th>
<th>Stand. dev</th>
<th>Mean PDI</th>
<th>Stand. dev</th>
<th>Mean Zeta Potential (mV)</th>
<th>Stand. dev</th>
<th>LE, %</th>
<th>Stand. dev</th>
</tr>
</thead>
<tbody>
<tr>
<td>F10</td>
<td>1</td>
<td>44.4</td>
<td>165</td>
<td>87.9</td>
<td>7.0</td>
<td>0.475</td>
<td>0.09</td>
<td>12.0</td>
<td>0.8</td>
<td>-3.3</td>
<td>-1.7</td>
</tr>
<tr>
<td>F11</td>
<td>1</td>
<td>44.4</td>
<td>331</td>
<td>66.6</td>
<td>0.9</td>
<td>0.507</td>
<td>0.11</td>
<td>11.8</td>
<td>0.6</td>
<td>-.65</td>
<td>-0.33</td>
</tr>
<tr>
<td>F12</td>
<td>1</td>
<td>44.4</td>
<td>496</td>
<td>55.5</td>
<td>6.2</td>
<td>0.479</td>
<td>0.10</td>
<td>13.5</td>
<td>1.1</td>
<td>2.5</td>
<td>1.3</td>
</tr>
<tr>
<td>F13</td>
<td>1</td>
<td>47.4</td>
<td>165</td>
<td>106.1</td>
<td>6.8</td>
<td>0.513</td>
<td>0.08</td>
<td>12.2</td>
<td>0.1</td>
<td>-1.3</td>
<td>-0.63</td>
</tr>
<tr>
<td>F14</td>
<td>1</td>
<td>47.4</td>
<td>331</td>
<td>84.7</td>
<td>1.0</td>
<td>0.545</td>
<td>0.10</td>
<td>12.0</td>
<td>-0.1</td>
<td>1.4</td>
<td>0.70</td>
</tr>
<tr>
<td>F15</td>
<td>1</td>
<td>47.4</td>
<td>496</td>
<td>73.6</td>
<td>6.1</td>
<td>0.517</td>
<td>0.09</td>
<td>13.7</td>
<td>0.4</td>
<td>4.6</td>
<td>2.3</td>
</tr>
<tr>
<td>F16</td>
<td>1</td>
<td>50</td>
<td>165</td>
<td>85.4</td>
<td>3.0</td>
<td>0.515</td>
<td>0.09</td>
<td>13.6</td>
<td>0.8</td>
<td>-1.5</td>
<td>-0.74</td>
</tr>
<tr>
<td>F17</td>
<td>1</td>
<td>50</td>
<td>331</td>
<td>64.0</td>
<td>4.8</td>
<td>0.558</td>
<td>0.10</td>
<td>13.4</td>
<td>0.6</td>
<td>1.2</td>
<td>0.59</td>
</tr>
<tr>
<td>F18</td>
<td>1</td>
<td>50</td>
<td>496</td>
<td>53.0</td>
<td>2.2</td>
<td>0.519</td>
<td>0.10</td>
<td>15.2</td>
<td>1.1</td>
<td>4.3</td>
<td>2.2</td>
</tr>
<tr>
<td>F19</td>
<td>2</td>
<td>44.4</td>
<td>165</td>
<td>97.2</td>
<td>8.3</td>
<td>0.499</td>
<td>0.03</td>
<td>14.7</td>
<td>0.7</td>
<td>.23</td>
<td>0.12</td>
</tr>
<tr>
<td>F20</td>
<td>2</td>
<td>44.4</td>
<td>331</td>
<td>75.9</td>
<td>0.5</td>
<td>0.431</td>
<td>0.04</td>
<td>14.5</td>
<td>0.5</td>
<td>2.9</td>
<td>1.4</td>
</tr>
<tr>
<td>F21</td>
<td>2</td>
<td>44.4</td>
<td>496</td>
<td>64.8</td>
<td>7.6</td>
<td>0.402</td>
<td>0.04</td>
<td>16.3</td>
<td>1.0</td>
<td>6.0</td>
<td>3.0</td>
</tr>
<tr>
<td>F22</td>
<td>2</td>
<td>47.4</td>
<td>165</td>
<td>115.3</td>
<td>8.2</td>
<td>0.436</td>
<td>0.02</td>
<td>14.9</td>
<td>0.1</td>
<td>2.3</td>
<td>1.1</td>
</tr>
<tr>
<td>F23</td>
<td>2</td>
<td>47.4</td>
<td>331</td>
<td>94.0</td>
<td>0.4</td>
<td>0.469</td>
<td>0.03</td>
<td>14.7</td>
<td>-0.2</td>
<td>4.9</td>
<td>2.5</td>
</tr>
<tr>
<td>F24</td>
<td>2</td>
<td>47.4</td>
<td>496</td>
<td>82.9</td>
<td>7.4</td>
<td>0.440</td>
<td>0.03</td>
<td>16.5</td>
<td>0.4</td>
<td>8.1</td>
<td>4.0</td>
</tr>
<tr>
<td>F25</td>
<td>2</td>
<td>50</td>
<td>165</td>
<td>94.7</td>
<td>4.4</td>
<td>0.439</td>
<td>0.03</td>
<td>16.4</td>
<td>0.8</td>
<td>2.1</td>
<td>1.0</td>
</tr>
<tr>
<td>F26</td>
<td>2</td>
<td>50</td>
<td>331</td>
<td>73.3</td>
<td>3.4</td>
<td>0.471</td>
<td>0.04</td>
<td>16.2</td>
<td>0.6</td>
<td>4.7</td>
<td>2.4</td>
</tr>
<tr>
<td>F27</td>
<td>2</td>
<td>50</td>
<td>496</td>
<td>62.2</td>
<td>3.6</td>
<td>0.443</td>
<td>0.03</td>
<td>17.9</td>
<td>1.1</td>
<td>7.9</td>
<td>3.9</td>
</tr>
<tr>
<td>F28</td>
<td>3</td>
<td>44.4</td>
<td>165</td>
<td>173.2</td>
<td>23.1</td>
<td>0.405</td>
<td>0.03</td>
<td>14.6</td>
<td>0.8</td>
<td>-1.1</td>
<td>-0.55</td>
</tr>
<tr>
<td>F29</td>
<td>3</td>
<td>44.4</td>
<td>331</td>
<td>151.9</td>
<td>15.3</td>
<td>0.437</td>
<td>0.05</td>
<td>14.4</td>
<td>0.6</td>
<td>1.6</td>
<td>0.78</td>
</tr>
<tr>
<td>F30</td>
<td>3</td>
<td>44.4</td>
<td>496</td>
<td>140.8</td>
<td>22.4</td>
<td>0.409</td>
<td>0.04</td>
<td>16.1</td>
<td>1.1</td>
<td>4.7</td>
<td>2.4</td>
</tr>
<tr>
<td>F31</td>
<td>3</td>
<td>47.4</td>
<td>165</td>
<td>191.3</td>
<td>23.0</td>
<td>0.442</td>
<td>0.02</td>
<td>14.7</td>
<td>0.2</td>
<td>.95</td>
<td>0.48</td>
</tr>
<tr>
<td>F32</td>
<td>3</td>
<td>47.4</td>
<td>331</td>
<td>170.0</td>
<td>15.2</td>
<td>0.475</td>
<td>0.04</td>
<td>14.5</td>
<td>-0.1</td>
<td>3.6</td>
<td>1.8</td>
</tr>
<tr>
<td>F33</td>
<td>3</td>
<td>47.4</td>
<td>496</td>
<td>158.9</td>
<td>22.2</td>
<td>0.446</td>
<td>0.03</td>
<td>16.3</td>
<td>0.5</td>
<td>6.8</td>
<td>3.4</td>
</tr>
<tr>
<td>F34</td>
<td>3</td>
<td>50</td>
<td>165</td>
<td>170.7</td>
<td>19.2</td>
<td>0.445</td>
<td>0.03</td>
<td>16.2</td>
<td>0.9</td>
<td>.73</td>
<td>0.36</td>
</tr>
<tr>
<td>F35</td>
<td>3</td>
<td>50</td>
<td>331</td>
<td>149.3</td>
<td>11.4</td>
<td>0.477</td>
<td>0.04</td>
<td>16.0</td>
<td>0.6</td>
<td>3.4</td>
<td>1.7</td>
</tr>
<tr>
<td>F36</td>
<td>3</td>
<td>50</td>
<td>496</td>
<td>138.2</td>
<td>18.4</td>
<td>0.449</td>
<td>0.04</td>
<td>17.8</td>
<td>1.2</td>
<td>6.5</td>
<td>3.3</td>
</tr>
</tbody>
</table>
4.2.2.3. Development of Inhalable Gemcitabine Loaded Gelatin Nanocarriers using the Taguchi Orthogonal Array Method

Type B anionic gelatin nanocarriers were prepared using the 2-step desolvation technique with some modifications, followed by subsequent genipin crosslinking (Figure 4.5) [1058, 1059, 1067]. Gem was added to each batch at a concentration of 1 mg/mL. The desolvating agent, ethanol, was added dropwise at a rate of approximately 2 mL per minute to the 0.5%, 1% or 1.5% w/v gelatin solution at 40°C under constant stirring at 600 rpm, and the genipin solution (0.2%, 0.6%, or 1.0% w/w) was added dropwise (1 mL/min) immediately following the ethanolic solution addition. Ethanol was selected over other desolvating agents, like acetone, because gemcitabine is soluble in water and acetone, but not soluble in ethanol. Therefore, Gem would associate in the hydrophilic gelatin polymeric nanocarrier. The solution was stirred at 600 rpm for an additional 60 min, at that point the stirring rate and temperature were dropped to 200 rpm and 30°C, respectively, and allowed to stir until the ethanol had completely evaporated. The GNC colloidal suspension was corrected for volume with distilled deionized water at the process end stage. The particle size and zeta potential of the resultant GNCs in suspension were obtained by dynamic light scattering and electrophoretic mobility, respectively.

![Figure 4.5](image)

**Figure 4.5.** Schematic showing the formulation of Gem loaded gelatin nanocarriers (Gem-GNC).

For the preparation of Gem-GNC, gelatin concentration, volume ratio of desolvating agent (90% v/v aqueous Ethanol) added to gelatin solution batch volume, and genipin concentration were selected as control factors and their levels were determined as shown in Table 4.4 [581, 582]. Gelatin concentration was selected as the orthogonally distributed parameter. The orthogonal array
(L₉, 3³) was utilized to determine the optimal parameters and to analyze the effect of these parameters [1068]. The formulation parameters were assigned to each column and nine batches (F37-F45) were formed as shown in Table 4.4. The orthogonal array is configured in respect to the total degrees of freedom of the targeted function. The degrees of freedom (DF=9-1=8) for L₉ orthogonal array can be equal to or more than the determined process parameters. The particle size error values were measured via the experimental design for each combination of the control factors. The determination of the quality characteristics of the measured control factors was provided by signal-to-noise (S/N) ratios, as shown in Figure 4.6A-B, where the lowest S/N ratios were inferred to have better quality. Formulation parameter selection was based on the target particle size of 150 nm and the regression analysis of the Taguchi dataset batches. The three leveled parameters within the designed experiments were the volume ratio (v:v) of 90% Ethanol to total batch volume, genipin weight to gelatin percent (% w/w), and gelatin weight to batch percent volume (% w/v). The largest variation of the S/N ratio of the genipin concentration suggests that it has the largest influence on the particle size.
Table 4.4. Experimental matrix and responses from the L$_9$ orthogonal array and the S/N ratios of experimental results for particle size the Taguchi Orthogonal Array Gem-GNC batches. Particle size was determined with PBS pH 7.4 at 25°C by dynamic light scattering as described within the methods section. The gelatin concentration was expressed as a weight volume percent (% w/v) of gelatin weight to batch volume. The desolvating agent addition was expressed as volume ratio of 90 % v/v ethanolic aqueous solution to volume of gelatin batch. The genipin concentration was expressed as weight percent (% w/w) of genipin to gelatin weight. Experiments were performed in triplicate (n=3).

<table>
<thead>
<tr>
<th>Batch no.</th>
<th>Gelatin (% w/v)</th>
<th>90% v/v Ethanol addition: Batch volume</th>
<th>Genipin (% w/w)</th>
<th>Average particle size (nm)</th>
<th>Standard deviation of particle size (nm)</th>
<th>S/N ($\eta_i, i=1-9$) (dB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F37</td>
<td>0.5</td>
<td>7:10</td>
<td>0.2</td>
<td>155.2</td>
<td>42.16</td>
<td>-43.72</td>
</tr>
<tr>
<td>F38</td>
<td>1</td>
<td>7:10</td>
<td>0.6</td>
<td>167.03</td>
<td>21.93</td>
<td>-46.89</td>
</tr>
<tr>
<td>F39</td>
<td>1.5</td>
<td>7:10</td>
<td>1</td>
<td>559.67</td>
<td>300.89</td>
<td>-53.64</td>
</tr>
<tr>
<td>F40</td>
<td>1</td>
<td>8:10</td>
<td>0.2</td>
<td>292.73</td>
<td>72.71</td>
<td>-47.43</td>
</tr>
<tr>
<td>F41</td>
<td>1.5</td>
<td>8:10</td>
<td>0.6</td>
<td>355.37</td>
<td>208.63</td>
<td>-51.6</td>
</tr>
<tr>
<td>F42</td>
<td>0.5</td>
<td>8:10</td>
<td>1</td>
<td>357.73</td>
<td>104.36</td>
<td>-53.7</td>
</tr>
<tr>
<td>F43</td>
<td>1.5</td>
<td>9:10</td>
<td>0.2</td>
<td>156.77</td>
<td>68.44</td>
<td>-46.81</td>
</tr>
<tr>
<td>F44</td>
<td>0.5</td>
<td>9:10</td>
<td>0.6</td>
<td>250.6</td>
<td>99.07</td>
<td>-46.33</td>
</tr>
<tr>
<td>F45</td>
<td>1</td>
<td>9:10</td>
<td>1</td>
<td>365.5</td>
<td>243.67</td>
<td>-52.08</td>
</tr>
</tbody>
</table>

dB=Decibels.
Figure 4.6. Experimental analysis of Taguchi designed experiments: (A) Taguchi main effects plot for mean particle size and (B) main effects plot for particle size S/N ratios of the Gem-GNC.

Taguchi S/N ratio was calculated for the particle size responses to understand the effect of the gelatin concentration, volume ratio of desolvating agent (90% v/v aqueous Ethanol) added to gelatin solution batch volume, and genipin concentration factor levels on the particle size response (Figure 4.6B). A higher S/N ratio infers higher influence of the parameter on the particle size. The S/N ratios were analyzed under the nominal is best condition, dependent on the target particle size of 150 nm. The S/N ratio was calculated using equation 1, as follows:

\[
\text{Nominal is best: } S_N = 10 \times \log\left(\frac{\bar{y}^2}{\sigma^2}\right) \quad \text{Equation 4.6}
\]
Where $\bar{Y}$ is the response mean and $\sigma$ is the variance. The “Nominal is best” was applied because a targeted response of 150 nm particle size was desired. The relative influence of each factor level was determined by comparing the S/N ratios of the particle size. This analysis determines which factor has more effect on the particle size by finding the largest range of the S/N ratio.

4.2.2.3.1. Preparation and Purification of Gemcitabine Loaded Gelatin Nanocarriers (Gem-GNC)

Following the same method as described above, Gem (1 mg/mL) was added to the 10 mL gelatin solution prior to the dropwise desolvating ethanol addition. The resultant gelatin nanocarriers were purified by one cycle of centrifugation at 10,000 $\times$g for 30 min to remove any crystalized Gem followed by dialysis against PBS pH 7.4 using a 12-14 kDa dialysis membrane for 1 hr with 3 medium changes (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA). Then, 1% w/v lactose monohydrate and 1% v/v Tween 20 (CRODA, Inc., Columbus, NJ, USA) were used as a cryoprotectant and surfactant, respectively, and the batch was frozen to -80°C for 3 hr. The frozen Gem-GNC suspension was lyophilized for 48 hr under vacuum at <0.133 mBar at -84°C using a Freezone 12 Plus Lyophilizer (Labconco, Kansas City, MO, USA). The Gem-GNC formulation suspension for nebulization was prepared by resuspending the lyophilized formulation in deionized water for in vitro characterization. The formulation, as prepared, will be reconstituted in 0.9% normal saline solution and administered as a suspension via the inhalation route of administration.

4.2.3. Particle Size and Zeta Potential Measurements

The particle size of the prepared S6S-GNC, S6S-GNC-P, and Gem-GNC were determined by dynamic light scattering using a NICOMP ZLS 380 analyzer (PSS-NICOMP, Santa Barbara, USA) [942, 1069]. The technique uses dynamic light scattering (DLS) to infer the particle size of materials within a solution or suspension by analyzing the random movements, or change, observed in the intensity of light scattering off of the randomly moving particles. The technique makes use of the relation between particle size and Brownian particle motion, as described by the Stokes-Einstein equation (Equation 4.7.):

\[ D_h = \frac{k_B T}{3\pi \eta D_c} \]  

Equation 4.7.
where, $D_h$ is the hydrodynamic diameter, $D_t$ is the translational diffusion coefficient, $k_B$ is Boltzmann’s constant, $T$ is the thermodynamic temperature, and $\eta$ is the dynamic viscosity. The NICOMP Particle Sizer relies upon this equation, but the user is responsible for inputting key parameters, such as temperature and viscosity. The particle size derived from dynamic light scattering is the hydrodynamic size; therefore, it assumes the shape of the particle is spherical.

Zeta potential is a measure of the electro kinetic potential in colloidal systems, which represent the potential within the interface of the slipping plane of the nanoparticle and the fluid at the interface. Therefore, it is a measure of the electrical charge at the double layer, but it is not equal to the electric surface potential or Stern potential in the double layer [1070]. A zeta potential analysis system measures the sample conductivity, applies an electric field, and then measures the motion of the particles using electrophoretic light scattering. The direction of the particle movement determines if the charge is positive or negative, and the speed determines the magnitude of the charge, or zeta potential, with respect to size. The movement of the nanoparticles in response to the electric field allows for the calculation of their electric charge. The $\zeta$ potential was determined by the electrophoretic mobility ($\mu$) measurements. The mobility $\mu$ of the nanoparticles at 25°C was converted to $\zeta$ potential by the Smoluchowski’s equation (Equation 4.8.):

$$\zeta \text{ potential} = \frac{\mu \eta}{\varepsilon}$$

Equation 4.8.

where $\eta$ is the viscosity of the dispersion solvent and $\varepsilon$ is the permittivity of the solution [1071, 1072].

The particle size of the S6S-GNC, S6S-GNC-P, and Gem-GNC were assessed by dispersion in Phosphate Buffered Saline (PBS) pH 7.4 at a concentration of 1-part gelatin nanocarrier suspension to 2 parts buffer for a total volume of 600 µl [942, 1069]. The zeta potential of the S6S-GNC, S6S-GNC-P, and Gem-GNC were assessed by dispersion of 100 µl gelatin nanocarrier suspension within 2 ml of distilled deionized water containing sodium chloride (1 µM) and was placed into the photoelectric cell. The zeta ($\zeta$) potential was calculated by Smoluchowski’s equation (Equation 4.7.) from the electrophoretic mobility of the S6S-GNC, S6S-GNC-P, and Gem-GNC at 25°C. For each batch, at least 3 independent samples were taken, with each recorded in triplicate (n=3).
4.2.4. Determination of Entrapment Efficiency (EE%) and Loading Efficiency (LE%)

The entrapment efficiency was determined by employing Vivaspin500 ultracentrifuge filters (MWCO100 kDa, Viva Products, Inc., Littleton, MA, USA) using UV spectrophotometry to quantify the free siRNA in a sample. For STAT6 siRNA entrapment determination, 100 µl of the formulation and 400 µl distilled deionized water was placed on the top of the Vivaspin filter membrane and centrifuged at 16,200×g for 20 min. The aqueous filtrate was then subjected to UV spectrophotometric analysis to determine the free STAT6 siRNA content using a BioSpek-nano Micro-volume UV-Vis Spectrophotometer (Shimadzu, Columbia, MD, USA). Sample sizes of 2 µl were loaded onto the sample mount and then analyzed using a pathlength of 0.7 mm. The entrapment of S6S within the developed formulations was calculated using the following equation (Equation 4.9.):

\[
\text{Entrapment Efficiency (EE), } \% = \frac{X_1 - X_2}{X_1} \times 100\% \tag{Equation 4.9.}
\]

Where \(X_1\) = amount of total STAT6 siRNA initially added to the batch, normalized to sample size (mg) and \(X_2\) = amount of free Gem detected after ultracentrifugation (mg).

Loading efficiency (LE) of STAT6 siRNA within the GNC was calculated with the following equation:

\[
\text{Loading Efficiency (LE), } \% = \frac{X_1 - X_2}{\text{Total mass GNC (mg)}} \times 100\%
\tag{Equation 4.10}
\]

Where \(X_1\) = total mass of S6S initially added to the batch, normalized to sample size (mg) and \(X_2\) = mass of free S6S detected after filtration centrifugation (mg).

For Gem entrapment determination, Gem loaded formulations (0.5 mL) were placed on top of the Vivaspin filters and centrifuged at 16,200 ×g for 15 min. The aqueous filtrate generated at the bottom of the Vivaspin 500 ultracentrifuge tubes was then subjected to HPLC analysis in triplicate (n=3), as described above, to determine the concentration of unloaded Gem (\(\lambda_{\text{max}}= 268\) nm). The entrapment efficiency of the Gem within the developed formulation was calculated using Equation 4.9. Where \(X_1\) = total mass of Gem initially added to the batch, normalized to sample size (mg) and \(X_2\) = mass of free Gem detected after filtration centrifugation (mg). Loading efficiency (LE) of Gem within the GNC was calculated with the following Equation 4.10. Where
X₁ = total mass of Gem initially added to the batch, normalized to sample size (mg) and X₂ = mass of free Gem detected after filtration centrifugation (mg).

The entrapment efficiencies of gemcitabine within the Gem-GNC formulations were determined by employing Vivaspin500 ultracentrifuge filters with a molecular weight cutoff (MWCO) of 10kDa (Viva Products, Inc., Littleton, MA, USA) using HPLC with UV spectrophotometry to quantify the free Gem in the sample. HPLC analysis of Gem was performed with a Dionex Ultimate 3000 LC system including a pump, autosampler, column compartment and diode array detector and data was displayed on the Chromeleon 7 software (Dionex, Sunnyvale, CA, USA). Samples were isocratically eluted using a reverse-phase HPLC system (Dionex, Sunnyvale, CA, USA) with a C18 column (Phenomenex Inc., Torrance, CA, USA). An HPLC method was adapted and modified for gemcitabine quantification [1073]. Methanol and water (30:70, v/v) containing 0.01 M ammonium acetate (NH₄OAc) were used as the mobile phase and detection was performed at a wavelength of 268 nm.

4.2.5. Evaluation of Inhalable Gelatin Nanocarriers Containing Gemcitabine (Gem-GNC)

4.2.5.1. In vitro Release of Gem from Gem-GNC

The in vitro release of Gem from the developed formulation was assessed under physiological pH employing DPBS, pH 7.4 and within Gamble’s solution to simulate the interstitial conditions within the lung as release media. Gamble’s solution was prepared by dissolving the following within distilled deionized Milli-Q water (g/L): 0.095 magnesium chloride, 6.019 sodium chloride, 0.298 potassium chloride, 0.126 disodium hydrogen phosphate, 0.063 sodium sulfate, 0.368 calcium chloride dihydrate, 0.574 sodium acetate, 2.604 sodium hydrogen carbonate), and 0.097 sodium citrate dihydrate [1074]. To conduct the release experiment, 2 mL of Gem-GNC formulation was placed inside a dialysis bag (MWCO12-14kDa, Spectrum Laboratories Inc. (Rancho Dominguez, CA, USA). The membrane bags were placed in 100 mL of DPBS (pH 7.4) under constant agitation at 200 rpm at 37 ± 2°C. At predetermined time intervals (0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 24, 48, 72 hr), 1 mL of dissolution medium was collected and equal volume of fresh dissolution medium while maintaining sink conditions. Each formulation underwent release studies using 3 independent release vessels. The samples were analyzed at each time interval in triplicate (n=3) using the HPLC method described earlier. The time versus percent Gem release was plotted to evaluate the release profile of the developed formulation. In vitro release of Gem from Gem-GNC and Gem solution within DPBS and Gamble’s SLF were analyzed.

4.2.5.2. pH Stability of Gem-GNC

Since tumor interstitial tissues and cells have acidic pH, it was necessary to measure the influence of pH on the particle size and zeta potential as stability markers of the Gem-GNC at pH 5.4-7.4.[582]. The Gem-GNC was incubated in DPBS pH 5.4, 6.4, and 7.4 up to 72 hr to assess the influence of pH on the surface charge and size of the nanoformulations as indicators of stability. Samples were collected and analyzed in triplicate (n=3) every 24 hr.

4.2.5.3. Differential Scanning Calorimetry (DSC) of Gem-GNC and Excipients

The physical state of Gem within the gelatin matrix was evaluated using a TGA/DSC1 (METTLER TOLEDO, Columbus, OH, USA). Approximately 2 mg of the Gem-GNC, GNC placebo with Gen crosslinker and lactose monohydrate as cryoprotectant with and without Gem present outside the NCs. Genipin, Gem, and lactose monohydrate were weighed into an aluminum pan, hermetically sealed, pan lids were pinholed to allow for escape of any volatile components, and the sample was analyzed over the range of -10°C to 260°C at a heating rate of 10 °C min⁻¹. Transition temperatures were determined from the endothermic or exothermic peak minima while transition enthalpies were obtained by integration of the endothermic transitions using linear baselines. Placebo GNCs were prepared by the same process as the Gem-GNC samples, but prepared without the addition of the active ingredient Gem. The physical mixture of placebo GNC and gemcitabine were prepared in the same way, except that 100 mg of Gem was dissolved within the suspending agent prior to freezing and lyophilization.

4.2.5.4. Powder X-ray Diffraction (PXRD) of Gem-GNC and Excipients

X-ray diffraction measurements of Gem-GNC, GNC placebo with genipin crosslinker and lactose monohydrate as cryoprotectant with and without free Gem outside the NCs, placebo GNC non-crosslinked with unentrapped genipin and Gem, and Gem alone were conducted to compare their crystalline structure using a Bruker D8 Advance powder XRD diffractometer (AXS GmbH, Karlsruhe, Germany) over an angular range of 5–50°. The PXRD diffraction instrument is equipped with a vertical goniometer in the Bragg-Brentano geometry (θ-2θ). The signal was conditioned using a Gobel mirror and collected using a LYNXEYE linear detector. A Cu-Kα radiation source was used, and the scanning (2θ) rate was 5°/min. Approximately 0.25 g of powder sample was filled into a low background Si crystal cut on the 511 plane sample cell and gently
compressed with a glass slide to make the sample surface and holder surface coplanar. The divergence slit was 0.1 mm, with a step size of 0.01 and scan speed of 0.5 sec per step. The Gem-GNC, placebo-GNC, and type 2 gelatin were placed in a regular sample holder. The powder diffraction patterns of the various Gem-GNC, placebo GNC controls, and the individual excipient controls were analyzed for crystalline or amorphous characteristics by identifying the presence of large diffracted peaks or an amorphous halo.

4.2.5.5. In vitro Aerosol Characterization of Gem-GNC

Aerodynamic particle size distribution was measured using an 8-stage non-viable Andersen cascade impactor (Westech Scientific Instruments, Marietta, GA, USA) lined with plates on each stage and an end-stage filter. To prevent particle bounce during nebulization, each plate on the impactor was coated with polysorbate 20 (Tween 20). A compressor nebulizer, the Vios® Aerosol Delivery system (PARI Respiratory Equipment, Inc., Midlothian, VA, USA) was operated at a flow rate of 8 L/min and equipped with a Pari LC Sprint Nebulizer cup. The freeze dried Gem-GNC were redispersed in normal saline solution to a concentration of 50 mg/ml, which corresponded to a Gem dose of 100 µg/ml. The Gem-GNC formulation was nebulized for 10 min into the cascade impactor at a flow rate of 28.3 L/min after nebulization, the amount of formulation deposited on the throat, impactor stages (0-7) and filter was collected by washing with 2 mL of PBS pH 7.4. Samples were obtained and particle sized with the Nicomp 380ZLS as described above. The nebulization process did not affect the particle size and surface charge of the GNCs, as shown by the insignificant change in these properties before and after nebulization. Then the remaining samples underwent degradation experiments as follows. The samples from each plate that potentially contained Gem-GNC were dispersed in 2 mL of trypsin solution (0.025 g/mL) in a 10 mL volumetric flask, shaken, and incubated at 37°C until a transparent solution formed (approximately 10 min), indicating that complete digestion of the GNCs and release of all Gem encapsulated within the gelatin matrix had been achieved. Water was added to the flask and the solution was made up to volume, and filtered through a 0.22 µm filter. The amount of Gem in the supernatant was analyzed using a validated HPLC, as discussed in a previous section, with a Diode Array Detector UV absorbance detector (Ultimate 3000, Dionex, Sunnyvale, CA, USA).

The mass median aerodynamic diameter (MMAD), geometric standard deviation (GSD), and fine particle fraction (FPF) were obtained from impactor data using Microsoft Excel with appropriate formulas. The MMAD is the diameter at which 50% of the particles by mass are larger.
and 50% are smaller. The United States Pharmacopeia (USP) chapter <601> instructs to determine the MMAD by plotting the percentages of mass less than the stated aerodynamic diameters versus the aerodynamic diameters on log probability paper. The MMAD is the intersection of the line with the 50% cumulative percent. GSD is a measure of the spread of an aerodynamic particle size distribution and is calculated using equation 4, as follows:

\[
GSD = \left( \frac{d_{84}}{d_{16}} \right)^{\frac{1}{2}}
\]

Equation 4.15

Where \(d_{84}\) and \(d_{16}\) represent the diameters at which 84% and 16% of the aerosol mass are contained in diameters less than these diameters, respectively. The fraction of a dose that will deposit in the lung, because of its size, is known as the FPF. This is resembled by the portion of the mass that enters the impactor with an aerodynamic diameter smaller than 5 µm, defined as the FPF or “respirable” fraction [FPF<5 µm] [1076]. The optimal size for central airway deposition represents the upper limit used to define this fraction varies between 4-6µm, where particles of size of 2-4 µm maintain peaks peripheral lung deposition. There is no lower limit for FPF, although particles with sizes of less than 1 µm may be exhaled without deposition [1077].

The aerodynamic particle size distribution measurement is based on the amount of Gem deposited on each stage of the cascade impactor and represents a relative particle distribution. Respirable mass, and respirable fractions were calculated from the known amount of drug deposited on the various parts. Only droplets measuring less than 5 µm in aerodynamic diameter were included in the assessment of the respirable mass and fraction. All cascade impactor experiments were repeated in triplicate (n=3) and data are represented as mean ± standard deviation.

4.2.5.6. Cell Proliferation MTT Assay on A549 and H460 NSCLCs Treated with Gem-GNC

The effect of Gem-GNC on the viability of A549 and H460 cell lines was measured using the established MTT assay protocol [1078]. The MTT assay measures the metabolic activity of cells and is supposed to correlate with increasing cell numbers [1079, 1080]. A549 and H460 cells were seeded onto separate 96-well plates at a density of 5,000 cells/well, and incubated overnight. Cells were then treated with varying dosing levels of Gem-GNC, Placebo GNC, and Gem solution (n=4) for 24, 48, and 72 hr. The 96-well plates were incubated at 37 ± 0.2°C, and the cell viability was measured using the MTT assay [99]. Untreated cells were used as a control. Cell viability was plotted vs. concentration of Gem and dose of Gem-GNC.
4.2.6. Preparation of S6S Loaded Gelatin Nanocarriers (S6S-GNC) and S6S-GNC conjugated with IL4R targeting peptide

Type A cationic gelatin nanocarriers were prepared using the established 2-step desolvation technique with some modifications, followed by subsequent genipin crosslinking as illustrated schematically in Figure 4.7 [99]. The first desolvation commenced as the high molecular weight gelatin fraction was prepared by dissolving 5% w/v gelatin in 250 mL water and desolvating it with 250 mL acetone for 5 min at 30°C under gentle stirring within a metal beaker. When the first desolvation was complete, the acetone water and low molecular weight gelatin mixture was decanted and discarded. The high molecular weight fraction which precipitated was washed twice with cold distilled deionized water and dried in an oven overnight at 50°C. After the gelatin was completely dry, the gelatin was weighed by conducting mass balance before and after the desolvation. A 2% w/v high-molecular weight gelatin solution was prepared by dissolving the gelatin in distilled deionized water at 45°C under stirring at 300 rpm for 4 hr. The 2% w/v gelatin solution was cooled and aliquoted into 50 ml centrifuge tubes and stored at 4°C until use. Stock gelatin (2% w/v) was warmed to 35°C and 2.5 mL 2% w/v gelatin along with 200 µL 100 µM siRNA were mixed together for 30 min at room temperature to form a primary siRNA gelatin complex. After incubation, non-solvent acetone was added dropwise (1 ml/min, New Era Syringe Pumps, Inc, Farmingdale, NY, USA) to reach final acetone concentration of 50% v/v (2.5 mL acetone) for controlled precipitation of gelatin solution to form S6S-GNC. After acetone addition, gelatin nanoparticles were crosslinked. Genipin, a biocompatible and biodegradable crosslinking agent, was dissolved in acetone (10 mg/ml) and added at the last stage of desolvation followed by evaporation of acetone (0.02% w/v). At 2 hr after genipin addition, 2.5 mL distilled deionized water was added to the nanoparticles and the hot/stir plate temperature was reduced to 30°C. This volume adjustment of the total aqueous nanoparticle suspension to 5 ml and gelatin concentration

![Figure 4.7. Formulation of PEGylated S6S-GNC conjugated with IL4Rα targeting peptide (S6S-GNC-P).](image)
of 1% w/v was needed in order to reduce the inter-particulate interactions as the apparent volume decreased during acetone evaporation. The crosslinking reaction continued overnight as the acetone was completely evaporated off under stirring at 300 rpm at 30°C. Genipin crosslinking was considered successful if the gelatin nanoparticle suspension appeared blue in color due to the genipin amine reaction. Gelatin nanocarriers containing STAT6 siRNA were purified by dialysis against water in 100 kDa dialysis membranes for 1 hr with 5 complete medium changes to remove any free siRNA or genipin.

The STAT6 siRNA loaded gelatin nanocarriers were collected from the dialysis bags and placed into glass vials for further PEGylation and peptide conjugation. The STAT6 siRNA loaded gelatin nanocarriers were PEGylated to prepare the formulation termed S6S-GNCs. The NH2-PEG (MW 5000 Da)-COOH was reacted with the amine-sulfhydryl crosslinker, AMAS (Pierce Biotechnology, USA), to yield amine protected AMAS-PEG-COOH conjugate (Figure 4.7) [1081]. The 20% molar excess of EDC/NHS (1-ethyl-3(3 dimethylaminopropyl) carbodiimide hydrochloride/ N-hydroxysulfo succinimide) was used to activate the carboxyl group of AMAS-PEG-COOH and subsequently dialyzed [Molecular weight cut-off (MWCO) 12-14 kDa] to remove free EDC/NHS and AMAS. The resultant activated AMAS-PEG-COOH was conjugated to the gelatin amine group located on the surface of S6S-GNC to formulated PEGylated S6S-GNC. Surface PEGylation densities of 65-70% surface was selected based on reported optimal levels for stealth effect in vivo [157].

PEGylated S6S-GNCs were conjugated with IL4R targeting peptide at concentrations of 1-30% mole ratios of peptide to total PEG, which corresponds to 50-1400 copies of peptide per gelatin nanocarrier to formulate S6S-GNC-P. The cysteine-tagged IL4Rα targeting peptide (Genscript, Piscataway, NJ, USA) of 7.5, 10, 15, and 30 mol % was conjugated on the surface of PEGylated S6S-GNC via the linkage between amine groups present on the PEGylated nanocarriers and sulfhydryl group of cysteine-tagged IL4Rα peptide using AMAS (Figure 4.7). Resultant S6S-GNC-P formulation was dialyzed using MWCO 300 kDa membrane for 4 h with 3 medium changes to remove uncoupled PEG, PEG-peptide conjugate and free reagents. The supernatant from the nanocarriers formulation was collected using Vivaspin (MWCO 100 kDa) and to was analyzed for reagents using HPLC confirm complete removal [1081]. Unconjugated PEG in the dialysis medium was quantified using a Barium/Iodine colorimetric method [1082]. A barium solution was prepared in 1 M hydrochloride to a final concentration of 5% w/v barium chloride.
Potassium iodide solution at a concentration of 2% w/v that contained 0.13 g/ml iodine. The 200 µl PEG sample, 50 µl barium solution, and 25 µl iodine solution were mixed sequentially in a 96 well plate. A standard curve of PEG at 0, 10, 20, and 30 µg/ml concentrations was performed along with the unknown samples, as shown in Figure 4.8.

![Figure 4.8](image)

**Figure 4.8.** PEG standard curve obtained using the Barium/Iodine colorimetric method measured at 535 nm. Data are presented mean ± standard deviation as the dots and error bars and the line is the best linear fit. Samples were run in triplicate (n=3).

The percentage of surface PEGylation was determined by quantifying the unconjugated PEG concentration to determine the amount of surface conjugated PEG and the surface area of the nanocarriers [1082, 1083]. Nanocarriers were characterized by determining size distribution, zeta potential, polydispersibility index, entrapment efficiency, *in vitro* release, and stability studies. FITC fluorescent dye was conjugated on the amine groups of gelatin before preparing the nanocarriers used in the flow cytometry binding and internalization assays. FITC-tagged gelatin were purified by dialysis overnight against PBS using a 10 kDa dialysis membrane and the collected fluorescent gelatin was freeze dried for future use. Preparation of nanoparticles composed of fluorescent gelatin commenced in the same procedures stated above.

### 4.2.7. Determination of Surface Morphology and Particle Size via Scanning Electron Microscopy and Transmission Electron Microscopy

Images of lyophilized S6S-GNC-P and S6S-GNC were collected and evaluated for surface morphology, topography, particle size, and observable aggregation characteristics using a transmission electron microscope (120 kV Hitachi HT7700 Transmission Electron Microscope) available at the Biological Electron Microscope Facility at the UH Manoa. The fully digital TEM...
was outfitted with a video camera for fast scanning and alignment and an AMT XR-41 2048x2048 pixel bottom-mount camera for high-resolution imaging. The microscope was equipped with a high-tilt stage for electron tomography. For TEM analysis, 10 mg of freeze dried nanocarrier samples re-dispersed in 5 ml distilled deionized water were applied to the top of a carbon-coated-Formvar-coated grid (200 mesh; 3.05 mm diameter). Also, gelatin nanocarriers were imaged from the original formulation that had not underwent freeze drying within lactose. SEM imaging was performed on the freeze dried S6S-GNC-P with and without lactose monohydrate cryoprotectant. The images were scanned with 100 kV accelerating voltage and 8000x direct magnification. Micrograph images of the Gem-GNC were obtained with a Hitachi S-4800 Field Emission Scanning Electron Microscope with an Oxford INCA X-Act EDS System and a 120 kV Hitachi HT7700 Transmission Electron Microscope (Chiyoda, Tokyo, Japan). Images were analyzed using ImageJ® software (1.48v, National Institutes of Health, USA).

4.2.8. Determination of the Number of Surface Bound Peptides per GNC

The number of peptide molecules attached to the surface of a particle was calculated using the Equation 4.11. [1083, 1084].

$$q = \frac{[(C_i - C_t)V]}{m}$$

Equation 4.11.

where q is the amount of peptide attached to one-unit mass of the nanoparticles (mg/mg); Ci and Ct are the concentrations of peptide in the initial solution and in the supernatant after the conjugation reaction, respectively (mg/ml), V is the volume of the aqueous phase (ml), and m is the mass of the nanoparticles (mg). Ci and Ct was quantified using the bicinchoninic acid assay (BCA) assay (Pierce Chemical Company) [1085]. The BCA assay is a detergent-compatible biochemical assay for determining the total concentration of a protein in a solution from 0.5 ng/µl to 1.5 mg/ml. This method has a similar determination range as the Lowry protein assay, Bradford protein assay, or biuret reagent. It combines the well-known reduction of Cu2+ to Cu1+ by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu1+) using a unique reagent containing BCA. First, the unconjugated protein was isolated by Vivaspin filtration using a centrifuge to remove the free peptide from the nanocarriers. The absorbance for the samples were measured following a 30 min incubation at 37°C at 562 nm. Since the BCA assay is not a true-endpoint method, the final color
will continue to develop upon longer incubation times. But the rate of color development at room temperature is slow enough to measure a large number of samples together.

The number of peptide bonds and the presence of the amino acids cysteine, cystine, tryptophan, and tyrosine are responsible for the color formation when combined with BCA. Since studies with di-, tri-, and tetrapeptides have shown that the color formation depends on more than the sum of the individual color causing functional groups, protein concentrations are determined and reported with reference to standards of a common protein like BSA. Standard BCA samples with known protein concentrations at 0, 1, 2.5, 5, 10, 20, and 40 µg/µl were prepared and analyzed to prepare a standard curve, as shown in **Figure 4.9**, for every BCA assay performed. The protein concentration is determined by monitoring the color change of the sample solution from green to purple in proportion to protein concentration, which was then measured using colorimetric techniques in a plate reader at 562 nm. The blank sample average absorbance at 562 nm was subtracted from the absorbance for all of the individual standard and unknown replicates. Then, a standard curve was prepared by plotting the average blank-corrected absorbance measurement at 562 nm for each BSA standard versus its concentration in µg/µl. The data was fitted with a four-parametric, or quadratic, to provide the best fit curve. The unknown protein sample concentrations were determined based on the standard curve prepared during each assay. For all BCA assay measurements performed, the microplate procedure was used in order to require a smaller volume of protein samples.

**Figure 4.9.** BCA protein assay representative standard curve of absorbance at 562 nm versus BSA concentration. Dots show the mean ± standard deviation with n=3.
The average mass of a particle was determined by **Equation 4.12**.

$$m_i = \frac{C}{N}$$

**Equation 4.12.**

where $m_i$ is the average mass of one nanoparticle (mg), $C$ is the concentration of the nanoparticle suspension (mg/ml), $N$ is the number of nanoparticles in a unit volume of suspension liquid (particles/ml) [1084]. The number of GNC per ml of suspension will be calculated using the size of the GNC determined using the following formula (**Equation 4.13.**) [1086, 1087],

$$N = \frac{\varphi}{\left[\frac{4}{3} \pi \left(\frac{d}{2}\right)^3\right]}$$

**Equation 4.13.**

where $N$ is the number of GNC/volume, $\varphi$ is the volume fraction of particles determined by viscosity, $4/3\pi(d/2)^3$ is the average volume of a GNC, and $d$ is the volume-weighted diameter determined by light scattering. The volume fraction, $\varphi$, of the particles estimated by viscosity is calculated as:

$$\varphi = \frac{\left(\frac{h}{h_0} - 1\right)}{2.5}$$

**Equation 4.14.**

where $h$ is the viscosity of the nanoparticle suspension; $h_0$ is the viscosity of the solvent without nanoparticles. Viscosity of the nanoparticle suspension was determined using a Cannon-Fenske glass capillary viscometer. The concentration of conjugated peptide per mL was determined by calculating the free peptide using BCA assay kit. The number of molecules of peptide per S6S-GNC-P will be determined by multiplying the molar concentration of conjugated peptide by Avogadro’s number and dividing by the number of GNC per ml.

### 4.2.9. *In vitro* Release Profile of S6S from S6S-GNC and S6S-GNC-P and Kinetic Analysis

S6S release from S6S-GNC formulation intended for use in the proof-of-concept adenocarcinoma *in vitro* assays was assessed under physiological pH employing phosphate-buffered saline (PBS; pH 7.4) as release milieu. To set up the release experiment, 2 ml of S6S-GNC formulation was placed inside a dialysis bag (MWCO 100 kDa, Fisher Scientific, USA). The membrane bags were placed in 50 ml of PBS pH 7.4 under constant agitation condition (300 rpm) at 37°C. At predetermined time intervals (0.5, 1, 2, 3, 4, 5, 6, 7, 24, 48, 72 hr), 0.5 ml of dissolution medium was collected and equal volume of fresh dissolution medium was replaced to simulate
perfect sink conditions. The samples were analyzed at each time interval using the S6S from the developed formulations.

The \textit{in vitro} release of S6S solution and S6S-GNC-P was performed using a cellulose membrane (MW cut off 100,000 Daltons) and PBS as dissolution medium with appropriate maintenance of sink conditions as described in a previous section. Sink conditions are defined as having a saturation solubility of a drug in the dissolution medium that is at least three times as much than the drug concentration. The 0.5 ml S6S-GNC-P was placed into an HPLC vial with the lid lined with 100 kDa dialysis membrane (Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA). The HPLC vials were inverted and placed membrane side down into a 20 ml scintillation vial containing 10 ml DPBS (pH 7.4) under constant agitation at 200 rpm at 37 °C. At predetermined time intervals (0, 0.5, 1, 2, 3, 4, 5, 24, 48, 72, 96 hr), 0.5 ml of dissolution medium was collected and an equal volume of fresh dissolution medium was replaced while maintaining sink conditions. Each formulation underwent release studies using three independent release vessels. The samples were analyzed at each time point in triplicate (n=3) using the BioSpec Nano UV method described above. The time point versus percent STAT6 siRNA release was plotted to evaluate the release profile of the developed formulation. Release kinetics of the S6S-GNC-P were analyzed using the free open source software, KinetDS 3 rev 2010, available at \url{http://sourceforge.net/projects.kinetds/} [1075].

Drug release kinetics from the GNC’s were assumed to be governed by diffusion within a matrix system [1088, 1089]. Therefore the release kinetics of the STAT6 siRNA from the S6S-GNC-P were confirmed by fitting the release data into five kinetic models: zero order, first order, Weibull, Higuchi, Baker Lonsdale, and Korsmeyer-Peppas models [1090, 1091]. The STAT6 siRNA release in DPBS were fitted to these models and their respective R$^2$ values were compared.

\textbf{4.2.10. Stability Studies under Serum and pH medium}

Stability of S6S-GNC formulated for proof-of-concept studies under conditions of varying pH and serum level was also assessed to investigate the stability of developed formulation under different environments [1092]. The 200 µl S6S-GNC was incubated in 400 µl PBS pH 6.4, 7.4 and 8.4 for 1 hr (n=3) to assess the influence of pH on the surface charge and size of the nanoformulations. Furthermore, to develop proof that the S6S-GNC will eventually prevent \textit{in vivo} degradation of S6S, stability studies were also performed under the presence of 10% v/v FBS at pH 7.4.
The S6S-GNC and S6S-GNC-P underwent similar stability assessments within DPBS (pH 7.4), DPBS (pH 7.4) containing 10% v/v FBS, and distilled deionized water containing 10% v/v FBS. Maintaining a particle size of less than 200 nm, but more than 50 nm, as well as relatively constant PDI were inferred to imply that the particles were stable. Samples were analyzed using the NICOMP 380 ZLS dynamic light scattering particle sizer at 1, 24, 48, 72, and 96 hr after incubation with the respective media.

4.2.11. S6S Stability Study: Agarose Gel Electrophoretic Mobility Assay

Stability of encapsulated S6S was assessed by agarose gel electrophoresis as described previously [1093], with slight modifications. The assay was performed to examine the stability of encapsulated siRNA in its loaded as well as solution form. To mimic siRNA exposure under in vivo condition, stability was also assessed in the presence of RNAse, an enzyme that degrades siRNA [1094, 1095]. The 1% (w/v) agarose gel was electrophoresed at a constant voltage of 70 V until the bromophenol blue marker bands were well separated.

The S6S-GNC-P stability using agarose gel electrophoretic mobility assays were conducted in two methods. First, the N:P ratio, the amount of amino groups of the gelatin to the phosphate groups of the siRNA, was evaluated by using the agarose gel retardation assay. Gelatin nanocarriers with increasing siRNA concentrations, thus decreasing the N:P ratio at levels of 3000, 800, and 500, were applied to the wells of a 1% agarose gel. It was expected that a high siRNA concentration, where the N:P ratio is low, that unprotected siRNA would not be hindered by the gelatin polymer and transverse the gel freely. Therefore, siRNA concentrations that are adequately protected by the gelatin within the GNC would be located only around their respective wells, while the sample buffer, DNA ladder, and control siRNA would freely separate on the agarose gel. siRNA concentration used were relevant to the in vitro cell studies in Th2 TIB-224 cells and siRNA levels were not elevated above what was deemed necessary for in vitro assays to avoid wasting the siRNA. Therefore, siRNA concentration or N:P ratio limits were not determined in this study.

The second way the S6S-GNC-P were analyzed for stability under the agarose gel electrophoretic mobility assays were used to identify the stability of matrix-entrapped STAT6 siRNA after preparation method conditions, and in the presence of protease and RNase [99, 1096]. The samples run on the 1% ultra-low range agarose gel were the (1) DNA ladder, (2) STAT6 siRNA, (3) STAT6 siRNA + Protease, (4) STAT6 siRNA + RNase, (5) STAT6 siRNA + Protease + RNase, (6) S6S-GNC-P, (7) S6S-GNC-P + Protease, (8) S6S-GNC-P + RNase, (9) S6S-GNC-
P + Protease + RNase (sequential), (10) S6S-GNC-P + RNAse + Protease (sequential). The sequence of enzyme addition was either protease followed by RNAse to degrade the gelatin matrix and release the siRNA or RNAse followed by protease to show that the siRNA was physically encapsulated in the gelatin matrix and did not undergo degradation. This method can illustrate if the method of preparation, nanoparticle crosslinking, surface PEGylation, and peptide conjugation had an influence on the entrapped siRNA with the GNC. Also, this method may show if the prepared S6S-GNC-P physically protect the entrapped siRNA from RNase in vivo during systemic circulation or cellular internalization.

4.2.12. In vitro Cytotoxicity of S6S-GNC in A549 Adenocarcinoma Cells and S6S-GNC and S6S-GNC in Mouse Th2 Cells

The effect of S6S-GNC on viability of A549 cell lines was measured using established MTT assay protocol [1078]. A549 cells were seeded into 96-well plates at a density of $10^4$ cells/well and incubated overnight. After this, cells were treated with S6S, S6S Lipofectamine complex, placebo GNC and S6S loaded GNCs for 24 and 48 hr at concentrations between 0-15 nM. The 96 well-plates were incubated at 37±0.2°C and the cell viability was measured using MTT assay [1078]. The untreated cells were used as control.

The intended goal of the S6S-GNC-P formulation was to deliver STAT6 siRNA effectively while not eliciting antiproliferative or cytotoxic effects in Th2 cells. To assess the safety of the S6S-GNC and S6S-GNC-P formulation in Th2 cells, treatments of varying levels of the formulations were conducted and the cell viability of the Th2 cells were quantified using the MTT assay [1078]. TIB-224 Th2 cells were plated onto 96-well plates at a concentration of 200,000 cells/mL with 100 µl per well. The cells were then treated with the various concentration of S6S-GNC and S6S-GNC-P, as well as controls including untreated cells, Scrambled siRNA-GNC, and Scrambled siRNA-GNC-P. The 96-well plates were incubated at 37±0.2°C for 24, 48, and 72 hr and the cell viability was measured using the MTT assay.

The MTT colorimetry assay measures cell viability through cell metabolic activity. The number of viable cells present are determined through NAD(P)H-dependent cellular oxireductase enzymes. These enzymes reduce the water soluble tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to water insoluble formazan [1097-1099]. For the suspended (non-adherent) Th2 cell line, the 96-well microplate was centrifuged prior to removing the treatment medium and adding 100 µl of fresh medium. Then, 10 µl of 12 mM MTT solution
was added to each well, including a negative control of 10 µl of the MTT solution to 100 µl of medium alone. The plate was incubated for 4 hr at 37°C within a cell culture incubator. Another round of centrifugation was performed before removing 85 µl of the medium from each well. The purple formazan crystals formed at this point may be dissolved in DMSO (150 µl/well) and the concentration determined by optical density at 540 nm after incubating the plate at 37°C for 10 min [1100]. The MTT assay has excellent linearity up to 10^6 cells per well, with certain cell lines.

4.2.13. Cellular Internalization Assay of S6S-GNC in A549 adenocarcinoma cells using inverted fluorescence microscopy

The cell internalization was evaluated by treating Nile red dye loaded GNC in A549 cells [655]. A549 cells were seeded in 24-well plates (25,000/well) and were incubated with Nile red-loaded GNC followed by imaging of cells at 0, 0.25, and 1 hr. The cellular uptake of the S6S-GNC A549 lung cancer cells were investigated with fluorescence microscopy using a Zeiss Axiovert 40 CFL inverted microscope with an appropriate filter set (Carl Zeiss Microscopy, LLC, USA). The fluorescent light source was an Exfo X-Cite series 120 (Lumen Dynamics Group, Inc, Mississauga, Ontario, Canada).

4.2.14. Upregulation of IL4Rα in Asthmatic Mouse Lung Tissue and Th2 cells

IL4Rα relative protein expression in Th2 cells (D10.G4.1, ATCC® TIB-224™), ovalbumin (OVA) treated allergic asthma mouse lung tissue protein lysates, and normal lung tissue protein lysates were evaluated using an established Western blot analysis. IL4Rα antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Total protein in tissue and cell culture lysates were measured with the BCA assay kit from Pierce™ (Thermo Scientific™, Waltham, MA, USA). Lysates were then normalized to 10 µg samples per well and diluted with 2X Laemmli sample buffer (Bio-Rad Laboratories, Inc.) containing β-mercaptoethanol. The protein samples were electrophoresed on 1.5 mm 8% SDS-PAGE gels at 45 V for 30 min and then 120 V until the markers were well separated. The protein gels were transferred to nitrocellulose membranes using a semi-dry electrophoresis transfer system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were then washed with TBS-T and blocked using 5% BSA solution prepared in TBS-T. Following blocking, the membranes were incubated with primary antibody overnight within 5% BSA TBS-T. The following day, the primary antibody was decanted from the membranes and the membranes were washed in TBS-T for 2 hr. Secondary antibody was prepared in 5% low-fat milk to reduce background and placed onto the membranes to incubate at
room temperature for 2 hr. Membranes were imaged using a Bio-Rad ChemiDoc system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

4.2.15. Determination of Optimum Surface Peptide Density Using STAT6 ELISA Assays

The Thermo Scientific™ Pierce™ In-Cell ELISA Colorimetric Detection Kit (Waltham, MA, USA) was used to determine the intracellular STAT6 protein expression following treatment with S6S-GNC with different surface targeting peptide densities. To perform the assay, Th2 cells (50,000 cells/well/150 µl) were plated on a 96-well plate, treated with 75 nM STAT6 siRNA gelatin nanocarrier equivalents and appropriate controls for 72 hr, and then fixed with formalin. Expression of STAT6 protein was monitored in a 96-well plate using target-specific primary antibodies and a horseradish peroxidase (HRP)-conjugated detection reagent. The kit was supplied with a whole-cell stain to control for differences in plating, which was important since the relative levels of STAT6 protein with different treatments were measured. After staining, the results were analyzed by normalizing the absorbance (HRP activity) values to cell number, which adjusts for the cell plating differences among the wells.

4.2.16. Flow Cytometry Experiments for Cell Binding and Cellular Internalization using FITC-S6S-GNC and FITC-S6S-GNC-P

A Beckman Quanta MPL flow cytometer (Beckman Coulter, Brea, CA, USA) was used to determine the extent of cellular binding and internalization of the S6S-GNC-P prepared with gelatin conjugated to FITC fluorescent dye. For the binding assay, cells were counted and the cell concentration was normalized to 1,500,000 cells/mL in complete growth medium. Then, 1 mL of cell suspension was placed into 1.5 ml microcentrifuge tubes and incubated at 4°C for 15 min. The various treatments (200 µl of each), including medium control, plain S6S-GNC, PEGylated S6S-GNC, 7.5% mol ratio S6S-GNC-P (360 copies of peptide per gelatin nanocarrier), 10% mol ratio S6S-GNC-P (480 copies of peptide per gelatin nanocarrier), and 15% mol ratio S6S-GNC-P (720 copies of peptide per gelatin nanocarrier) were placed into the microcentrifuge tubes containing 1.5 million cells. The cells were returned to 4°C for 1 hr to prevent any active or passive cellular internalization and promote receptor bound peptide. This procedure was conducted with three independent batches and runs (n=3). One batch of 30% mol ratio of peptide to total surface PEG (1000 peptide/GNC) S6S-GNC-P was included in the binding assay primarily to assess the selected range of peptide concentrations. Following incubation, the cells were washed 2x with cold DPBS to remove any free nanoparticles. The cells were then re-suspended within DPBS containing 50
mM glucose and plated onto a 96-well plate for flow cytometry analysis using the Beckman Quanta using laser excitation at 488 nm wavelength.

The cellular internalization assay was performed using TIB-224 cells treated with FITC-loaded gelatin nanoparticles and incubated at 37°C for timepoints of 5 min, 10 min, 15 min, 30 min, 1 hr, and 12 hr. TIB-224 cells were treated with the medium control, plain S6S-GNC, PEGylated S6S-GNC, 7.5% mol ratio S6S-GNC-P, 10% mol ratio S6S-GNC-P, and 15% mol ratio S6S-GNC-P within serum free Th2 culture medium. Upon reaching those specified timepoints, the samples were removed from the 6-well plate and placed into 1.5 ml microcentrifuge tubes and washed 2x with DPBS glucose 50 mM. The cells were then re-suspended in DPBS containing 50 mM glucose and plated onto a 96-well plate for flow cytometry analysis. Again, one batch of 30% mol ratio S6S-GNC-P was included in a preliminary experiment to confirm the range of selected peptide concentrations.

Flow cytometry data was analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA). Gating was performed to minimize peak aberrations, but not to the point of shifting peak location. Full data without gating is provided in the Appendix C.

4.2.17. Protein and mRNA Downregulation by S6S-GNC-P via Western Blot and RT-PCR

It is best to monitor protein and mRNA expression levels in therapeutic siRNA experiments because mRNA reduction may occur without a reduction in protein levels. This is a case that indicates that protein turnover is slow. Protein reduction observed in the absence of mRNA reduction, however, may indicate that an siRNA is mediating its effects at the translation level like a microRNA [1101].

In order to assess the efficiency of STAT6 silencing in A549 adenocarcinoma cells by the various formulations prepared, expression levels of STAT6 protein was monitored following nanoformulation treatment with appropriate controls. The untreated control, S6S+Lipofectamine complex (15 nM STAT6 siRNA) and S6S-GNC (15 nM STAT6 siRNA equivalent) treated A549 cells were lysed using RIPA buffer and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as per previously described [51, 1102]. The 8% SDS-PAGE gel was electrophoresed at 45 V for 30 min and then 120 V until the bromophenol blue markers were well separated. Human STAT6 and β-actin proteins (Cell Signaling, USA) were detected using rabbit polyclonal primary antibodies (Santa Cruz Biotechnology Inc., USA). The STAT6 antibody utilized was capable of detecting endogenous levels of total STAT6 protein. The primary
antibodies were tagged with secondary anti-rabbit IgG antibody horseradish peroxidase (HRP)-linked antibody. The affinity purified goat anti-rabbit IgG (H&L) antibody was conjugated to horseradish peroxidase by the supplier/manufacturer for use as a secondary antibody in chemiluminescent western blotting applications. Proteins were visualized using Luminol Reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

The STAT6 siRNA protein downregulation caused by S6S-GNC and S6S-GNC-P in Th2 cells was assessed similarly as above, but with some modifications. Lipofectamine RNAiMax was used as a transfection agent for the scrambled and STAT6 siRNA for negative and positive controls (Thermo Fisher Scientific, Waltham, MA, USA). 6-well plates were used for Th2 transfection and treatment with the siRNA complexes, S6S-GNC, or S6S-GNC-P formulations using medium or scrambled siRNA equivalents as controls (Greiner Bio-One). Protein was extracted from the Th2 cells 48 or 72 hr post treatment using RIPA buffer with protease inhibitors (Teknova). Total protein concentrations were quantified using a Pierce BCA assay kit (Pierce™ Thermo Scientific™, Waltham, MA, USA). Protein samples were normalized to specific levels per well of a SDS-PAGE gel by calculating the exact volume and volume of sample loading buffer. Sample loading buffer was prepared using Laemmli sample buffer from Bio-Rad Laboratories, Inc. (Cat #161-0737) with a final concentration of 5% β-mercaptoethanol, 710 mM. The protein sample was diluted using 1-part sample with 1 part Laemmli sample buffer. Samples were loaded into 1.5 mm SDS-PAGE gels composed of 30% acrylamide, water, buffer, 10% SDS, ammonium persulfide, and TEMED as the resolving and stacking gels with either 10- or 15- wells, depending on the experiment requirements. The gels were loaded into a Bio-Rad Electrophoresis tank containing the recommended amount of 1X running buffer and samples were pipetted into the wells using the Bio-Rad Kaleidoscope Ladder for each gel. Gels were electrophoresed for 30 min at 45 V and then 120 V just until the markers fell off the gel.

The proteins embedded in the SDS-PAGE gels were transferred to nitrocellulose membranes using a semi-dry transfer system (Bio-Rad Laboratories, Inc.) containing transfer buffer. The transfer was conducted at 25 V for 1 hr. Adequate protein transfer was inferred by complete transfer of the Kaleidoscope ladder which is visible in either the SDS-PAGE gel or nitrocellulose membrane, but will be absent from the SDS-PAGE gel if it is completely transferred. The membranes are then cut, washed in TBS-T, and placed into incubation containers. The blocking step commenced with the addition of 5% w/v BSA in TBS-T and the membranes were
placed on a shaker for 1 hr. Primary antibodies were diluted in 5% w/v BSA in TBS-T at levels suggested by the suppliers. STAT6 antibody (Cell Signaling) was diluted at a 1:1000 dilution. Beta actin antibody (C-4, mouse monoclonal, sc-47778, lot # H1914, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was diluted at 1: 10,000 dilutions in 5% BSA in TBS-T. After the 1 hr blocking step, the membranes were washed two times with TBS-T and then the primary antibodies were added to the membranes and incubated overnight at 4°C on a plate shaker. Secondary antibodies, goat anti-mouse (sc-2005, lot # E3113, Santa Cruz Biotechnology) and goat anti-rabbit (sc-2054, lot # H2112, Santa Cruz Biotechnology) were prepared in 5% nonfat dry milk in TBS-T. The following morning, the primary antibodies were removed from the membranes and TBS-T was used to rinse the membranes. Following shaking with TBS-T for 30 min to remove all of the primary antibodies, the secondary antibodies were added to the membranes for 2 hr while shaking at room temperature. After the secondary antibody incubation, the membranes were washed twice with TBS-T and then shaked for 30 min in TBS-T. The membranes were developed using Bio-Rad Clarity™ Western ECL Substrate luminol/enhancer solution and peroxide solution mixed in equal part and following the manufacturers protocol (Control 102030610, Expiration Date: 2017-01-16). The membranes were imaged using a Bio-Rad Chemidoc system. Bands were normalized to the loading control, Beta Actin, and the band densities were analyzed using ImageJ software. Relative protein expressions were normalized to a medium control unless stated otherwise.

The STAT6 siRNA Lipofectamine RNAiMax complex positive control concentration was optimized. Lipofectamine RNAiMax volumes per 200 µl well were 0, 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 µl (0, 0.08, 0.17, 0.25, 0.33, 0.42, and 0.5% v/v) placed into wells of a 96-well plate containing Th2 cells. Cell viability was measured at 24 hr after treatment using the MTT assay. Two RNAiMax concentrations were selected based on the cell viability curve. Concentrations of 0.17% and 0.25% v/v RNAiMax were selected based on their low impact on cell viability. The formation of the STAT6 siRNA complex was expected to lessen their reduction on cell viability through reducing the overall charge of the cationic lipoplexes. Then, STAT6 siRNA concentrations were varied at concentrations of 12.5 nM, 25 nM, 50 nM, 100 nM, 150 nM, and 200 nM using each of the two RNAiMax concentrations (0.17% and 0.25% v/v) and STAT6 protein relative expression will be quantified using Western blot protein densitometry analysis. Controls included medium only, RNAiMax control, and a Scrambled siRNA control at 50 nM. STAT6 protein expression was normalized to Beta actin loading controls and then were normalized relative to the medium.
control. STAT6 siRNA and Lipofectamine concentrations was selected based on the levels that provided the most STAT6 protein downregulation.

Next, S6S-GNC and S6S-GNC-P formulation concentration selection for STAT6 protein downregulation Western blot analysis was selected based on dose dependency experiments. S6S-GNC and S6S-GNC-P at concentrations of 25 nM, 50 nM, 100 nM, 150 nM, and 200 nM STAT6 siRNA were placed in 6-well plates containing Th2 cells. STAT6 relative protein expression and Beta Actin loading controls were analyzed using Western blot and quantified with densitometry. STAT6 protein expression was normalized to the Beta Actin loading controls and then were normalized relative to the Scrambled siRNA-GNC or Scrambled siRNA-GNC-P (200 nM) control. Two S6S-GNC-P STAT6 siRNA equivalent concentrations were selected based on the level that provided the most STAT6 protein downregulation.

S6S-GNC and S6S-GNC-P at 100 nM were analyzed for their effect on STAT6 protein downregulation at 48 and 72 hr post treatment using medium as a control, as well as scrambled siRNA, STAT6 siRNA, Scrambled-GNC, and Scrambled-GNC-P at 100 nM siRNA concentrations. Relative STAT6 protein expression was measured using Western blot and densitometry analysis as described above.

Total RNA was extracted from the TIB-224 cells using the High Pure RNA Isolation Kit (Roche Diagnostics, Risch-Rotkreuz, Switzerland) following the manufacturer’s protocol. cDNA was then prepared in the presence of reverse transcriptase using iScript™ Reverse Transcription Supermix for RT-qPCR following the manufacturer’s protocol (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Total RNA and cDNA concentrations were determined by using a BioSpek-nano Micro-volume UV-Vis Spectrophotometer (Shimadzu, Columbia, MD, USA). The mRNA levels were determined by STAT6, GATA3, and Beta Actin primer sets provided by Sigma Aldrich prepared using the iQ™ SYBR® Green Supermix protocol (Bio-Rad Laboratories, Inc., Hercules, CA, USA). RT-PCR was conducted using a Bio-Rad CFX384 Touch™ Real-Time PCR Detection System (Hercules, CA, USA).

4.2.18. Statistical Analysis

One-way analysis of variance (ANOVA) with Tukey’s multiple comparison post-test was used in the analysis of differences between the physicochemical properties of nanocarrier formulations. The least significant difference post-hoc ANOVA analysis was used in the comparison of particle sizes between different formulations. The experiments were conducted in
triplicate with data reported as mean ± standard deviation. The significance level was set at \( p<0.05 \). Statistical analysis of the data was performed using Minitab 16 Statistical Software (State College, Benton, PA, USA).
CHAPTER 5. RESULTS AND DISCUSSION

5.1. STAT6 siRNA Loaded Gelatin Nanocarriers

S6S-GNC were developed and tested in a lung cancer model cell line in terms of their cellular uptake and STAT6 protein downregulation capabilities to ascertain the feasibility of the development of a peptide targeted STAT6 siRNA loaded gelatin nanocarrier formulation for asthma therapy. The aim of this sub-hypothesis was to provide proof of concept that gelatin polymer (FDA approved polymer) based nanocarrier formulations of STAT6 siRNA (S6S-GNC) will provide an alternate therapeutic approach in siRNA delivery for cancer therapy [110]. Newer and more effective pharmacological interventions for cancer therapy are necessary because surgery and radiotherapy are not viable options in some patients while chemotherapy results in low response rates with detrimental adverse effects [1103, 1104].

5.1.1. Development of STAT6 siRNA loaded Gelatin Nanocarriers

Gelatin is a biodegradable and biocompatible FDA-approved polymer for i.v. administration. Gelatin is denatured collagen protein obtained through either acid (Type A gelatin) or alkaline (Type B gelatin) hydrolysis and contains cationic anionic, and hydrophobic amino acid groups within its polypeptide [1105]. The gelatin polypeptide is ~11% hydrophobic, ~13% positive charged, and ~12% negative charged [108, 1106]. Gelatin is cheaper to obtain than synthetic or other natural polymers and gelatin nanoparticle preparation methods have potential for pilot- and industrial scale-up [108]. Gelatin-based nanoparticles represent an attractive strategy, since a significant amount of bioactive material can be incorporated into the protein based nanoparticle matrix [100, 1107]. In addition, gelatin nanoparticles have shown low cytotoxicity [108]. Among the two subtypes of gelatin (Type A and B), type A gelatin is positively charged at pH 5 and above, and hence, type A gelatin was used to elicit pH dependent protonation efficiency of gelatin [126, 1108]. The type B gelatin has been previously used for siRNA delivery [100], however reports comparing type A and type B gelatin clearly infer type A gelatin to be best for siRNA delivery. The gelatin type A has net positive charge that allows the formulation of efficient primary complexation with negatively charged siRNAs [1109-1111]. Therefore, type A gelatin has been selected to formulate the STAT6 siRNA encapsulated nanocarriers.

For the gelatin nanocarrier preparation, a two-step desolvation technique was utilized, where in the first step, the type A gelatin was fractionated to remove the LMW fraction using acetone as a desolvating agent and then the second step was performed to form the nanocarriers.
using the remaining solidified fractions [125, 1060]. The electrostatic interactions between the negatively charged siRNA and positive charged gelatin was used to formulate the STAT6 siRNA matrix-loaded gelatin nanocarriers. The formulation strategy followed here differs from the previously described methods, for example, by Kommareddy et al. and Lemieux et al., where neutral or negative charged non-condensing lipids or polymers and the negatively charged oligonucleotide payload was encapsulated by the physical entanglement of nucleic acid constructs within the matrix or through hydrogen bonds between the polymer and nucleic acid bases [1112, 1113]. Electrostatic interaction as a means of oligonucleotide or siRNA loading has been used successfully in previous studies [126, 1114]. Nanocarriers with particle size of <100 nm have the ability to pass through the pores of tumor vasculature based on the enhanced permeation and retention (EPR) effect and also fulfill the prerequisite of having long circulation lifetimes [1115-1118]. An optimization of the formulation parameters has not been accomplished to reduce the particle size to a more desirable range for enhanced cancer targeting (size<100 nm) [1119, 1120].

The effect of varying gelatin molecular weight on formulation of gelatin nanocarriers were also studied by Coester et al. in 2000, wherein molecular weight of gelatin was reported to be greatly influential on the particle size and stability of the developed gelatin nanocarriers [125]. A systematic study on the influence of low, medium, and high molecular weight fractions, isolated by controlled timing of the first desolvation step, was performed. This exploration of varying molecular weight fractions of gelatin illustrated that the HMW fraction had apparent advantages over the whole gelatin in respect to producing lower particle size and PDI of the resultant nanocarriers, which was in agreement with previously reported findings [125, 1060]. Since HMW gelatin fraction produced smaller particle sized nanoparticles with an average particle size of approximately 82 ± 5 nm, it was anticipated that the medium molecular weight (MMW) fraction might produce further reduce particle size. Typically, in nanocarrier formulation the LMW polymers lead to formation of smaller sized nanocarriers [1121, 1122]. The gelatin nanocarriers formulated with MMW fraction resulted in non-significantly different (p>0.05) nanocarriers with particle size of 85 ± 5 nm as compared to HMW, but the variance, or the polydispersity index (PDI), was significantly higher in case of MMW at 0.69 compared to the HMW PDI of 0.6 (Figure 4.2). However, it can be evinced that there is a non-significant difference between the HMW and MMW gelatin fractions based nanocarriers formulation (p>0.05; Figure 4.2). The larger PDI was unexpected since the LMW fraction was anticipated to have a lower molecular weight distribution
and therefore the resultant nanocarriers were expected to have a lower PDI. It was possible that the unique combination of gelatin molecular weights remaining after the desolvation process allowed for tighter packing in the spherical gelatin nanocarrier, similar to the tighter molecule packing between two different molecular weight fractions in co-crystals compared to pure crystals [1123]. Conclusively, as shown in Figure 4.2, the HMW fraction generated more robust nanocarriers with a lower PDI. Therefore, the HMW fraction was selected for further development of the S6S-GNC formulation.

Taguchi orthogonal array design of experiments was used to identify the optimum formulation parameters for those selected: stirring rate (300, 600, 700 rpm), ethanol volume (41, 44, 47 % v/v or 7, 8, 9 ml), and glutaraldehyde concentration (100, 150, 300 µl 10% GTA), as shown in Table 4.1. The dependent outcome variable was particle size, where a particle size of >20 nm but <100 nm was most desirable. The outcomes of these experiments are presented in Figure 5.1. The optimized parameters were found to be 600 rpm stirring rate, 7 ml of ethanol added as desolvating agent, and 300 µl of 10% glutaraldehyde (GTA). The stir rates of 300 and 600 rpm lead to similar particle size means. Stir rate of 700 rpm generated a much higher particle size means compared to the gelatin nanocarriers prepared at 300 and 600 rpm. The crosslinker concentration in interaction with stir rate did not influence on the particle size. The ethanol volume added had great influence on the particle size means with interaction with the crosslinker concentration. The formula optimized using APAP (pKa=9.4; protonated acid at pH 7.4, neutral) as a model drug was then applied to formulate S6S-GNC with slight modifications. Since the optimized ethanol percent volume added to the gelatin solution was 80% v/v, a 9:1 (v/v) ethanol to water solution was prepared and the final percent volume of total aqueous ethanol desolvating agent to be added was increased to 90% (v/v).
A modified two-step desolvation technique was used to prepare the gelatin nanocarriers as a colloidal delivery system while key factors effecting formulation of gelatin nanocarriers were considered in the preparation of the formulation. Particle size is highly influential on nanoparticle cellular uptake and tissue or organ distribution [1124]. Following intravenous administration, nanocarriers must travel to the target tumor tissue location through the circulation, while avoiding elimination by the liver and spleen [582]. Nanocarriers with particle sizes of >5 µm and 1-5 µm are trapped and phagocytosed in the capillary beds and Kupffer cells in the liver, respectively [118]. Nanocarriers with particle sizes of 100-200 nm undergo rapid clearance by the spleen when compared to nanocarriers with particle sizes of 20-100 nm [1115, 1116]. The circulation lifetime of a nanocarrier depends on the particle size and shape because these are characteristics which influence the nanocarrier fluid dynamics within the blood [582]. Spherical nanocarriers with particle sizes of 100-150 nm flows through the middle of the vascular flow due to van der Waals forces between the nanoparticle and the endothelial lining and hemodynamic fluidics. Therefore, nanocarriers maintain a further distance from the endothelial lining, resulting in less likelihood to non-specifically bind to endothelial cells, and thus increases the circulatory lifetime [1117].
Nanocarriers with particle sizes of <30 nm have advantages like increased cellular uptake rates than larger particles, however, nanocarriers with particle sizes of <20 nm undergo rapid clearance via hepatic and renal routes of elimination. Nanocarriers with particle sizes <5 nm rapidly transverse and equilibrate in the extravascular space, thus also effectively clearing them from the circulation and reducing their bioavailability [1125, 1126]. In addition, *in vivo* distribution studies of intravenously delivered nanoparticles with particle size of > 230 nm accumulate in the spleen because of capillary diameters within this organ [1127]. Nanocarriers with particle size of < 100 nm and >20 nm were shown to have an improved efficacy due to improved pharmacokinetics [1128].

**Figure 5.2.** Particle size and zeta potential of the S6S-GNC and placebo-GNC batches. The bars represent the mean particle size (nm), the square markers represent the zeta potential (mV), and the error bars represent their standard deviation. Particle size measurements were conducted in PBS pH 7.4 medium at a concentration of 25% v/v nanoparticle dispersion to buffer medium concentration. The zeta potential measurements were conducted in water containing 10μM NaCl at a concentration of 5% v/v nanoparticle dispersion. Measurements were conducted in triplicate (n=3).

Increased circulation lifetimes, which allows the nanocarrier to accumulate at higher concentration via the enhanced permeation and retention (EPR) effect. The EPR effect occurs because tumor tissue vasculature contains “leaky” blood vessels containing fenestrae which have poor cutoff sizes between 380-780 nm [1129, 1130]. The EPR effect is the preferential uptake of nanoparticles within the tumor tissue and their accumulation compared to normal tissues [1129]. The longer circulating nanocarriers have increased probability to pass into the tumor tissue through the tumor vasculature fenestrae. Therefore, optimization of gelatin nanoparticles should be
performed critically to achieve the desired properties and therapeutic effects [582]. Nanocarriers with particle sizes of 20-100 nm have the ability to pass into tumor tissue via the EPR effect while maintaining a prolonged circulation lifetime, therefore, a target particle size was set to 20-100 nm. The particle size and zeta potential of the S6S-GNC and placebo GNC formulations are shown in Figure 5.2. The particle size and zeta potential of the S6S-GNC formulation were observed to be 69.6 ± 6.5 nm and +10 ± 0.56 mV, respectively. Other studies that aimed to formulate gelatin nanoparticles have shown the particle size of > 100 nm [125, 1114, 1131]. The entrapment efficiency (EE%) of the S6S-GNC formulation was found to be 85±2.87%. The S6S-GNC’s were found to be within the desired formulation characteristics range (<100 nm particle size, > +5 mV surface charge, >80% EE%, and X% LE%).

5.1.2. In vitro Release of STAT6 siRNA from STAT6 siRNA loaded GNC (S6S-GNC)

The in vitro release profile of STAT6 siRNA from the S6S-GNC formulations compared to STAT6 siRNA solution in PBS media was shown in Figure 5.3. The developed S6S-GNC formulation demonstrated a sustained release of the entrapped STAT6 siRNA, inferring efficient cargo retention of the developed formulation (Figure 6). The S6S-GNC showed < 15% STAT6 siRNA release at 24 hr, ~ 50% release at 48 hr and ~ 84% release at 72 hr. Burst release of approximately 5.0% was observed upon incubation of the formulation to the PBS pH 7.4 inferring that a small fraction of loaded STAT6 siRNA is associated with the surface of the gelatin nanocarrier, while the majority of STAT6 siRNA was embedded within the gelatin matrix of the formulated gelatin nanocarriers [1132]. A sustained release of loaded bioactive from gelatin nanoparticles were also observed by other investigators and these results are in agreement with reported findings [1119, 1133].
Figure 5.3. *In vitro* STAT6 siRNA release profile for the S6S-GNC formulation compared to the STAT6 siRNA solution. Lyophilized formulation was re-suspended in PBS pH 7.4 and filled inside dialysis membrane bags with MWCO of 300 kDa (Sigma, USA). The membrane bags were placed in 50 ml of PBS medium maintained at a temperature of 37±2°C with continuous gentle stirring at 300 rpm on a magnetic heating and stirring plate. At specific time intervals, 0.5 ml aliquots of dissolution medium were withdrawn and analyzed using a Biospek UV spectrophotometer. Results are represented as mean ± standard deviations (where n=3).

An integral challenge in the development of a clinically relevant nanoparticle dosage form is the proper retention of the drug until it reaches the target site. Burst release from nanoparticles compromises the delivery of the intended dose of the drug. Newer formulation approaches integrate stimuli responsive materials that allow a triggered drug release. Therefore, the drug remains within the carrier until it is triggered by local *in vivo* changes in temperature, electrolyte concentrations, or pH due to the physiopathology of the intended target site. The drug release may also be triggered by a magnetic field or by the illumination of light outside of the body [1134]. Nanoparticles which have these properties are prepared using stimuli-responsive polymers [1135].

5.1.3. Serum and pH Stability of STAT6 siRNA Loaded Gelatin Nanocarriers

It was widely reported that active therapeutic ingredient incorporation into nanoparticles significantly prevents their degradation [1120, 1136]. In order to generate proof behind our hypothesis that gelatin nanocarriers will eventually prevent *in vivo* degradation of STAT6 siRNA, stability studies were performed in the presence of buffer solutions and 10% v/v FBS. S6S-GNC showed a non-significant (*p* >0.05) change in the size and charge in the presence of buffer solutions in the pH range of 6.4 to 8.4 (Figure 5.4). Similarly, a non-significant (*p* >0.05) change in the size
and charge was observed in presence of FBS/PBS. The degree of gelatin nanocarrier and serum protein interaction depends highly on the size and charge characteristics of the gelatin nanocarriers [1137]. The developed S6S-GNC formulation, were stable in terms of size in the presence of these simulated physiological media.

![Graph showing stability of S6S-GNC formulation](image)

**Figure 5.4.** Stability of S6S-GNC formulation. Outcome is as expressed by size (nm) and zeta potential (mV) under the influence of varying pH between 5.4 to 8.4 and 10 %v/v FBS at physiological pH 7.4 to mimic the serum found in human blood. Results are represented as mean ±SD (n=3).

5.1.4. STAT6 siRNA Loaded Gelatin Nanocarrier Agarose Gel Electrophoretic Mobility Assay

The agarose gel electrophoretic mobility shift assay was performed to assess the stability of entrapped siRNA during formulation conditions and exposure to RNase A. **Figure 5.5** shows the electrophoretic mobility pattern of siRNA from the S6S-GNC compared to that of appropriate controls. The bands indicated that the naked STAT6 siRNA had been enzymatically degraded by the RNAses and therefore moved through the 1% ultra-low range agarose gel more rapidly compared to that of STAT6 siRNA entrapped within the gelatin nanocarrier matrix (Figure 5.5).
Figure 5.5. Agarose Gel Electrophoretic Mobility shift assay. The scrambled siRNA control, scrambled siRNA treated with RNAse control, S6S-GNC, S6S-GNC treated with RNAse, filtrate, filtrate treated with RNAse, and the placebo-GNC treated with RNAse were loaded onto a 1% w/v agarose gel and electrophoresed at a constant voltage of 70 V. This study was performed to examine the stability of the encapsulated siRNA due to preparation conditions and the stability in the presence of RNAse. The arrow head indicates the distance traveled by the cleaved siRNA fragments.

Intact super coiled RNA was recovered from the native scrambled siRNA-loaded lane. Treatment with RNAse only enzymatically degraded the STAT6 siRNA alone and this effect was not seen for S6S-GNC. This suggests that the prepared S6S-GNC protected the STAT6 siRNA from RNAse degradation. The filtrate collected from the bottom of the Vivaspin centrifuge filter was found to contain only a negligible amount STAT6 siRNA and this suggests the STAT6 siRNA entrapment within the gelatin matrix. This can be correlated with the observed STAT6 siRNA entrapment efficiency of ~ 85 %. In further experiments, S6S-GNC were subjected to 10% v/v Trypsin to intentionally degrade the gelatin matrix to release the STAT6 siRNA to confirm that the STAT6 siRNA was entrapped within the gelatin nanocarriers. Intact siRNA was recovered from the degraded gelatin nanocarriers. In agreement with our findings, Kriegel and Amiji showed protection of encapsulated siRNA from degradation using gelatin nanoparticles [1093]. Our results demonstrate the formulation of a stable and functional siRNA loaded gelatin nanocarriers (S6S-GNC) that can protect the siRNA from nucleases during systemic circulation.
5.1.5. In vitro Cytotoxicity of STAT6 siRNA Loaded Gelatin Nanocarriers

After physiochemical characterization of the gelatin nanocarriers formulation, the in vitro cytotoxicity of the STAT6 siRNA loaded formulation was evaluated against A549 lung cancer cells using the MTT cell viability assay [205, 1138, 1139]. Human adenocarcinoma A549 cells were selected for this investigation based on their reported expression of STAT6 protein [193]. We observed that the percent cell kill was increased significantly ($p<0.001$) with S6S-GNC (55±4.1 %) compared to native STAT6 siRNA (2±0.55%) and STAT6 siRNA with Lipofectamine (40±3.1 %) (Figure 5.6).

**Figure 5.6.** Cytotoxicity of the developed S6S-GNC and STAT6 siRNA with Lipofectamine on A549 lung cancer cells. The graph shows the percent cell viability observed after 24 and 48 hr following treatment. Cell viability was performed using $5\times10^3$ A549 (human adenocarcinoma cell line) cells in F12-K medium supplemented with 10% FBS and an antibiotic solution of penicillin (5000 U/ml), streptomycin (0.1 mg/ml) and neomycin (0.2 mg/ml) (PSN). Cell incubation was conducted within a humidified atmosphere of 5% CO$_2$ at a temperature of 37±0.5°C. The formulation and STAT6 siRNA Lipofectamine complexes were applied as freshly prepared solutions between 0 to 15 nM concentrations. The absorbance of the formazan crystals dissolved in DMSO was read at 540 nm on a Biospek Synergy H1 plate reader. Values are represented as Mean ± SD (where n=3).

Placebo GNC treatment showed >97% viability of cells demonstrating non-toxicity and safety of gelatin used in the gelatin nanocarrier formulation. In agreement with these findings, Shah et al. found that the HIF-1α siRNA loaded gelatin or PEG-modified gelatin nanoparticle treatment of the known HIF-1α overexpressing cell lines, SKOV3 and MDA-MB-231 cells significantly inhibited the expression of HIF-1α compared to that of naïve siRNA, therefore reversing the aggressive phenotype of these tumors [100, 128, 1140].
5.1.6. Cellular Internalization Assay of STAT6 siRNA and Nile Red Loaded Gelatin Nanocarriers

Effective cell internalization of nanoparticles plays important roles in eliciting therapeutic effects [1141]. Therefore, cellular uptake studies were performed employing Nile Red loaded gelatin nanocarriers to understand the uptake of nanocarriers within the cells [1142]. Nile red is capable of fluorescent staining of intracellular lipid droplets that excites and emits at wavelengths similar to red fluorescent protein at approximately 485 nm and 525 nm, respectively [1143]. Figure 5.7 shows the fluorescence images of the control Nile-Red solution and the Nile-Red loaded gelatin nanocarriers (Nile-GNC) taken after 15 min and 1 hr of treatment in A549 cells at 40X magnification. The Nile red loaded gelatin nanocarriers clearly outperformed the Nile red solution at 15 min and 1 hr time points as shown by the apparent increase in the cellular fluorescence associated with gelatin nanocarriers (Figure 5.10). The cell internalization studies of Nile red loaded gelatin nanocarriers formulations showed that the developed formulation gets rapidly internalized within cells compared to the free dye solution.

![Image of fluorescence images](image_url)

Figure 5.7. Fluorescence Images of control and Nile-red loaded-GNC (Nile-GNC) taken after 15 min and 1 h of treatment in A549 cells (at 40x magnification). For this assay, lung cancer A549 cells (5 × 10^4) were seeded on 24 well plates and incubated at 37±0.5°C under 5% CO₂ for 24 h. The cells were treated with the Nile Red solution (control) or Nile Red loaded formulations for 15 min and then treated for an addition 45 min for a total treatment time of 1 hr. After 15 min or 60 min, media was removed and the resulting cells were washed with PBS.
5.1.7. STAT6 Protein Downregulation by STAT6 siRNA Loaded Gelatin Nanocarriers

STAT6 protein, activated by phosphorylation by Janus kinases (JAKs) within cells, is involved in tumor progression and apoptosis resistance. Therefore downstream expression of the activated pSTAT6, in addition to other downstream proteins, IFNγ, TGF-β, Foxp3, IgE, and GATA3 plays important roles in tumor progression due to the resultant favoring of Th2 differentiation, cell cycle promotion, anti-apoptotic and pro-metastatic properties [1144]. The phosphorylation of STAT6 to pSTAT6 leads to increased expression of GATA3 and hampered expression of IFNγ, TGF-β, and Foxp3 [40]. Thus, inhibition of STAT6 using S6S-GNC will inhibit the effects of IL4, and promote upregulation of IFNγ and TGF-β/Foxp3 [1145]. In addition, an inverse relationship between STAT6 protein expression and cholesterol biosynthesis has been identified along with the FOXJ2 binding sites in the upstream region of the HMGCR, HMGCS1, and IdI1 genes within the cholesterol synthesis pathway [193]. STAT6 silencing leads to ER stress induced apoptosis in lung cancer cells through a C/EBP homologous protein (CHOP) induction, alteration of NH3 only proteins expression and ROS production [1146]. STAT6 is constitutively upregulated in cancer cells, which transcriptionally upregulates COX-2 expression [191]. COX-2 overexpression is associated with apoptosis resistance and malignancy in NSCLC [191]. Therefore, STAT6 protein downregulation or knockdown leads to apoptosis of cancer cells via a COX-2 dependent ER stress induced mechanism [1146, 1147]. A Western blot experiment was performed to determine the effect on STAT6 protein downregulation in response to S6S-GNC treatment compared to the S6S-Lipofectamine complex and the negative control. Figure 5.8 shows the effect of S6S-GNC on the expression of STAT6 protein in A549 cells.
Figure 5.8. Effect of nanocarriers on the STAT6 protein expression by Western Blot. (A) Image of Western Blot and (B) Densitometry of the bands normalized to beta actin. The effect of STAT6 siRNA-GNC on the expression of STAT6 in A549-treated lung cancer cells was shown. A549 cells were pre-incubated with S6S-GNC, STAT6 siRNA-Lipofectamine Complex, and without any treatment (control). The cells were lysed and STAT6 protein expression was analyzed by Western blot of whole cell lysates. The β-Actin expression was analyzed as a loading control. Bars show mean ± standard deviation (n=3). ***p<0.001.

The developed S6S-GNC formulation was able to successfully downregulate STAT6 protein expression in A549 cells thereby supporting the effectiveness of the developed formulation. In support of these results, Kriegel et al. demonstrated the downregulation of TNF-α using a combination of TNF-α and CyD1 siRNA loaded type B Gelatin nanoparticles [1148]. The strategy outlined in this study successfully developed a safer and efficacious STAT siRNA loaded gelatin nanocarrier formulation.

Stable and effective non-PEGylated STAT6 siRNA gelatin nanocarriers (S6S-GNC) formulation with a particle size of < 80 nm and encapsulation efficiency of > 85% were successfully developed. In addition, the formulation was found to be stable in presence of buffers
solutions, serum solution and RNase. The S6S-GNC formulation showed sustained release of S6S, which is highly desirable considering long term effect of formulation with allowing for reduced dosing intervals. S6S-GNC evaluated in A549 lung cancer cell line inferred significantly (p<0.001) higher percent cell kill with S6S-GNC compared to that of medium control and STAT6 Lipofectamine complex. The cell internalization studies showed that the developed gelatin nanocarrier formulation gets rapidly internalized within cells and these results support the successful delivery of siRNA within tumor cells. The Western blot results confirmed the successful downregulation of STAT6 protein expression by developed S6S-GNC formulation in A549 cells. The developed S6S-GNCs were found to be effective in protecting STAT6 siRNA from degradation and were able to deliver STAT6 siRNA within tumor cells to exert anticancer activity. This provides evidence that a peptide targeted and PEGylated S6S-GNC-P is a feasible strategy for intravenous delivery of STAT6 siRNA to cells associated with asthma progression, such as T lymphocytes.

5.2. Gemcitabine Loaded Gelatin Nanocarriers (Gem-GNC) for Pulmonary Delivery

Cancer is the second leading cause of death in the United States, with lung cancer contributing to the highest number of estimated cancer deaths [226]. Current treatments for lung cancer include surgery, chemotherapy, and radiation therapy [226]. However, surgery does not completely remove the cancer in most patients, while radiotherapy and chemotherapy causes severe adverse effects and low response rates [1149, 1150]. A nanocarrier mediated delivery of a chemotherapeutic agent to the lung cancer tumor mass may increase the response rates and decrease the associated systemic adverse effects with conventional chemotherapeutic options. The purpose was to formulate a Gem-GNC designed to be delivered via the inhalation route of administration and to evaluate the physical characteristics, stability, in vitro efficacy using A549 and H460 non-small cell lung cancer cells, and aerodynamic particle size distribution.

5.2.1. Preparation and Purification of Gem loaded Gelatin Nanocarriers (Gem-GNC)

Nanocarriers used for the delivery of Gem to cancerous tissues would allow for control of the release of the drug within local environment of the lung and avoid systemic exposure of drug. The limitations of conventional Gem(pKa= 11.52; protonated base at pH 7.4, positively charged when ionized) delivery are due to its low molecular weight, high hydrophilicity, short half-life (30-90 min), and fast decomposition to inactive products post administration [235]. Therefore, the use of inhalable Gem-GNC could potentially reduce local cancerous lung tissue and minimize side-
effects associated with intravenous delivery. Other methods for overcoming the limitations and increasing the efficacy of Gem include the bioconjugation of Gem with PEG and folic acid moieties, thus forming a polymeric carrier for the targeted therapy of folate-receptor expressing cancer [1151, 1152]. Another example was the synthesis of saturated and monounsaturated C18 and C20 long chain 4-(N)-acyl derivatives and 5I-esters of Gem (Eli Lilly), which increased the cytotoxicity of Gem in vitro [1153].

Gelatin-based nanocarriers are an attractive drug delivery system since the polymer matrix that is formed during the nanocarrier formation allows for the incorporation of therapeutic cargo. There are two different types of gelatin, type A and type B. Type A is positively charged at physiological pH due to its isoelectric point in the range of 7 to 9 and Type B is negatively charged at physiological pH since its isoelectric point is in the range of 4.7 to 5.4 [1108, 1154]. When preparing nanoparticles with type A gelatin, the zeta potential of the nanoparticles will be positive [1155]. In contrast, preparing nanoparticles with type B gelatin will create nanoparticles with a negative zeta potential [1060]. Type B gelatin has been selected for the Gem-GNC formulation. A gelatin hydrogel containing cisplatin developed by Konishi et al. illustrated sustained release properties and resulted in an increased anticancer effect [1156].

For the preparation of gelatin nanocarriers, a 2-step desolvation method was used, where the first step was the fractionation of gelatin to obtain the high molecular weight fraction with the use of bulk addition of anti-solvent to a final concentration of 50% v/v and the second step was the formation of the GNC by dropwise addition of non-solvent. A schematic showing the formation process is outlined in Figure 4.4. To better understand the interrelationships between the dependent parameters and their optimization using a full factorial design is both time and labor intensive. Therefore, a Taguchi method with an L9 orthogonal array design was selected to optimize the experimental conditions.

Preliminary trials were conducted to select factors and their working ranges that have major influence on the efficiency of the Gem-GNC formulation. The experimental range of gelatin concentration (% w/v), volume ratio of 90% v/v aqueous ethanol solution, and genipin concentration (% w/w) were found to be 0.5-1.5 % w/v, 7:10-9:10 v/v, and 0.2-1.0 % w/w, respectively. In order to minimize the number of trials necessary and to effectively study the effect of the factors in all possible combinations, a Taguchi orthogonal array design was utilized.
The Taguchi method was developed by Genuchi Taguchi to improve product quality [1157]. The Taguchi method is a powerful tool in the design of a high quality system. It employs an orthogonal array design to determine the effects of the entire constitutive parameters through a smaller number of experiments. The use of the Taguchi method can allow for a reduction in the time required for experimental procedures, while being an effective means of investigating the effects of multiple factors and individual factors on a process performance [1158, 1159]. With this method, it is possible to reduce the number of experiments required to study the influence of multiple and individual factors influence compared to the full factorial designs. The Taguchi orthogonal array design has been used previously to design hydrogel nanoparticle preparations composed of chitosan, gelatin, and PLGA [1160-1162]. The Taguchi method involves a design of experiments, followed by a technique for high quality systems design.

The selected parameters and their levels and the batch experiments and the resultant particle sizes are shown in Table 5.1. These effects of the levels of each factor on the product quality, in this case, particle size, are defined and evaluated according to the total mean values of experimental trial results or S/N ratios. Distribution of the means of S/N ratio for particle size are shown in Figure 4.5. The main effects plot shows an increasing and then decreasing particle size (Figure 4.5 A) and the opposite for the S/N ratio with increasing volume ratio of 90% v/v aqueous ethanol solution (Figure 4.5 B). This is in accordance with the formation of small particles observed with the use of high organic solvent to aqueous solution ratios (final concentrations of >40% v/v), imparted by the prevention of coalescence between particles due to the availability of large amounts of solvent for diffusion [1163]. The main effects plot for the particle size results for the genipin concentration (% w/w) indicate that increasing the crosslinker concentration increases particle size (Figure 4.5 A). At lower crosslinker concentration of 0.2 % w/w genipin to total gelatin, a nanoparticle was formed that had ideal particle size of approximately 100 nm-200 nm. For factor A, gelatin concentration (% w/v), the main effects plot (Figure 4.5 A-B) show increased particle size and decreased S/N ratio with increasing polymer concentration. At high polymeric concentrations, the viscosity of the polymeric solution increases to the point where resistance against nanoparticles in the external aqueous phase which reduces the Gem-GNC dispersibility. This results in larger particles that often have higher EE% due to low diffusion of the drug from the polymeric solution to the external phase. The effects of the three factors on the particle size of the Gem-GNC were summarized in Equation 5.1.
The Taguchi method L0 type orthogonal array design was used to optimize the formulation parameters. The independent factors were the gelatin concentration (0.5, 1.0, and 1.5 % \( w/v \)), volume ratio of 90% \( v/v \) aqueous ethanol to gelatin solution batch volume (7:10, 8:10, and 9:10 \( v:v \)), and genipin to gelatin weight percent (0.2, 0.6, and 1.0 % \( w/w \)). Particle size (nm) was the key dependent factor measured. The signal to noise (S/N) ratio was calculated for the particle size response to establish significance of each factor and their optimum levels for the optimized formulation. S/N ratios of particle size were shown in Table 4.4.

The responses were interpreted by considering nominal S/N ratios for better accuracy of the measured data. As shown in Table 1, the formulation parameters A: Gelatin (% \( w/v \)), B: Volume ratio \( v:v \) of 90% \( v/v \) aqueous ethanol, and C: Genipin (% \( w/w \)) were discriminated by considering different levels and possible effects according to the selected orthogonal array. The “main effects plot” was drawn for each factor at different levels by taking levels as x-coordinates and S/N ratios as y-coordinates (Figure 4.5 A-B). The plots show the effects of the individual factors on multiple responses in terms of their S/N ratios. Genipin concentration was inferred to be the most influential parameter on particle size since it has the largest S/N ratio range.

The predictive equations generated via linear regression for particle size are given in Equation 5.1.

\[
\text{Particle size (nm)} = 169 - 1.82(\text{volume ratio of 90\% ethanol}) + 283(\text{Genipin \%w / w}) + 103(\text{Gelatin \%w / v})
\]

Equation 5.1

Where \( R^2 \) coefficient of determination values for the particle size were calculated as 85.9%.

The target particle size value was 150 nm, so the volume ratio of 90% \( v/v \) ethanol was selected by fixing the gelatin and genipin concentrations to 1% \( w/v \) and 0.02% \( w/w \) respectively. This led to the theoretical optimized parameter levels of 1.0% \( w/v \) gelatin to total batch volume, 7:10 \( v:v \) of 90% \( v/v \) aqueous ethanol to gelatin solution volume, and 0.2% \( w/w \) genipin to gelatin weight \%. These levels were selected for further evaluation based of the Taguchi orthogonal array design and analysis.

5.2.2. Preparation and Evaluation of Lyophilized Gem-GNC

As shown in Table 5.1, the Gem-GNC average particle size was 166 ± 6.7 nm before lyophilization and 178 ± 7.1 nm after lyophilization. The Gem-GNC formulation had non-significantly different zeta potentials (p>0.05) before and after lyophilization and corresponded to
-9.15 ± 0.45 mV and -9.07 ± 0.49 mV, respectively. In addition, the EE% and LE% of the Gem-GNC formulation remained stable throughout the lyophilization process, as inferred by the insignificant (p>0.05) change from 93.7% to 92.5% and 9.2% to 9.1%, respectively. The placebo GNC had an average particle size of 152 ± 7.8 nm before lyophilization and of 167 ± 7.9 nm after lyophilization. The difference of particle size, zeta potential, EE% and LE% before and after lyophilization were statistically non-significant (p>0.05) and the final reconstituted particle size was within our target value of particle size <200 nm. The process validation batches (n=3) had no significant differences between batches (p>0.05). Therefore, the formulation process was considered successfully robust at preparing stable Gem-GNC.

Table 5.1. Gem-GNC particle size, zeta potential, Entrapment Efficiency (EE%) and Loading Efficiency (LE%). The Gem-GNC and Placebo GNC batches were subjected to dynamic light scattering and electrophoretic mobility analysis to determine their particle size and zeta potential before lyophilization using the prepared nanoparticle suspension and after lyophilization using 10 mg of freeze dried nanocarriers re-dispersed in 10 ml of distilled deionized water and equilibrated for 1 hr.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Characterization parameters</th>
<th>Before Lyophilization</th>
<th>After Lyophilization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gem-GNC</td>
<td>Particle Size (nm)</td>
<td>166 ± 6.7</td>
<td>178 ± 7.1</td>
</tr>
<tr>
<td></td>
<td>Zeta Potential (mV)</td>
<td>-9.15 ± 0.45</td>
<td>-9.07 ± 0.49</td>
</tr>
<tr>
<td></td>
<td>EE %</td>
<td>93.7 ± 2.3</td>
<td>92.5 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>LE%</td>
<td>9.2 ± 0.4</td>
<td>9.1 ± 0.3</td>
</tr>
<tr>
<td>Placebo GNC</td>
<td>Particle Size (nm)</td>
<td>152 ± 7.8</td>
<td>167 ± 7.9</td>
</tr>
<tr>
<td></td>
<td>Zeta Potential (mV)</td>
<td>-10 ± 0.51</td>
<td>-9.8 ± 0.56</td>
</tr>
</tbody>
</table>
Nanoparticle drug delivery systems, like many other pharmaceutical products, are stored preferably in the dried state. In general, nanoparticles are prepared within a liquid medium. Transformation of the liquid dispersion into a dried power may be achieved by freeze drying (lyophilization) or spray-drying [1164, 1165]. These methods can be performed at laboratory, pilot, or industrial scales. For freeze-drying, cryoprotectants are often added to the nanoparticle dispersion to prevent aggregation of nanoparticles during the reconstitution of the dispersion from the dried powder before administration. Optimization of the freeze drying cycle improves the quality of the freeze dried product [1164]. Unlike freeze drying, which is based on per batch or per unit dose, spray drying is a continuous process. Another advantage of spray drying is the reduced cost due to less energy requirements compared to the process of freeze drying. Spray drying requires the addition of drying auxiliary compounds in the nanoparticle dispersion before being processed through the spray dryer [1166].

Three batches were prepared using the optimized parameters to evaluate the model and to determine inter-batch variability. The three batches showed non-significant (p<0.05) differences in particle size and zeta potential before and after lyophilization (Table 5.1). There was a slight increase in particle size when comparing the Gem-GNC to the placebo GNC, possibly due to the entrapment of the Gem within the gelatin matrix.

Gelatin-based nanoparticles have demonstrated safety and efficacy in vitro and in vivo by studies performed by Tseng et al., who developed gelatin nanoparticles containing cisplatin coated with EGF tumor-specific ligand that was administered by simple aerosol delivery [259]. Although this formulation was successful at encapsulating cisplatin and delivering it to the cancer tissue while not eliciting inflammation, the particles had a size of 200-300 nm thus making them susceptible to alveolar macrophage uptake [241, 259]. However, it was demonstrated that the cisplatin accumulation in the cancerous tissue of the lung following inhalation was more pronounced than compared to the aerosolized cisplatin solution in a mouse model [258, 1167]. Another gelatin nanoparticle was developed for the targeted treatment of pancreatic cancer that involved a redox-responsive nature for the delivery of wt-p53 plasmid DNA and Gem [1168]. A PLGA nanoparticle loaded with celecoxib was recently optimized by Taguchi design of experiments and evaluated for in vitro cytotoxicity [1162]. Gelatin nanocarriers have previously demonstrated anti-cancer effects, sustained drug release kinetics, low toxicity, ease of scale-up, and low cost, making them ideal candidates for an aerosolized nanocarrier delivery system.
5.2.3. Gem-GNC Stability

The stability of the Gem-GNC was assessed in the presence varying pH DPBS solutions (pH 5.4, 6.4, and 7.4). Figure 5.9 A shows the particle size of the Gem-GNC formulations when in DPBS solutions of varying pH over the time points of 0, 1, 2, and 3 days. Particle size at the initial time point and at day 1 differ significantly (p<0.05) from days 2 and 3 due to apparent gelatin nanocarrier matrix erosion occurring between days 1 and 2. The PDI of the Gem-GNCs within the buffers at pH 5.4, 6.4, and 7.4 were not statistically significantly different (p>0.05) (Figure 5.9 B). These non-significant (p>0.05) differences in particle size and PDI of the Gem-GNC within varying pHs infer stability over varying pHs within a biologically relevant medium and a possible release mechanism of the Gem-GNC.

Figure 5.9. Stability of Gem-GNC at pH 6.4, 7.4 and 8.4 in DPBS as assessed by the (A) Particle size and (B) polydispersity index after reconstitution of the three independent Gem-GNC batches. Time after reconstitution of 0 days refers to 1 hr after rehydration with normal saline solution. Results are shown as mean ± SD (n=3). Statistical significance was determined at p<0.05.

The stability of the Gem-GNC batches were evaluated in water and DPBS at varying pH buffer solutions. The Gem-GNC shows good stability in water as inferred by the non-significant (p<0.05) change in particle size up to 72 hr. The pathogenesis of lung cancer is partly due to accelerated oxidative stress associated with reduced airway pH [1169, 1170]. The pH in healthy lungs is in the range of 7.38-7.42, equal to the blood traveling through the body. However, in cancerous lungs the pH drops to about 6.7 [1171]. Therefore, it was pertinent to examine the stability of the Gem-GNC under acidic pH 5.4 and 6.4 conditions, as well as at the normal physiological pH 7.4. The formulation also showed no significant change (p<0.05) in particle size...
up to 3 days at pH 5.4, 6.4, and 7.4. A study by Menon et al. screened and compared the degradation and release from gelatin nanoparticles and other polymeric nanoparticles [597]. This study found that glutaraldehyde crosslinked gelatin nanoparticles with particle size of 191 nm were stable in distilled deionized water, 10% v/v FBS and in Gamble’s simulated lung fluid for over 5 days, as inferred by no significant aggregation or change in particle size [597].

5.2.4. In vitro release from Gem-GNC

The Gem release profile within DPBS pH 7.4 and Gamble’s simulated lung fluid (pH 7.4) of the Gem-GNC and Gem solution are shown in Figure 5.10. The Gem solution in both release mediums shows a rapid release that achieved 100% release in <6 hr. The Gem-GNC formulation displayed a sustained release of entrapped Gem, suggesting that the formulation had efficient retention and entrapment. The Gem-GNC showed ~20% release at 5 hr compared to the total release of the Gem solution at the same time. The release of the Gem-GNC at 24 to 72 hr showed a controlled release and attained 65% release by 72 hr. The release of Gem from Gem-GNC within the Gamble’s simulated lung fluid showed initially a similar rate of release that progress to an increased rate and overall release at 72 hr compared to Gem-GNC release within DPBS. At 72 hr the Gem-GNC within Gamble’s SLF achieved 81% release, as shown in Figure 5.10.

![Figure 5.10. In vitro release of Gem solution and Gem-GNC within DPBS (pH 7.4) and SLF (Gamble’s Solution; pH 7.4). Lyophilized formulations were re-suspended in distilled deionized water and placed into a dialysis membrane bag with molecular weight cut-off of 12-14 kDa. The membrane bags were then placed into 100 mL of DPBS or Gamble’s SLF medium maintained at a temperature of 37°C with continuous stirring at 300 rpm. At specified time](image-url)
intervals, 0.5 mL dissolution medium was sampled and analyzed for Gem content using HPLC with a UV diode array detector. The amount of Gem-GNC filled into the membrane bag was 10 mg/mL at 0.48% w/w Gem content. The Gem solution filled into the membrane bag was 0.1 mg/ml. Total volume of the Gem-GNC suspension and the Gem solution was 0.5 ml. Results are represented as mean ± SD (n=3).

Drug release kinetics were assumed to be governed by diffusion within a matrix system. Therefore, the release kinetics of the Gem from the Gem-GNC were confirmed by fitting the release data into five kinetic models: zero order, first order, Weibull, Higuchi, Baker-Lonsdale, and Korsmeyer-Peppas models. The Gem-GNC release in DPBS and SLF media were fitted to these models and their respective R² values are presented in Table 5.2. For release within the DPBS (pH 7.4) medium, the Baker Lonsdale, Weibull, and Korsmeyer-Peppas models were observed to have the best fit of the release profile with R² values of 0.979, 0.994, and 0.992, respectively. This indicated a matrix diffusion based release. Release of Gem from the Gem-GNC within the Gamble’s SLF had best fit with the Baker-Lonsdale, Weibull, and Korsmeyer-Peppas models that had corresponding R2 values of 0.944, 0.995, and 0.993, respectively. The Korsmeyer-Peppas power law was used to determine the drug release mechanism. For a spherical system, an exponent value of 0-0.42 indicates Fickian diffusion, while a value of 0.43 to <0.85 indicates anomalous transport or non-Fickian diffusion release, and an exponent value of >0.85 indicates Case II transport [1172]. In this study, the n values of 1.02 and 1.03 within the DPBS and Gamble’s SLF suggests a Case II mode of release due to diffusion and erosion mechanisms. Case II transport occurs when the sorption is entirely controlled by stress induced relaxations occurring at a boundary separation on an outer swollen shell essentially at equilibrium penetrant concentration, from an unpenetrated glassy core. Ideally, this sharp boundary moves through the polymer at a constant velocity during Case II transport [1173]. This study confirmed that the mechanism of release is based on Case II transport and diffusion mechanisms from a matrix-based gelatin nanocarrier.

Model independent descriptions of the release curves were calculated as dissolution efficiency (DE) and mean dissolution time (MDT). DE was calculated using Equation 5.2:

\[
DE = \frac{\int_0^t Q \, dt}{Q_{max} \times t} \times 100
\]

Equation 5.2.

Where \( Q \) is the amount of drug released, \( t \) is time, and \( Q_{max} \) is the maximum amount of drug released (≈100%). The MDT was calculated using Equation 5.3.
\[ MDT = \frac{\sum_{j=1}^{n} t_j^{AV} \times Q_j}{\sum_{j=1}^{n} \Delta Q_j} \]  

Equation 5.3.

Where \( \Delta Q = Q_t - Q_{(t-1)} \), \( t_j^{AV} = (t_1+t_{i-1})/2 \), and \( n \) is the number of timepoints.

**Table 5.2.** *In vitro* release models showing correlation coefficient (\( R^2 \)) values for Gem release from the Gem-GNC within DPBS and SLF (pH 7.4). Variables are defined in the text.

<table>
<thead>
<tr>
<th>Model Name</th>
<th>Model</th>
<th>DPBS (pH 7.4) Release Medium ( R^2 )</th>
<th>SLF (pH 7.4) Release Medium ( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero Order</td>
<td>( C_t = C_0 + K_0 t )</td>
<td>0.939</td>
<td>0.971</td>
</tr>
<tr>
<td>First Order</td>
<td>( \log C = \log C_0 - \frac{Kt}{2.303} )</td>
<td>0.112</td>
<td>0.125</td>
</tr>
<tr>
<td>Higuchi</td>
<td>( f_t = Q = A \sqrt{(2C_0 - 2C)t} )</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Baker-Lonsdale</td>
<td>( f_1 = \frac{3}{2} [1 - \left(1 - \frac{M_t}{M_\infty}\right)^\frac{2}{3} M_t = k_t )</td>
<td>0.979</td>
<td>0.944</td>
</tr>
<tr>
<td>Weibull</td>
<td>( M = M_0 \left[1 - e^{-\left(C-t\right)^b} \right] )</td>
<td>0.994</td>
<td>0.995</td>
</tr>
<tr>
<td>Korsmeyer-Peppas</td>
<td>( \frac{M_t}{M_\infty} = k t^n )</td>
<td>0.992</td>
<td>0.994</td>
</tr>
</tbody>
</table>

In zero-order release kinetics, the dissolution of a drug molecule is only dependent on time. This model is true only in the case of very slow drug release. The zero-order release is modeled as:

\[ M_0^{GNC} - M_t^{GNC} = k_0 t \]  

Equation 5.4.
Where $M_0^{GNC}$ is the initial concentration of the drug present in the gelatin nanocarriers, $M_t^{GNC}$ is the concentration of drug in the gelatin nanocarriers at time $t$, and $k_0$ is the zero-order release constant with units of concentration per unit time.

The first-order model is commonly used to describe the absorption or elimination of certain drugs in the body. It is derived from the first-order release kinetics, in which the change of concentration with respect to time is dependent on concentration:

$$\frac{dM^{GNC}}{dt} = -kM^{GNC}$$

Equation 5.5.

Where $M^{GNC}$ is the concentration of drug in the drug molecule and $k$ is the first-order release constant. Integrating this equation gives:

$$\ln\left(\frac{M_0^{GNC}}{M_t^{GNC}}\right) = kt$$

Equation 5.6.

Where $M_0^{GNC}$ is the initial concentration of drug in the gelatin nanocarriers and $M_t^{GNC}$ is the concentration of siRNA in the gelatin nanocarriers at time $t$.

The Higuchi model or mathematical fitting of drug dissolution from matrix systems was originally derived for planar systems. This model relates the drug concentration to the square root of time. Many assumptions follow with the use of this model, such as the drug concentration in the matrix is initially higher than the solubility of the drug, the edge effects are negligible so diffusion is unidirectional, the thickness of the dosage form is much larger than the size of the drug molecules, the swelling and dissolution of the matrix is negligible, the diffusivity of the drug is constant, and that perfect sink conditions are attained in the release environment. The S6S-GNC and the S6S-GNC-P do not exhibit any correlation to this model because of their spherical geometry and the role of matrix swelling in the siRNA release (Table 5.6.). The Higuchi model best describes modified release dosage forms, such as transdermal systems and matrix tablets with water soluble drugs [1174].

The Baker-Lonsdale model was developed from the Higuchi model to describe the release from spherical matrices according to the equation:

$$f_1 = \frac{3}{2} \left[1 - \left(1 - \frac{M_t}{M_\infty}\right)^\frac{2}{3} \frac{M_L}{M_\infty}\right] = kt$$

Equation 5.7.

Where the release rate constant, $k$, corresponds to the slope. To study the release kinetics, *in vitro* release data obtained were plotted as $[d(M_t/M_\infty)]/dt$ with respect to the inverse root of time.
The Weibull model is useful for comparing the release profiles of matrix type drug release [1175]. The model’s equation is as follows:

\[ M = M_0 \left[ 1 - e^{-\frac{(t-T)^b}{a}} \right] \]  

Equation 5.8.

Where \( M \) is the amount of siRNA in the release medium as a function of time \( t \), \( M_0 \) is the total amount of siRNA being released, \( T \) accounts for the lag time measured as a result of the dissolution process, \( a \) is the scale parameter that describes the time dependence, and \( b \) is the shape of the dissolution curve progression. For \( b=1 \), the shape of the curve corresponds exactly to the shape of an exponential profile with the constant \( k=1/a \). If \( b>1 \), then the shape of the curve is sigmoidal with a turning point, whereas the shape of the curve with \( b<1 \) will show a steep increase than with one with \( b=1 \). The time when 50% w/w and 90% w/w of the total released siRNA in the formulation is released, can be calculated using the inverse function of the Weibull equation:

\[ t_{(50\% \text{ resp.}, 90\% \text{ dissolved})} = (-aln\frac{M_0-M}{M_0})^{1/b} + T \]  

Equation 5.9.

The Korsemeyer-Peppas model was developed to specifically model the release of a drug from a polymeric matrix hydrogel. The equation is as follows:

\[ \frac{M_t^{sol}}{M_{\infty}^{sol}} = k_{KP}t^n \]  

Equation 5.10.

Where \( M_t^{sol} \) is the concentration of the drug in the release solution at time \( t \), \( M_{\infty}^{sol} \) is the equilibrium concentration of drug in the release solution, \( k_{KP} \) is the drug release rate constant, and \( n \) is the diffusional exponent.

The Korsemeyer-Peppas power law, based on the value of \( n \), characterizes the release mechanism of the drug. The exponent \( n \) should only be calculated based on the portion of the release curve where \( \frac{M_t^{sol}}{M_{\infty}^{sol}} < 0.6 \) as plotted as log cumulative percentage siRNA release versus log time. For a spherical release system, when \( n<0.43 \) it indicates Fickian diffusion, when \( n>0.42 \) and \( n<0.85 \) it indicated anomalous transport or non-Fickian diffusion, and for \( n<0.85 \) it indicates Case II transport [1172].

Since the Gem-GNC is to be delivered via the inhalation route of administration, it was necessary to evaluate the \textit{in vitro} release in the presence of a simulated lung fluid. The alveolar region of the lungs does not contain mucus, but the viscoelastic layer in the tracheobronchial region does. There are many simulated lung fluids that are formulated for specific purposes, such as, artificial lysosomal fluid (pH 4.5) and Gamble’s solution (pH 7.4) that are used to simulate fluid
that particles would encounter after phagocytosis by alveolar and interstitial macrophages and deep interstitial fluid of the lung, respectively [1074]. Other simulated lung fluids are variations of Gamble’s solution, in that they mimic the extracellular lung fluids or the interstitial fluid.

The *in vitro* release of Gem from the Gem-GNC was investigated under physiological pH and within Gamble’s SLF (Figure 5.10). The *in vitro* release of the neat Gem solution showed complete release within 5 hr. In contrast, the Gem-GNC crosslinked with genipin showed Gem release of approximately 30% at 24 hr in both dissolution media and displayed a biphasic release profile consisting of an initial burst release followed by a prolonged release. Of the kinetic models that were fitted to the Gem-GNC release data within the Gamble’s SLF, the data best fit Weibull and Korsmeyer-Peppas with R² values of 0.990 and 0.983, respectively. The Weibull model is useful for comparing drug release profiles of matrix type drug delivery, while the Korsmeyer-Peppas model has been used previously to describe release from several modified release dosage forms. The Korsmeyer-Peppas power law was utilized in order to elucidate the drug release mechanism. A release exponent value of <0.43 represents Fickian diffusion, 0.43 to <1 represents non-Fickian diffusion, and >1 represents case II transport for a spherical particle. The exponent values of 0.58 and 0.59 for the release within the respective DPBS and Gamble’s SLF suggests non-Fickian diffusion. Model independent values for Gem release from Gem-GNC in DPBS were calculated using 11 timepoint values and were DE=41% and MDT=27 hr and in SLF the values were DE=48% and MDT=29 hr. Thus, the main mechanisms that drive the release of Gem from the Gem-GNC are believed to be diffusion and erosion from a matrix-based nanocarrier. Gem release from Gem-GNC within SLF was increased by 7% compared to release in DPBS [1075]. The Gem-GNC formulations exhibited the desired controlled release that will allow for the drug to be delivered to the tumor tissue in a constant manner.

### 5.2.5. Gem-GNC Imaging with Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)

The TEM uses a high voltage electron beam produced by an electron gun with a tungsten filament cathode as the electron source to create an image. The electron beam is accelerated by an anode at between 40-400 keV with respect to the cathode, focused by electrostatic and electromagnetic lenses, and transmitted through the specimen that is in part transparent to electrons and scatters them out of the beam. A transmission electron microscope (TEM) is capable of better than 50 pm resolution and magnifications up to 10,000,000x. TEM provides details on the internal
composition of a sample such as morphology, stress, magnetic domains, or crystallographic information. The wavelength of an electron is up to 100,000 times shorter than visible light photons, therefore electron microscopes have a much higher resolving power than a light microscope, thus revealing smaller structures. SEM images specimens by focusing an electron beam scattered across a rectangular area of the specimen, also known as raster scanning. When the electron beam interacts with the specimen, it loses energy. The energy lost is converted to signals which hold information on the specimen’s topography and composition properties in the form of heat, emission of low-energy secondary electrons, and high-energy backscattered electrons, cathodoluminescence (light emission), or x-ray emission. SEM identifies the sample surface and its composition, showing the 3D morphology of the sample.

The SEM and TEM micrographs of the Gem-GNC are shown in Figure 5.11 A and B, respectively. The SEM images show the Gem-GNC’s morphology, indicating that the particles have a smooth surface. The number mean particle size observed within the SEM micrograph was calculated as 229 ± 68 nm (n=7). The number mean particle size calculated using the TEM micrograph was 197 ± 18 nm (n=6). The particles appeared spherical within both the SEM and TEM images and the particle sizes corresponded to the average particle sizes and PDIs found with dynamic light scattering.

![SEM and TEM Micrographs of the Gem-GNCs](image)

**Figure 5.11. SEM and TEM Micrographs of the Gem-GNCs.** (A) SEM micrograph with a scale bar of 3.0 µm and (B) TEM micrograph with a scale bar of 500 nm. The Gem-GNC particle size measured from the SEM micrograph is 229 ± 68 nm, while the particle size measured from this TEM micrograph is 197 ± 18 nm. SEM: HV= 5.0 kV. TEM: 0.002427 um/pixel, HV=100.0 kV, Direct Magnification 6000X.
The morphology and particle size of the Gem-GNC was examined using scanning electron microscopy (SEM) and transmission electron microscopy (TEM), as shown in Figure 5.11. SEM images obtained are in agreement with other SEM images of gelatin nanoparticles [1176]. Negative staining was used for contrasting the Gem-GNC from the optically opaque fluid, where the background was stained and the Gem-GNC was unstained. In TEM, opaqueness to electrons is directly related to atomic number, or the number of protons. Negative stains are chosen due to their ability to scatter electrons strongly and to adsorb biological matter. The method for negatively staining a sample for TEM is a mild preparation method. The interpretation of the morphology of the particle gives insight on the shape, structure, and size of the particles within the Gem-GNC formulation. The analysis of the TEM images showed formation of homogenous, smooth, and spherical nanoparticles with no aggregation (Figure 5.11). These results are in agreement with other polymeric nanoparticle TEM images obtained from a gelatin nanoparticle formulation containing polymerized siRNA that found that the formulated nanoparticles had a particle size of approximately 150 nm and that the natural gelatin was composed of 12.3 nm amorphous granules [1177].

5.2.6. Differential Scanning Calorimetry (DSC)

DSC was performed on lyophilized samples of physical mixture of excipients, Gem-GNC, and placebo GNC to evaluate the thermal behavior of the sample in order to assess the physical state of Gem within the nanoparticle matrix. The endothermic events shown in Figure 5.12. are summarized in Table 5.3.
Figure 5.12. DSC thermograms of the Gem-GNCs, physical mixture of actives and excipients, Placebo GNCs and unentrapped Gem, Placebo GNCs, lactose monohydrate, gelatin, genipin, and the active ingredient Gem. The samples were placed into the pinholed 40 μL aluminum crucibles, sealed, and the change of heat flux was analyzed with increasing temperature from 25-300 °C compared to an empty control crucible.

The thermogram of Gem shows an endothermic peak at 290°C that correlates with literature values of 292°C for Gem melting point and degradation (Figure 5.12) [1178]. The thermogram for the Gem-GNC shows no corresponding endotherm for Gem. The physical mixture had three endothermic events with onsets and peaks at 143.8°C and 150.2°C, 205.4°C and 211.2°C, and 231.4°C and 234.2°C (Figure 5.12), respectively. The physical mixture also exhibited one exothermic degradation event with an onset temperature of 293.4°C preceded by an endothermic inflection consistent with melting of Gem. The endothermic peaks at 151.2°C and 223.6°C are attributed to the lactose monohydrate and genipin in the physical mixture. The lactose monohydrate thermogram had two endothermic events with onset temperatures of 143.5°C and 224.9°C and peak temperatures of 143.6°C and 226.0°C consistent with literature values for α-lactose monohydrate [1179]. The placebo GNC and Gem mixture had one endothermic event at an onset temperature of 223.9°C and peak of 224.1°C that can be associated with lactose monohydrate and a melting and degradation event at an onset temperature of 292.1°C and peak of 300°C that was associated with the Gem. The placebo GNC alone displayed one endothermic event.
with an onset temperature of 219.7°C and peak of 220.4°C that may be attributed to the lactose monohydrate cryoprotectant. Genipin had two endothermic events with onset temperatures of 122.5°C and 207.8°C and peak temperatures of 122.7°C and 208.6°C. The endotherm at 122.5°C corresponds to genipin crystal form I as described previously [1180]. Gelatin exhibited broad endothermic peak onset at 50-75°C that occurred due to the release of water [1181]. The Gem-GNC thermogram also did not show any endothermic or exothermic properties in this temperature range, indicating that the Gem and formulation excipients were molecularly dispersed.

### Table 5.3. DSC determination of endothermic events of the Gem-GNCs, physical mixture of actives and excipients, Placebo GNCs and unentrapped Gem, Placebo GNCs, lactose monohydrate, gelatin, genipin, and the active ingredient Gem.

<table>
<thead>
<tr>
<th></th>
<th>Tg (°C)</th>
<th>Endothermic onset (°C)</th>
<th>Endothermic Peak (°C)</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gem-GNC</td>
<td>75</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Physical Mixture</td>
<td>-</td>
<td>143.8, 205.4, 231.4</td>
<td>150.2, 211.2, 234.2</td>
<td>293.4</td>
</tr>
<tr>
<td>Placebo GNC with unentrapped Gem</td>
<td>-</td>
<td>223.9</td>
<td>224.1</td>
<td>292.1</td>
</tr>
<tr>
<td>Placebo GNC</td>
<td>-</td>
<td>219.7</td>
<td>220.4</td>
<td>-</td>
</tr>
<tr>
<td>Lactose monohydrate</td>
<td>-</td>
<td>143.5, 224.9</td>
<td>143.6, 226</td>
<td>-</td>
</tr>
<tr>
<td>Gelatin</td>
<td>-</td>
<td>50</td>
<td>75</td>
<td>-</td>
</tr>
<tr>
<td>Genipin</td>
<td>-</td>
<td>122.5, 207.8</td>
<td>122.7, 208.6</td>
<td>-</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>290</td>
</tr>
</tbody>
</table>

DSC analysis can be used to analyze chemical and physical stability of the Gem and crosslinker (genipin) with the gelatin polymeric matrix. DSC may detect polymorphic structural changes within a polymeric matrix, which may give some insight on the formulation stability over time. It has well been established that melting and recrystallization curves can be associated with polymorphic changes in lipid matrices [1182]. Thus it may determine and provide evidence of the incorporation of drugs within the nanoparticles through the examination of enthalpy changes. DSC
is a thermo-analysis method that measures the heat flow associated with a change of temperature with transitions of materials as a function of time. Results from DSC may provide information on the endothermic or exothermic phenomena that occur due to physical and chemical changes or changes in heat capacity [1183].

The Gem-GNC displayed a glass transition temperature (Tg) peak of gelatin at 75°C, which suggests that the pure Gem or the excipient genipin was present within the nanoparticles as an amorphous, disordered crystalline, or solid solution state. It also suggests that the Gem-GNC preparation method did not affect the properties of the gelatin.

5.2.7. Powder X-ray Diffraction (PXRD)

To investigate the degree of order of the gelatin nanocarriers matrix-associated Gem, the diffraction patterns of Gem, the lyophilized physical mixture of formulation constituents, lyophilized placebo GNCs, and lyophilized Gem-GNC were obtained (Figure 5.13). The PXRD pattern of Gem showed peaks that were sharp and intense, indicating its crystalline state. The Gem-GNC showed few sharp peaks of low intensities. The Gem and the physical mixture of the formulation components exhibited more crystallinity, whereas the lyophilized Gem-GNC and placebo-GNC showed amorphous or molecularly dispersed properties.
Figure 5.13. PXRD Diffraction Patterns. (A) Gelatin, (B) Lactose monohydrate, (C) Gem, (D) Genipin, (E) Physical mixture of actives and excipients compared to the placebo GNC and Gem, (F) Gem-GNC compared to the placebo GNC and Gem mixture.
X-ray diffraction is necessary for the analysis of the presence of crystal structure and spacing in a polymeric lattice [1184, 1185]. The incorporation of therapeutic agents influences the polymer structure and spacing. PXRD provides information of the patterns and crystallinity which may be confirmed with DSC. We have evaluated the thermal and powder diffraction characteristics using DSC and PXRD. In order to evaluate the interaction of Gem within the genipin crosslinked Gem-GNC, DSC was conducted on various samples including the lyophilized Gem-GNC, physical mixture of excipients, placebo GNC with unentrapped Gem, genipin, lactose monohydrate, and Gem (Figure 5.13). The thermogram of Gem has an endothermic melting followed by degradation at approximately 290°C that was not observed in the Gem-GNC thermogram. This is in agreement with another study that found that the cisplatin was molecularly dispersed within a gelatin matrix without significant changes in the physical and chemical profile as shown by DSC thermograms of the drug loaded nanoparticles compared to individual components [1186].

The crystalline or amorphous nature of the Gem entrapped within the gelatin matrix was investigated by comparison to the powder x-ray diffraction patterns of Gem (Figure 5.13). A diffraction pattern that is missing the characteristic peaks for Gem or have a significant reduction in the intensity and sharpness of characteristic peaks and a change of baseline indicate more disordered state of the drug [1187]. The crystalline or amorphous drug entrapped within the gelatin nanoparticle matrix was evaluated in order to ascertain the Gem entrapment within the hydrophilic polymeric matrix of the polymeric nanoparticle. The gelatin sample showed a highly disordered molecular distribution (Figure 5.13 A) as displayed by the lack of sharp peaks. Lactose monohydrate, Gem, and genipin (Figure 5.13 B, D-E) displayed sharp well defined peaks, illustrating that they are in a highly ordered crystalline state. There is a strong reduction in characteristic peaks in the placebo GNC and Gem mixture when compared to the physical mixture, possibly due to missing lactose monohydrate and genipin crystalline peaks (Figure 5.13 C). The Gem-GNC shows an amorphous-like molecularly dispersed diffraction pattern that has significant reduction in most characteristic peaks of the placebo GNC and Gem mixture (Figure 5.13 F). These results are consistent with another study that performed PXRD analysis on self-assembled gelatin-oleic acid nanoparticles [1188]. This study found that characteristic peaks that were associated with the highly crystalline pure drugs were absent in diffraction patterns for the drugs encapsulated within the nanoparticles [1188].
5.2.8. Cell Viability Assay of Gem-GNC treated A549 and H460 NSCLC Cell Lines

The results of the MTT cell viability assay of the A549 human lung adenocarcinoma and H460 human large cell lung carcinoma cell lines treated with Gem-GNC and Gem solution are shown in Figure 5.14. The A549 cells treated with Gem-GNC did not achieve 50% cell kill after 48 hr, but obtained an IC$_{50}$ of 0.013 and 0.023 μM after treatment with Gem solution and Gem-GNC at 72 hr, respectively. The H460 cells treated with the Gem solution achieved IC$_{50}$ values of 230 μM after a 48 hr and 59 μM after 72 hr. H460 cells treated with the Gem-GNC formulation significantly outperformed the Gem solution, corresponding to IC$_{50}$ values of 41 μM after 48 hr (** p<0.01) and 5.7 μM after 72 hr (** p<0.01). The H460 cells treated at 48 hr with Gem-GNC had a 5 fold lower IC$_{50}$ value than with the Gem solution (** p<0.01), whereas at 72 hr the Gem-GNC had a 10 fold lower IC$_{50}$ value than the Gem solution (** p<0.01).

Figure 5.14. Cell viability over time and concentration of A549 cells (A) and NCI-H460 cells (B) treated with Gem-GNC and Gem solution. Non-treated cells were used as controls. A549 cells
treated with Gem-GNC had an IC$_{50}$ value of 0.023 µM after 72 hr, whereas the Gem solution had an IC$_{50}$ of 0.013 µM. The H460 cells treated with the Gem solution achieved IC$_{50}$ values of 230 µM after a 48 hr and 59 µM after 72 hr. Results are shown as mean ± SD (n=3).

Gelatin based nanocarriers have been used previously to deliver chemotherapeutics to lung cancer tissue via the inhalation route of administration. For example, Tseng et al. prepared cisplatin-loaded epidermal growth factor (EGF)-modified gelatin nanocarriers for targeting cancerous cells in the lung that highly express EGF receptor (EGFR) [264]. This formulation showed higher cisplatin concentration and associated cytotoxicity on A549 cells that show high EGFR expression and showed lower cisplatin concentration on the HFL1 cells that have relatively lower EGFR expression. Post-inhalation, nanoparticle accumulation in murine lung tissue that contains EGFR-overexpressing cells indicated that the formulation was capable of prolonged residence times within lung tumor tissue. In another study, EGF modified gelatin nanoparticle delivered doxorubicin via inhalation and exhibited controlled release [260]. These findings reflected dose-dependent cytotoxicity in A549 and H226 cells with IC$_{50}$ values of 0.56 µg/ml and 0.47 µg/ml, respectively [260]. In vivo biocompatibility of the gelatin nanoparticles may be inferred by the lack of inflammatory or immune response elicited by the formulation. Similar to the studies conducted by Tseng et al., lung tumor tissue nanoparticle accumulation of the EGF ligand surface modified gelatin nanoparticles was observed [260]. After Gem is internalized by the cell, it is phosphorylated by deoxycytidine kinase thereby generating the nucleotide monophosphate. The occurrence of further phosphorylation is rate limited by this first phosphorylation step. Further phosphorylation leads to the active diphosphate and triphosphate derivatives of Gem that have anticancer activity [1189]. The Gem diphosphate indirectly inhibits ribonucleotide reductase, which results in the depletion of the deoxyribonucleotide pool needed for DNA synthesis [1190]. The resultant Gem triphosphosphate (dFdCTP) binds DNA polymerase, and competes with the natural substrate deoxycytidine triphosphate. This single deoxynucleotide addition causes the termination of the DNA helix as a result of binding another nucleotide due to steric hindrance [1191]. This terminal dFdCTP and DNA complex is not recognized by normal cell repair factors which then causes cell cycle arrest and apoptosis [1191]. Also, Gem mediates within the cell cycle before cell proliferation stage, specifically between the G1 and S phases [237]. Gem blocks the de novo synthesis pathway and decreases the intracellular concentrations of normal deoxynucleotide triphosphate pools [1189]. Therefore, Gem must be
delivered to the cytoplasm of the cell following internalization. The developed Gem-GNC formulation with free surface amine groups, allows for endosomal escape by eliciting the proton-sponge effects that lyses the endosomal, thus releasing its contents within the cytoplasm of the cell.

The cell viability studies were performed in A549 and H460 cells lines with the use of the MTT viability assay. Time and dose dependency was studied by comparing the change in cell viability in response to Gem in the concentration range of 0.001 µM to 1000 µM and corresponding Gem-GNC concentrations at 48 and 72 hr timepoints. The placebo GNC treatment showed >95% viability of cells over placebo GNC concentration comparable to the Gem-GNC concentrations. This demonstrated the safety and non-toxicity of the gelatin nanocarrier core that contained gelatin and genipin, the cryoprotectant lactose monohydrate, and the surfactant polysorbate 20. In other studies, gelatin nanocarriers have shown more than 90% cell viability to a nanoparticle concentration up to 1000 µg/ml and approximately 80% cell viability at 2000 µg/ml, illustrating their biocompatibility at high concentrations [597].

The A549 cells treated with Gem-GNC did not achieve 50% cell kill after 48 hr, but after 72 hr the Gem-GNC obtained a 2-fold increase in the IC₅₀ of Gem-GNC treatment when compared to the Gem solution. This increase of Gem-GNC IC₅₀ from the IC₅₀ of the Gem solution, may have been due to the negative charge on the gelatin nanocarriers. The H460 cells treated with the Gem solution achieved IC₅₀ values of 230 µM after a 48 hr and 59 µM after 72 hr. H460 cells treated with the Gem-GNC formulation outperformed the Gem solution, corresponding to IC₅₀ values of 41 µM at 48 hr and 5.7 µM at 72 that represents a 5-fold and 10-fold decrease from 48 hr to 72 hr (** p<0.01), respectively. A previous study found IC₅₀ values of nebulized Gem treatment on H460 cells to be 5.72 nM and treatment on A549 cells resulted in an IC₅₀ value of 29.9 nM at 72 after treatment [1192]. Theoretically, the negatively charged Gem-GNC has lower potential of cellular uptake in in vitro monolayer cell uptake studies due to electrostatic interactions between the negatively charged cellular membrane. The cell viability was decreased after a 72 hr treatment when compared to the 48 hr treatment, which inferred a dose and time dependency in both A549 and H460 cell lines.
5.2.9. *In vitro* Aerosol Characterization

The Gem-GNC formulation was nebulized into an Andersen Mark-II cascade impactor. The stage numbers pre-separator, 0, 1, 2, 3, 4, 5, 6, and 7 had the cut-off diameters of 10, 9, 5.8, 4.7, 3.3, 2.1, 1.1, 0.7, and 0.4, respectively. The log cumulative probability plot of particle size versus cumulative weight percent frequency of nebulized Gem-GNC impaction is shown in Figure 5.15. The average net weight of Gem on each the pre-separator, stages 0-7, and filter were \(0.31 \pm 0.04\), \(0.81 \pm 0.1\), \(0.59 \pm 0.2\), \(0.46 \pm 0.1\), \(0.51 \pm 0.5\), \(0.71 \pm 0.8\), \(2.3 \pm 0.1\), \(0.5 \pm 0.4\), \(2.6 \pm 0.3\), and \(0.18 \pm 0.04\) µg respectively. The total average emitted dose was \(325 \pm 20\) µg and the percent emitted dose was \(65 \pm 0.04\%\) as calculated from 3 different cascade impactor runs. The percent emitted dose was The Gem-GNC formulation showed an average MMAD of \(1.99 \pm 0.16\) µm, GSD of \(2.7 \pm 0.16\), and FPF of 76%.

![Figure 5.15](image_url)

**Figure 5.15.** Log cumulative probability plot of particle size versus cumulative weight percent (%) frequency of Gem-GNC impaction within an 8-stage non-viable Andersen cascade impactor. Data markers represent particle diameter (µm) of the nebulized nanocarrier containing droplets at a given cumulative probability less than stated size and the blue line is best fit linear curve.
Aerosolized Gem delivered via inhalation has been previously investigated. When aerosolized Gem (1 and 4 mg/kg) was administered, one out of four patients showed pulmonary toxicity, while no patients developed hematologic toxicity [1193]. Animal studies on Gem administration by intratracheal instillation by tracheotomy (i.t.t.) or orotracheal route (i.t.o.) was performed in rats [1194]. Pulmonary toxicity was evaluated by comparing lung morphology, histopathology, coefficient, wet/dry weight ratio, cells related with inflammation, and inflammatory cytokines. The i.t.t. and i.t.o. administrations displayed good absolute bioavailability and similar acute lung injury compared with intravenous route. Preclinical studies on the use of Gem aerosol in osteosarcoma-bearing dogs found that Gem aerosol formulation induced increased apoptotic effect with enhanced Fas expression against lung metastatic foci [1195]. These studies indicated that aerosolized Gem is an effective treatment for lung cancer. However, aerosol delivery of Gem still caused local and systemic side effects of bronchospasm, fatigue, vomiting, dyspnea, cough. These adverse effects may be avoided if Gem was delivered via a sustained release manner.

Inhalation therapy allows for the delivery of chemotherapeutics with a local deposition at the target site with reduced side effects associated with systemic delivery. Administration of 5-fluoracil, doxorubicin, paclitaxel, docetaxel, platinum analogues, cetuximab, 9-nitrocamptothecin, interleukins, granulocyte stimulating growth factor and bavazismab via the inhalation route have shown efficacy and safety in past studies [1196]. In the 1980’s, 5-fluorouracil (5-FU), the first chemotherapeutic agent, was studied in inhalation therapy [1197]. It was shown that there was increased 5-FU concentrations within the tumor than in other tissues and that high concentrations of 5-FU were found in the main bronchus and associated lymph nodes [1198]. Further formulations of 5-FU involved lipid-coated nanoparticles or difluoromethylornithine were created in order to provoke a sustained release and enhance anticancer properties [1199, 1200]. Liposomal carriers, nanoparticles, polymeric micelles, and lipid nanocapsules have proven to increase therapeutic index of taxanes by prolonging the residence time and subsequent regional action in the lung [1201]. This study indicated that the mononuclear phagocyte system attacks the colloidal drug, therefore a PEGylated lipid nanocapsule was needed to help prolong regional action. Other studies have shown increased efficacy of entrapped taxanes within nanoparticle and linked to human albumin [1201-1203]. Neurotoxicity and other adverse effects were observed to be dose-dependent, but were accompanied by decreased tumor size [1201]. Cyclosporine A added to the paclitaxel aerosol was found to increase the anticancer efficacy of the therapy [1204]. Taxane
compounds when compared to doxorubicin have less toxicity to the lung parenchyma and do not exhibit the cardiotoxicity associated with doxorubicin treatment [242, 1205]. In order to streamline the treatment methods for doxorubicin inhaled chemotherapy to human subjects, protocols were created by Otterson et al. [1206, 1207] These phase I and I/II studies showed that the aerosol treatment resulted in adverse effects including mild bronchospasm and moderate reduction of pulmonary function [1207]. Platinum analog delivery by inhalation has also been studied and was found to have similar effects as inhaled doxorubicin [1208, 1209]. On the forefront of targeted inhaled chemotherapy was a biotinylated EGF-modified gelatin nanoparticle that was found to increase the anticancer activity of cisplatin [259, 264, 1210]. Lung cancer cells that overexpressed the EGF had increased uptake of the targeted gelatin nanoparticle containing cisplatin, which reduced nephrotoxicity due to the avoidance of the systemic circulation [259]. Inhalable Gem formulations were found to be effective against metastatic osteosarcoma lesions in animal models [1195]. Gem has been delivered by an aerosol or instillation within the lung parenchyma in patients with lung cancer and have shown efficacy [1193, 1194]. It is important to note that even though this formulation did not contain ingredients incompatible with aerosol delivery and did not induce fibrotic lesions within the lung parenchyma, the animal model treatments resulted in some deaths from pulmonary edema after the aerosol administration of Gem [1211, 1212]. Inhaled chemotherapy is feasible if delivered by a nebulization system, as has been shown by the previously published conclusions of this treatment modality. However, a missing link in the development of aerosol chemotherapy is the understanding of long term adverse effects to the lung and more trials are needed to prove safety and efficacy when compared to the conventional intravenous administration.

Aerodynamic particle size distribution is regarded by the FDA as a Critical Quality Attribute (CQA). Cascade impaction measures the aerodynamic, instead of the geometric size of the particles, the mass of the active pharmaceutical ingredient of the drug, and the mass of the entire emitted dose. Particle size distribution impacts inhalation delivery by determining the efficiency of the particles to get to the deep alveolar region of the lung. There are different techniques of particle sizing used to analyze aerosol particles, including optical microscopy, laser light scattering, laser Doppler methods, and the cascade impactor method. Cascade impaction does have limitation as an in vitro test for the determination of the actual aerodynamic particle size distribution. For example, the cascade impactor uses a fixed flow-rate as oppose to a variable
inhalation flow rate that patients will be more likely to do, the USP throat is not a good reflection of the oropharyngeal pathway and dry powder particle may re-entrain during the administration to the cascade impactor. Also the deposition in the cascade impactor is based off of impaction with distinct cut-offs per stage as oppose to the more in vivo relevant impaction, diffusion, and sedimentation over the whole lung surface lung deposition.

Drug delivery to the lungs via the inhalation route of administration does have its own set of limitations in physiological efficacy. Aerosols must reach the intended site of action in order to be effective. The physical characteristics that impact the distribution and retention of inhaled aerosols are particle size, velocity, charge, destiny and hygroscopicity. In addition, deposition is influenced by the physiological factors of respiration rate, airway diameter, presence of excessive mucus, and respiratory volume. Therefore, the drug delivery system for the administration of a therapeutic agent via the inhalation route of administration should be designed with these points in mind.

The cascade impactor may provide information on particles within the aerodynamic diameter range of 0.5-32 μm. Variables that will be determined are the total mass of drug released from the inhalation aerosol, the quantity of drug collected at each location of the cascade impactor device, the MMAD, and the GSD. The cascade impactor consists of a sampling chamber, the cascade impactor, a vacuum pump and a flow meter. In order to determine the aerodynamic behavior, Gem-GNC were suspended in water at a concentration of 25 mg/mL, and delivered via a nebulizer into an Andersen Mark-II cascade impactor. The Gem-GNC formulation displayed a MMAD of 1.99 ± 0.16 μm, GSD of 2.73 ± 0.16, and FPF of 75.2 ± 2.4%. These results are in good accordance with a nebulizer nanoliposomal celecoxib formulation developed recently that had a fine particle fraction, MMAD, and GSD of approximately 76%, 1.6 μm, and 1.2 μm, respectively [249]. An important parameter in inhalation delivery is the particle size distribution because it, in part, determines the efficiency of the delivery of particles to the deep alveolar region of the lung. Aerosol particles with aerodynamic diameters between 1 and 5 μm are optimal for inhalation delivery, therefore the obtained characteristics are suitable for pulmonary deposition within the respiratory zone [1213]. These aerosolized gelatin nanocarriers are within suitable characteristics for airway deposition, therefore they have potential applications for inhalation delivery of drugs and siRNA for a range of different pulmonary disorders, such as lung cancer and asthma.
5.3. STAT6 siRNA loaded Gelatin Nanocarriers for Asthma Treatment

5.3.1. Upregulation of IL-4Rα in Asthmatic Mouse Lung Tissue and Th2 cells

The IL4Rα expression was measured using Western blot analysis of Th2 cell and lung tissue protein lysates in order to validate IL4Rα as an asthma biomarker and to check the TIB-224 cell line expression. The IL4Rα relative protein expression in Th2 cells (D10.G4.1, ATCC® TIB-224™), ovalbumin (OVA) treated allergic asthma mouse lung protein lysates, and normal lung tissue protein lysates were evaluated using an established Western blot analysis. IL4Rα was highly expressed in the Th2 cells (p<0.001) and in OVA treated allergic asthma mouse lung (p<0.01) compared to normal lung protein lysates (Figure 5.16 A-B). The mouse lung tissue lysates contained Th2 cells, amongst airway epithelial, B cells, and other lung tissue associated cells.

![Figure 5.16](image)

**Figure 5.16.** IL4Rα relative protein expression in Th2 cells, normal lung tissue protein lysates, and OVA treated allergic asthma mouse lung protein lysates. A) Western blot analysis and B) quantitative densitometry of the IL4Rα Western Blot bands normalized with β-Actin. Data are shown as mean ± standard deviation (n=3). **p<0.01, ***p<0.001.

According to BioGPS, a database of expression profiling by array, showed that the tissue specific pattern of IL4Rα mRNA expression is higher in asthma associated cells types as shown in Figure 5.17 [1214, 1215].
The IL4R are single-pass transmembrane proteins that part of the hematopoietic cytokine receptor super-family [483]. In this receptor family, ternary ligand-receptor complexes along with conformational changes constitute a prerequisite for proximity-driven cross-activation of receptor-chain-associated cytoplasmic Janus kinases (JAKs) [174, 1216, 1217]. IL4Rα and the common γ-chain (IL2Rγ) diffuse as monomers in the plasma membrane. Therefore, in the IL4R, a diffusion-controlled recruitment of the second receptor chain triggers JAK activation and signal transduction at the plasma membrane [1217]. Since the common γ-chain is shared amongst different cytokine receptor complexes, signaling output may be as a result of local dynamic complex equilibrium [1218-1220].

IL4 specifically binds to the IL4Rα chain, enabling the recruitment of either the common γ-chain to form a type-1 receptor complex or IL13Rα1 to form a type-2 receptor complex. Type-2 receptor complex may also be induced by IL13 bound IL13Rα-1, making both receptor subunits of the type-2 complex capable of servings as a ligand-presenting or recruited-receptor subunits [151, 1217, 1221]. Since the dimerization of the IL4 subunits is a pivotal step for JAK/STAT signal transduction [153, 1222-1224]. It IL4R subunits all accumulate within a subpopulation of early endosomes that are stably anchored just underneath the plasma membrane, termed cortical endosomes [1217, 1225]. The IL4R is internalized through a Rac1- and Pak-regulated clathrin-independent endocytosis route that has been suggested to be related to the fast endophilin-mediated
endocytosis (FEME) [1226-1230]. The current model of IL4R pathway activation lists endocytosis as an essential process that is upstream of receptor dimerization [1226]. Taken together, the S6S-GNC-P are expected to be internalized via type 1 and type 2 IL4R complexes in a clathrin independent, Ras-related C3 botulinum toxin substrate 1 (Rac1) and p21-activated kinase- (Pak) mediated endocytosis pathway.

Recently, IL4R targeting has been reported by Kim et al., where hydrophobic modified glycol chitosan nanoparticles were surface conjugated with IL4R binding peptides and delivered to mice with IL4R+ expressing tumors [1231]. The IL4R targeted nanoparticles had improved IL4R dependent cellular uptake within the tumors than with plain nanoparticles, which resulted in improved therapeutic and imaging efficacy of the theranostic. The authors found that the IL4R binding peptide facilitated the cellular uptake of nanoparticles within the tumor tissues [1231]. In a study by Al Faraj et al., superparamagnetic iron oxide nanoparticles were conjugated with anti-IL4Rα blocking antibodies via polyethylene glycol (PEG) polymers [681]. The delivery of these blocking antibodies to the inflammatory sites in the lung via the developed nanocarriers was assessed using noninvasive free-breathing pulmonary MRI. Targeting of the developed nanocarriers to areas rich in IL4Rα positive inflammatory cells was observed. These anti-IL4Rα-conjugated nanocarriers were confirmed to be efficient in targeting key inflammatory cells during chronic lung inflammation following intrapulmonary administration [681].

These studies suggest the feasibility of using a IL4R binding peptide to increase cellular internalization of a nanocarrier. IL4R targeting S6S-GNC-P is the first account of delivering STAT6 siRNA to Th2 cells for asthma therapy via the IL4R receptor mediated internalization. Considering the differential expression of IL4Rα, the IL4Rα guided delivery of STAT6 siRNA will be greatly beneficial for the treatment of asthma.

5.3.2. Optimization of RNAiMax and STAT6 siRNA Concentrations

Since transfection efficiency is not always guaranteed, even with commercially available transfection reagents, a transfection protocol was optimized for the Th2 cell line TIB-224. Important considerations in siRNA transfection experiments are the health of the cultured cells, the transfection method and conditions, and the quality and amount of siRNA. The aim of this experiment was to optimize the transfection using Lipofectamine RNAiMax so that a clear positive control exhibits significant gene downregulation without impacting the Th2 cell viability. First, the Th2 cells were treated with varying levels of RNAiMax (cationic liposome) to obtain the dose
response curve, where the response it loss of cellular viable as measured using the MTT assay. The MTT results are shown in Figure 5.18. A RNAiMax concentrations of 0.17% and 0.25% v/v associated with 100% and 92% cell viability, respectively, were selected based on the cell viability curve. Upon complexation of the cationic liposome and the negative charged siRNA, the it is expected that the charge-based cellular cytotoxicity will be reduced.

![Graph showing cell viability vs RNAiMax concentration](image)

**Figure 5.18.** Cell viability of Th2 cells (TIB-224) following treatment with non-complexed RNAiMax cationic liposomes. Non-treated cells were used as a control. Results are shown as mean ± SD (n=4).

The next step was to identify the STAT6 siRNA concentration to use with the two different RNAiMax levels. The Western blot results are shown in Figure 5.19. It is apparent that the 0.17% v/v RNAiMax concentration was not effective at all STAT6 siRNA concentrations due to a lack of transfection efficiency with those levels in the TIB-224 Th2 cell line (Figure 5.19 A-C). The 0.25% v/v RNAiMax STAT6 siRNA complexes produced STAT6 protein downregulation in a dose-dependent manner (Figure 5.19 A, D-E). Figure 5.19 B and D shows the densitometry analysis of the band densities normalized to the β-actin loading controls and expressed relative to the medium control band density for the 0.17% v/v and 0.25% v/v RNAiMax concentrations, respectively. Figure 5.19 C and E shows the densitometry analysis of the 0.17% w v/v and 0.25%
v/v RNAiMax concentrations, respectively. Due to the apparently lower STAT6 protein basal expression level compared to the RNAiMax control, this additional densitometry analysis and relative STAT6 protein expression was presented to better illustrate the effect of the STAT6 siRNA transfection. For future STAT6 protein downregulation studies, the Th2 cells were pre-incubated with IL4 protein to upregulate the STAT6 protein expression, with understanding of the events that occur in IL4 mediated asthma pathophysiology and as discussed in Chapter 4 Cell Culture. The STAT6 siRNA concentration EC\textsubscript{50} value here was found to be 150 nM, based on results normalized to the RNAiMax treated control. Assessment of the STAT6 siRNA RNAiMax complex cytotoxicity was conducted using the MTT cell viability assay in Th2 cells (Figure 5.20.). The cell viability assay confirmed that the 150 nM STAT6 siRNA concentration would not lead to substantial loss in cell viability. The STAT6 siRNA concentrations of 25-100 nM led to loss in cell viability due to the cationic charges of the RNAiMax transfection regent.
Figure 5.19. STAT6 siRNA concentration was optimized using Western blot protein analysis following treatment at varying doses. Western blot of Th2 cell lysates which were treated with varying levels of STAT6 siRNA in complex 0.17% v/v or 0.25% v/v RNAiMax (A), (B-C) shows the densitometric relative STAT6 protein expression in the 0.17% v/v RNAiMax STAT6 siRNA complexes normalized to the medium or RNAiMax treated controls respectively, and (D-E) shows the densitometry relative STAT6 protein expression in the 0.25% v/v RNAiMax STAT6 siRNA complexes normalized to the medium or RNAiMax treated controls respectively. Western blot band density was determined by densitometry using ImageJ software and was normalized to the beta actin loading control and normalized relative to either the medium control or the RNAiMax control as noted above. Bars show means ± standard deviations, with n=3.
5.3.3. Preparation of S6S Loaded Gelatin Nanocarriers (S6S-GNC) and S6S-GNC conjugated with IL4R targeting peptide (S6S-GNC-P)

S6S-GNC were prepared using the 2-step desolvation technique, where HMW gelatin was fractionated from the GELITA Medella Pro low endotoxin gelatin using 50% v/v rapid addition of acetone for 5 minutes. The resultant precipitated HMW gelatin was dried overnight at 50°C and underwent mass reconciliation to determine the required volume to prepare a 2% w/v HMW gelatin solution. Then, 5 mL of 2% w/v gelatin was placed into a beaker along with 200 µl 100 µM STAT6 siRNA (or Scrambled siRNA for controls). This is 20 nmol total siRNA to final batch volume of 10 mL containing 1% w/v gelatin nanocarriers. The siRNA and gelatin polymer were mixed at 300 rpm for 15 min to produce siRNA gelatin complexes. The temperature was increased to 40°C and 5 mL acetone was added at a rate of 1 mL/minute using a syringe pump. The S6S-GNC were crosslinked with the addition of 40 µl of 10 mg/ml genipin. Acetone was evaporated overnight while stirring at 300 rpm and with a temperature of 30°C. After 2 hr of acetone evaporation, 5 mL distilled deionized water was added to make up an aqueous volume of 10 ml. The particles were PEGylated and peptide conjugated using 7.5-30% mol ratio of peptide to total PEG (360-1400 copies of peptide per gelatin nanocarrier) as described in Chapter 4 Section 4.2.6.
The particle size, PDI, zeta potential, entrapment efficiency (EE%), and loading efficiency (LE%) are shown in Table 5.5.

As shown in Table 5.4, there was a non-significant (p>0.05) increase in particle size of the non-PEGylated S6S-GNC at 93 ± 3.9 nm, the PEGylated S6S-GNC at 97 ± 3.6 nm, and the S6S-GNC-P at 98 ± 3.6 nm. The PDI between the non-PEGylated and the PEGylated S6S-GNC did not undergo a significant change (p>0.05), but the PDI of the S6S-GNC-P formulation had a very statistically significant (p<0.01) difference from the PEGylated S6S-GNC. This change in PDI could be explained by the peptide conjugation procedure, which consists of cysteimide containing peptide to the gelatin nanocarriers PEGylated with reactive maleimide terminated functional groups and extensive dialysis to remove conjugation agents and free peptide. The zeta potential of the PEGylated S6S-GNC and the S6S-GNC-P had a significant reduction (p<0.001) in zeta potential to +6.8 ± 0.74 mV and +6.3 ± 0.54 mV, respectively compared to the zeta potential of the non-PEGylated S6S-GNC of +17.1 ± 0.48 mV. According to the manufacturer, the IL4Rα targeting peptide consisting of an amino acid sequence of QRLFRAFR was estimated to have a net charge at pH 7 of 3, with an isoelectric point at pH 12.7. All S6S-GNC formulations maintained high entrapment efficiencies of approximately 99% and loading efficiencies of approximately 19%.

Table 5.4. Particle size, zeta potential, entrapment and loading efficiencies of the non-PEGylated S6S-GNC, PEGylated S6S-GNC, and S6S-GNC-P.

<table>
<thead>
<tr>
<th></th>
<th>Particle Size (nm)</th>
<th>PDI</th>
<th>Zeta Potential (mV)</th>
<th>EE%</th>
<th>LE%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-PEGylated S6S-GNC</td>
<td>93 ± 3.9</td>
<td>0.58 ± 0.02</td>
<td>+17.1 ± 0.48</td>
<td>99 ± 0.01</td>
<td>19 ± 0.001</td>
</tr>
<tr>
<td>S6S-GNC</td>
<td>97 ± 3.6</td>
<td>0.55 ± 0.01</td>
<td>+6.8 ± 0.74</td>
<td>99 ± 0.02</td>
<td>19 ± 0.002</td>
</tr>
<tr>
<td>S6S-GNC-P</td>
<td>98 ± 3.6</td>
<td>0.66 ± 0.02</td>
<td>+ 6.3 ± 0.54</td>
<td>98 ± 0.02</td>
<td>19 ± 0.002</td>
</tr>
</tbody>
</table>
The resultant S6S-GNC-P were subjected to SEM and TEM microscopy to confirm morphology and particle size. An electron microscope accelerated electrons in a beam as an illumination source.

The SEM and TEM images of S6S-GNC-P are shown in Figure 5.21. The gelatin nanocarriers were analyzed by SEM to characterize the surface of dry, non-dispersed particles (Figure 5.21 A), and to characterize the surface of the lactose-cryoprotected non-dispersed particles (Figure 5.21 B). The SEM of the non-dispersed particles without cryoprotectant reveals an aggregate structure of the particles, while the SEM of the particles with cryoprotectant shows the amorphous-like structure of the lactose cryoprotectant [1232]. The TEM images show individual S6S-GNC-P which have particles sizes of $170 \pm 40$ nm (n=25), which are larger than the sizes found using DLS due to sample preparation for imaging (Figure 5.21 C). For this image, the freeze-dried S6S-GNC-P using lactose was rehydrated and diluted. Upon inspection, with the sample grid applied to the tilt stage for topography analysis (Figure 5.21 D), the nanocarriers appear to be flattened, which could account for the larger particle sizes observed in Figure 5.21 C. For DLS particle size analysis, the gelatin nanocarriers are dispersed in sterile filtered water or PBS, where in SEM image detection the gelatin nanocarrier samples are dispersed onto a sample grid and dried under vacuum conditions. These conditions may cause shrinking or collapsing of the polymeric nanoparticles prior to SEM analysis [1232]. On the other hand, DLS measures the hydrodynamic diameter (d(H)) of particles, which tend to be higher than their real diameter [1232, 1233].

TEM images of the S6S-GNC-P reveal particles of $90 \pm 26$ nm (n=5), which is comparable to the obtained DLS measurements (Figure 5.21 E-F.). Due to the apparent collapsing of the gelatin nanocarriers during the SEM sample preparation, these samples were kept dispersed within distilled deionized water at a gelatin nanocarrier solids concentration of 0.5% w/v prior to imaging in order to keep the polymeric matrix hydrated. The particles were then applied to the sample grid and dried as previously mentioned. The particles were also applied to a lacey grid and imaged as shown in Figure 5.21 F.
Figure 5.21. Representative SEM and TEM images of S6S-GNC-P. (A) S6S-GNC-P non-dispersed freeze dried without cryoprotectant and imaged with SEM, (B) S6S-GNC-P non-dispersed freeze dried with lactose and imaged with SEM, (C) S6S-GNC-P dispersed and imaged with TEM, (D) S6S-GNC dispersed and imaged on SEM with tilted stage, (E) TEM image of dispersed S6S-GNC-P using Formvar grid, and (F) TEM image of dispersed S6S-GNC-P using a lacey grid.
A number of considerations must be addressed when designing a novel nanoparticle drug delivery system, such as the methods of purification, storage, and scale-up. The most detailed data regarding the scale up of production of pharmaceutical grade nanoparticle dispersions are available for pilot scale production of nanoparticles prepared by the emulsification-solvent diffusion method and by the nanoprecipitation method. A pilot-scale production is of an intermediate size compared to the laboratory and the industrial scale production batches. Pilot size production is geared to simulate the conditions of an industrial scale production as much as possible, and therefore needs to integrate all of the parameters that need to be optimized before reaching the industrial production batch sizes [958].

The two step desolvation method is the mostly widely reported method for preparing gelatin nanoparticles, and has been reported to outperform the nanoprecipitation method in terms of smaller particle size for gelatin nanoparticles [1234]. The nanoprecipitation method is analogous to the desolvation method, except that the polymer solution is in the organic phase in nanoprecipitation but in the desolvation method it is in the aqueous phase. These solvent displacement methods are based on forced nucleation and nanoparticle precipitation of a polymer due to the displacement of the polymer solvent by a non-solvent which is miscible with the polymer solvent [965, 1235]. In the desolvation method, a polymer solution is prepared by dissolving the polymer within its solvent. Then the non-solvent is added to the polymer solution using a controlled rate of addition and mixing to produce polymer colloids. Polymers with low amphiphilic properties precipitate forming nanoparticles within a narrow range of the phase diagram with respect to the polymer solvent and non-solvent system [1236]. To successfully prepare polymeric nanoparticles by the desolvation method, one must consider the basic requirements: (1) the polymer solution should be dilute, (2) the polymer solvent and the non-solvent should be miscible, and (3) the polymer solvent should be easily removed at the end stage of the process. The preferred polymer solvent is usually water and the non-solvents may be acetone, ethanol, or tetrahydrofuran.

At the laboratory scale of nanoparticle production, the preparation of a batch containing only a few ml inherently maintains the same conditions from the beginning to the end of the process. In contrast, the production of large nanoparticle batches within a reactor is not ideal because the conditions for desolvation change from the beginning to the end while the two phases mix. To maintain homogenous conditions for desolvation, the two phases need to be unified under equal conditions throughout the process. Two-phase mixing devices which continuously feed
specific flow rates with the polymer solution and the non-solvent phases while the nanoparticle dispersion flows out of the mixing device, have been previously suggested [1237]. These mixing conditions are constant no matter what volume of nanoparticle dispersion is being produced. The mixing device is integral to the scale-up production of polymeric nanoparticles which maintain homogenous conditions at any timepoint of the process. Mixing devices that have been previously designed have been based on a T-shaped tube [1238, 1239]. An example of a pilot plant for polymeric nanoparticle preparation may include a reservoir with a capacity of several liters of the polymer solution, another reservoir with similar capacity for the non-solvent, a large capacity receiver, and the mixing device and pumps necessary to feed the mixing device in a controlled fashion [1240]. It has been reported that a pilot plant with 3 L reservoirs and a T-shaped mixing device can convert 5 g of polymer into nanoparticles within 2 hr with high reproducibility [1165, 1241].

5.3.4. *In vitro* Release of STAT6 siRNA from S6S-GNC and S6S-GNC-P

![Graph](image_url)

**Figure 5.22.** *In vitro* release of STAT6 siRNA from S6S-GNC (red), S6S-GNC-P (green), and STAT6 siRNA solution (blue) in DPBS pH 7.4 medium at 37°C using a 100 kDa dialysis membrane (STAT6 siRNA MW 13.3 kDa).
In vitro siRNA release from S6S-GNC and S6S-GNC-P were compared to that of STAT6 siRNA in solution from a 100 kDa dialysis membrane in DPBS pH 7.4 release medium. The results are shown in Figure 5.22. The S6S-GNC and S6S-GNC-P formulation underwent <20% cumulative percent siRNA within 1 hr. At 24 hr, the S6S-GNC released 77 ± 15% siRNA, while the S6S-GNC-P released 71 ± 10% siRNA. At 48 hr both formulation had approximately 92% cumulative siRNA release and by 72 hr both formulation had complete siRNA release. This represents a sustained release compared to the free siRNA solution, which had complete siRNA release within 1 hr. Drug release kinetics were assumed to be based on release from a matrix-based system based on the in vitro release findings for the Gem-GNC formulation. Therefore, the release kinetics of the STAT6 siRNA from the gelatin nanocarriers were confirmed by filling the release data into six kinetic models: zero order, first order, Weibull, Higuchi, Baker-Lonsdale, and Korsemeyer-Peppas models (Table 5.5). Several issues exist when fitting dissolution data to available models. The theoretical calculations should be compared with the experimental results to perform a simulation adjustment of the model parameters. It is possible to obtain a good fit even if the model is inappropriate for the drug delivery system. Therefore, if adequate experimental evidence is provided, then one should use those models no matter how well theoretical models agree with the dissolution results. Other models may be applicable are the regression models of linear (first order regression model) and quadratic model (second order regression model), the non-linear regression models, and the model independent approach using similarity factor [1175].

Model independent measurements of DE and MDT were conducted for the STAT6 siRNA solution, S6S-GNC, and S6S-GNC-P. The STAT6 siRNA solution had a DE of 57% and a MDT of 0.43 hr, indicating that is was quickly released within 1 hr. The S6S-GNC formulation had a DE of 76% and a MDT of 17.4 hr, while the S6S-GNC-formulation had a comparable DE of 74% and MDT of 18.5 hr. The long MDT of the S6S-GNC and S6S-GNC-P formulation indicates a controlled release of the siRNA from the gelatin matrix.
Table 5.5. *In vitro* release models showing correlation coefficient (R²) values for STAT6 siRNA release from the S6S-GNC and S6S-GNC-P within DPBS (pH 7.4). Variables are defined in the text.

<table>
<thead>
<tr>
<th>Model Name</th>
<th>Model</th>
<th>STAT6 siRNA Solution</th>
<th>S6S-GNC (R²)</th>
<th>S6S-GNC-P (R²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero Order</td>
<td>( M_0^{\text{GNC}} - M_t^{\text{GNC}} = k_0 t )</td>
<td>0.981</td>
<td>0.882</td>
<td>0.900</td>
</tr>
<tr>
<td>First Order</td>
<td>( \ln\left(\frac{M_0^{\text{GNC}}}{M_t^{\text{GNC}}}\right) = k t )</td>
<td>0.532</td>
<td>0.124</td>
<td>0.119</td>
</tr>
<tr>
<td>Higuchi</td>
<td>( f_t = Q = A\sqrt{D(2C - 2C^2)Cs * t} )</td>
<td>N/A</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Baker-Lonsdale</td>
<td>( f_1 = \frac{3}{2} \left[ 1 - \left( 1 - \frac{M_t}{M_\infty} \right)^{\frac{2}{3}} \frac{M_t}{M_\infty} = k_t \right) )</td>
<td>N/A</td>
<td>0.999</td>
<td>0.995</td>
</tr>
<tr>
<td>Weibull</td>
<td>( M = M_0 \left[ 1 - e^{-\frac{t-\tau}{\alpha}} \right] )</td>
<td>N/A</td>
<td>0.991</td>
<td>0.989</td>
</tr>
<tr>
<td>Korsmeyer-Peppas</td>
<td>( \frac{M_t^{\text{sol}}}{M_\infty^{\text{sol}}} = k_{\kappa P} t^n )</td>
<td>N/A</td>
<td>0.977 n=1.1</td>
<td>0.974 n=1.1</td>
</tr>
</tbody>
</table>

The Baker-Lonsdale model and the Weibull model best fit the S6S-GNC and S6S-GNC-P release kinetics as shown in Table 5.5. This implies that the release is best described as diffusion through a matrix-based spherical system. The Korsemeyer-Peppas power law analysis of the kinetic release data shown case II transport given a spherical particle at \( n=1.1 \). Taken together, the siRNA release kinetics from the S6S-GNC and the S6S-GNC-P were found to be defined by the Korsemeyer-Peppas model using Case II transport and diffusion mechanisms from a matrix-based spherical nanocarriers. This is in direct agreement with results obtained for the release from gelatin nanocarriers kinetic analysis performed by Zuo et al. [1242].
5.2.5. Stability of S6S-GNC and S6S-GNC-P

The stability of the S6S-GNC and S6S-GNC-P formulations were assessed in the presence of DPBS with and without 10% v/v FBS, as well as within water containing 10% v/v FBS. **Figure 5.23.** shows the particle size of the formulations following incubations at time 1, 24, 48, 72, and 96 hr. Amongst all of the simulated physiologic media, the particle size and PDI of the developed S6S-GNC and S6S-GNC-P formulations are non-significantly different at the same timepoints. Within the DPBS pH 7.4 medium (**Figure 5.23 A**), the particle size of both the S6S-GNC and S6S-GNC-P remained unchanged until day 5, where the mean particle size was reduced to approximately 70-80 nm from the original 90-100 nm. The S6S-GNC particle size had no significant (p>0.05) change in particle size from time 0 to 96 hr within DPBS containing 10% FBS (pH 7.4) (**Figure 5.23 B**), inferring the stability of the nanoparticles in the presence serum at room temperature. It is important to note that the stability of the gelatin nanocarriers within these media at body temperature (37°C) may lead to more drastic reduction in particle size due to degradation. The S6S-GNC and S6S-GNC-P formulations underwent a gradual reduction in particle size when incubated within water containing 10% FBS over the time period of 0-96 hr (**Figure 5.23 C**). The particle size of the formulation at time 0 hr was between 90-110 nm, while from 24 to 96 hr the particle size was between 80-90 nm.

**Figure 5.23.** Stability of S6S-GNC and S6S-GNC-P in DPBS pH 7.4 (A), DPBS pH 7.4 containing 10% FBS (B), and water containing 10% v/v FBS (C). Results are shown as mean ± SD (n=5).
5.3.6. Optimization of IL4Ra Peptide Concentration Conjugated onto the Nanocarrier Surface.

The number of nanocarriers per ml batch volume was calculated to be $2.4 \times 10^{13}$ based on kinematic viscosity of gelatin nanocarrier suspensions given a volume-weighted hydrodynamic diameter of 100 nm [1083, 1086, 1087]. The kinematic viscosity was measured using a Cannon Fiske viscometer and the percent formulation versus time in seconds is shown in Figure 5.24. The data was fitted with a regression line that had a corresponding $R^2$ value of 0.998. The viscosities of the GNC suspension and water was then applied to Equation 4.14 to calculate the volume fraction of particles ($\varphi$). The volume fraction of particles, ($\varphi$), and the volume-weighted hydrodynamic diameter of the GNC were then applied to Equation 4.13 to calculate the number of GNC per ml of suspension. The results of these calculations can be found in Table 5.6.

PEG conformation on the surface of a nanoparticle is described in terms of the Flory radius (F) according to Equation 5.11:

$$F = \frac{3}{5} \alpha n$$  \hspace{1cm} \text{Equation 5.11.}

Where $n$ is the quantity of monomers within the polymeric PEG chain and $\alpha$ is the length of one monomer in Angstroms (for PEG $\alpha = 3.5\text{Å}$) [130, 1243, 1244]. There are two PEG conformations, the first is the mushroom conformation when PEG density is low (the distance between the polymer attachment points on the surface, $D$ is larger than $F$). In the mushroom conformation, the polymer chain occupies a semi-sphere where the radius is similar to the Flory radius. The second conformation occurs when the grafting density is high ($D<F$). Here the PEG polymer will form a brush-border configuration, where long bristles of PEG can extend from the polymer surface. When the distance between the PEG molecules on the surface of the nanocarrier ($D$) nears the Flory radius ($F$), the mushroom conformation changes to the brush-border conformation [1245]. A brush-border conformation is ideal since nanoparticles with this conformation tend to have longer circulation times due to the better shielding from the reticuloendothelial system (RES) [1246, 1247].

The number of PEG molecules per GNC was then calculated based on the 1 mg/mL PEG5000 addition to the gelatin nanocarrier batch. The PEGylation efficiency was determined to be 96% by quantifying the unreacted PEG using the Barium/Iodine colorimetric method [1082]. Therefore, the number of PEG molecules per GNC is 4800, which is above the necessary number of PEG molecules per gelatin nanocarrier in order to achieve a brush-border PEG
The theoretical number of peptide per gelatin nanocarrier was calculated based on the addition of 7.5%, 10%, 15%, and 30% mole ratios of peptide to conjugated PEG corresponds to 360, 480, 720 and 1400 copies of peptide per gelatin nanocarrier.

![Graph](image)

**Figure 5.24.** Kinematic viscosity of gelatin nanocarriers as measured by a Cannon Fiske viscometer.

**Table 5.6.** S6S-GNC suspension characteristics.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume Fraction of Particles ($\phi$)</td>
<td>0.125</td>
</tr>
<tr>
<td>Number of GNC/ml</td>
<td>$2.4 \times 10^{13}$</td>
</tr>
<tr>
<td>Gelatin concentration</td>
<td>1% w/v</td>
</tr>
<tr>
<td>Gelatin concentration (nM)</td>
<td>40</td>
</tr>
<tr>
<td>Gelatin molecules/ml</td>
<td>$1.3 \times 10^{17}$</td>
</tr>
<tr>
<td>Gelatin molecules/GNC</td>
<td>~81</td>
</tr>
<tr>
<td>Distance between PEG (D)</td>
<td>10</td>
</tr>
<tr>
<td>Flory Radius (F)</td>
<td>16</td>
</tr>
<tr>
<td>GNC surface area per ml (mm$^2$)</td>
<td>31</td>
</tr>
<tr>
<td>PEG ligands per GNC needed for Brush-border configuration</td>
<td>3100</td>
</tr>
</tbody>
</table>

Before proceeding to *in vitro* evaluation of the S6S-GNC-P formulation, the surface conjugated peptide density was determined and optimized. To determine the optimal amount of peptide to surface conjugate onto the PEGylated S6S-GNC two methods were used: (1) STAT6 in cell ELISA quantification following Th2 treatment with 75 nM STAT6 siRNA with varying
surface peptide concentrations (Figure 5.25) and (2) using flow cytometry to determine the cellular associated fluorescence of Th2 cells treated with FITC conjugated S6S-GNC-P at varying surface peptide concentrations (Figure 5.26 and 5.27). For the ELISA assay, 50,000 Th2 TIB-224 cells were plated onto a 96-well plate and treated with 75 nM Scrambled siRNA RNAiMax complex (negative control), STAT6 siRNA RNAiMax complex (positive control), and 75 nM STAT6 siRNA equivalents of S6S-GNC, and S6S-GNC-P containing 1, 2.5, 5, 7.5, 10, 15, and 30 mole ratio % of the total conjugated PEG. Medium treatments were used as controls. In the STAT6 Elisa assay, the positive STAT6 siRNA control led to significantly (p<0.01) reduced STAT6 protein downregulation when compared to the medium control, while the negative control did not produce any significant STAT6 downregulation. (p>0.05). According to the ELISA results, the peptide to PEG mole ratios of 2.5-15% (120 -720 copies of peptide per gelatin nanocarrier) had significantly (p<0.01) more efficient STAT6 protein downregulation than the PEGylated S6S-GNC. The peptide to PEG mol ratios of 1 and 30% (48 and 1400 copies peptide per gelatin nanocarrier, respectively) did not downregulate STAT6 protein significantly better (p>0.05) than the PEGylated S6S-GNC. The PEGylated S6S-GNC did not significantly downregulate the STAT6 protein when compared to the medium control (p>0.05). However, peptide conjugated formulations containing 48-120 copies of peptide per gelatin nanocarrier (1-2.5% mol ratio peptide to PEG) had achieved significantly reduced STAT6 expression compared to the medium control (p<0.05). The peptide conjugated formulation containing 240-720 copies of peptide per gelatin nanocarrier (5-15% mol ratio peptide to PEG) was able to more significantly reduce the STAT6 protein expression compared to the medium control at p<0.01.
Figure 5.25. STAT6 protein quantification using ELISA assay following treatment with 75 nM STAT6 siRNA equivalents within S6S-GNC-P containing varying concentrations of surface conjugated peptide. Data represented as mean ± standard deviation. *p<0.05, **p<0.01, ***p<0.001 with n=4.

Flow cytometry binding and internalization assays were performed to verify this observation. Concentrations selected for flow cytometry binding and internalization assays were 7.5%, 10%, and 15% mole ratio peptide to total surface conjugated PEG. The flow cytometry binding assay experiments shown that upon incubation at 4°C for 1 hr, the 7.5% mole ratio of peptide to PEG GNC-P formulation had significantly higher binding (p<0.001) compared to the 10-30% mol ratio peptide conjugated formulations (Figure 5.26 A and B). Additionally, the peptide conjugated gelatin nanocarrier formulations which contained 7.5, 10, and 15% mol ratio peptide (360-720 copies of peptide per gelatin nanocarrier) had significantly higher Th2 cell binding (p<0.001) when compared to the medium control. The 30% mol ratio S6S-GNC-P (1400 copies of peptide per gelatin nanocarrier) did not have a significant difference from the control. On the other hand, incubation at 4°C for 4 hr showed that the peptide concentrations ranging from 7.5%, 10%, and 15% mole ratio of peptide to PEG concentrations had no statistically significant differences (p>0.05) between them, but had a significantly higher (p<0.001) Th2 cell associated
fluorescence when compared to the control (Figure 5.26 C and D). This implies that the 4 hr cellular incubation time with the formulations led to gelatin nanocarrier binding or internalization mediated through something other than the IL4Rα targeting peptide. This binding most likely had occurred because the maleimide-terminated PEG on the surface of the PEGylated S6S-GNC had elicited enhanced cellular binding or uptake via a thiol-mediated transport mechanism [1248]. The S6S-GNC-P formulations at all peptide concentrations had statistically increased cellular binding compared to the PEGylated S6S-GNC (p<0.05). From this study, peptide concentrations of 7.5% and 15% mole ratio of peptide to PEG, or 360 and 720 copies of peptide per gelatin nanocarrier, were selected for comparison under the flow cytometry internalization assays and were compared to untreated control, plain gelatin nanoparticles, and the PEGylated S6S-GNC formulation.

Figure 5.26. Binding assay flow cytometry relative fluorescent intensities following incubation at 4°C with the various S6S-GNC and S6S-GNC-P formulations. (A) Cellular fluorescence intensity following 4°C incubation for 1 hr (n=3), (B) Quantification of cellular fluorescence intensity following 4°C incubation for 1 hr (n=3), (C) cellular fluorescence intensity following 4°C incubation for 4 hr (n=2), and (D) quantification of cellular fluorescence intensity following 4°C incubation for 4 hr (n=2).
In the area of targeted nanotherapeutics, it is generally accepted that determining the optimum ligand density is necessary for sufficient cellular or tissue targeting [1249]. Elias et al. had developed a site-specific bioconjugation strategy which allows for control of ligand density on superparamagnetic iron oxide nanoparticle with particle sizes between 25-28 nm through expressed protein ligation and copper-free click chemistry in order to determine if an optimal ligand density in the range of 0-36 Her2/new targeting antibody ligands per nanoparticle existed [1250]. Similar to the results presented here, an intermediate amount of ligand density was more optimal than a low or high ligand density in terms of cellular binding [1250].

The flow cytometry cellular internalization assays of the S6S-GNC-P containing 360 and 720 copies of peptide per gelatin nanocarrier were compared to non-surface modified (plain) cationic S6S-GNC, PEGylated S6S-GNC, and the medium control cells (Figure 5.27 A-H). Following incubation at 37°C for 5 min (Figure 5.27 A-B), the S6S-GNC-P treated Th2 cells had significantly (p<0.05) higher cellular associated fluorescent compared to the medium control, possibly due to a maleimide mediated uptake mechanism [1248]. The PEGylated S6S-GNC was surface conjugated with maleimide terminated PEG. The S6S-GNC-P containing 7.5% mol ratio peptide (360 copies of peptide per gelatin nanocarrier) treatment group had a significantly increased cellular associated fluorescence (p<0.001) similar to that of the non-surface modified (plain) S6S-GNC (p<0.001) when compared to the medium treated control. The 15% mol ratio surface peptide conjugated S6S-GNC-P formulation did not have a significant effect on cell associated fluorescence when compared to the medium treated control (p>0.05) and also had a statistically significant (p<0.01) decrease in cell associated fluorescence when compared to the 7.5% mol ratio surface peptide conjugated S6S-GNC-P (Figure 5.27 B). At 15, 30, and 60 min (1 hr) incubation times at 37°C (Figure 5.27 C-J), all of the gelatin nanocarrier formulations had an extremely significant (p<0.001) statistical difference from the medium control. At an incubation time of 15 min (Figure 5.27 C-D), the Th2 cells treated with the S6S-GNC-P formulations containing 360 and 720 copies of peptide 15 gelatin nanocarrier each had a statistically significant increase (p<0.001) in cell associated fluorescence when compared to the PEGylated S6S-GNC treated cells (Figure 5.27 D). At 30 min of incubation at 37°C (Figure 5.27 E-F), the peptide conjugated S6S-GNC-P at 7.5% mol ratio (360 copies of peptide per gelatin nanocarrier) had a significant decrease (p<0.001) in cell associated fluorescence when compared to the cells treated with the PEGylated S6S-GNC (Figure 5.27 F). However, the S6S-GNC-P formulation containing
15% mol ratio of peptide to total PEG (720 copies of peptide per gelatin nanocarrier) treated cells had a significantly increased (p<0.001) cell associated fluorescence when compared to the PEGylated S6S-GNC, inferring that the 15% mol ratio S6S-GNC-P had increased internalization at 30 min incubation. At a timeframe of 1 hr at 37°C (Figure 5.27 G-H), the S6S-GNC-P at 7.5% mol ratio peptide to PEG (360 and 720 copies of peptide per gelatin nanocarrier) treated Th2 cells, had significantly (p<0.001) less cell-associated fluorescence compared to the PEGylated S6S-GNC treated cells (Figure 5.27 H). The cell associated fluorescence and inferred cellular internalization of the S6S-GNC-P containing 15% mol ratio peptide, or 720 copies of peptide per gelatin nanocarrier, was reduced, but not significantly (p>0.05) compared to the PEGylated S6S-GNC treated cells after a 1 hr incubation. The PEGylated S6S-GNC formulation compared similarly to the S6S-GNC-P at 15% mol ratio peptide, possible due to the maleimide mediated uptake and the high concentration of nanoparticles applied to the cells [1248]. In the in vitro evaluation of the S6S-GNC and S6S-GNC-P, a lower concentration of nanoparticles will be applied to the cells, with respect to STAT6 siRNA concentration, and therefore the effects of the maleimide mediated uptake of the S6S-GNC are not expected to outperform the peptide targeted S6S-GNC-P in those studies.

The cellular internalization assay conducted using flow cytometry shown that the 15% mol ratio of peptide to total PEG molecules, or 720 copies of peptide per gelatin nanocarrier, produced higher internalization when compared to the 7.5% mol ratio at 30 min and 1 hr after treatment in Th2 cells. The ELISA results had shown that peptide concentrations within the range of 5-15% mol ratio peptide to PEG (240-720 copies of peptide per gelatin nanocarrier) had the most significant effect on STAT6 protein downregulation following a 48 treatment with 75 nM STAT6 siRNA equivalents of the various S6S-GNC and S6S-GNC-P formulations. Therefore, a 15% mol ratio for the preparation of S6S-GNC-P was selected for further in vitro evaluation in Th2 cells. These experiments require repetition with other cell lines, such as airway epithelial cells or B cells, to further validate the surface binding peptide density on the S6S-GNC-P. In vivo studies should contain a range of peptide densities validated using binding assays and internalization assays in all desired cellular targets including Th2 cells, airway epithelial cells, and B cells.
Figure 5.27. Internalization assay by flow cytometry. Fluorescent internalization comparison between medium control cells, plain gelatin nanocarriers (GNC plain), S6S-GNC, 7.5% mol ratio S6S-GNC-P, and 15% mol ratio GNC-P under 37°C incubation for 5 min (A), 15 min (C), 30 min (E), and 1 hr (G). The fluorescent means and standard deviations are plotted for the time points of 5 min (B), 15 min (D), 30 min (F), and 1 hr (H). * p<0.05, **p<0.01, ***p<0.001. Results are shown as mean ± SD (n=3).
5.3.7. Agarose Gel Electrophoretic Mobility Shift Assay for Stability Assessments

An agarose gel electrophoretic mobility shift assay (gel retardation assay) was performed to determine the affinity between the STAT6 siRNA and the gelatin polymeric nanocarrier [1251]. To evaluate that the selected siRNA concentrations within the prepared S6S-GNC-P required for Th2 in vitro STAT6 protein downregulation analysis, a gel electrophoretic mobility shift assay was performed (Figure 5.28). The STAT6 siRNA that we have selected for this formulation is resistant to serum degradation as discussed by Healey et al. [1056, 1057]. This excludes the potential of siRNA degradation during the running of the gel, which would affect its gel shift. S6S-GNC-P were prepared containing different concentrations of siRNA, no siRNA (Placebo GNC-P), 1 nmol STAT6 siRNA, 4 nmol STAT6 siRNA, and 7 nmol STAT6 siRNA per 1 mL of 2% w/v gelatin during the sRNA polymer complexation stage before the desolvation step. The S6S-GNC-P were adjusted to identical volumes with DEPC treated RNAse free water, and then loaded onto a 1% ultra-low range agarose ethidium bromide gel and separated for 50 min at a constant voltage of 70 V. UV imaging revealed that the STAT6 siRNA at all concentration used was protected by the gelatin nanocarrier. This suggests that the concentration range needed for the in vitro work discussed here produces stable S6S-GNC-P with adequate N:P ratio. The N:P ratio is the theoretical charge ratio between the amino groups of gelatin and the phosphate groups of STAT6 siRNA [1252].

![Figure 5.28. Agarose gel electrophoretic mobility shift assay of cationic S6S-GNC-P. This data demonstrated that the gelatin nanocarriers entrap the STAT6 siRNA effectively.](image_url)
The results of the agarose gel electrophoretic mobility shift assay where the formulation was subjected to protease and RNase enzymes is shown in Figure 5.29. The STAT6 siRNA which was treated with protease (S6S + protease) was not affected and shown an intact band comparable to the siRNA control (S6S). The STAT6 siRNA treated with RNase I (S6S + RNase) resulted in degradation of the siRNA and similar results were seen in the lane containing STAT6 siRNA with protease and RNase (S6S + Protease + RNase). S6S-GNC-P was also subjected to protease and RNase. The S6S-GNC-P treated with protease alone (S6S-GNC-P + Protease) shows intact STAT6 siRNA that was released when the gelatin nanocarrier polymeric matrix was degraded by the protease. The S6S-GNC-P formulation treated with RNase (S6S-GNC + RNase), did not show an siRNA band, suggesting that the siRNA remained entrapped within the gelatin nanocarrier. The sequence of enzyme addition was either protease followed by RNase to degrade the matrix and release the STAT6 siRNA, or RNase followed by protease to show that the siRNA was physically encapsulated in the matrix and did not undergo degradation until protease was added. Lanes with S6S-GNC-P with protease and then RNase (S6S-GNC-P + Protease + RNase) appears to have less siRNA than the lane with the S6S-GNC-P with RNase and then protease (S6S-GNC-P + RNase + Protease), inferring that the pre-treatment with protease allowed for gelatin nanocarrier matrix degradation, and thus upon RNase addition, more siRNA was degraded because of the enzyme kinetics. The results suggest that the method of S6S-GNC-P preparation, nanoparticle crosslinking, surface PEGylation, and peptide modification did not have any detectable negative effects on the siRNA entrapped within the gelatin nanocarriers [1253].
Figure 5.29. Agarose gel electrophoresis shift assay to determine the stability of STAT6 siRNA entrapped within the gelatin nanocarrier matrix. This data demonstrates that the gelatin nanocarrier binds the siRNAs and effectively prevents them from RNAse degradation.

5.2.8. S6S-GNC and S6S-GNC-P Safety Determination by MTT Cell Proliferation Assay

The PEGylated S6S-GNC and peptide conjugated PEGylated S6S-GNC-P formulation are not expected to produce any significant cell cytotoxicity because they are composed of biocompatible and biodegradable gelatin [108]. In addition, the genipin crosslinker, PEG, IL4Rα targeting peptide, and STAT6 siRNA are not expected to cause significant cell cytotoxicity [133, 1056, 1254-1256]. The STAT6 siRNA was purified by the manufacturer to remove synthesis reagents, including ethanol and salts. Also, designed STAT6 siRNA has a total of 21 base pairs, so presumably it would not be capable of activating the nonspecific interferon response which causes cytotoxicity. Potentially, using high concentrations of siRNA (above 100 nM) could result in nonspecific (off-target) downregulation effects, so therefore an upper limit of 250 nM was selected to identify if the selected STAT6 siRNA had potential for off-target downregulation when loaded into the S6S-GNC or S6S-GNC-P. To test the safety of the STAT6 siRNA and the designed S6S-GNC and S6S-GNC-P formulation, a MTT cell viability assay was performed on Th2 cells.
treated with S6S-GNC (Figure 5.30 A) and S6S-GNC-P (Figure 5.30 B) for 48 hr at concentrations of 0-250 nM STAT6 siRNA equivalents.

The results of the MTT cell viability assay on the Th2 TIB-224 cells treated with varying levels of STAT6 siRNA equivalents of S6S-GNC and S6S-GNC-P formulations are shown in Figure 5.30 A and B, respectively. Results were statistically analyzed using Graphpad prism software, where a one-way ANOVA and Tukey’s post test revealed no significant differences in the data. This data suggests that the formulations, S6S-GNC (Figure 5.30 A) and S6S-GNC-P (Figure 5.30 B) at STAT6 siRNA equivalent concentrations of 25, 50, 75, 100, 125, 150, 175, 200, and 250 nM induced no significant (p>0.05) cell kill during the treatment time of 48 hr.

Figure 5.30. Cell viability of Th2 cells treated with different levels of STAT6 siRNA equivalents of S6S-GNC (A) and S6S-GNC-P (B) conducted at 48 hr post-treatment. No significant differences exist, p>0.05. Results are shown as mean ± SD (n=3). No statistically significant differences exist between the medium control and the gelatin nanocarrier formulations.
5.3.8. STAT6 Protein and mRNA Downregulation by S6S-GNC-P by Western Blot Protein Analysis and RT-PCR

First, S6S-GNC and S6S-GNC-P dose dependent effect on STAT6 protein downregulation was determined by Western blot analysis of Th2 cell lysates (Figure 5.31).

**Figure 5.31.** S6S-GNC and S6S-GNC-P dose dependent downregulation of STAT6 protein in Th2 cells. Representative Western blots of S6S-GNC treated Th2 cell lysates (A) and S6S-GNC-P treated Th2 cell lysates (B), as well as, densitometry analysis of the S6S-GNC bands (C) and of the S6S-GNC-P bands (D) are shown. Densitometry was performed by first normalizing each band with their corresponding beta actin loading controls. Densitometry results are shown relative to the scrambled siRNA control. *p<0.05, **p<0.01, and ***p<0.001. Data are reported as mean ± SD (n=3).

The S6S-GNC-P formulation obtained an approximate IC$_{50}$ at 100 nM STAT6 siRNA equivalents (Figure 5.31 B and D). For the S6S-GNC formulation, a statistically significant (p<0.001) downregulation in STAT6 protein occurred following treatment with a 25 nM STAT6 siRNA equivalent concentration of S6S-GNC compared to the scrambled siRNA loaded PEGylated gelatin nanocarrier control (Figure 5.31 A and C). This increased STAT6 protein downregulation suggests that the lower concentration of S6S-GNC used was better at cellular...
uptake than at concentrations of 50, 100, or 150 nM STAT6 siRNA equivalents. However, at 200 nM STAT6 siRNA equivalent concentration of S6S-GNC, there was a significant (p<0.05) downregulation in STAT6 protein expression. At a concentration of 200 nM STAT6 siRNA equivalents of PEGylated S6S-GNC, there appears to be some STAT6 siRNA downregulation despite the inferred poor cellular uptake. As shown in Figure 5.31 B and D, the S6S-GNC-P had significantly increased STAT6 protein downregulation at STAT6 siRNA equivalent concentration of 25 nM (p<0.01), and at concentrations of 50-200 nM (p<0.001) compared to the scrambled siRNA loaded peptide conjugated gelatin nanocarrier. An increase in the concentration to 150 nM or 200 nM STAT6 siRNA equivalent of S6S-GNC-P seemed to saturate the nanoparticle cellular uptake or RNAi machinery, as evidenced by the reduced STAT6 downregulation in these treatment groups (Figure 5.31 B and D). The reduced STAT6 protein downregulation of the S6S-GNC, in comparison, suggests that the PEGylation density is inhibiting the Th2 cellular internalization of these particles (Figure 5.31 A and C) [1257].

The protein lysates of Th2 cells treated with 100 nM STAT6 siRNA equivalents were analyzed by Western blot for STAT6 protein expression (Figure 5.32 A) and the bands densitometry was performed (Figure 5.32 B). The STAT6 siRNA sequence selected for development was designed by Healey et al., who developed an in silico and in vitro system to screen the STAT6 gene for siRNA targeting and identified siRNA sequences for pre-clinical development [1057]. The modified siRNA, having an IC50 value of 0.4 nM in treated A549 cells, was found to have improved in vivo circulation, without loss of STAT6 knockdown efficiency or induction of interferon responses [1057]. The difference in IC50 values may be due to the use of different cells lines, different cell plating densities, or different transfection reagents. The STAT6 siRNA RNAiMax transfection positive control showed a significant (p<0.001) downregulation in STAT6 protein expression. The STAT6 protein downregulation following 72 hr treatment with 100 nM STAT6 siRNA equivalents of the PEGylated S6S-GNC formulation did not lead to a significant downregulation of STAT6 protein expression, agreeing with the poor cellular binding and internalization results shown above. The S6S-GNC-P formulation at a concentration of 100 nM STAT6 siRNA equivalents had a statistically significant (p<0.001) effect on the STAT6 protein expression. Total cell RNA was extracted from Th2 cells treated in a parallel experiment. The results of the relative STAT6 mRNA expression normalized to β-actin an expressed relative to the medium control are shown in Figure 5.33.
Figure 5.32. Western blot analysis of STAT6 in Th2 cells after 100 nM STAT6 siRNA treatment (A), quantification by densitometry of STAT6 protein expression normalized using β-actin (B), and STAT6 relative mRNA expression in Th2 cells following 50 nM STAT6 siRNA treatments. Data are presented as mean ± SD (n=3). *** p<0.001.

Figure 5.33. RT-PCR on total RNA extracts from Th2 cells treated with 100 nM equivalents of S6S-GNC and S6S-GNC-P using medium and scrambled siRNA controls were normalized to beta actin mRNA expression. ** p<0.01 and *** p<0.001. Data are presented as mean ± SD (n=3).
Stable, sustained release, and effective S6S-GNC-P with average particle size of 98 ± 3.6 nm, DPI of 0.66, zeta potential of +6.3 ± 0.54 mV, and 19% LE% were successfully prepared. The S6S-GNC-P were able to bind and facilitate the uptake within IL4Rα expressing Th2 cells to downregulate STAT6 protein and mRNA expression significantly at a 100 nM STAT6 siRNA equivalent concentration of formulation. The developed S6S-GNC-P potentially could become a viable candidate for in vivo asthma mouse model studies for development of a novel safe and effective nanoparticle-based asthma therapy. The results of the inhalable Gem-GNC aerodynamic analysis suggest stability of a gelatin nanocarrier formulation under nebulization and therefore suggests the potential use of S6S-GNC-P for inhalation delivery.
CHAPTER 6. SUMMARY AND FUTURE DIRECTIONS

6.1. Summary

Stable and effective non-PEGylated STAT6 siRNA gelatin nanocarriers (S6S-GNC) formulation with a particle size of < 80 nm and encapsulation efficiency of > 85% were successfully developed. In addition, the formulation was found to be stable in presence of buffers solutions, serum solution and RNase. The S6S-GNC formulation showed sustained release of S6S, which is highly desirable considering long term effect of formulation with allowing for reduced dosing intervals. S6S-GNC evaluated in A549 lung cancer cell line inferred significantly (p<0.001) higher percent cell kill compared to that of medium control and STAT6 Lipofectamine complex. The cell internalization studies showed that the developed gelatin nanocarrier formulation gets rapidly internalized within cells within 15 min and these results support the successful delivery of siRNA within tumor cells. The Western blot results confirmed the downregulation of STAT6 protein expression by developed S6S-GN formulation in A549 cells. The developed S6S-GNCs were found to be effective in protecting STAT6 siRNA from degradation and were able to deliver STAT6 siRNA within tumor cells to exert anticancer activity.

Controlled release Gem-GNC with particle size of approximately 180 nm, EE% >90%, and LE% of ~9% were successfully developed for pulmonary delivery by the inhalation route of administration. The target particle size value was 150 nm, so the employed predicted equation generated via linear regression modeling for particle size led to the volume ratio of 7:10 of 90% v/v ethanol, which was selected by fixing the gelatin and genipin concentrations to 1% w/v and 0.02% w/w, respectively. The SEM and TEM images had shown that the developed Gem-GNC were uniform in particle size and were of a smooth spherical morphology. The Gem-GNC particle size measured from the SEM micrograph is 229 ± 68 nm, while the particle size measured from this TEM micrograph is 197 ± 18 nm. The Taguchi analysis determined that genipin concentration was the most influential parameter on particle size since it had the largest S/N ratio range. DSC of lyophilized Gem-GNC indicated that the Gem and excipients where molecularly dispersed. PXRD analysis of the lyophilized Gem-GNC found that Gem within the Gem-GNC had an amorphous configuration. The formulation was found to be stable in the presence of pH 6.4-8.4 DPBS solutions. The release mechanisms of Gem from the Gem-GNC were found to be non-Fickian diffusion and erosion from a matrix-based nanocarrier. The Gem-GNC exhibited a controlled release of Gem, which is highly desirable for the long term constant delivery of the formulation
with reduced dosing intervals. The nebulized Gem-GNC exhibited a mass median aerodynamic diameter (MMAD) of 2.0 ± 0.16 µm, geometric standard deviation (GSD) of 2.7 ± 0.16, and fine particle fraction (FPF) of 75.2 ± 2.4%. A549 cells treated with Gem-GNC obtained IC$_{50}$ of 0.023 µM, however the Gem solution obtained an IC$_{50}$ of 0.013 µM at 72 hr. The H460 cells treated with Gem-GNC obtained 5–fold lower IC$_{50}$ at 48 hr when compared to the Gem solution, whereas at 72 hr the IC$_{50}$ for Gem-GNC was 10-fold lower than the IC$_{50}$ of Gem solution (** p<0.01). The developed Gem-GNC was found to be effective in protecting Gem from degradation and was able to delivery Gem within the tumor cells to exert anticancer activity. The development and evaluation of the Gem-GNC provides evidence that an aerosolized GNC approach may be useful for the delivery of therapeutics to the lungs, possibly for lung cancer treatment. More studies are warranted to fully illustrate the safety profile in order to form risk and benefit comparisons.

A novel formulation, S6S-GNC-P, for the targeted delivery of STAT6 siRNA to Th2 cells was successfully optimized and evaluated within in vitro studies. This formulation was intended to be delivered by the intravenous route of administration, however the previous study of aerosolized Gem-GNC depicts the potential of pulmonary delivery of S6S-GNC-P. More studies are necessary to evaluate the effects of pulmonary administered gelatin, because it has potential for antigenic or immunogenic effects in the highly sensitive lungs of asthmatic patients. The formulation was optimized using Taguchi orthogonal array design of experiments, regression analysis, and desirability factor determination. This lead to optimized formulation parameters of 2% w/v gelatin, 50% v/v acetone addition, and 0.02% w/v genipin addition. STAT6 siRNA was incorporated into the formulation in-place of APAP and the gelatin nanocarriers were PEGylated using carbodiimide crosslinking of a maleimide terminated PEG5000. The S6S-GNC were then peptide conjugated using a cysteamide containing IL4R targeted peptide forming a stable thioether linkage using 15% mol ratio of or approximately 720 peptides per gelatin nanocarrier as optimized by efficient STAT6 protein downregulation with increased binding and internalization efficiencies suggested by flow cytometry binding and internalization assays. The S6S-GNC-P had a particle size of 98 ± 3.6 nm, PDI of 0.66 ± 0.02, zeta potential of +6.3 ± 0.54, EE% > 90%, and LE% of 19%. SEM and TEM confirmed the freeze dried and dispersed gelatin nanocarrier spherical morphology and particle size. The siRNA release kinetics from the S6S-GNC-P was best described by the Baker-Lonsdale and Weibull models, suggesting that the mechanism of release is diffusion through a matrix-based spherical system. The Korsemeyer-Peppas power law suggested a Case II
transport mechanism given a spherical particle. The S6S-GNC was found to be stable in the presence of simulated physiological media and under electrophoretic mobility assays in the presence of RNase.

Modified STAT6 siRNA and RNAiMax concentrations were optimized at 150 nM siRNA using 0.25% v/v Lipofectamine RNAiMax transfection agent. The S6S-GNC-P dose dependency studies found that 100 nM STAT6 siRNA equivalent doses were superior than the other concentrations studied for maximal STAT6 protein downregulation. The surface peptide density range for the flow cytometry binding assay was selected to range between 7.5-30% mol ratio of peptide to PEG (360-1400 copies of peptide per gelatin nanocarrier) using ELISA STAT6 protein quantification following a 48 hr treatment with 75 nM STAT6 siRNA equivalents of S6S-GNC and S6S-GNC-P formulation. The binding assay results had shown that the peptide concentration of 7.5 % mol ratio peptide to PEG (360 peptides per gelatin nanocarrier) had significantly higher Th2 binding than with the higher concentrations of 10-30% mol ratio peptide to PEG during a 1 hr incubation at 4°C. However, at a 4 hr incubation at 4°C, the 15% mol ratio peptide to PEG S6S-GNC (720 copies of peptide per gelatin nanocarrier) had increased cellular binding than the other formulations, though not significantly (p>0.05). Therefore, for the flow cytometry internalization assays, peptide concentrations of 7.5% and 15% mol ratio peptide to PEG S6S-GNC-P (360 and 720 copies of peptide per gelatin nanocarrier) were selected to analyze their cellular internalization in comparison with the medium control, unmodified plain S6S-GNC, and PEGylated S6S-GNC. The cellular internalization assay determined that 15% mol ratio of peptide to PEG on S6S-GNC-P, or 720 copies of peptide on each gelatin nanocarrier, was the best concentration for the in vitro efficacy evaluation in Th2 cells.

The dose dependent efficacy of the S6S-GNC-P formulation on the STAT6 protein downregulation was determined by Western blot of the protein lysates collected from Th2 cells following a 72 hr treatment with scrambled siRNA loaded PEGylated gelatin nanocarriers and scrambled siRNA loaded PEGylated and peptide conjugated gelatin nanocarriers as controls to the S6S-GNC and S6S-GNC-P at 25-200 nM STAT6 siRNA equivalents. The S6S-GNC-P formulation had an EC50 value of 100 nM under these treatments within the TIB-224 Th2 cell line. While the S6S-GNC-P did not impart any significant cell cytotoxicity with Th2 cells, they did promote statistical significant STAT6 protein downregulation (p<0.001) compared to the medium, STAT6 siRNA positive, and Scrambled-GNC-P controls, as well as the PEGylated S6S-
GNC. The STAT6 mRNA expression levels as determined by RT-PCR following treatment with S6S-GNC-P formulation at a 100 nM STAT6 siRNA equivalent concentration had a statistically significant STAT6 mRNA downregulation (p<0.01) when compared to the medium control and the PEGylated S6S-GNC treated cells (p<0.001). This development of a STAT6 siRNA loaded gelatin nanocarrier, targeted to pathophysiological biomarker targets in asthma, represents the first critical steps in the pre-clinical development of a newer asthma therapy.

6.2. Future Directions

The optimized S6S-GNC-P require further efficacy evaluation using B and co-culture cell based assay and in vivo asthma models. The uptake of S6S-GNC-P will be compared between a mixture of normal airway epithelial cells with the GFP expressing Th2 cells to demonstrate specificity of targeting of S6S-GNC-P. IL4Rα is highly expressed on Th2 cells and IL4Rα binding peptide conjugation on the surface of PEGylated S6S-GNC will specifically deliver S6S-GNC–P to cells involved in asthma progression via IL4R endocytosis. The dose dependent efficacy of S6S-GNC-P will be investigated using Th2 cell differentiation assay under pre- and post-cytokine exposure.

Pharmacokinetic studies will determine the extent of targeting and improvement in the biodistribution of STAT6 siRNA. PEGylation of nanocarriers will prolong circulation life time by avoiding reticuloendothelial clearance. The use of Cy5.5 conjugated STAT6 siRNA may interfere in the biological effect and biodistribution and therefore, we cy5.5 amine conjugated gelatin will be used. The dose dependent efficacy of the S6S-GNC-P will be evaluation in pre- and post-allergen exposure based mouse model of asthma to determine the therapeutic and prophylactic effect. The targeted delivery of STAT6 siRNA using clinically validated biopolymer based S6S-GNC-P will selectively deliver STAT6 siRNA to effectively silence the STAT6 and exert anti-asthmatic effects. Furthermore, the transcriptional gene silencing of STAT6 will downregulate GATA3 mediated production of IgE, mast cells, eosinophils and basophils, which are chief mediators of asthma. The safety and mechanism of action of S6S-GNC-P will also be evaluated. These studies will confirm the therapeutic superiority of IL4Rα targeting nanocarriers compared to passively targeted formulation (S6S-GNC) and will also avoid off-site effects. These studies will establish the importance of receptor guided nanocarriers and STAT6 blockade in the treatment and/or prevention of asthma. The S6S-GNC-P may be further evaluated in dust-mite/cockroach allergen based asthma models followed by clinical trials. These studies offer high impacts on
human health by providing a framework for preclinical and human clinical settings after attaining successful outcomes in mouse models.

The data produced in this dissertation work provides important information about an innovative way to combat asthma via a new targeted delivery approach that will be used for the site-specific delivery of S6S to T cells, as well as other cells mitigating asthma pathogenesis and pathophysiology. Thus, this research work has provided unique strategy to drive the project forward will concentrate on further optimization of the delivery system, siRNA panel delivery expansion and associated mechanistic studies. Due to the versatile applicability, this delivery system may also be adapted to deliver other siRNAs with allied roles in asthma and allergy. Furthermore, the IL4Rα targeting peptide may be replaced by other innovative class of cell target synthetic peptide and a comparative assessment of targeting potential of NC under influence of IL4Rα peptide and IL4Rα antibody ligands may be deduced under similar sets of conditions to establish as well as affirm potency with targeted NC.

These proposed studies offer high impact on human health by providing framework for preclinical and human clinical settings after attaining successful outcomes in mouse models. For future human clinical trials, the human IL4Rα specific peptide (Met-His-Lys-Cys-Asp; Genscript, USA) will be employed to conjugate on surface of GNC to deliver the S6S selectively to T immune cells). As an alternate approach human monoclonal IL4Rα antibody (Miltenyi Biotec Inc. USA) will also be engaged for formulation of GNC for targeting IL4Rα receptors on T immune cells. The use of clinically validated polymer will facilitate the translation of developed nanocarriers for further human use. For future clinical trials, the human recombinant gelatin (Fibrogen Inc.) and the human IL4Rα specific peptide (MHKCD; Genscript, USA) will be employed to conjugate on surface of STAT6 siRNA loaded nanocarriers. As an alternate approach human monoclonal IL4Rα antibody (Miltenyi Biotec Inc. USA) will also be engaged for formulation of nanocarriers. Furthermore, the S6S-GNC-P may be further developed for pulmonary delivery and the aerosolized nanocarriers will be evaluated for their aerodynamic properties and efficacy. The results of the aerosolized S6S-GNC-P and the intravenously delivered formulation will be compared their effects in asthma models. The S6S-GNC-P will also be tested in combination with FDA-approved asthma therapies, such as leukotriene inhibitors and bronchodilators. It will also be interesting to test the S6S-GNC-P in combination with IL4 or IL13 receptor antagonists.
APPENDIX A: PUBLICATIONS

Peer-Reviewed Articles


Book Chapter
### Appendix B: Data for Objective 1

- **Started with a 1% for the 1 step desolvation**
- **Dec-12**
- **Started with a 5% for the 2 and 2+ step desolvation (for HMW and MMW fractions)**
- **Initially ignored the HMMW and LMMW fractions**

<table>
<thead>
<tr>
<th></th>
<th>2-step desolvation using HMW fraction (1% w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HMW</strong></td>
<td></td>
</tr>
<tr>
<td>no xlink</td>
<td>ps (nm)1  ps (nm)2  ps ave (nnm ps std dev)</td>
</tr>
<tr>
<td>pH 5.4</td>
<td>265.8  247.9  256.85  12.65721  244  214.4  229.2  20.93036  0.843  0.748  0.7955  0.067175</td>
</tr>
<tr>
<td>pH 6.4</td>
<td>266.7  256.7  261.7  7.071068  222.9  219  220.95  2.757716  0.699  0.728  0.7135  0.020506</td>
</tr>
<tr>
<td>pH 7.4</td>
<td>340.8  338.8  339.8  1.414214  280.8  295.8  288.3  10.6066  0.679  0.762  0.7205  0.05869</td>
</tr>
<tr>
<td>pH 8.4</td>
<td>288.8  292.1  290.45  2.333452  241.7  247.1  244.4  3.818377  0.701  0.716  0.7085  0.010607</td>
</tr>
<tr>
<td>10% FBS</td>
<td>708.3  848  778.15  98.78282  385.3  554.6  469.95  119.7132  0.296  0.428  0.362  0.093338</td>
</tr>
<tr>
<td>purified w</td>
<td>740.7  728  734.35  8.980256  399.3  363.3  381.3  25.45584  0.291  0.249  0.27  0.029698</td>
</tr>
</tbody>
</table>

| xlink          | ps (nm)1  ps (nm)2  ps ave (nnm ps std dev) | std dev1  std dev2  ave pop st dev pop var PI 1 | var PI2  ave var PI  std dev vazeta (mV) |
|----------------|-----------------------------------------------|
| pH 5.4         | 5.4 151.1  145.3  148.2  4.101219  122.7  114.7  118.7  5.656854  0.659  0.623  0.641  0.025456 |
| pH 6.4         | 6.4 127.8  123.3  125.5  3.181981  109.3  103.2  106.25  4.313351  0.731  0.701  0.716  0.021213 |
| pH 7.4         | 7.4 124.3  121.1  122.7  2.262742  104.3  99.5  101.9  3.394113  0.704  0.676  0.69  0.019799 |
| pH 8.4         | 8.4 116  117.7  116.85  1.202082  93.6  96.5  95.05  2.05061  0.651  0.672  0.6615  0.014849 |
| 10% FBS        | 10% FBS 154.7  92.4  123.55  44.05275  125.4  79.6  102.5  32.38549  0.658  0.743  0.7005  0.060104 |
| H2O            | H2O 396.9  369.8  383.35  19.16259  239.3  244.8  242.05  3.889087  0.364  0.438  0.401  0.052326 |

<table>
<thead>
<tr>
<th></th>
<th>2-step desolvation using HMW fraction (1% w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MMW</strong></td>
<td></td>
</tr>
<tr>
<td>no xlink</td>
<td>ps (nm)1  ps (nm)2  ps ave (nnm ps std dev)</td>
</tr>
<tr>
<td>pH 5.4</td>
<td>5.4 229.3  188  208.65  29.20351  191.3  156.6  173.95  24.53661  0.696  0.694  0.695  0.001414</td>
</tr>
<tr>
<td>pH 6.4</td>
<td>6.4 198.2  202.7  200.45  3.181981  170  175.8  172.9  4.101219  0.736  0.752  0.744  0.011314</td>
</tr>
<tr>
<td>pH 7.4</td>
<td>7.4 233.1  258.1  245.6  17.67767  201.2  215  208.1  9.758074  0.745  0.694  0.7195  0.036062</td>
</tr>
<tr>
<td>pH 8.4</td>
<td>8.4 241.1  205.5  223.3  25.173  196.3  175.3  185.8  14.84924  0.663  0.728  0.6955  0.045962</td>
</tr>
<tr>
<td>10% FBS</td>
<td>10% FBS 338.2  193.2  265.7  102.5305  235.7  160  197.85  53.52798  0.486  0.686  0.586  0.141421</td>
</tr>
<tr>
<td>H2O</td>
<td>H2O 646.1  658.7  652.4  8.909545  320.5  334.6  327.55  9.970206  0.246  0.258  0.252  0.008485</td>
</tr>
</tbody>
</table>

| xlink          | ps (nm)1  ps (nm)2  ps ave (nnm ps std dev) | std dev1  std dev2  ave pop st dev pop var PI 1 | var PI2  ave var PI  std dev vazeta (mV) |
|----------------|-----------------------------------------------|
| pH 5.4         | 5.4 150.1  148.9  149.5  0.848528  114.8  113.8  114.3  0.707107  0.585  0.584  0.5845  0.000707 |
| pH 6.4         | 6.4 146.2  143.6  144.9  1.838478  112.3  110  111.15  1.626346  0.59  0.587  0.5885  0.002121 |
| pH 7.4         | 7.4 135.3  136.9  136.1  1.131371  107.4  108.6  108  0.848528  0.63  0.629  0.6295  0.000707 |
| pH 8.4         | 8.4 122.4  121.5  121.95  0.636396  104.4  97.3  100.85  5.020458  0.728  0.642  0.685  0.068011 |
| 10% FBS        | 10% FBS 76  92.2  84.1  11.45313  63.6  76.6  70.1  1.932388  0.699  0.689  0.694  0.007071 |
| H2O            | H2O 337.2  294.4  315.8  30.26417  184.5  205.8  195.15  15.06137  0.299  0.489  0.394  0.13435 |
2-step desolvation using HMW fraction (1% w/v)
Compare the whole, HMW, and MMW gelatin fractions in their particle size and entrapment efficiency (%).

20 mg APAP used for 1% gelatin solution

<table>
<thead>
<tr>
<th>Buffer pH or medium type</th>
<th>WHOLE no xlink</th>
<th>WHOLE MMW xlink</th>
<th>MMW no xlink</th>
<th>MMW xlink</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.4</td>
<td>109.8</td>
<td>109.95</td>
<td>112.4</td>
<td>110.9</td>
</tr>
<tr>
<td>6.4</td>
<td>112</td>
<td>110</td>
<td>108</td>
<td>107.2</td>
</tr>
<tr>
<td>7.4</td>
<td>109</td>
<td>109.95</td>
<td>110</td>
<td>108.7</td>
</tr>
<tr>
<td>8.4</td>
<td>95.1</td>
<td>101.5</td>
<td>98.3</td>
<td>112.4</td>
</tr>
<tr>
<td>10% FBS</td>
<td>93.6</td>
<td>89.1</td>
<td>91.35</td>
<td>109.95</td>
</tr>
<tr>
<td>H2O</td>
<td>205.6</td>
<td>205.6</td>
<td>205.6</td>
<td>205.6</td>
</tr>
<tr>
<td>washed, 10% FBS pH7.4</td>
<td>100.3</td>
<td>98</td>
<td>99.15</td>
<td>99.15</td>
</tr>
<tr>
<td>pH 5.4</td>
<td>87.4</td>
<td>86.7</td>
<td>87.05</td>
<td>87.05</td>
</tr>
<tr>
<td>pH 6.4</td>
<td>83.6</td>
<td>84</td>
<td>83.8</td>
<td>83.8</td>
</tr>
<tr>
<td>pH 7.4</td>
<td>80.7</td>
<td>84</td>
<td>82.35</td>
<td>82.35</td>
</tr>
<tr>
<td>pH 8.4</td>
<td>72.1</td>
<td>73.7</td>
<td>72.9</td>
<td>72.9</td>
</tr>
<tr>
<td>10% FBS</td>
<td>65.4</td>
<td>60.4</td>
<td>62.9</td>
<td>62.9</td>
</tr>
<tr>
<td>H2O</td>
<td>213.4</td>
<td>206.4</td>
<td>209.9</td>
<td>209.9</td>
</tr>
<tr>
<td>washed, 10% FBS pH7.4</td>
<td>82.2</td>
<td>80.8</td>
<td>81.5</td>
<td>81.5</td>
</tr>
<tr>
<td>pH 5.4</td>
<td>86.5</td>
<td>88.3</td>
<td>87.4</td>
<td>87.4</td>
</tr>
<tr>
<td>pH 6.4</td>
<td>92.3</td>
<td>83.2</td>
<td>87.75</td>
<td>87.75</td>
</tr>
<tr>
<td>pH 7.4</td>
<td>83.6</td>
<td>87.1</td>
<td>85.35</td>
<td>85.35</td>
</tr>
<tr>
<td>pH 8.4</td>
<td>75.7</td>
<td>75.1</td>
<td>75.4</td>
<td>75.4</td>
</tr>
<tr>
<td>10% FBS</td>
<td>59.7</td>
<td>54.9</td>
<td>57.3</td>
<td>57.3</td>
</tr>
<tr>
<td>H2O</td>
<td>256.4</td>
<td>237.9</td>
<td>247.15</td>
<td>247.15</td>
</tr>
<tr>
<td>washed, 10% FBS pH7.4</td>
<td>66.7</td>
<td>74.6</td>
<td>70.65</td>
<td>70.65</td>
</tr>
</tbody>
</table>

Analysis Medium/Gelatin fraction vs. Particle Size

Particle Size (nm)
Analysis Medium/Gelatin fraction vs. Particle Size
APAP-GNC-A

Polydispersity Index (PDI) %

Particle size (nm)

<table>
<thead>
<tr>
<th>Analysis Medium/Gelatin fraction</th>
<th>Whole PS</th>
<th>HMW PS</th>
<th>MMW PS</th>
<th>Whole PDI</th>
<th>HMW PDI</th>
<th>MMW PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 5.4</td>
<td>112.4</td>
<td>87.05</td>
<td>87.4</td>
<td>0.574</td>
<td>0.5625</td>
<td>0.7005</td>
</tr>
<tr>
<td>pH 6.4</td>
<td>110</td>
<td>83.8</td>
<td>87.75</td>
<td>0.5785</td>
<td>0.616</td>
<td>0.681</td>
</tr>
<tr>
<td>pH 7.4</td>
<td>109.95</td>
<td>82.35</td>
<td>85.35</td>
<td>0.6005</td>
<td>0.596</td>
<td>0.6805</td>
</tr>
<tr>
<td>pH 8.4</td>
<td>98.3</td>
<td>72.9</td>
<td>75.4</td>
<td>0.6</td>
<td>0.5755</td>
<td>0.6295</td>
</tr>
<tr>
<td>10% FBS</td>
<td>91.35</td>
<td>62.9</td>
<td>57.3</td>
<td>0.631</td>
<td>0.611</td>
<td>0.69</td>
</tr>
<tr>
<td>H2O</td>
<td>205.6</td>
<td>209.9</td>
<td>247.15</td>
<td>0.4785</td>
<td>0.5715</td>
<td>0.4685</td>
</tr>
<tr>
<td>washed, 10% FBS + pH 7.4</td>
<td>99.15</td>
<td>81.5</td>
<td>70.65</td>
<td>0.647</td>
<td>0.637</td>
<td>0.794</td>
</tr>
</tbody>
</table>
ALL 1 step desolvation

Constants: 600 rpm stir rate, xlink was performed 5 min after etoh addition, constant stirring for 1 hour, then the stir rate was dropped to 200 rpm until etoh evaporated

<table>
<thead>
<tr>
<th>pH 5.4</th>
<th>PH 6.4</th>
<th>PH 7.4</th>
<th>PH 8.4</th>
<th>10% FBS</th>
<th>H2O</th>
<th>washed, 10% FBS</th>
<th>pH 7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>no xlink</td>
<td>ps (nm) 1</td>
<td>ps (nm) 2</td>
<td>ps ave (nm)</td>
<td>ps std dev</td>
<td>std dev 1</td>
<td>std dev 2</td>
<td>ave pop std</td>
</tr>
<tr>
<td>5.4</td>
<td>92.7</td>
<td>98.4</td>
<td>95.5</td>
<td>4.030509</td>
<td>75.5</td>
<td>83.3</td>
<td>78.9</td>
</tr>
<tr>
<td>6.4</td>
<td>90.3</td>
<td>84.6</td>
<td>87.45</td>
<td>4.030509</td>
<td>76.8</td>
<td>69.7</td>
<td>73.25</td>
</tr>
<tr>
<td>7.4</td>
<td>88.1</td>
<td>81.1</td>
<td>84.6</td>
<td>4.049747</td>
<td>74.2</td>
<td>64.6</td>
<td>69.4</td>
</tr>
<tr>
<td>8.4</td>
<td>79.1</td>
<td>76.2</td>
<td>77.65</td>
<td>2.05061</td>
<td>61.6</td>
<td>61.7</td>
<td>61.65</td>
</tr>
<tr>
<td>10% FBS</td>
<td>43.1</td>
<td>51.9</td>
<td>47.5</td>
<td>6.22254</td>
<td>33.9</td>
<td>45.4</td>
<td>39.65</td>
</tr>
<tr>
<td>H2O</td>
<td>301.4</td>
<td>354.5</td>
<td>327.95</td>
<td>37.54737</td>
<td>276.4</td>
<td>319</td>
<td>297.7</td>
</tr>
</tbody>
</table>

| xlink | ps (nm) 1 | ps (nm) 2 | ps ave (nm) | ps std dev | std dev 1 | std dev 2 | ave pop std | stdev pop std | stdev pop | PI 1 | ave PI 2 | var PI 2 | ave var PI | std dev var | zeta (mV) |
|-------|--------|--------|-------------|------------|-----------|-----------|-------------|----------------|----------|------|--------|--------|----------|-----------|-------------|----------|
| 5.4 | 83 | 84.5 | 83.75 | 1.06066 | 61.1 | 61.6 | 61.35 | 0.35355339 | 0.542 | 0.531 | 0.5365 | 0.007778175 | -1.78 |
| 6.4 | 83.3 | 79.6 | 81.45 | 2.616295 | 62.8 | 56.9 | 59.85 | 4.1713001 | 0.569 | 0.511 | 0.54 | 0.041012193 | 0.03 |
| 7.4 | 81.5 | 78 | 79.75 | 2.474874 | 59.7 | 55.7 | 57.7 | 2.82842712 | 0.537 | 0.51 | 0.5235 | 0.019091883 | -0.14 |
| 8.4 | 77.7 | 78.7 | 78.2 | 0.707107 | 55.3 | 56.3 | 55.8 | 0.70710678 | 0.507 | 0.511 | 0.509 | 0.002828427 | 0.09 |
| 10% FBS | 65.3 | 57.4 | 61.35 | 5.586144 | 56.3 | 44.6 | 50.45 | 8.27314934 | 0.741 | 0.604 | 0.6725 | 0.096873629 | -0.18 |
| H2O | 141.3 | 156.8 | 149.05 | 10.96016 | 106.1 | 114.6 | 110.35 | 6.01040764 | 0.564 | 0.534 | 0.549 | 0.021213203 | 6.87 |
1-Step Desolvation of Type A gelatin (1% w/v) with EtOH

- Polydispersity Index (PDI)
- Particle size (nm)
- SD
- Buffer pH
- 5.4, 6.4, 7.4, 8.4
- 0, 20, 40, 60, 80, 100, 120

1-Step Desolvation of Type A gelatin (1% w/v) with EtOH

- Polydispersity Index (PDI)
- Particle size (nm)
- SD
- Buffer pH
- 5.4, 6.4, 7.4, 8.4, 10% FBS, H2O
- 0, 50, 100, 150, 200, 250, 300, 350, 400
<table>
<thead>
<tr>
<th>pH</th>
<th>PS (nm) ± SD</th>
<th>Zeta (mV)</th>
<th>pH</th>
<th>PS (nm) ± SD</th>
<th>Zeta (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.4</td>
<td>8.12</td>
<td>6.34</td>
<td>5.4</td>
<td>93.472</td>
<td>3.99</td>
</tr>
<tr>
<td>5.4</td>
<td>n/a</td>
<td>7.33</td>
<td>6.4</td>
<td>115.4</td>
<td>2.61</td>
</tr>
<tr>
<td>6.4</td>
<td>4.89</td>
<td>3.65</td>
<td>7.4</td>
<td>151</td>
<td>1.69</td>
</tr>
<tr>
<td>7.4</td>
<td>n/a</td>
<td>3.65</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**zeta vs pH**

\[ y = -1.585x + 9.96 \]

\[ y = -1.533x + 7.49 \]

**GNC**

<table>
<thead>
<tr>
<th>pH</th>
<th>Particle Size (nm)</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.4</td>
<td>100</td>
<td>3</td>
</tr>
<tr>
<td>5.4</td>
<td>150</td>
<td>2.5</td>
</tr>
<tr>
<td>6.4</td>
<td>200</td>
<td>2</td>
</tr>
<tr>
<td>7.4</td>
<td>300</td>
<td>1</td>
</tr>
</tbody>
</table>
Gelatin type A --1% (w/v)-- one step desolvation

Stir rate, ethanol and GTA variation

<table>
<thead>
<tr>
<th>stir rate</th>
<th>Batch ID</th>
<th>Batch vol (ml)</th>
<th>ETOH (mL)</th>
<th>10% GTA (ul)</th>
<th>particle size (nm)</th>
<th>Stnd Dev (nm)</th>
<th>Var (P.I.)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>300 RPM</td>
<td>1a</td>
<td>10</td>
<td>8</td>
<td>100</td>
<td>194.1</td>
<td>163.6</td>
<td>0.711</td>
<td>11.27</td>
</tr>
<tr>
<td></td>
<td>1b</td>
<td>10</td>
<td>8</td>
<td>150</td>
<td>198.7</td>
<td>169.9</td>
<td>0.731</td>
<td>3.51</td>
</tr>
<tr>
<td></td>
<td>1c</td>
<td>10</td>
<td>8</td>
<td>300</td>
<td>202.8</td>
<td>169.1</td>
<td>0.696</td>
<td>8.1</td>
</tr>
<tr>
<td></td>
<td>2a</td>
<td>10</td>
<td>9</td>
<td>100</td>
<td>222.8</td>
<td>195.4</td>
<td>0.769</td>
<td>1.34</td>
</tr>
<tr>
<td></td>
<td>2b</td>
<td>10</td>
<td>9</td>
<td>150</td>
<td>211.1</td>
<td>178.4</td>
<td>0.714</td>
<td>4.73</td>
</tr>
<tr>
<td></td>
<td>2c</td>
<td>10</td>
<td>9</td>
<td>300</td>
<td>216.3</td>
<td>181.9</td>
<td>0.672</td>
<td>7.42</td>
</tr>
<tr>
<td>600 RPM</td>
<td>3a</td>
<td>10</td>
<td>10</td>
<td>100 large particulates formed out of range</td>
<td>12.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3b</td>
<td>10</td>
<td>10</td>
<td>150</td>
<td>large particulates formed out of range</td>
<td>11.94</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3c</td>
<td>10</td>
<td>10</td>
<td>300</td>
<td>large particulates formed</td>
<td>12.83</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4a</td>
<td>10</td>
<td>8</td>
<td>100</td>
<td>359</td>
<td>251.7</td>
<td>0.491</td>
<td>12.97</td>
</tr>
<tr>
<td></td>
<td>4b</td>
<td>10</td>
<td>8</td>
<td>150</td>
<td>364.2</td>
<td>248</td>
<td>0.464</td>
<td>3.32</td>
</tr>
<tr>
<td></td>
<td>4c</td>
<td>10</td>
<td>8</td>
<td>300</td>
<td>361.3</td>
<td>234.1</td>
<td>0.604</td>
<td>12.38</td>
</tr>
<tr>
<td>900 RPM</td>
<td>5a</td>
<td>10</td>
<td>9</td>
<td>100</td>
<td>289.2</td>
<td>200.4</td>
<td>0.48</td>
<td>12.57</td>
</tr>
<tr>
<td></td>
<td>5b</td>
<td>10</td>
<td>9</td>
<td>150</td>
<td>268.6</td>
<td>185.6</td>
<td>0.477</td>
<td>6.91</td>
</tr>
<tr>
<td></td>
<td>5c</td>
<td>10</td>
<td>9</td>
<td>300</td>
<td>301.9</td>
<td>199.6</td>
<td>0.571</td>
<td>10.71</td>
</tr>
<tr>
<td></td>
<td>6a</td>
<td>10</td>
<td>10</td>
<td>100</td>
<td>132.5</td>
<td>99</td>
<td>0.558</td>
<td>13.89</td>
</tr>
<tr>
<td></td>
<td>6b</td>
<td>10</td>
<td>10</td>
<td>150</td>
<td>135.7</td>
<td>105.3</td>
<td>0.602</td>
<td>3.32</td>
</tr>
<tr>
<td></td>
<td>6c</td>
<td>10</td>
<td>10</td>
<td>300</td>
<td>133.9</td>
<td>105</td>
<td>0.617</td>
<td>12.38</td>
</tr>
<tr>
<td>1000 RPM</td>
<td>7a</td>
<td>10</td>
<td>8</td>
<td>100</td>
<td>111.6</td>
<td>84.9</td>
<td>0.579</td>
<td>12.97</td>
</tr>
<tr>
<td></td>
<td>7b</td>
<td>10</td>
<td>8</td>
<td>150</td>
<td>115.2</td>
<td>88.8</td>
<td>0.594</td>
<td>6.91</td>
</tr>
<tr>
<td></td>
<td>7c</td>
<td>10</td>
<td>8</td>
<td>300</td>
<td>121.1</td>
<td>97.2</td>
<td>0.645</td>
<td>10.71</td>
</tr>
<tr>
<td></td>
<td>8a</td>
<td>10</td>
<td>9</td>
<td>100</td>
<td>103.1</td>
<td>83.7</td>
<td>0.654</td>
<td>11.06</td>
</tr>
<tr>
<td></td>
<td>8b</td>
<td>10</td>
<td>9</td>
<td>150</td>
<td>102.9</td>
<td>82.3</td>
<td>0.638</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>8c</td>
<td>10</td>
<td>9</td>
<td>300</td>
<td>121.1</td>
<td>102.8</td>
<td>0.697</td>
<td>8.8</td>
</tr>
<tr>
<td></td>
<td>9a</td>
<td>10</td>
<td>10</td>
<td>100</td>
<td>359</td>
<td>251.7</td>
<td>0.491</td>
<td>12.57</td>
</tr>
<tr>
<td></td>
<td>9b</td>
<td>10</td>
<td>10</td>
<td>150</td>
<td>364.2</td>
<td>248</td>
<td>0.464</td>
<td>6.91</td>
</tr>
<tr>
<td></td>
<td>9c</td>
<td>10</td>
<td>10</td>
<td>300</td>
<td>361.3</td>
<td>234.1</td>
<td>0.604</td>
<td>10.71</td>
</tr>
</tbody>
</table>

Note: Stir rates: 100, 300, 500, 700 RPM

- cloudy
- large precip
<table>
<thead>
<tr>
<th>Stir Rate</th>
<th>Batch ID</th>
<th>Batch vol (ml)</th>
<th>EtOH (mL)</th>
<th>10% GTA (ul)</th>
<th>Particle size (nm)</th>
<th>sd dev</th>
<th>avg sd dev</th>
<th>ave PI</th>
<th>stdev pi</th>
<th>zeta</th>
<th>stdev on readout</th>
</tr>
</thead>
<tbody>
<tr>
<td>300 RPM</td>
<td>1a</td>
<td>10</td>
<td>8</td>
<td>100</td>
<td>194.6333333</td>
<td>3.827967</td>
<td>164.7666667</td>
<td>0.716333</td>
<td>11.27</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1b</td>
<td>10</td>
<td>8</td>
<td>150</td>
<td>196.8333333</td>
<td>13.66614</td>
<td>162.3333333</td>
<td>0.679</td>
<td>3.51</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1c</td>
<td>10</td>
<td>8</td>
<td>300</td>
<td>212.2333333</td>
<td>10.04805</td>
<td>180.9666667</td>
<td>0.726333</td>
<td>8.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2a</td>
<td>10</td>
<td>9</td>
<td>100</td>
<td>157.4666667</td>
<td>4.617719</td>
<td>128.4333333</td>
<td>0.665333</td>
<td>1.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2b</td>
<td>10</td>
<td>9</td>
<td>150</td>
<td>166.6333333</td>
<td>13.70752</td>
<td>137.180952</td>
<td>0.685333</td>
<td>4.73</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2c</td>
<td>10</td>
<td>9</td>
<td>300</td>
<td>154.1333333</td>
<td>18.5511</td>
<td>138.84202</td>
<td>0.812667</td>
<td>7.42</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3a</td>
<td>10</td>
<td>10</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3b</td>
<td>10</td>
<td>10</td>
<td>150</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3c</td>
<td>10</td>
<td>10</td>
<td>300</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>600 RPM</td>
<td>4a</td>
<td>10</td>
<td>8</td>
<td>100</td>
<td>134.0333333</td>
<td>1.604161</td>
<td>103.1966667</td>
<td>0.591667</td>
<td>13.89</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4b</td>
<td>10</td>
<td>8</td>
<td>150</td>
<td>115.9666667</td>
<td>7.961719</td>
<td>90.3596667</td>
<td>0.606</td>
<td>3.32</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4c</td>
<td>10</td>
<td>8</td>
<td>300</td>
<td>109.8333333</td>
<td>11.49319</td>
<td>89.5666667</td>
<td>0.663</td>
<td>12.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5a</td>
<td>10</td>
<td>9</td>
<td>100</td>
<td>341.4666667</td>
<td>34.96875</td>
<td>244.6166667</td>
<td>0.519667</td>
<td>12.97</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5b</td>
<td>10</td>
<td>9</td>
<td>150</td>
<td>271.8333333</td>
<td>15.99698</td>
<td>189.5666667</td>
<td>0.486667</td>
<td>12.57</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5c</td>
<td>10</td>
<td>9</td>
<td>300</td>
<td>313.9333333</td>
<td>12.98704</td>
<td>200.7333333</td>
<td>0.410333</td>
<td>6.91</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6a</td>
<td>10</td>
<td>10</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6b</td>
<td>10</td>
<td>10</td>
<td>150</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6c</td>
<td>10</td>
<td>10</td>
<td>300</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>900 RPM</td>
<td>7a</td>
<td>10</td>
<td>8</td>
<td>100</td>
<td>213.180952</td>
<td>7.402027</td>
<td>138.516667</td>
<td>0.423</td>
<td>11.58</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7b</td>
<td>10</td>
<td>8</td>
<td>150</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7c</td>
<td>10</td>
<td>8</td>
<td>300</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>8a</td>
<td>10</td>
<td>9</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>8b</td>
<td>10</td>
<td>9</td>
<td>150</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>8c</td>
<td>10</td>
<td>9</td>
<td>300</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>9a</td>
<td>10</td>
<td>10</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>9b</td>
<td>10</td>
<td>10</td>
<td>150</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>9c</td>
<td>10</td>
<td>10</td>
<td>300</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**GNCA Stir speed, EtOH, and GTA variation Study**

- **Stir Rate**:
  - 300 rpm
  - 600 rpm
  - 900 rpm
- **EtOH added to 10 ml batch (ml)**:
  - 100 ul GTA
  - 300 ul GTA
  - 150 ul GTA

![GNCA Stir speed, EtOH, and GTA variation Study](image-url)
1-Step Desolvation of 1% gelatin

Stir rate: 600 rpm, ethanol vol and 10% GTA vol variation
600 rpm regular 2 step desolvation 1 batch

16 ml ethanol added dropwise to the hmw fraction of 1% gelatin solution. (first desolvated with 20 ml of acetone, 20 ml batch size)

600 rpm and 600 ul 10% GTA added to batch 5 minutes after end of dropwise addition

<table>
<thead>
<tr>
<th>before x link</th>
<th>particle size (nm)</th>
<th>mean ps</th>
<th>stdev ps</th>
<th>stdev</th>
<th>mean stdev</th>
<th>PI</th>
<th>mean PI</th>
<th>zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>105.7</td>
<td>106.3</td>
<td>3.7</td>
<td>88.9</td>
<td>89.4</td>
<td>0.8</td>
<td>0.707</td>
<td>0.002646</td>
</tr>
<tr>
<td>2</td>
<td>102.9</td>
<td>90.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.702</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>110.2</td>
<td>89.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.706</td>
<td></td>
</tr>
</tbody>
</table>

5 min after

<table>
<thead>
<tr>
<th>before x link</th>
<th>particle size (nm)</th>
<th>mean ps</th>
<th>stdev ps</th>
<th>stdev</th>
<th>mean stdev</th>
<th>PI</th>
<th>mean PI</th>
<th>zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50.4</td>
<td>33.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.445</td>
<td>14.72</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

end process

<table>
<thead>
<tr>
<th>before x link</th>
<th>particle size (nm)</th>
<th>mean ps</th>
<th>stdev ps</th>
<th>stdev</th>
<th>mean stdev</th>
<th>PI</th>
<th>mean PI</th>
<th>zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>61.6</td>
<td>65.9</td>
<td>4.4</td>
<td>37.5</td>
<td>40.0</td>
<td>2.1</td>
<td>0.371</td>
<td>0.369</td>
</tr>
<tr>
<td>2</td>
<td>70.3</td>
<td>41.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.343</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>65.7</td>
<td>41.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.393</td>
<td></td>
</tr>
</tbody>
</table>

Crosslinking 2-step desolvation GNCA

Mean Particle Size
Crosslinking 2-step desolvation GNCA
Population Standard Deviation

Particle Size (nm)

Before

89.4

After

40.0

Crosslinking HMW 2-step desolvation GNCA
Polydispersity Index (PDI)

Before

0.705

After

0.369
Particle size and zeta potential of final selected batch for publication

<table>
<thead>
<tr>
<th></th>
<th>Particle size</th>
<th>Ps sd</th>
<th>Zeta</th>
<th>Zeta sd</th>
</tr>
</thead>
<tbody>
<tr>
<td>S6S-GNC</td>
<td>70</td>
<td>6.5</td>
<td>10</td>
<td>1.5</td>
</tr>
<tr>
<td>Placebo-GNC</td>
<td>90</td>
<td>7.4</td>
<td>9</td>
<td>1.2</td>
</tr>
</tbody>
</table>

![Graph showing particle size and zeta potential comparison between S6S-GNC and Placebo-GNC.](image-url)
<table>
<thead>
<tr>
<th>time (hr)</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>average (ng/ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>0.5</td>
<td>1.02</td>
<td>0</td>
<td>0.506667</td>
</tr>
<tr>
<td>0.5</td>
<td>0.9</td>
<td>0.45</td>
<td>0.16</td>
<td>0.503333</td>
</tr>
<tr>
<td>1</td>
<td>1.8</td>
<td>0.13</td>
<td>0.3</td>
<td>0.703333</td>
</tr>
<tr>
<td>2</td>
<td>1.66</td>
<td>0.78</td>
<td>0.61</td>
<td>1.016667</td>
</tr>
<tr>
<td>3</td>
<td>1.35</td>
<td>1.248</td>
<td>0.47</td>
<td>0.91</td>
</tr>
<tr>
<td>4</td>
<td>1.91</td>
<td>1.48</td>
<td>0.79</td>
<td>1.393333</td>
</tr>
<tr>
<td>5</td>
<td>1.47</td>
<td>0.64</td>
<td>0.68</td>
<td>0.93</td>
</tr>
<tr>
<td>6</td>
<td>2.12</td>
<td>0.88</td>
<td>2.29</td>
<td>1.763333</td>
</tr>
<tr>
<td>7</td>
<td>2.75</td>
<td>0.66</td>
<td>0.13</td>
<td>1.18</td>
</tr>
<tr>
<td>8</td>
<td>2.41</td>
<td>1.33</td>
<td>0.28</td>
<td>1.34</td>
</tr>
<tr>
<td>24</td>
<td>3.65</td>
<td>1.44</td>
<td>0.62</td>
<td>1.903333</td>
</tr>
<tr>
<td>48</td>
<td>11.82</td>
<td>9.32</td>
<td>8.89</td>
<td>9.99</td>
</tr>
<tr>
<td>72</td>
<td>19.08</td>
<td>10.9</td>
<td>11.92</td>
<td>13.96667</td>
</tr>
</tbody>
</table>

Uncorrected ng in 50 ml vessel

<table>
<thead>
<tr>
<th>correctionngaverage</th>
<th>sd</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.25</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>48</td>
<td>0</td>
</tr>
<tr>
<td>72</td>
<td>0</td>
</tr>
</tbody>
</table>

Corrected dissolution

<table>
<thead>
<tr>
<th>time (hr)</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>average</th>
<th>sd</th>
<th>% I</th>
<th>% II</th>
<th>% III</th>
<th>sd dev of ?</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>50</td>
<td>102</td>
<td>0</td>
<td>76</td>
<td>51.00327</td>
<td>4.984067</td>
<td>4.187328</td>
<td>2.754821</td>
<td>5.619835</td>
</tr>
<tr>
<td>0.5</td>
<td>90.5</td>
<td>46.02</td>
<td>16</td>
<td>68.26</td>
<td>37.48315</td>
<td>4.746749</td>
<td>3.760882</td>
<td>4.986226</td>
<td>2.535537</td>
</tr>
<tr>
<td>1</td>
<td>181.4</td>
<td>14.47</td>
<td>18.16</td>
<td>97.935</td>
<td>95.32972</td>
<td>6.422561</td>
<td>5.395868</td>
<td>9.994497</td>
<td>0.797245</td>
</tr>
<tr>
<td>2</td>
<td>169.2</td>
<td>79.6</td>
<td>61.34</td>
<td>124.4</td>
<td>57.72834</td>
<td>8.158131</td>
<td>6.853994</td>
<td>9.323214</td>
<td>4.385675</td>
</tr>
<tr>
<td>3</td>
<td>139.8</td>
<td>127.18</td>
<td>47.95</td>
<td>133.52</td>
<td>49.80901</td>
<td>8.756219</td>
<td>7.356474</td>
<td>7.705785</td>
<td>7.007163</td>
</tr>
<tr>
<td>5</td>
<td>155.12</td>
<td>69.108</td>
<td>70.21</td>
<td>112.114</td>
<td>49.34401</td>
<td>7.352418</td>
<td>6.17708</td>
<td>8.546556</td>
<td>3.807603</td>
</tr>
<tr>
<td>6</td>
<td>221.59</td>
<td>93.748</td>
<td>231.89</td>
<td>157.669</td>
<td>76.95548</td>
<td>10.33991</td>
<td>8.686997</td>
<td>12.20882</td>
<td>5.165179</td>
</tr>
<tr>
<td>7</td>
<td>286.71</td>
<td>72.628</td>
<td>18.18</td>
<td>179.669</td>
<td>141.9531</td>
<td>11.78266</td>
<td>9.899118</td>
<td>15.79699</td>
<td>4.001543</td>
</tr>
<tr>
<td>8</td>
<td>255.46</td>
<td>140.288</td>
<td>33.31</td>
<td>197.874</td>
<td>111.1002</td>
<td>12.97654</td>
<td>10.90215</td>
<td>14.07493</td>
<td>7.729366</td>
</tr>
<tr>
<td>24</td>
<td>381.87</td>
<td>152.618</td>
<td>67.59</td>
<td>267.244</td>
<td>162.568</td>
<td>52.5282</td>
<td>14.72419</td>
<td>21.03967</td>
<td>8.408705</td>
</tr>
<tr>
<td>48</td>
<td>1202.52</td>
<td>942.058</td>
<td>889.21</td>
<td>1072.289</td>
<td>167.7282</td>
<td>70.32053</td>
<td>59.07928</td>
<td>66.25455</td>
<td>51.90402</td>
</tr>
<tr>
<td>72</td>
<td>1940.34</td>
<td>1109.378</td>
<td>1207.04</td>
<td>1524.859</td>
<td>454.1961</td>
<td>100</td>
<td>84.04127</td>
<td>106.90586</td>
<td>61.12275</td>
</tr>
</tbody>
</table>
### Table 1: A549 experiment S6S-GNC type A

<table>
<thead>
<tr>
<th>Medium</th>
<th>Placebo</th>
<th>S6S 5 nM</th>
<th>S6S 10 nM</th>
<th>S6S 15 nM</th>
<th>soln 5 nM</th>
<th>soln 10 nM</th>
<th>soln 15 nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hours</td>
<td>0.495</td>
<td>0.221</td>
<td>0.197</td>
<td>0.179</td>
<td>0.362</td>
<td>0.336</td>
<td>0.33</td>
</tr>
<tr>
<td>48 hours</td>
<td>0.437</td>
<td>0.216</td>
<td>0.211</td>
<td>0.181</td>
<td>0.344</td>
<td>0.333</td>
<td>0.349</td>
</tr>
<tr>
<td>72 hours</td>
<td>0.418</td>
<td>0.206</td>
<td>0.156</td>
<td>0.149</td>
<td>0.337</td>
<td>0.341</td>
<td>0.358</td>
</tr>
<tr>
<td></td>
<td>0.361</td>
<td>0.211</td>
<td>0.153</td>
<td>0.145</td>
<td>0.265</td>
<td>0.253</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>0.366</td>
<td>0.194</td>
<td>0.149</td>
<td>0.146</td>
<td>0.284</td>
<td>0.323</td>
<td>0.277</td>
</tr>
<tr>
<td></td>
<td>0.335</td>
<td>0.177</td>
<td>0.149</td>
<td>0.149</td>
<td>0.254</td>
<td>0.286</td>
<td>0.295</td>
</tr>
<tr>
<td></td>
<td>0.688</td>
<td>0.183</td>
<td>0.153</td>
<td>0.168</td>
<td>0.265</td>
<td>0.328</td>
<td>0.384</td>
</tr>
<tr>
<td></td>
<td>0.59</td>
<td>0.21</td>
<td>0.183</td>
<td>0.192</td>
<td>0.265</td>
<td>0.286</td>
<td>0.373</td>
</tr>
<tr>
<td></td>
<td>0.553</td>
<td>0.21</td>
<td>0.183</td>
<td>0.201</td>
<td>0.286</td>
<td>0.373</td>
<td>0.355</td>
</tr>
<tr>
<td></td>
<td>0.471444</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nM</td>
<td>average</td>
<td>Std dev</td>
<td>average</td>
<td>Std dev</td>
<td>Average</td>
<td>Std dev</td>
<td>average</td>
</tr>
<tr>
<td>15</td>
<td>0.201</td>
<td>0.2904</td>
<td>0.204</td>
<td>0.2428</td>
<td>0.20614</td>
<td>0.216</td>
<td>0.298</td>
</tr>
<tr>
<td>10</td>
<td>0.323</td>
<td>0.303</td>
<td>0.303</td>
<td>0.303</td>
<td>0.303</td>
<td>0.303</td>
<td>0.303</td>
</tr>
<tr>
<td>5</td>
<td>0.323</td>
<td>0.303</td>
<td>0.303</td>
<td>0.303</td>
<td>0.303</td>
<td>0.303</td>
<td>0.303</td>
</tr>
<tr>
<td>placebo</td>
<td>0.298</td>
<td>0.258</td>
<td>0.258</td>
<td>0.258</td>
<td>0.258</td>
<td>0.258</td>
<td>0.258</td>
</tr>
</tbody>
</table>

### Table 2: A549 experiment S6S-Soln type A

<table>
<thead>
<tr>
<th>Medium</th>
<th>Placebo</th>
<th>S6S-GNC</th>
<th>S6S-Soln</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hours</td>
<td></td>
<td>48 hours</td>
<td>72 hours</td>
</tr>
<tr>
<td>0.398</td>
<td></td>
<td>0.361</td>
<td>0.335</td>
</tr>
<tr>
<td>0.398</td>
<td></td>
<td>0.361</td>
<td>0.335</td>
</tr>
<tr>
<td>0.398</td>
<td></td>
<td>0.361</td>
<td>0.335</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.361</td>
<td>0.335</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.361</td>
<td>0.335</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.361</td>
<td>0.335</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.361</td>
<td>0.335</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.361</td>
<td>0.335</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.361</td>
<td>0.335</td>
</tr>
</tbody>
</table>

### Table 3: A549 experiment Average placebo

<table>
<thead>
<tr>
<th>Medium</th>
<th>Placebo</th>
<th>S6S-GNC</th>
<th>S6S-Soln</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hours</td>
<td></td>
<td>48 hours</td>
<td>72 hours</td>
</tr>
<tr>
<td>0.398</td>
<td></td>
<td>0.361</td>
<td>0.335</td>
</tr>
<tr>
<td>0.398</td>
<td></td>
<td>0.361</td>
<td>0.335</td>
</tr>
<tr>
<td>0.398</td>
<td></td>
<td>0.361</td>
<td>0.335</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.361</td>
<td>0.335</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.361</td>
<td>0.335</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.361</td>
<td>0.335</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.361</td>
<td>0.335</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.361</td>
<td>0.335</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.361</td>
<td>0.335</td>
</tr>
</tbody>
</table>
Appendix C: Data for Objective 2

IL4R Expression in Human, mRNA expression by array, BioGPS.org

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>average</th>
<th>stdev</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD19+ B</td>
<td>926.9</td>
<td>421.0114</td>
</tr>
<tr>
<td>Dendritic</td>
<td>29.3</td>
<td>2.828427</td>
</tr>
<tr>
<td>CD8+ T</td>
<td>17.8</td>
<td>3.11127</td>
</tr>
<tr>
<td>CD4+ T</td>
<td>67.5</td>
<td>9.263099</td>
</tr>
<tr>
<td>NK</td>
<td>15.1</td>
<td>2.899138</td>
</tr>
<tr>
<td>Myeloid</td>
<td>23.5</td>
<td>4.030509</td>
</tr>
<tr>
<td>Monocytes</td>
<td>31.1</td>
<td>1.767767</td>
</tr>
<tr>
<td>Bronchial epithelial</td>
<td>14.9</td>
<td>3.040559</td>
</tr>
<tr>
<td>Other tissues</td>
<td>6.8</td>
<td>2.046042</td>
</tr>
</tbody>
</table>

Heart, kidney, liver, brain, bone marrow, and Endothelial cells CD105+

![Bar chart showing Log Expression Level for different cell types with average and standard deviation values.](chart.png)
## Western Blot Densitometry of IL4R Protein Expression

### POLYCLONAL

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>beta actin</th>
<th>IL4Ra pab</th>
<th>IL4Ra Pab/Beta act</th>
<th>Norm Pab/beta</th>
<th>IL4Ra alpha pab</th>
<th>IL4Ra alpha pab/Beta act</th>
<th>Norm Pab/beta</th>
<th>ave norm</th>
<th>stdev norm</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIB24</td>
<td>2763.522</td>
<td>7713.877</td>
<td>0.278645073</td>
<td>100</td>
<td>7908.341</td>
<td>0.280207355</td>
<td>100</td>
<td>0.511514756</td>
<td>100</td>
</tr>
<tr>
<td>RAW264.7</td>
<td>21917.572</td>
<td>8591.25</td>
<td>0.391980006</td>
<td>140.6735824</td>
<td>8124.037</td>
<td>0.370663183</td>
<td>131.414117</td>
<td>0.489569146</td>
<td>95.70986188</td>
</tr>
<tr>
<td>Normal Lung Tissue (mouse)</td>
<td>21689.714</td>
<td>5034.723</td>
<td>0.232124914</td>
<td>83.30486949</td>
<td>5258.601</td>
<td>0.242446765</td>
<td>85.95654777</td>
<td>0.303570992</td>
<td>59.3475551</td>
</tr>
<tr>
<td>OVA Lung Tissue (mouse)</td>
<td>21830.765</td>
<td>4438.472</td>
<td>0.203312711</td>
<td>72.96476063</td>
<td>4537.108</td>
<td>0.207830092</td>
<td>73.68392183</td>
<td>0.436695095</td>
<td>85.37292231</td>
</tr>
<tr>
<td>BEAS-2B</td>
<td>16712.572</td>
<td>6290.551</td>
<td>0.376396344</td>
<td>135.089258</td>
<td>6660.208</td>
<td>0.398514843</td>
<td>141.2985837</td>
<td>0.507769315</td>
<td>114.907972</td>
</tr>
<tr>
<td>AS49/LUC</td>
<td>18406.572</td>
<td>6693.915</td>
<td>0.363669835</td>
<td>130.5136428</td>
<td>6519.844</td>
<td>0.354212832</td>
<td>125.5818455</td>
<td>0.515458446</td>
<td>100</td>
</tr>
</tbody>
</table>

### MONOCLONAL

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>beta actin</th>
<th>IL4Ra mab</th>
<th>IL4Ra Mab/Beta act</th>
<th>Norm IL4Ra Mab</th>
<th>IL4Ra alpha mab</th>
<th>IL4Ra alpha mab/Beta act</th>
<th>Norm IL4Ra Mab</th>
<th>ave norm</th>
<th>stdev norm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th2 Cells</td>
<td>2793.522</td>
<td>8543.731</td>
<td>0.308621353</td>
<td>1.336168477</td>
<td>5878.217</td>
<td>0.212336313</td>
<td>1.362943009</td>
<td>0.5393.338</td>
<td>0.200095132</td>
</tr>
<tr>
<td>RAW264.7</td>
<td>21917.572</td>
<td>7373.589</td>
<td>0.336426324</td>
<td>1.455636868</td>
<td>6411.208</td>
<td>0.29214518</td>
<td>1.877590369</td>
<td>6247.229</td>
<td>0.285032895</td>
</tr>
<tr>
<td>Normal Lung Tissue (mouse)</td>
<td>21689.714</td>
<td>5009.782</td>
<td>0.230975014</td>
<td>1</td>
<td>3379.095</td>
<td>0.155792511</td>
<td>1</td>
<td>3068.267</td>
<td>0.14161847</td>
</tr>
<tr>
<td>OVA Lung Tissue (mouse)</td>
<td>21830.765</td>
<td>6983.853</td>
<td>0.319008762</td>
<td>1.385036225</td>
<td>5320.602</td>
<td>0.243720364</td>
<td>1.564390761</td>
<td>5196.551</td>
<td>0.238037911</td>
</tr>
<tr>
<td>BEAS-2B</td>
<td>16712.572</td>
<td>2627.376</td>
<td>0.157209955</td>
<td>0.680634462</td>
<td>845.406</td>
<td>0.050950333</td>
<td>0.326434983</td>
<td>906.406</td>
<td>0.054234946</td>
</tr>
<tr>
<td>AS49/LUC</td>
<td>18406.572</td>
<td>2918.154</td>
<td>0.1585387</td>
<td>0.683590862</td>
<td>1253.891</td>
<td>0.08121919</td>
<td>0.437265548</td>
<td>1091.062</td>
<td>0.059275676</td>
</tr>
</tbody>
</table>

### IL-4Ra (mAb sc-165974)

**IL-4Ra Relative Protein Expression (%)**

- **TIB24**
  - ave norm: 2.12
  - stdev norm: 0.35
- **RAW264.7**
  - ave norm: 1.62
  - stdev norm: 0.42
- **Normal Lung Tissue (mouse)**
  - ave norm: 1.5
  - stdev norm: 0.28
- **OVA Lung Tissue (mouse)**
  - ave norm: 1.4
  - stdev norm: 0.27
- **BEAS-2B**
  - ave norm: 0.75
  - stdev norm: 0.15
- **AS49/LUC**
  - ave norm: 0.65
  - stdev norm: 0.09
S6S-GNC and S6S-GNC-P Characterization
### Glycine (µg/mL)

<table>
<thead>
<tr>
<th>1° Amine</th>
<th>Glycine</th>
<th>Corr Abs</th>
<th>MW glycine</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.518333333</td>
<td>0.033333</td>
<td>75.0666 g/mol</td>
</tr>
<tr>
<td>1</td>
<td>1.4436E+15</td>
<td>0.535166667</td>
<td>1µg glycine=8.02E15 molecules</td>
</tr>
<tr>
<td>2</td>
<td>3.609E+15</td>
<td>0.538</td>
<td>conc in µg/mL</td>
</tr>
<tr>
<td>3</td>
<td>7.218E+15</td>
<td>0.561833333</td>
<td>placed 90 µl=0.09 mL sample</td>
</tr>
<tr>
<td>4</td>
<td>1.0827E+16</td>
<td>0.579</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1.4436E+16</td>
<td>0.601833333</td>
<td></td>
</tr>
</tbody>
</table>

### Gelatin (µg/mL)

<table>
<thead>
<tr>
<th>1°NH/molecule</th>
<th>Gelatin</th>
<th>Molecules</th>
<th>Molecules 50 kDa</th>
<th>Corr Abs</th>
<th>MW Glycine</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>2.7099E+13</td>
<td>0.518333</td>
<td>0.00216</td>
<td>0.016833</td>
<td>75.0666 g/mol</td>
</tr>
<tr>
<td>50</td>
<td>6.7748E+13</td>
<td>0.505616</td>
<td>0.016618</td>
<td>0.031833</td>
<td>100.1549 g/mol</td>
</tr>
<tr>
<td>100</td>
<td>1.355E+14</td>
<td>0.603667</td>
<td>0.066282</td>
<td>0.085333333</td>
<td>150.3199 g/mol</td>
</tr>
<tr>
<td>150</td>
<td>2.0324E+14</td>
<td>0.626</td>
<td>0.00405</td>
<td>0.107666667</td>
<td>200.4839 g/mol</td>
</tr>
<tr>
<td>200</td>
<td>2.7099E+14</td>
<td>0.6744</td>
<td>0.007849</td>
<td>0.155666667</td>
<td>250.6479 g/mol</td>
</tr>
</tbody>
</table>

### Glycine Standard Curve Corrected Abs vs. µg/mL

\[ y = 228.19x + 0.8432 \]
\[ R^2 = 0.9934 \]

### Glycine Standard Curve Corrected Abs vs. 1° Amine

\[ y = 2E+17x + 6E+14 \]
\[ R^2 = 0.9934 \]
Edible gelatin contains nine of the ten essential amino acids. Gelatin contains non-essential amino acids also. It contains specific amounts of 18 different amino acids (AA) which are joined together in sequences to form polypeptide chains of ca. 1000 AA per chain, scientifically known as the primary structure. Three of the polypeptide chains formed this way join together as a left-hand spiral to give the secondary structure. In the tertiary structure, the spiral winds and folds itself to a right-hand spiral (triple helix). This results in a rod-shaped molecule, the so-called proto fibril.

Theoretically, if 1 gelatin chain contains 1000 AA and 15% of this comes from arginine, histidine, lysine, then 150 primary amines should be present. This represents the value that the graph is approaching.

The approximate amino acid composition of gelatin is: **glycine** 21%, **proline** 12%, **hydroxyproline** 12%, glutamic acid 10%, **alanine** 9%, arginine 8%, aspartic acid 6%, lysine 4%, serine 4%, **leucine** 3%, **valine** 2%, **phenylalanine** 2%, threonine 2%, **isoleucine** 1%, hydroxylysine 1%, **methionine** and **histidine** <1% and **tyrosine** <0.5%.

Calculate n/p ratio based on 127 amines within 200 µg/ml gelatin solution.
### Alanine
8.6

### Arginine
8.3

### Aspartic Acid
6.2

### Cystine
0.1

### Glutamic Acid
11.3

### Glycine
26.4

### Histidine
0.9

### Hydroxylysine
1

### Isoleucine
1.4

### Leucine
3.1

### Lysine
4.1

### Methionine
0.8

### Phenylalanine
2.1

### Proline
16.2

### Serine
2.9

### Threonine
2.2

### Tyrosine
0.4

### Valine
2.5

### Glycine
0.26

### Proline
0.16

### Hydroxyproline
0.14

### Glutamic acid
0.11

### Alanine
0.09

### Arginine
0.08

### Histidine
0.09

### Aspartic Acid
0.06

### Lysine
0.04

### Serine
0.03

### Leucine
0.03

### Valine
0.025

### Phenylalanine
0.02

### Threonine
0.02

### Methionine
0.01

### Hydroxylysine
0.01

### Cystine
0.001

### Tyrosine
0.005

### Histidine
0.009

### Methionine
0.008

### Isoleucine
0.01

Gelatin manufacturers institute of america

Gelatin is denatured form of collagen

Essentially, the tertiary structure of gelatin is broken when in solution
Viscosity

| Placebo-GNC | 50/50 | 05:07.2 | 05:06.5 | 05:05.3 | 05:07.2 | 05:06.3 | 1.07E-05 | 257.2 | 307.2 | 306.5 | 305.3 | 307.2 | 305.4 | 0.931128 | 50 | 3.125 | 212.54 | 0.896103 |
| 12.5/6.25 | 04:32.6 | 04:31.3 | 04:33.5 | 04:32.1 | 04:32.3 | 1.17E-05 | 272.6 | 271.3 | 273.5 | 271.2 | 271.3 | 272.7 | 306.3 | 0.931128 | 50 | 3.125 | 212.54 | 0.896103 |
| 12.5/6.25 | 04:11.6 | 04:12.9 | 04:12.5 | 04:13.1 | 04:13.3 | 1.17E-05 | 251.6 | 252.9 | 252.5 | 251.2 | 251.3 | 251.9 | 306.3 | 0.931128 | 50 | 3.125 | 212.54 | 0.896103 |
| 12.5/6.25 | 04:01.4 | 04:01.7 | 04:02.2 | 04:01.4 | 04:01.7 | 1.17E-05 | 241.4 | 241.7 | 241.4 | 241.4 | 241.4 | 241.4 | 306.3 | 0.931128 | 50 | 3.125 | 212.54 | 0.896103 |
| 1.125/3.125 | 03:52.3 | 03:53.8 | 03:53.3 | 03:52.6 | 03:52.7 | 1.17E-05 | 232.3 | 233.8 | 231.3 | 232.6 | 232.7 | 232.7 | 306.3 | 0.931128 | 50 | 3.125 | 212.54 | 0.896103 |

| S6S-GNC | 50/50 | 05:03.4 | 05:00.3 | 05:04.1 | 05:02.1 | 05:04.1 | 1.86E-05 | 303.4 | 300.3 | 304.1 | 302.1 | 304.1 | 302.8 | 1.618641 | 50 | 3.125 | 212.54 | 0.896103 |
| 25/75 | 04:38.6 | 04:36.2 | 04:37.4 | 04:37.2 | 04:36.7 | 2.03E-05 | 278.6 | 276.2 | 277.4 | 274.7 | 276.6 | 276.6 | 1.723949 | 50 | 3.125 | 212.54 | 0.896103 |
| 12.5/6.25 | 04:10.5 | 04:09.9 | 04:10.2 | 04:10.4 | 04:10.6 | 4.25E-06 | 250.5 | 249.9 | 250.2 | 250.8 | 250.6 | 250.4 | 1.353553 | 50 | 3.125 | 212.54 | 0.896103 |
| 12.5/6.25 | 04:03.9 | 03:59.8 | 03:57.7 | 03:58.4 | 03:59.9 | 2.77E-05 | 243.9 | 239.8 | 237.7 | 238.4 | 239.9 | 239.9 | 2.402707 | 50 | 3.125 | 212.54 | 0.896103 |
| 1.125/3.125 | 03:53.1 | 03:53.8 | 03:53.5 | 03:53.7 | 03:53.5 | 3.28E-05 | 233.1 | 233.8 | 233.3 | 233.7 | 233.3 | 233.48 | 2.402707 | 50 | 3.125 | 212.54 | 0.896103 |

Volume of particle

\[ V = \frac{4}{3} \pi r^3 \]


\[ \phi = \frac{H - 1}{2.5} \]

\[ N = \phi \left[ \frac{4\pi}{3} \left( \frac{d}{2} \right)^3 \right] \]

\[ y = 1.551x + 231.21 \]

\[ \text{R}^2 = 0.992 \]

\[ y = 1.4511x + 230.55 \]

\[ \text{R}^2 = 0.998 \]

\[ N = \frac{\phi}{4} \]

\[ 3 \pi d^2 \]

\[ 3 \]

\[ \text{conc gel} = 1\% \text{ w/v} \]

\[ g/mL = 0.01 \]

\[ \text{mol/ml} = 3.975151 \times 10^{-11} \]

\[ \text{mol/l} = 3.975151 \times 10^{-10} \]

\[ \mu M = 0.03975514 \]

\[ nM = 39.75514508 \]

\[ D = 10 \]

\[ F = 16 \]

\[ \text{Brush} \]

\[ \text{Np surface area nm}^2 = 31415.92654 \]

\[ \text{peg ligands per np #} = 3141.592654 \]

\[ \text{total np in 1 ml} = 2.39405 \times 10^{13} \]

\[ \text{total peg ligands 1 ml} = 7.52115 \times 10^{16} \]
STABILITY in DPBS, DPBS + 10% FBS, and Water + 10% FBS up to 96 hr.
*10% FBS in water, DPBS, and 10% FBS in DPBS. FBS solutions were preserved with 0.01% sodium azide

**x-GNC refers to PEGylated; x-GNC-I refers to peptide conjugated formulation

**DPBS**

<table>
<thead>
<tr>
<th></th>
<th>Placebo-GNC</th>
<th>Placebo-GNC-Peg</th>
<th>Placebo-GNC-I</th>
<th>S6S-GNC</th>
<th>S6S-GNC-Peg</th>
<th>S6S-GNC-I</th>
</tr>
</thead>
<tbody>
<tr>
<td>ps 1</td>
<td>80.8</td>
<td>94.5</td>
<td>99.2</td>
<td>95.5</td>
<td>82.9</td>
<td>91.6</td>
</tr>
<tr>
<td>ps 2</td>
<td>82.4</td>
<td>92</td>
<td>102.6</td>
<td>94.7</td>
<td>85.6</td>
<td>90.1</td>
</tr>
<tr>
<td>ps 3</td>
<td>87.3</td>
<td>91.3</td>
<td>105.7</td>
<td>96.6</td>
<td>82.1</td>
<td>97.4</td>
</tr>
<tr>
<td>ave ps</td>
<td>83.5</td>
<td>92.6</td>
<td>102.5</td>
<td>95.6666</td>
<td>81.533333</td>
<td>93.033333</td>
</tr>
<tr>
<td>std dev</td>
<td>3.86738844</td>
<td>1.682260384</td>
<td>3.25153661</td>
<td>1.0016628</td>
<td>1.83939294</td>
<td>3.85529393</td>
</tr>
<tr>
<td>pdi</td>
<td>0.607</td>
<td>0.576</td>
<td>0.618</td>
<td>0.621</td>
<td>0.598</td>
<td>0.546</td>
</tr>
<tr>
<td>pdi</td>
<td>0.620</td>
<td>0.546</td>
<td>0.643</td>
<td>0.619</td>
<td>0.575</td>
<td>0.567</td>
</tr>
<tr>
<td>pdi</td>
<td>0.518</td>
<td>0.521</td>
<td>0.54</td>
<td>0.606</td>
<td>0.557</td>
<td>0.608</td>
</tr>
<tr>
<td>zeta ave</td>
<td>7.27</td>
<td>5.29</td>
<td>13.46</td>
<td>5.92</td>
<td>6.75</td>
<td>5.3</td>
</tr>
<tr>
<td>zeta 1</td>
<td>99.2</td>
<td>2.06</td>
<td>13.96</td>
<td>7.64</td>
<td>6.75</td>
<td>3.3</td>
</tr>
<tr>
<td>zeta 2</td>
<td>9.89</td>
<td>7.97</td>
<td>12.72</td>
<td>9.06</td>
<td>6.75</td>
<td>3.3</td>
</tr>
<tr>
<td>zeta 3</td>
<td>7.27</td>
<td>6.38</td>
<td>7.32333333</td>
<td>6.47</td>
<td>3.09666667</td>
<td>16.833333</td>
</tr>
<tr>
<td>zeta stdev</td>
<td>3.0375924</td>
<td>2.024672813</td>
<td>6.23171805</td>
<td>1.959362382</td>
<td>0.04957426</td>
<td>0.020005316</td>
</tr>
<tr>
<td><strong>After 0.2µm filtration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ps 1</td>
<td>n/a</td>
<td>n/a</td>
<td>62.1</td>
<td>n/a</td>
<td>67.8</td>
<td>75.6</td>
</tr>
<tr>
<td>ps 2</td>
<td>n/a</td>
<td>n/a</td>
<td>60.5</td>
<td>n/a</td>
<td>65.2</td>
<td>74.9</td>
</tr>
<tr>
<td>ps 3</td>
<td>n/a</td>
<td>n/a</td>
<td>71.3</td>
<td>n/a</td>
<td>71.2</td>
<td>65.9</td>
</tr>
<tr>
<td>ave ps</td>
<td>n/a</td>
<td>n/a</td>
<td>74.83333333</td>
<td>n/a</td>
<td>67.93333333</td>
<td>72.13333333</td>
</tr>
<tr>
<td>std dev</td>
<td>n/a</td>
<td>n/a</td>
<td>14.23828951</td>
<td>n/a</td>
<td>3.035347317</td>
<td>5.40959944</td>
</tr>
<tr>
<td>pdi</td>
<td>n/a</td>
<td>n/a</td>
<td>0.479</td>
<td>n/a</td>
<td>0.438</td>
<td>0.537</td>
</tr>
<tr>
<td>pdi</td>
<td>n/a</td>
<td>n/a</td>
<td>0.785</td>
<td>n/a</td>
<td>0.422</td>
<td>0.501</td>
</tr>
<tr>
<td>pdi</td>
<td>n/a</td>
<td>n/a</td>
<td>0.554</td>
<td>n/a</td>
<td>0.461</td>
<td>0.461</td>
</tr>
<tr>
<td>pdi stdev</td>
<td>n/a</td>
<td>n/a</td>
<td>0.606</td>
<td>n/a</td>
<td>0.45666667</td>
<td>0.49966667</td>
</tr>
<tr>
<td>zeta 1</td>
<td>n/a</td>
<td>n/a</td>
<td>18.42</td>
<td>n/a</td>
<td>18.7</td>
<td>14.56</td>
</tr>
<tr>
<td>zeta 2</td>
<td>n/a</td>
<td>n/a</td>
<td>18.4</td>
<td>14.43</td>
<td>19.29</td>
<td>15.06</td>
</tr>
<tr>
<td>zeta 3</td>
<td>n/a</td>
<td>n/a</td>
<td>17.28</td>
<td>12.72</td>
<td>17.77</td>
<td>14.96</td>
</tr>
<tr>
<td>zeta ave</td>
<td>n/a</td>
<td>n/a</td>
<td>18.03333333</td>
<td>n/a</td>
<td>18.62</td>
<td>14.86</td>
</tr>
<tr>
<td>zeta stdev</td>
<td>n/a</td>
<td>n/a</td>
<td>0.6528482439</td>
<td>n/a</td>
<td>0.6528482439</td>
<td>0.294575111</td>
</tr>
</tbody>
</table>

**DPBS + 10% FBS**

<table>
<thead>
<tr>
<th></th>
<th>Placebo-GNC</th>
<th>Placebo-GNC-Peg</th>
<th>Placebo-GNC-I</th>
<th>S6S-GNC</th>
<th>S6S-GNC-Peg</th>
<th>S6S-GNC-I</th>
</tr>
</thead>
<tbody>
<tr>
<td>ps 1</td>
<td>99.5</td>
<td>75.4</td>
<td>67.9</td>
<td>80.93333333</td>
<td>16.5107036</td>
<td>0.976</td>
</tr>
<tr>
<td>ps 2</td>
<td>114.7</td>
<td>86.3</td>
<td>89.9</td>
<td>96.66666667</td>
<td>15.7601616</td>
<td>0.696</td>
</tr>
<tr>
<td>ps 3</td>
<td>102.3</td>
<td>88.2</td>
<td>100.1</td>
<td>96.86666667</td>
<td>7.58732221</td>
<td>0.625</td>
</tr>
<tr>
<td>ave ps</td>
<td>104.5</td>
<td>83</td>
<td>89.6</td>
<td>92.36666667</td>
<td>11.0137925</td>
<td>0.65</td>
</tr>
<tr>
<td>std dev</td>
<td>25.1</td>
<td>24.2</td>
<td>24</td>
<td>24.43333333</td>
<td>0.589946528</td>
<td>0.658</td>
</tr>
<tr>
<td>pdi</td>
<td>25.1</td>
<td>24.2</td>
<td>24</td>
<td>24.43333333</td>
<td>0.589946528</td>
<td>0.658</td>
</tr>
<tr>
<td>pdi</td>
<td>24.1</td>
<td>23.6</td>
<td>23.4</td>
<td>23.7</td>
<td>0.642</td>
<td>0.618</td>
</tr>
<tr>
<td>pdi</td>
<td>29.9</td>
<td>29.2</td>
<td>22.7</td>
<td>22.76666667</td>
<td>0.846</td>
<td>0.823</td>
</tr>
<tr>
<td>pdi</td>
<td>22.8</td>
<td>21.1</td>
<td>21.4</td>
<td>21.76666667</td>
<td>0.908377173</td>
<td>0.619</td>
</tr>
<tr>
<td>zeta ave</td>
<td>22.8</td>
<td>21.1</td>
<td>21.4</td>
<td>21.76666667</td>
<td>0.908377173</td>
<td>0.619</td>
</tr>
<tr>
<td>zeta 1</td>
<td>25.1</td>
<td>24.2</td>
<td>24</td>
<td>24.43333333</td>
<td>0.589946528</td>
<td>0.658</td>
</tr>
<tr>
<td>zeta 2</td>
<td>24.1</td>
<td>23.6</td>
<td>23.4</td>
<td>23.7</td>
<td>0.642</td>
<td>0.618</td>
</tr>
<tr>
<td>zeta 3</td>
<td>29.9</td>
<td>29.2</td>
<td>22.7</td>
<td>22.76666667</td>
<td>0.846</td>
<td>0.823</td>
</tr>
<tr>
<td>zeta ave</td>
<td>22.8</td>
<td>21.1</td>
<td>21.4</td>
<td>21.76666667</td>
<td>0.908377173</td>
<td>0.619</td>
</tr>
<tr>
<td>zeta stdev</td>
<td>24.1</td>
<td>23.6</td>
<td>23.4</td>
<td>23.7</td>
<td>0.642</td>
<td>0.618</td>
</tr>
</tbody>
</table>

**Water + 10% FBS**

<table>
<thead>
<tr>
<th></th>
<th>Placebo-GNC</th>
<th>Placebo-GNC-Peg</th>
<th>Placebo-GNC-I</th>
<th>S6S-GNC</th>
<th>S6S-GNC-Peg</th>
<th>S6S-GNC-I</th>
</tr>
</thead>
<tbody>
<tr>
<td>ps 1</td>
<td>40.1</td>
<td>40</td>
<td>40</td>
<td>40.03333333</td>
<td>0.507730027</td>
<td>0.734</td>
</tr>
<tr>
<td>ps 2</td>
<td>37.4</td>
<td>40.2</td>
<td>41.8</td>
<td>39.8</td>
<td>2.227105745</td>
<td>0.699</td>
</tr>
<tr>
<td>ps 3</td>
<td>37.2</td>
<td>37.9</td>
<td>30.5</td>
<td>36.2</td>
<td>4.083398643</td>
<td>0.666</td>
</tr>
<tr>
<td>ave ps</td>
<td>48.5</td>
<td>43.7</td>
<td>43.5</td>
<td>45.23333333</td>
<td>2.830783166</td>
<td>0.643</td>
</tr>
<tr>
<td>std dev</td>
<td>314</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Placebo-GNC</td>
<td>Placebo-GNC-I</td>
<td>S6S-GNC</td>
<td>S6S-GNC-I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------</td>
<td>-------------</td>
<td>---------------</td>
<td>---------</td>
<td>-----------</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>DPBS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ps 1</td>
<td>84.8</td>
<td>93.3</td>
<td>96.9</td>
<td>71.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ps 2</td>
<td>77.2</td>
<td>73.5</td>
<td>100.2</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ps 3</td>
<td>75.4</td>
<td>68.6</td>
<td>105.2</td>
<td>76</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ps ave</td>
<td>79.1333</td>
<td>78.46667</td>
<td>100.7667</td>
<td>89.00667</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ps stdev</td>
<td>4.989322</td>
<td>13.07759</td>
<td>4.178915</td>
<td>8.210562</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pdi 1</td>
<td>0.724</td>
<td>0.702</td>
<td>0.134</td>
<td>0.501</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pdi 2</td>
<td>0.646</td>
<td>0.56</td>
<td>0.258</td>
<td>0.359</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pdi 3</td>
<td>0.637</td>
<td>0.569</td>
<td>0.288</td>
<td>0.511</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pdi ave</td>
<td>0.669</td>
<td>0.610333</td>
<td>0.226667</td>
<td>0.457</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pdi stdev</td>
<td>0.047843</td>
<td>0.079513</td>
<td>0.081641</td>
<td>0.085018</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| **DPBS + 10% FBS** |             |               |         |           |
| ps 1          | 21.2        | 21.7          | 23.5    | 32.1      |
| ps 2          | 21          | 21.7          | 24.1    | 23.4      |
| ps 3          | 21.3        | 21.1          | 22.1    | 21.7      |
| ps ave        | 21.16667    | 21.5          | 23.2333 | 25.73333  |
| ps stdev      | 0.152753    | 0.34641       | 1.02632 | 5.578829  |
| pdi 1         | 0.527       | 0.572         | 0.697   | 0.872     |
| pdi 2         | 0.527       | 0.566         | 0.643   | 0.613     |
| pdi 3         | 0.528       | 0.534         | 0.573   | 0.558     |
| pdi ave       | 0.528       | 0.534         | 0.573   | 0.558     |
| pdi stdev     | 0.001732    | 0.020429      | 0.062172 | 0.167681  |

| **Water + 10% FBS** |             |               |         |           |
| ps 1          | 23.8        | 18.5          | 20.7    | 26.2      |
| ps 2          | 22.9        | 18.5          | 26.1    | 25        |
| ps 3          | 23          | 18.5          | 29.5    | 24.5      |
| ps ave        | 23.23333    | 0.493288      | 25.43333 | 25.23333  |
| ps stdev      | 0.593       | 0.564         | 0.581   | 0.584     |
| pdi 1         | 0.564       | 0.573         | 0.757   | 0.572     |
| pdi 2         | 0.573       | 0.576667      | 0.835   | 0.576667  |
| pdi 3         | 0.572       | 0.576667      | 0.724333 | 0.595333  |
| pdi ave       | 0.572       | 0.576667      | 0.724333 | 0.595333  |
| pdi stdev     | 0.014844    | 0.009849      | 0.130113 | 0.030616  |

<table>
<thead>
<tr>
<th></th>
<th>Placebo-GNC</th>
<th>Placebo-GNC-I</th>
<th>S6S-GNC</th>
<th>S6S-GNC-I</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DPBS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ps 1</td>
<td>115.6</td>
<td>109.6</td>
<td>109.3</td>
<td>62.6</td>
</tr>
<tr>
<td>ps 2</td>
<td>108.6</td>
<td>113.6</td>
<td>127.6</td>
<td>53.5</td>
</tr>
<tr>
<td>ps 3</td>
<td>110.8</td>
<td>107.9</td>
<td>127.2</td>
<td>52.3</td>
</tr>
<tr>
<td>ps ave</td>
<td>111.6667</td>
<td>110.3667</td>
<td>101.376</td>
<td>88.1333</td>
</tr>
<tr>
<td>ps stdev</td>
<td>3.579572</td>
<td>2.926317</td>
<td>10.45195</td>
<td>5.632347</td>
</tr>
<tr>
<td>pdi 1</td>
<td>0.694</td>
<td>0.501</td>
<td>0.578</td>
<td>0.601</td>
</tr>
<tr>
<td>pdi 2</td>
<td>0.667</td>
<td>0.604</td>
<td>0.577</td>
<td>0.406</td>
</tr>
<tr>
<td>pdi 3</td>
<td>0.672</td>
<td>0.576</td>
<td>0.586</td>
<td>0.377</td>
</tr>
<tr>
<td>pdi ave</td>
<td>0.677667</td>
<td>0.560333</td>
<td>0.580333</td>
<td>0.461333</td>
</tr>
<tr>
<td>pdi stdev</td>
<td>0.014364</td>
<td>0.053257</td>
<td>0.004933</td>
<td>0.121821</td>
</tr>
</tbody>
</table>

| **DPBS + 10% FBS** |             |               |         |           |
| ps 1          | 21.1        | 22.7          | 23.1    | 20.4      |
| ps 2          | 21.3        | 24.5          | 21      | 20.9      |
| ps 3          | 20.9        | 38            | 22.1    | 22.9      |
| ps ave        | 21.1        | 28.4          | 22.06667 | 21.4      |
| ps stdev      | 0.2         | 8.362416      | 1.050397 | 1.322876  |
| pdi 1         | 0.573       | 0.558         | 0.555   | 0.542     |
| pdi 2         | 0.579       | 0.558         | 0.482   | 0.563     |
| pdi 3         | 0.575       | 0.52         | 0.516   | 0.63      |
| pdi ave       | 0.57        | 0.52         | 0.517667 | 0.578333  |
| pdi stdev     | 0.010817    | 0.061011      | 0.036529 | 0.04596   |

<p>| <strong>Water + 10% FBS</strong> |             |               |         |           |
| ps 1          | 18.6        | 18.7          | 21.5    | 21.6      |
| ps 2          | 20.1        | 18.5          | 23.6    | 21.1      |
| ps 3          | 19.2        | 18.3          | 21.2    | 20.1      |
| ps ave        | 19.3        | 18.5          | 22.1    | 20.93333  |
| ps stdev      | 0.754983    | 0.2           | 1.30767 | 0.763763  |
| pdi 1         | 0.508       | 0.486         | 0.627   | 0.585     |
| pdi 2         | 0.575       | 0.496         | 0.687   | 0.555     |
| pdi 3         | 0.52        | 0.491         | 0.634   | 0.524     |
| pdi ave       | 0.534333    | 0.546467      | 0.649333 | 0.554667  |
| pdi stdev     | 0.035726    | 0.030501      | 0.032808 | 0.005001  |</p>
<table>
<thead>
<tr>
<th></th>
<th>ps 1</th>
<th>ps 2</th>
<th>ps 3</th>
<th>ps ave</th>
<th>ps stdev</th>
<th>pdi 1</th>
<th>pdi 2</th>
<th>pdi 3</th>
<th>pdi ave</th>
<th>pdi stdev</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DPBS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo-GNC</td>
<td>122.8</td>
<td>111.4</td>
<td>146.5</td>
<td>126.9</td>
<td>17.90559</td>
<td>0.632</td>
<td>0.539</td>
<td>0.773</td>
<td>0.648</td>
<td>0.117818</td>
</tr>
<tr>
<td>Placebo-GNC-I</td>
<td>142.7</td>
<td>142</td>
<td>141</td>
<td>141.9</td>
<td>0.8544</td>
<td>0.711</td>
<td>0.738</td>
<td>0.725</td>
<td>0.72467</td>
<td>0.013503</td>
</tr>
<tr>
<td>S6S-GNC</td>
<td>84.34</td>
<td>74.5</td>
<td>94.1</td>
<td>84.3133</td>
<td>9.80027</td>
<td>0.623</td>
<td>0.734</td>
<td>0.583</td>
<td>0.64667</td>
<td>0.078233</td>
</tr>
<tr>
<td>S6S-GNC-I</td>
<td>78.8</td>
<td>77.5</td>
<td>99.8</td>
<td>85.3667</td>
<td>12.51652</td>
<td>0.476</td>
<td>0.494</td>
<td>0.496</td>
<td>0.48867</td>
<td>0.011015</td>
</tr>
<tr>
<td><strong>DPBS + 10% FBS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo-GNC</td>
<td>22.1</td>
<td>20.7</td>
<td>21</td>
<td>21.2667</td>
<td>0.737111</td>
<td>0.511</td>
<td>0.534</td>
<td>0.540</td>
<td>0.54033</td>
<td>0.03296</td>
</tr>
<tr>
<td>Placebo-GNC-I</td>
<td>19.8</td>
<td>32.6</td>
<td>25.1</td>
<td>25.8333</td>
<td>6.431433</td>
<td>0.531</td>
<td>0.616</td>
<td>0.592</td>
<td>0.57967</td>
<td>0.043822</td>
</tr>
<tr>
<td>S6S-GNC</td>
<td>21.2</td>
<td>20.6</td>
<td>20.9</td>
<td>20.9</td>
<td>0.3</td>
<td>0.588</td>
<td>0.563</td>
<td>0.564</td>
<td>0.57167</td>
<td>0.014154</td>
</tr>
<tr>
<td>S6S-GNC-I</td>
<td>21.5</td>
<td>19.9</td>
<td>23.5</td>
<td>21.6333</td>
<td>1.8037</td>
<td>0.545</td>
<td>0.42</td>
<td>0.527</td>
<td>0.527</td>
<td>0.099232</td>
</tr>
<tr>
<td><strong>Water + 10% FBS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo-GNC</td>
<td>17.1</td>
<td>17</td>
<td>16.7</td>
<td>16.9333</td>
<td>0.208167</td>
<td>0.507</td>
<td>0.496</td>
<td>0.503</td>
<td>0.50333</td>
<td>0.006351</td>
</tr>
<tr>
<td>Placebo-GNC-I</td>
<td>16.2</td>
<td>16.5</td>
<td>16.3</td>
<td>16.3333</td>
<td>0.152753</td>
<td>0.472</td>
<td>0.473</td>
<td>0.470</td>
<td>0.47033</td>
<td>0.003786</td>
</tr>
<tr>
<td>S6S-GNC</td>
<td>18</td>
<td>19.9</td>
<td>17.2</td>
<td>18.3667</td>
<td>1.38843</td>
<td>0.561</td>
<td>0.531</td>
<td>0.580</td>
<td>0.58067</td>
<td>0.06189</td>
</tr>
<tr>
<td>S6S-GNC-I</td>
<td>19.4</td>
<td>18.9</td>
<td>17.2</td>
<td>18.5</td>
<td>1.153256</td>
<td>0.548</td>
<td>0.488</td>
<td>0.527</td>
<td>0.52867</td>
<td>0.046918</td>
</tr>
<tr>
<td><strong>DPBS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo-GNC</td>
<td>111.6</td>
<td>112.3</td>
<td>123.1</td>
<td>115.667</td>
<td>6.446963</td>
<td>0.859</td>
<td>0.852</td>
<td>0.953</td>
<td>0.888</td>
<td>0.0564</td>
</tr>
<tr>
<td>Placebo-GNC-I</td>
<td>144</td>
<td>138.4</td>
<td>146.7</td>
<td>143.033</td>
<td>4.233596</td>
<td>0.845</td>
<td>0.852</td>
<td>0.852</td>
<td>0.828</td>
<td>0.035679</td>
</tr>
<tr>
<td>S6S-GNC</td>
<td>64.94</td>
<td>78.13</td>
<td>63.52</td>
<td>68.8633</td>
<td>8.056515</td>
<td>0.634</td>
<td>0.641</td>
<td>0.539</td>
<td>0.53467</td>
<td>0.178147</td>
</tr>
<tr>
<td>S6S-GNC-I</td>
<td>77.9</td>
<td>80.9</td>
<td>66.3</td>
<td>75.0333</td>
<td>7.710599</td>
<td>0.573</td>
<td>0.594</td>
<td>0.453</td>
<td>0.54</td>
<td>0.076072</td>
</tr>
<tr>
<td><strong>DPBS + 10% FBS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo-GNC</td>
<td>20.8</td>
<td>20.1</td>
<td>20.2</td>
<td>20.3667</td>
<td>0.378594</td>
<td>0.575</td>
<td>0.54</td>
<td>0.54</td>
<td>0.55167</td>
<td>0.020207</td>
</tr>
<tr>
<td>Placebo-GNC-I</td>
<td>22.1</td>
<td>23.7</td>
<td>24.8</td>
<td>23.5333</td>
<td>1.357694</td>
<td>0.572</td>
<td>0.626</td>
<td>0.654</td>
<td>0.61733</td>
<td>0.041681</td>
</tr>
<tr>
<td>S6S-GNC</td>
<td>22.1</td>
<td>20.2</td>
<td>19.7</td>
<td>20.6667</td>
<td>1.266228</td>
<td>0.615</td>
<td>0.537</td>
<td>0.529</td>
<td>0.56033</td>
<td>0.047511</td>
</tr>
<tr>
<td>S6S-GNC-I</td>
<td>21.3</td>
<td>26.4</td>
<td>20.9</td>
<td>22.8667</td>
<td>3.066486</td>
<td>0.588</td>
<td>0.771</td>
<td>0.569</td>
<td>0.64267</td>
<td>0.111545</td>
</tr>
<tr>
<td><strong>Water + 10% FBS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo-GNC</td>
<td>16.8</td>
<td>16.6</td>
<td>16.3</td>
<td>16.5667</td>
<td>0.251661</td>
<td>0.504</td>
<td>0.498</td>
<td>0.468</td>
<td>0.49</td>
<td>0.019287</td>
</tr>
<tr>
<td>Placebo-GNC-I</td>
<td>17</td>
<td>17.1</td>
<td>17.2</td>
<td>17.1</td>
<td>0.1</td>
<td>0.477</td>
<td>0.479</td>
<td>0.496</td>
<td>0.484</td>
<td>0.01044</td>
</tr>
<tr>
<td>S6S-GNC</td>
<td>18.4</td>
<td>18.5</td>
<td>18.4</td>
<td>18.4333</td>
<td>0.057735</td>
<td>0.526</td>
<td>0.529</td>
<td>0.53</td>
<td>0.52833</td>
<td>0.002082</td>
</tr>
<tr>
<td>S6S-GNC-I</td>
<td>17.8</td>
<td>16.7</td>
<td>18.5</td>
<td>17.6667</td>
<td>0.907377</td>
<td>0.489</td>
<td>0.388</td>
<td>0.581</td>
<td>0.486</td>
<td>0.096535</td>
</tr>
<tr>
<td>concentration ng/µl sample</td>
<td>55S-GNC</td>
<td>55S-GNC-Peg</td>
<td>55S-GNC-P</td>
<td>55S-GNC-P-P</td>
<td>65S-GNC-P-P</td>
<td>55S-GNC-C-P</td>
<td>55S-GNC-C</td>
<td>55S-C</td>
<td>55S</td>
<td>55S</td>
</tr>
<tr>
<td>---------------------------</td>
<td>---------</td>
<td>-------------</td>
<td>-----------</td>
<td>-------------</td>
<td>-------------</td>
<td>-------------</td>
<td>-----------</td>
<td>------</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>time (µg/10 ml)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.25</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>0.34</td>
<td>0</td>
<td>0.71</td>
<td>0.69</td>
<td>1.24</td>
<td>0.91</td>
<td>0.53</td>
<td>2.09</td>
<td>0.36</td>
<td></td>
</tr>
<tr>
<td>0.75</td>
<td>3.41</td>
<td>3.98</td>
<td>4.35</td>
<td>3.73</td>
<td>4.65</td>
<td>3.79</td>
<td>4.01</td>
<td>3.53</td>
<td>3.04</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4.59</td>
<td>5.04</td>
<td>5.85</td>
<td>4.76</td>
<td>6.56</td>
<td>4.46</td>
<td>5.06</td>
<td>3.88</td>
<td>5.72</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>3.34</td>
<td>3.45</td>
<td>3.28</td>
<td>2.5</td>
<td>2.99</td>
<td>2.33</td>
<td>2.45</td>
<td>3.74</td>
<td>2.55</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>12.76</td>
<td>10.57</td>
<td>13.67</td>
<td>11.11</td>
<td>13.54</td>
<td>11.11</td>
<td>12.76</td>
<td>11.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>11.37</td>
<td>10.73</td>
<td>12.41</td>
<td>11.06</td>
<td>12.39</td>
<td>11.06</td>
<td>11.37</td>
<td>10.73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>12.52</td>
<td>11.24</td>
<td>12.74</td>
<td>11.06</td>
<td>12.39</td>
<td>11.06</td>
<td>12.52</td>
<td>11.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sampling correction</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.25</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.75</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>46.13</td>
<td>45.95</td>
<td>45.76</td>
<td>45.58</td>
<td>45.76</td>
<td>45.58</td>
<td>46.13</td>
<td>45.95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>128.23</td>
<td>127.98</td>
<td>128.23</td>
<td>127.98</td>
<td>128.23</td>
<td>127.98</td>
<td>128.23</td>
<td>127.98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>114.98</td>
<td>114.98</td>
<td>114.98</td>
<td>114.98</td>
<td>114.98</td>
<td>114.98</td>
<td>114.98</td>
<td>114.98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>122.38</td>
<td>122.38</td>
<td>122.38</td>
<td>122.38</td>
<td>122.38</td>
<td>122.38</td>
<td>122.38</td>
<td>122.38</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**100% for S55-GNC** 124.0530802 15.9519233
**100% for PEG** 44.6510615 13.7451796
**100% for peptide** 68.4496623 8.8207055
**100% for siRNA** 57.87387411 4.123310295
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>time</td>
<td>ave</td>
<td>stdev</td>
<td>ave</td>
<td>stdev</td>
<td>ave</td>
<td>stdev</td>
<td>ave</td>
<td>stdev</td>
<td>ave</td>
<td>stdev</td>
</tr>
<tr>
<td>0.25</td>
<td>0</td>
<td>2.741159776</td>
<td>0</td>
<td>15.8684444</td>
<td>0</td>
<td>18.1167466</td>
<td>0</td>
<td>13.2953543</td>
<td>0</td>
<td>3.743448127</td>
</tr>
<tr>
<td>0.5</td>
<td>27.4784952</td>
<td>32.1472291</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.75</td>
<td>13.19002564</td>
<td>40.85412874</td>
<td>107.0693713</td>
<td>147.674159</td>
<td>81.7718234</td>
<td>74.5407692</td>
<td>54.33678893</td>
<td>84.0393603</td>
<td>107.3740548</td>
<td>99.50932474</td>
</tr>
<tr>
<td>1</td>
<td>27.30722748</td>
<td>28.23944631</td>
<td>26.92498918</td>
<td>57.03532602</td>
<td>68.3461407</td>
<td>86.9408499</td>
<td>36.78044231</td>
<td>55.48508964</td>
<td>38.14497114</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>103.3900139</td>
<td>85.78375415</td>
<td>110.826232</td>
<td>115.7214379</td>
<td>63.0630751</td>
<td>82.1103593</td>
<td>101.9309883</td>
<td>116.646794</td>
<td></td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>137.5570904</td>
<td>117.2287433</td>
<td>99.4165486</td>
<td>88.80328085</td>
<td>87.39206538</td>
<td>123.804538</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>96</td>
<td>120</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cumulative release (%) | ave     | stdev   | ave     | stdev     | ave     | stdev     | ave     | stdev     | ave     | stdev     |
| 0.25                   | 0       | 0       | 0       | 0         | 0       | 0         | 0       | 0         | 0       | 0         |
| 0.5                    | 11.3185712 | 2.21652002 | 12.454 | 13.5823576 | 19.282618 | 3.8575476 | 55.1126862 | 10.3198581 |
| 0.75                   | 13.6133458 | 3.823783608 | 16.4447531 | 25.82086794 | 15.817548 | 7.12273721 | 85.63457998 | 0.051599293 |
| 1                      | 15.54848 | 5.17256494 | 18.25455565 | 33.25851136 | 22.473259 | 15.16941424 | 100 | 7.143171009 |
| 24                     | 88.542354 | 0.676130077 | 76.74612874 | 14.60461253 | 70.97230572 | 10.42707332 |
| 48                     | 100       | 12.86081528 | 93.08462298 | 27.1070367 | 92.22944 | 17.3310859 |
| 72                     | 100       | 19.0849856 | 100       | 20.6270683 |

| time                   | ave     | stdev   | ave     | stdev     | ave     | stdev     | ave     | stdev     | ave     | stdev     |
| 0.25                   | 0       | 0       | 0       | 0         | 0       | 0         | 0       | 0         | 0       | 0         |
| 0.5                    | 11.385712 | 2.21652002 | 12.454 | 13.5823576 | 19.282618 | 3.8575476 | 55.1126862 | 10.3198581 |
| 0.75                   | 13.6133458 | 3.823783608 | 16.4447531 | 25.82086794 | 15.817548 | 7.12273721 | 85.63457998 | 0.051599293 |
| 1                      | 15.54848 | 5.17256494 | 18.25455565 | 33.25851136 | 22.473259 | 15.16941424 | 100 | 7.143171009 |
| 24                     | 88.542354 | 0.676130077 | 76.74612874 | 14.60461253 | 70.97230572 | 10.42707332 |
| 48                     | 100       | 12.86081528 | 93.08462298 | 27.1070367 | 92.22944 | 17.3310859 |
| 72                     | 100       | 19.0849856 | 100       | 20.6270683 |

318
### Peg assay

#### Before Dialysis

<table>
<thead>
<tr>
<th>Peg conc (µg/ml)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.258</td>
<td>0.576</td>
<td>0.905</td>
<td>1.234</td>
<td>2.282</td>
<td>2</td>
<td>2.04</td>
<td>1.871</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.253</td>
<td>0.575</td>
<td>0.921</td>
<td>1.201</td>
<td>2.057</td>
<td>2.092</td>
<td>2.014</td>
<td>1.534</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.279</td>
<td>0.592</td>
<td>0.921</td>
<td>1.233</td>
<td>2.109</td>
<td>1.931</td>
<td>2.277</td>
<td>1.542</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>0.2033333</td>
<td>0.581</td>
<td>0.9156667</td>
<td>1.2226667</td>
<td>2.041333</td>
<td>0.808</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>0.01379613</td>
<td>0.00953939</td>
<td>0.0092376</td>
<td>0.018771</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### After dialysis

<table>
<thead>
<tr>
<th>Peg conc (µg/ml)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.255</td>
<td>0.565</td>
<td>0.915</td>
<td>1.187</td>
<td>1.01</td>
<td>0.808</td>
<td>0.945</td>
<td>0.77</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.254</td>
<td>0.575</td>
<td>0.924</td>
<td>1.227</td>
<td>0.907</td>
<td>0.8</td>
<td>0.829</td>
<td>0.623</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.276</td>
<td>0.596</td>
<td>0.924</td>
<td>1.253</td>
<td>0.853</td>
<td>0.783</td>
<td>0.824</td>
<td>0.62</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>0.2033333</td>
<td>0.581</td>
<td>0.9156667</td>
<td>1.2226667</td>
<td>2.041333</td>
<td>0.808</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

This is evidence that 2 hr with 5 complete medium changes is necessary to remove more free PEG. Proposal: 4 hr with 3 complete medium changes.
Before Dialysis

\[ y = 31.119x - 0.0098 \]
\[ R^2 = 0.9998 \]

PEG Concentration (µg/mL)
Absorbance, x nm

After Dialysis

\[ y = 31.277x - 0.0679 \]
\[ R^2 = 0.9991 \]

PEG Concentration (µg/mL)
Absorbance, x nm
Before dislysis is important because we can find the amount of PEG actually conjugated onto the GNC.

10 mg PEG was added to 10 mL batch.

0.5 mL sample of formulation was passed through a 100,000 da vivaspin.

| Plac-GNC | 2.14933333 | 1.886 | 58.680634 | 29.34032 | 586.80634 | 10000 | 9413.194 | 94.1319366 | 1.13373E+18 |
| Plac-GNC-I | 2.00766667 | 1.74433333 | 54.272109 | 27.13605 | 542.72109 | 10000 | 9457.279 | 94.5727891 | 1.13903E+18 |
| S6S-GNC | 2.11033333 | 1.847 | 57.466993 | 28.7335 | 574.66993 | 10000 | 9425.33 | 94.2533007 | 1.13519E+18 |
| S6S-GNC-I | 1.649 | 1.38566667 | 43.110761 | 21.55538 | 431.10761 | 10000 | 9568.892 | 95.6889239 | 1.15248E+18 |

### Peg conc (µg/ml) absorbance, 535 nm, A.U.

<table>
<thead>
<tr>
<th>Peg conc (µg/ml)</th>
<th>0</th>
<th>0.26333333</th>
<th>0.581</th>
<th>0.91566667</th>
<th>1.22266667</th>
<th>0.33125</th>
<th>0.58466667</th>
<th>0.608</th>
<th>0.59633333</th>
<th>0.53533333</th>
</tr>
</thead>
<tbody>
<tr>
<td>corr abs</td>
<td>0.01739613</td>
<td>0.00953939</td>
<td>0.0092376</td>
<td>0.01877054</td>
<td>0.00980000</td>
<td>0.00980000</td>
<td>7.8767325</td>
<td>8.02338325</td>
<td>8.23932825</td>
<td>6.34106925</td>
</tr>
<tr>
<td>stdev</td>
<td>0.01379613</td>
<td>0.00953939</td>
<td>0.0092376</td>
<td>0.01877054</td>
<td>0.00980000</td>
<td>0.00980000</td>
<td>7.8767325</td>
<td>8.02338325</td>
<td>8.23932825</td>
<td>6.34106925</td>
</tr>
</tbody>
</table>

### Peg conc (µg/ml) absorbance, 535 nm, A.U.

**Series1**

\[
y = 31.119x - 0.0098
\]

**R² = 0.9998**

Absorbance, 535 nm, A.U.

PEG, µg/ml

96.14% PEGylation efficiency
### Before PEGylation/Peptide conjugation

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td>0</td>
<td>5</td>
<td>10</td>
<td>15</td>
<td>20</td>
<td>placebo</td>
<td>S6S-GNC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.205</td>
<td>0.375</td>
<td>0.249</td>
<td>0.27</td>
<td>1.525</td>
<td>1.251</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.181</td>
<td>0.196</td>
<td>0.215</td>
<td>0.217</td>
<td>0.219</td>
<td>1.064</td>
<td>0.975</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>0.186</td>
<td>0.247</td>
<td>0.242</td>
<td>0.252</td>
<td>0.43</td>
<td>1.04</td>
<td>1.268</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>0.202</td>
<td>0.22</td>
<td>0.236</td>
<td>0.223</td>
<td>0.241</td>
<td>0.511</td>
<td>1.287</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>0.183</td>
<td>0.211</td>
<td>0.24</td>
<td>0.238</td>
<td>0.267</td>
<td>0.433</td>
<td>1.496</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Glycine concentration (µg/mL)

<table>
<thead>
<tr>
<th>µg/mL</th>
<th>abs</th>
<th>con</th>
<th>stdev</th>
<th>number of amines</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.1914</td>
<td>0</td>
<td>0.011238</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>0.2168</td>
<td>0.0254</td>
<td>0.018939</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>0.2334</td>
<td>0.0422</td>
<td>0.010761</td>
<td>10</td>
</tr>
<tr>
<td>20</td>
<td>0.2854</td>
<td>0.0844</td>
<td>0.083464</td>
<td>20</td>
</tr>
</tbody>
</table>

placebo: 1.209667, 1.018267, 0.27335, 218.425
S6S-GNC: 1.3255, 1.1341, 0.114614, 243.2608

**Correct based on glycine molecular weight and actual amount of amines (molecule=1 amine)**

90 µL of sample formulation present
90 µL of 1% gelatin has

1g/100mL = 0.1 g/10 mL = 0.01 g/1 mL = 10 µg/1000 µL
10/1000 * 90 = 9 µg/90 µL

0.0000009 g
2E-11 mol
1.204E+13 molecules

*Note: the nanoparticle concentration at 1% gelatin and 90 µL of formulation is too high for this assay

### After dialysis

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.203</td>
<td>0.229</td>
<td>0.249</td>
<td>0.313</td>
<td>0.261</td>
<td>0.956</td>
<td>0.846</td>
<td>1.103</td>
<td>0.856</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.279</td>
<td>0.229</td>
<td>0.226</td>
<td>0.274</td>
<td>0.294</td>
<td>0.866</td>
<td>0.82</td>
<td>1.147</td>
<td>0.698</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.198</td>
<td>0.229</td>
<td>0.339</td>
<td>0.271</td>
<td>0.345</td>
<td>0.823</td>
<td>0.867</td>
<td>0.867</td>
<td>0.79</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>0.212</td>
<td>0.227</td>
<td>0.289</td>
<td>0.264</td>
<td>0.32</td>
<td>1.919</td>
<td>1.979</td>
<td>0.858</td>
<td>1.877</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>0.21</td>
<td>0.273</td>
<td>0.253</td>
<td>0.266</td>
<td>0.337</td>
<td>0.206</td>
<td>1.235</td>
<td>1.186</td>
<td>0.792</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Glycine molecular weight is 75.0666 g/mol

### Glycine concentration (µg/mL)

<table>
<thead>
<tr>
<th>µg/mL</th>
<th>abs</th>
<th>con</th>
<th>stdev</th>
<th>amines</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.2204</td>
<td>0</td>
<td>0.033231</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0.2374</td>
<td>0.017</td>
<td>0.01992</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>0.2652</td>
<td>0.0448</td>
<td>0.03107</td>
<td>10</td>
</tr>
<tr>
<td>15</td>
<td>0.2776</td>
<td>0.0572</td>
<td>0.020182</td>
<td>15</td>
</tr>
<tr>
<td>20</td>
<td>0.3114</td>
<td>0.091</td>
<td>0.034283</td>
<td>20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>µg/mL</th>
<th>abs</th>
<th>con</th>
<th>stdev</th>
<th>amines</th>
<th>stdev</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo-GN: 0.882333</td>
<td>0.061933</td>
<td>0.067649</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo-GN: 0.884333</td>
<td>0.029393</td>
<td>0.023544</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S6S-GNC: 1.0035</td>
<td>0.7831</td>
<td>0.166342</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S6S-GNC: 1.1035</td>
<td>0.784</td>
<td>0.065013</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Binding Assay – Flow Cytometry

### Binding Assay following incubation at 37°C for 1 hr.

![Graph showing binding assay results after 1 hour of incubation at 37°C.](image)

### Binding Assay following incubation at 37°C for 4 hr.

![Graph showing binding assay results after 4 hours of incubation at 37°C.](image)
Internalization Assay – Flow Cytometry

Internalization Assay following incubation at 37°C for 5 min.

<table>
<thead>
<tr>
<th>Sample Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 min Medium Control Imd</td>
</tr>
<tr>
<td>5 min non-PEGylated GNC Imd</td>
</tr>
<tr>
<td>5 min GNC Imd</td>
</tr>
<tr>
<td>5 min 7.5% mol ratio GNC-P Imd</td>
</tr>
<tr>
<td>5 min 15% mol ratio GNC-P Imd</td>
</tr>
</tbody>
</table>

FL1: FL1 Fluorescence

Count

Internalization Assay following incubation at 37°C for 15 min.

<table>
<thead>
<tr>
<th>Sample Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 min Medium Control Imd</td>
</tr>
<tr>
<td>15 min non-PEGylated GNC Imd</td>
</tr>
<tr>
<td>15 min GNC Imd</td>
</tr>
<tr>
<td>15 min 7.5% mol ratio GNC-P Imd</td>
</tr>
<tr>
<td>15 min 15% mol ratio GNC-P Imd</td>
</tr>
</tbody>
</table>

FL1: FL1 Fluorescence

Count
In vitro release

STAT6 siRNA Solution

Model order is 0
Start point is 1.00000000000000E-010  Point no =  1
End point is 1.00000000000000E+000  Point no =  5
a = 9.9808000070221E+001
b = 5.46599999451080E+000
r = 9.81348217389239E-001
standard error for a = 7.94425780471424E+000
standard error for b = 4.86484450166818E+000
percent standard error for $a = 7.95954012118799E+000$
percent standard error for $b = 8.900191384133E+001$

Fitting results

time   OBS    PRED
1.00000000000000E-010  1.00000000000000E-010  5.4660000044160E+000
2.50000000000000E-001  3.61100000000000E+001  3.0417999962663E+001
5.00000000000000E-001  5.51100000000000E+001  5.5369999980218E+001
7.50000000000000E-001  8.56300000000000E+001  8.0321999997774E+001
1.00000000000000E+000  9.99999999999900E+001  1.05274000001533E+002

$\text{RMSE} = 4.86484450127900E+000$
AIC = 2.78675370860607E+001
BIC = 2.7086429109289E+001

--------------------------------------------

Model independent description

$\text{DE} = 5.6712499982058E+001$
$\text{MDT} = 4.32875000017886E-001$
No of timepoints = 5

--------------------------------------------

Model order is 1
Start point is 1.00000000000000E-010  Point no = 1
End point is 1.00000000000000E+000  Point no = 5
$a = 2.24502032343541E+001$
$b = -1.25000504239720E+001$
$r = 5.3209709999821E-001$
standard error for $a = 1.21546414265921E+001$
standard error for $b = 7.44316737541122E+000$
percent standard error for $a = 5.41404516463024E+001$
percent standard error for $b = 5.95450988032580E+001$

Fitting results

time   OBS    PRED
1.00000000000000E-010  1.00000000000000E-010  3.72646527252687E-006
2.50000000000000E-001  3.61100000000000E+001  1.0204620171767E-003
5.00000000000000E-001  5.51100000000000E+001  2.79445273554419E-001
7.50000000000000E-001  8.56300000000000E+001  7.65238151696959E+001
1.00000000000000E+000  9.99999999999900E+001  2.09554243435268E+004

$\text{RMSE} = 9.32687640873737E+003$
AIC = 1.03453744039434E+002
BIC = 1.02672619864303E+002

--------------------------------------------
Model order is 0
Start point is 1.00000000000000E-010 Point no = 1
End point is 7.20000000000000E+001 Point no = 8
a = 1.40386220748964E+000
b = 1.5193023253397E+001
r = 8.81632094601077E-001
standard error for a = 2.10001377289931E-001
standard error for b = 6.66811211396858E+000
percent standard error for a = 1.49588311566169E+001
percent standard error for b = 4.38893034729116E+001

Fitting results

<table>
<thead>
<tr>
<th>time</th>
<th>OBS</th>
<th>PRED</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00000000000000E-010</td>
<td>1.00000000000000E-010</td>
<td>1.5193023253397E+001</td>
</tr>
<tr>
<td>2.50000000000000E-001</td>
<td>1.04400000000000E+000</td>
<td>1.55439888772121E+001</td>
</tr>
<tr>
<td>5.00000000000000E-001</td>
<td>1.22500000000000E+001</td>
<td>1.58949544290845E+001</td>
</tr>
<tr>
<td>7.50000000000000E-001</td>
<td>1.64400000000000E+001</td>
<td>1.62459199809569E+001</td>
</tr>
<tr>
<td>1.00000000000000E+000</td>
<td>1.82500000000000E+001</td>
<td>1.65968855328293E+001</td>
</tr>
<tr>
<td>2.40000000000000E+001</td>
<td>7.67500000000000E+001</td>
<td>4.88857630590101E+001</td>
</tr>
<tr>
<td>4.80000000000000E+000</td>
<td>9.30800000000000E+001</td>
<td>8.25784092848243E+001</td>
</tr>
<tr>
<td>7.20000000000000E+000</td>
<td>9.90000000000000E+001</td>
<td>1.1627102264594E+002</td>
</tr>
</tbody>
</table>

RMSE = 1.33434688602419E+001
AIC = 6.20919649924028E+001
BIC = 6.22508480757625E+001

Model independent description
DE = 7.58261631944357E+001
MDT = 1.74051625000008E+001
No of timepoints = 8

Model order is 1
Start point is 1.00000000000000E-010 Point no = 1
End point is 7.20000000000000E+001 Point no = 8
a = 1.19211867781722E-001
b = -2.05701920749794E+000
r = 1.23646009016873E-001
standard error for a = 1.29566760421595E-001
standard error for b = 4.11409532587064E+000
percent standard error for a = 1.08686125662281E+002
percent standard error for b = 2.00002766667154E+002

Fitting results
<table>
<thead>
<tr>
<th>time</th>
<th>OBS</th>
<th>PRED</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00000000000000E-010</td>
<td>1.00000000000000E-010</td>
<td>1.27834450519342E-001</td>
</tr>
<tr>
<td>2.50000000000000E-001</td>
<td>1.04400000000000E+001</td>
<td>1.3170163999082E-001</td>
</tr>
<tr>
<td>5.00000000000000E-001</td>
<td>1.22500000000000E+001</td>
<td>1.3568531750881E-001</td>
</tr>
<tr>
<td>7.50000000000000E-001</td>
<td>1.64400000000000E+001</td>
<td>1.39790513846263E-001</td>
</tr>
<tr>
<td>1.00000000000000E+000</td>
<td>1.82500000000000E+001</td>
<td>1.4401938420613E-001</td>
</tr>
<tr>
<td>2.40000000000000E+001</td>
<td>7.67500000000000E+001</td>
<td>2.2346075428717E+000</td>
</tr>
<tr>
<td>4.80000000000000E+001</td>
<td>9.30800000000000E+001</td>
<td>3.906201455464E+001</td>
</tr>
<tr>
<td>7.20000000000000E+001</td>
<td>9.99999999999900E+001</td>
<td>6.8282725236105E+002</td>
</tr>
</tbody>
</table>

RMSE = 2.08865798143044E+002
AIC = 1.06102603238608E+002
BIC = 1.06261486321968E+002

Korshmeyer-Peppas model
Model is ln(y) = a*ln(x)+b
Which is equal to y = K*x^A
K = 7.14928519008952E+000
A = 1.0469650924813E+000
Start point is 1.00000000000000E-010 Point no = 1
End point is 7.20000000000000E+001 Point no = 8
a = 1.0469650924813E+000
b = 1.96701237829419E+000
r = 9.76728647973613E-001
standard error for a = 6.59751553546218E-002
standard error for b = 5.59842507677859E-001
percent standard error for a = 6.30156208916774E+000
percent standard error for b = 2.8461560544792E+001

Fitting results
<table>
<thead>
<tr>
<th>time</th>
<th>OBS</th>
<th>PRED</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00000000000000E-010</td>
<td>1.00000000000000E-010</td>
<td>2.42444143047421E-010</td>
</tr>
<tr>
<td>2.50000000000000E-001</td>
<td>1.04400000000000E+001</td>
<td>1.6746606448139E+000</td>
</tr>
<tr>
<td>5.00000000000000E-001</td>
<td>1.22500000000000E+001</td>
<td>3.4601483928560E+000</td>
</tr>
<tr>
<td>7.50000000000000E-001</td>
<td>1.64400000000000E+001</td>
<td>5.2900552717628E+000</td>
</tr>
<tr>
<td>1.00000000000000E+000</td>
<td>1.82500000000000E+001</td>
<td>7.14928519008953E+000</td>
</tr>
<tr>
<td>2.40000000000000E+001</td>
<td>7.67500000000000E+001</td>
<td>1.9920287956275E+002</td>
</tr>
<tr>
<td>4.80000000000000E+001</td>
<td>9.30800000000000E+001</td>
<td>4.11588762951976E+002</td>
</tr>
<tr>
<td>7.20000000000000E+001</td>
<td>9.99999999999900E+001</td>
<td>6.2925244364653E+002</td>
</tr>
</tbody>
</table>

RMSE = 2.22753319163024E+002
AIC = 1.07132571806783E+002
BIC = 1.07291454890142E+002

Model is Weibull y=100*(1-exp[-((t-lag)^A)/K])
K = 6.4700212019096916E+000
A = 1.10140017871315E+000

328
lag = 0
Start point is 1.00000000000000E-010 Point no = 1
End point is 7.20000000000000E+001 Point no = 8
a = 1.10140017871315E+000
b = -1.86717938546412E+000
r = 9.90954275648220E-001
standard error for a = 4.29600110331262E-002
standard error for b = 3.64543898038264E-001
percent standard error for a = 3.90049065393468E+000
percent standard error for b = 1.9523774784735E+001

Fitting results

<table>
<thead>
<tr>
<th>time</th>
<th>OBS</th>
<th>PRED</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00000000000000E-010</td>
<td>1.00000000000000E-010</td>
<td>1.49655439950214E-010</td>
</tr>
<tr>
<td>2.50000000000000E-001</td>
<td>1.04400000000000E+001</td>
<td>3.3015319576444E-001</td>
</tr>
<tr>
<td>5.00000000000000E-001</td>
<td>1.22500000000000E+001</td>
<td>6.95010926409571E-001</td>
</tr>
<tr>
<td>7.50000000000000E-001</td>
<td>1.64900000000000E+001</td>
<td>1.0648004555482E+001</td>
</tr>
<tr>
<td>1.00000000000000E+000</td>
<td>1.82500000000000E+001</td>
<td>1.43207059212814E+001</td>
</tr>
<tr>
<td>2.40000000000000E+000</td>
<td>7.67500000000000E+001</td>
<td>9.4023219239760E+001</td>
</tr>
<tr>
<td>4.80000000000000E+000</td>
<td>9.30800000000000E+001</td>
<td>9.9983051731249E+001</td>
</tr>
<tr>
<td>7.20000000000000E+001</td>
<td>9.99999999999900E+001</td>
<td>9.9999965059467E+001</td>
</tr>
</tbody>
</table>

RMSE = 9.28051565044190E+000
AIC = 5.62822061099949E+001
BIC = 5.6441089135467E+001

--------------------------------------------
--------------------------------------------
Baker-Lonsdale 3/2[1-(1-y)^2/3]-y=Kt
Start point is 1.00000000000000E-010 Point no = 1
End point is 7.20000000000000E+001 Point no = 8
a = 6.8556988066032E-003
b = -8.94853649186022E-004
r = 9.99218870726711E-001
standard error for a = 7.8253113187732E-005
standard error for b = 2.48474808248538E-003
percent standard error for a = 1.1444638742536E+000
percent standard error for b = 2.77670888948775E+002

Fitting results

<table>
<thead>
<tr>
<th>time</th>
<th>OBS</th>
<th>PRED</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00000000000000E-010</td>
<td>1.00000000000000E-010</td>
<td>0.00000000000000E+000</td>
</tr>
<tr>
<td>2.50000000000000E-001</td>
<td>1.04400000000000E+001</td>
<td>0.00000000000000E+000</td>
</tr>
<tr>
<td>5.00000000000000E-001</td>
<td>1.22500000000000E+001</td>
<td>0.00000000000000E+000</td>
</tr>
<tr>
<td>7.50000000000000E-001</td>
<td>1.64900000000000E+001</td>
<td>0.00000000000000E+000</td>
</tr>
<tr>
<td>1.00000000000000E+000</td>
<td>1.82500000000000E+001</td>
<td>0.00000000000000E+000</td>
</tr>
<tr>
<td>2.40000000000000E+000</td>
<td>7.67500000000000E+001</td>
<td>0.00000000000000E+000</td>
</tr>
<tr>
<td>4.80000000000000E+000</td>
<td>9.30800000000000E+001</td>
<td>0.00000000000000E+000</td>
</tr>
<tr>
<td>7.20000000000000E+001</td>
<td>9.99999999999900E+001</td>
<td>0.00000000000000E+000</td>
</tr>
</tbody>
</table>

RMSE = 5.63658153271977E+001
AIC = 8.51453381498299E+001
BIC = 8.53042212331896E+001
--------------------------------------------
Model is Higuchi y=K*t^(1/2)
Which is equal to ln(y) = 1/2*ln(t)+b
K = exp(b) = 2.7325601953845087E+000
Start point is 1.00000000000000E-010 Point no = 1
End point is 7.20000000000000E+001 Point no = 8
a = 5.00000000000000E-001
b = 1.00523897024385E+000
r = -3.33008729001645E-001
standard error for a = 2.32840123667679E-001
standard error for b = 1.97580131522956E+000
percent standard error for a = 4.65680247335358E+001
percent standard error for b = 1.96550409774730E+002

Fitting results

time   OBS   PRED
1.00000000000000E-010 1.00000000000000E-010 2.73256019538451E-005
2.50000000000000E-001 1.04400000000000E+001 1.36628009769225E+000
5.00000000000000E-001 1.22500000000000E+001 1.93221184415682E+000
7.50000000000000E-001 1.64000000000000E+001 2.3664654657315E+000
1.00000000000000E+000 1.82500000000000E+001 2.73256019538451E+000
2.40000000000000E+001 7.67500000000000E+001 1.3387563402639E+001
4.80000000000000E+001 9.30800000000000E+001 1.893173275852E+001
7.20000000000000E+001 9.99999999999900E+001 2.31865421298819E+001
RMSE  = 4.47783680704791E+001
AIC    = 8.14631350092625E+001
BIC    = 8.16220180926221E+001
--------------------------------------------
S6S-GNC-P
--------------------------------------------
Model order is 0
Start point is 1.00000000000000E-010 Point no = 1
End point is 7.20000000000000E+001 Point no = 8
a = 1.36273471923065E+000
b = 1.627240245450830E+001
r = 9.00241318767036E-001
standard error for a = 1.851959880817275E-001
standard error for b = 5.88047386889857E+000
percent standard error for a = 1.35900249306250E+001
percent standard error for b = 3.61376716235418E+001
### Fitting results

<table>
<thead>
<tr>
<th>time</th>
<th>OBS</th>
<th>PRED</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00000000000000E-010</td>
<td>1.00000000000000E-010</td>
<td>1.6272420452193E+001</td>
</tr>
<tr>
<td>2.50000000000000E-001</td>
<td>1.30500000000000E+001</td>
<td>1.6613104138907E+001</td>
</tr>
<tr>
<td>5.00000000000000E-001</td>
<td>1.52800000000000E+001</td>
<td>1.695378136983E+001</td>
</tr>
<tr>
<td>7.50000000000000E-001</td>
<td>1.58200000000000E+001</td>
<td>1.7294714935060E+001</td>
</tr>
<tr>
<td>1.00000000000000E+000</td>
<td>2.24700000000000E+001</td>
<td>1.76351573136E+001</td>
</tr>
<tr>
<td>2.40000000000000E+001</td>
<td>7.09700000000000E+001</td>
<td>4.89780537156185E+001</td>
</tr>
<tr>
<td>4.80000000000000E+001</td>
<td>9.22300000000000E+001</td>
<td>8.1683689771540E+001</td>
</tr>
<tr>
<td>7.20000000000000E+001</td>
<td>9.9999999999900E+001</td>
<td>1.1438932023869E+002</td>
</tr>
</tbody>
</table>

RMSE = 1.17673366332190E+001  
AIC = 6.0087741145202E+001  
BIC = 6.0239657197899E+001

-------------

### Model independent description

DE = 7.43550868055450E+001  
MDT = 1.84643375000022E+001  
No of timepoints = 8

-------------

### Model order is 1

Start point is 1.00000000000000E-010  
End point is 7.20000000000000E+001  
a = 1.17054888917976E-001  
b = -1.95173628682741E+000  
r = 1.18757785527467E-001  
standard error for a = 1.30175883285674E-001  
standard error for b = 4.1334362544343E+000  
percent standard error for a = 1.11209266429608E+002  
percent standard error for b = 2.11782537084578E+002

-------------

### Fitting results

<table>
<thead>
<tr>
<th>time</th>
<th>OBS</th>
<th>PRED</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00000000000000E-010</td>
<td>1.00000000000000E-010</td>
<td>1.42027257323982E-001</td>
</tr>
<tr>
<td>2.50000000000000E-001</td>
<td>1.30500000000000E+001</td>
<td>1.4624494723230E+001</td>
</tr>
<tr>
<td>5.00000000000000E-001</td>
<td>1.52800000000000E+001</td>
<td>1.50587820152489E+001</td>
</tr>
<tr>
<td>7.50000000000000E-001</td>
<td>1.58200000000000E+001</td>
<td>1.5505962989629E+001</td>
</tr>
<tr>
<td>1.00000000000000E+000</td>
<td>2.24700000000000E+001</td>
<td>1.59664363065291E+001</td>
</tr>
<tr>
<td>2.40000000000000E+001</td>
<td>7.09700000000000E+001</td>
<td>2.3574512278665E+000</td>
</tr>
<tr>
<td>4.80000000000000E+001</td>
<td>9.22300000000000E+001</td>
<td>3.91303500199284E+001</td>
</tr>
<tr>
<td>7.20000000000000E+001</td>
<td>9.9999999999900E+001</td>
<td>6.4950365065916E+002</td>
</tr>
</tbody>
</table>

RMSE = 1.97048618862626E+002  
AIC = 1.05170740246171E+002  
BIC = 1.05329623329531E+002

-------------
**Korsmeyer-Peppas model**
Model is \( \ln(y) = a \ln(x) + b \)
Which is equal to \( y = K \cdot x^A \)

\[
K = 7.64001971143966E+0000 \\
A = 1.04730890843710E+0000 \\
\]
Start point is \( 1.00000000000000E-010 \)  Point no =  1
End point is \( 7.20000000000000E+0001 \)  Point no =  8
\[
a = 1.04730890843710E+0000 \\
b = 2.03340018320647E+0000 \\
r = 9.73645757803505E-001 \\
\]
standard error for \( a \) = \( 7.03434963869720E-002 \)
standard error for \( b \) = \( 5.96910749272711E-001 \)
percent standard error for \( a \) = \( 6.71659486711956E+000 \)
percent standard error for \( b \) = \( 2.93553012438231E+001 \)

**Fitting results**

<table>
<thead>
<tr>
<th>time</th>
<th>OBS</th>
<th>PRED</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00000000000000E-010</td>
<td>1.00000000000000E-010</td>
<td>2.57042771177614E-010</td>
</tr>
<tr>
<td>2.50000000000000E-0001</td>
<td>1.30500000000000E+0001</td>
<td>1.78875840173978E+0000</td>
</tr>
<tr>
<td>5.00000000000000E-0001</td>
<td>1.52800000000000E+0001</td>
<td>3.697754787108E+0000</td>
</tr>
<tr>
<td>7.50000000000000E-0001</td>
<td>1.58200000000000E+0001</td>
<td>5.6525799924397E+0000</td>
</tr>
<tr>
<td>1.00000000000000E+0000</td>
<td>2.24700000000000E+0000</td>
<td>7.6401971143970E+0000</td>
</tr>
<tr>
<td>2.40000000000000E+0001</td>
<td>7.09700000000000E+0001</td>
<td>2.13109106408901E+0002</td>
</tr>
<tr>
<td>4.80000000000000E+0001</td>
<td>9.22300000000000E+0001</td>
<td>4.40426461538606E+0002</td>
</tr>
<tr>
<td>7.20000000000000E+0001</td>
<td>9.99999999999900E+0001</td>
<td>6.73434479862445E+0002</td>
</tr>
</tbody>
</table>

RMSE = \( 2.42604538276050E+002 \)
AIC = 1.0849845584018E+002
BIC = 1.0865733867378E+002

**Model is Weibull \( y=100\times(1-\exp[-((t-lag)^A)/K]) \)**

\[
K = 6.1174481655845854E+0000 \\
A = 1.10072222674569E+0000 \\
\]
lag = 0
Start point is \( 1.00000000000000E-010 \)  Point no =  1
End point is \( 7.20000000000000E+0001 \)  Point no =  8
\[
a = 1.10072222674569E+0000 \\
b = -1.8114504316182E+0000 \\
r = 9.88529852090156E-001 \\
\]
standard error for \( a \) = \( 4.84051673249794E-002 \)
standard error for \( b \) = \( 4.10749624068668E-001 \)
percent standard error for \( a \) = \( 4.39758243713223E+000 \)
percent standard error for \( b \) = \( 2.26790024036398E+001 \)

**Fitting results**

<table>
<thead>
<tr>
<th>time</th>
<th>OBS</th>
<th>PRED</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00000000000000E-010</td>
<td>1.00000000000000E-010</td>
<td>1.60770897462970E-010</td>
</tr>
</tbody>
</table>
2.50000000000000E-001 1.30500000000000E+001 3.49167753117789E+000  
5.00000000000000E-001 1.52800000000000E+001 7.3389537884854E+000  
7.50000000000000E-001 1.58200000000000E+001 1.1227981720039E+001  
1.00000000000000E+000 2.24700000000000E+001 1.50805353207368E+001  
2.40000000000000E+001 7.09700000000000E+001 9.5498288692661E+001  
4.80000000000000E+001 9.22300000000000E+001 9.99990723458038E+001  
7.20000000000000E+001 9.99999999999900E+001 9.99999986309972E+001  
RMSE = 1.17647672722468E+001  
AIC = 6.00772801833456E+001  
BIC = 6.02361632667053E+001  

--------------------------------------------
--------------------------------------------

Model is Higuchi y=K*t^(1/2)
Which is equal to ln(y) = 1/2*ln(t)+b
K = exp(b) = 2.9183611697034407E+0000
Start point is 1.00000000000000E-010 Point no = 1
End point is 7.20000000000000E+001 Point no = 8
a = 5.00000000000000E-003
b = 1.07102221547200E+000
r = -3.44148724871237E-001
standard error for a = 2.34249256903222E-001
standard error for b = 1.98775873586765E+000
percent standard error for a = 4.68498513806444E+001
percent standard error for b = 1.85594538297383E+002

Fitting results

time OBS PRED
1.00000000000000E-010 1.00000000000000E+001 2.9183616970344E-005  
2.50000000000000E-001 1.30500000000000E+001 1.45918058485172E+000  
5.00000000000000E-001 1.52800000000000E+001 2.06359297304881E+000  
7.50000000000000E-001 1.58200000000000E+001 2.5273491038125E+000  
1.00000000000000E+000 2.24700000000000E+001 2.9183616970344E+000  
2.40000000000000E+001 7.09700000000000E+001 1.42969915018506E+001  
4.80000000000000E+001 9.22300000000000E+001 2.02189992830500E+001  
7.20000000000000E+001 9.99999999999900E+001 2.47631156765857E+001  
RMSE = 4.31943049650677E+001  
AIC = 8.08868708461982E+001  
BIC = 8.10457539295579E+001  

--------------------------------------------

Baker-Lonsdale 3/2(1-(1-y)^2/3)-y=Kt
Start point is 1.00000000000000E-010 Point no = 1
End point is 7.20000000000000E+001 Point no = 8
a = 6.73770880359223E-003
b = -3.5961502474689E-003
r = 9.94600860740571E-001
standard error for a = 2.0266299731726E-004
standard error for b = 6.43509868776606E-003
percent standard error for a = 3.00789189944919E+000
percent standard error for b = 1.78945852495889E+002

Fitting results

time OBS PRED
1.00000000000000E-010 1.00000000000000E-010 0.00000000000000E+000
2.50000000000000E-001 1.30500000000000E+001 0.00000000000000E+000
5.00000000000000E-001 1.52800000000000E+001 0.00000000000000E+000
7.50000000000000E-001 1.58200000000000E+001 0.00000000000000E+000
1.00000000000000E+000 2.24700000000000E+001 0.00000000000000E+000
2.40000000000000E+001 7.09700000000000E+001 0.00000000000000E+000
4.80000000000000E+001 9.22300000000000E+001 0.00000000000000E+000
7.20000000000000E+001 9.99999999999999E+001 0.00000000000000E+000

RMSE = 5.55676029715134E+001
AIC = 8.49171379504610E+001
BIC = 8.50760210338207E+001

--------------------------------------------

ELISA
<table>
<thead>
<tr>
<th></th>
<th>relative</th>
<th>sd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>0.515</td>
<td>0.221</td>
</tr>
<tr>
<td>Scrambled siRNA</td>
<td>0.534</td>
<td>0.515</td>
</tr>
<tr>
<td>STAT6 siRNA</td>
<td>0.092</td>
<td>0.089</td>
</tr>
<tr>
<td>S6S-GNC (PEGylated)</td>
<td>0.356</td>
<td>0.352</td>
</tr>
<tr>
<td>S6S-GNC-I (1 mol % peptide to PEG)</td>
<td>0.107</td>
<td>0.127</td>
</tr>
<tr>
<td>S6S-GNC-I (2.5% mol % peptide to PEG)</td>
<td>0.108</td>
<td>0.112</td>
</tr>
<tr>
<td>S6S-GNC-I (5% mol % peptide to PEG)</td>
<td>0.107</td>
<td>0.131</td>
</tr>
<tr>
<td>S6S-GNC-I (10% mol % peptide to PEG)</td>
<td>0.113</td>
<td>0.125</td>
</tr>
</tbody>
</table>
### Flow Cytometry

#### Binding Assay

<table>
<thead>
<tr>
<th>Protein</th>
<th>Medium</th>
<th>Scrambled</th>
<th>STAT6 siRNA</th>
<th>S6S-GNC</th>
<th>S6S-GNC-I (1 mol % peptide to PEG)</th>
<th>S6S-GNC-I (7.5% mol % peptide to PEG)</th>
<th>S6S-GNC-I (15% mol % peptide to PEG)</th>
<th>S6S-GNC-I (30% mol % peptide to PEG)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.28</td>
<td>0.462</td>
<td>0.297</td>
<td>0.32</td>
<td>0.61</td>
<td>0.47</td>
<td>0.46</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>0.457</td>
<td>0.389</td>
<td>0.297</td>
<td>0.36</td>
<td>0.47</td>
<td>0.47</td>
<td>0.46</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>0.203</td>
<td>0.389</td>
<td>0.248</td>
<td>0.297</td>
<td>0.47</td>
<td>0.47</td>
<td>0.46</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>0.266</td>
<td>0.422</td>
<td>0.276</td>
<td>0.297</td>
<td>0.47</td>
<td>0.47</td>
<td>0.46</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>0.372</td>
<td>0.364</td>
<td>0.289</td>
<td>0.297</td>
<td>0.47</td>
<td>0.47</td>
<td>0.46</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>0.21</td>
<td>0.211</td>
<td>0.289</td>
<td>0.297</td>
<td>0.47</td>
<td>0.47</td>
<td>0.46</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>0.388</td>
<td>0.389</td>
<td>0.248</td>
<td>0.297</td>
<td>0.47</td>
<td>0.47</td>
<td>0.46</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>0.507</td>
<td>0.42</td>
<td>0.347</td>
<td>0.26</td>
<td>0.35</td>
<td>0.48</td>
<td>0.45</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>0.518</td>
<td>0.42</td>
<td>0.347</td>
<td>0.26</td>
<td>0.35</td>
<td>0.48</td>
<td>0.45</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>0.488</td>
<td>0.26</td>
<td>0.347</td>
<td>0.26</td>
<td>0.35</td>
<td>0.48</td>
<td>0.45</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>0.46</td>
<td>0.3</td>
<td>0.347</td>
<td>0.26</td>
<td>0.35</td>
<td>0.48</td>
<td>0.45</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>0.518</td>
<td>0.42</td>
<td>0.347</td>
<td>0.26</td>
<td>0.35</td>
<td>0.48</td>
<td>0.45</td>
<td>0.35</td>
</tr>
</tbody>
</table>

**Graph:**

- **x-axis:** Medium, Scrambled siRNA, STAT6 siRNA, S6S-GNC (PEGylated), S6S-GNC-I (1 mol % peptide to PEG), S6S-GNC-I (7.5% mol % peptide to PEG), S6S-GNC-I (15% mol % peptide to PEG), S6S-GNC-I (30% mol % peptide to PEG)
- **y-axis:** STAT6 Protein Relative Expression

**Legend:**

- 0.518333 med
- 0.423333 scr
- 0.265333 sirna
- 0.359667 gnc
- 0.300333 gnc-1 1%
- 0.290333 gnc-1 7.5%
- 0.393667 gnc-1 15%
- 0.479 gnc-1 30%
<table>
<thead>
<tr>
<th>run</th>
<th>fluor</th>
<th>stdev</th>
<th>relative fluor intensity</th>
<th>rel stdev</th>
</tr>
</thead>
<tbody>
<tr>
<td>run 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium control</td>
<td>30.7</td>
<td>21</td>
<td>1</td>
<td>0.032573</td>
</tr>
<tr>
<td>GNC-P</td>
<td>13.4</td>
<td>8.68</td>
<td>2.175895765</td>
<td>0.031651</td>
</tr>
<tr>
<td>7.5% mol ratio GNC-P</td>
<td>66.8</td>
<td>44.4</td>
<td>1.495114007</td>
<td>0.030812</td>
</tr>
<tr>
<td>10% mol ratio GNC-P</td>
<td>45.9</td>
<td>29.7</td>
<td>1.495114007</td>
<td>0.030812</td>
</tr>
<tr>
<td>15% mol ratio GNC-P</td>
<td>44.2</td>
<td>29</td>
<td>1.495114007</td>
<td>0.031243</td>
</tr>
<tr>
<td>30% mol ratio GNC-P</td>
<td>29.2</td>
<td>19.5</td>
<td>1.495114007</td>
<td>0.030812</td>
</tr>
<tr>
<td>run 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium control</td>
<td>21.4</td>
<td>17.4</td>
<td>1</td>
<td>0.046729</td>
</tr>
<tr>
<td>GNC-P</td>
<td>365</td>
<td>330</td>
<td>17.05607477</td>
<td>0.05196</td>
</tr>
<tr>
<td>7.5% mol ratio GNC-P</td>
<td>522</td>
<td>420</td>
<td>24.39252336</td>
<td>0.046241</td>
</tr>
<tr>
<td>10% mol ratio GNC-P</td>
<td>604</td>
<td>554</td>
<td>28.22429907</td>
<td>0.052714</td>
</tr>
<tr>
<td>15% mol ratio GNC-P</td>
<td>683</td>
<td>618</td>
<td>31.9588785</td>
<td>0.052002</td>
</tr>
<tr>
<td>run 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium control</td>
<td>21.4</td>
<td>17.4</td>
<td>1</td>
<td>0.046729</td>
</tr>
<tr>
<td>GNC-P</td>
<td>285</td>
<td>204</td>
<td>13.31775701</td>
<td>0.041137</td>
</tr>
<tr>
<td>7.5% mol ratio GNC-P</td>
<td>421</td>
<td>308</td>
<td>19.6728972</td>
<td>0.042045</td>
</tr>
<tr>
<td>10% mol ratio GNC-P</td>
<td>475</td>
<td>404</td>
<td>22.19626168</td>
<td>0.048881</td>
</tr>
<tr>
<td>15% mol ratio GNC-P</td>
<td>567</td>
<td>468</td>
<td>26.4953271</td>
<td>0.047437</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium control</td>
<td>1</td>
<td></td>
<td>1</td>
<td>0.046729</td>
</tr>
<tr>
<td>GNC-P</td>
<td>15.18692</td>
<td>2.64339</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.5% mol ratio GNC-P</td>
<td>22.03271</td>
<td>3.33728</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% mol ratio GNC-P</td>
<td>25.21028</td>
<td>4.262466</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15% mol ratio GNC-P</td>
<td>29.20561</td>
<td>3.832915</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Binding Assaay</td>
<td>Flur Stdev</td>
<td>Relative Fluor Std dev</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium control</td>
<td>30.7</td>
<td>21</td>
<td>1</td>
<td>0.032573</td>
</tr>
<tr>
<td>7.5% mol ratio GNC-P</td>
<td>66.8</td>
<td>44.4</td>
<td>2.175895765</td>
<td>0.068869</td>
</tr>
<tr>
<td>10% mol ratio GNC-P</td>
<td>45.9</td>
<td>25.7</td>
<td>1.495114007</td>
<td>0.039864</td>
</tr>
<tr>
<td>15% mol ratio GNC-P</td>
<td>44.2</td>
<td>29</td>
<td>1.495114007</td>
<td>0.044982</td>
</tr>
<tr>
<td>30% mol ratio GNC-P</td>
<td>29.2</td>
<td>19.5</td>
<td>1.495114007</td>
<td>0.030247</td>
</tr>
</tbody>
</table>
## Internalization Assay

### Trial 1

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>fluor</td>
<td>stdev</td>
<td>relative fluor intensity</td>
<td>rel stdev</td>
</tr>
<tr>
<td>Medium</td>
<td>11.3</td>
<td>8.15</td>
<td>1</td>
<td>0.088496</td>
</tr>
<tr>
<td>GNC plain</td>
<td>17.8</td>
<td>8.86</td>
<td>1.575221</td>
<td>0.080923</td>
</tr>
<tr>
<td>S6S-GNC</td>
<td>12.6</td>
<td>8.31</td>
<td>1.115044</td>
<td>0.061074</td>
</tr>
<tr>
<td>7.5% mol ratio GNC-P</td>
<td>14.4</td>
<td>7.71</td>
<td>1.274336</td>
<td>0.065695</td>
</tr>
<tr>
<td>10% mol ratio GNC-P</td>
<td>14.6</td>
<td>7.77</td>
<td>1.292035</td>
<td>0.0653</td>
</tr>
<tr>
<td>15% mol ratio GNC-P</td>
<td>11.7</td>
<td>6.45</td>
<td>1.035398</td>
<td>0.067642</td>
</tr>
<tr>
<td>30% mol ratio GNC-P</td>
<td>14.1</td>
<td>9.54</td>
<td>1.247788</td>
<td>0.083018</td>
</tr>
</tbody>
</table>

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>6</td>
<td>3.4</td>
<td>1</td>
<td>0.16667</td>
</tr>
<tr>
<td>GNC plain</td>
<td>67.9</td>
<td>39.1</td>
<td>11.3</td>
<td>0.169367</td>
</tr>
<tr>
<td>S6S-GNC</td>
<td>67.4</td>
<td>38.4</td>
<td>11.23333</td>
<td>0.167569</td>
</tr>
<tr>
<td>7.5% mol ratio GNC-P</td>
<td>123</td>
<td>56</td>
<td>20.5</td>
<td>0.133907</td>
</tr>
<tr>
<td>10% mol ratio GNC-P</td>
<td>79.1</td>
<td>36.6</td>
<td>13.18333</td>
<td>0.13609</td>
</tr>
<tr>
<td>15% mol ratio GNC-P</td>
<td>119</td>
<td>53.6</td>
<td>19.83333</td>
<td>0.132477</td>
</tr>
<tr>
<td>30% mol ratio GNC-P</td>
<td>138</td>
<td>63.1</td>
<td>23</td>
<td>0.134484</td>
</tr>
</tbody>
</table>

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>10.1</td>
<td>6.88</td>
<td>1</td>
<td>0.09901</td>
</tr>
<tr>
<td>GNC plain</td>
<td>59.8</td>
<td>31.2</td>
<td>5.920792</td>
<td>0.075834</td>
</tr>
<tr>
<td>S6S-GNC</td>
<td>61.6</td>
<td>30.8</td>
<td>6.09901</td>
<td>0.072674</td>
</tr>
<tr>
<td>7.5% mol ratio GNC-P</td>
<td>41</td>
<td>20.4</td>
<td>4.059406</td>
<td>0.07232</td>
</tr>
<tr>
<td>10% mol ratio GNC-P</td>
<td>66.2</td>
<td>33.4</td>
<td>6.554455</td>
<td>0.073333</td>
</tr>
<tr>
<td>15% mol ratio GNC-P</td>
<td>72</td>
<td>34.1</td>
<td>7.128713</td>
<td>0.068839</td>
</tr>
<tr>
<td>30% mol ratio GNC-P</td>
<td>81</td>
<td>34.3</td>
<td>8.019802</td>
<td>0.061549</td>
</tr>
</tbody>
</table>

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>21.2</td>
<td>8.83</td>
<td>1</td>
<td>0.04717</td>
</tr>
<tr>
<td>GNC plain</td>
<td>40.3</td>
<td>22.3</td>
<td>1.900943</td>
<td>0.062667</td>
</tr>
<tr>
<td>S6S-GNC</td>
<td>78.2</td>
<td>36.6</td>
<td>3.688679</td>
<td>0.053005</td>
</tr>
<tr>
<td>7.5% mol ratio GNC-P</td>
<td>44.6</td>
<td>26.6</td>
<td>2.103774</td>
<td>0.067544</td>
</tr>
<tr>
<td>10% mol ratio GNC-P</td>
<td>56.4</td>
<td>31.7</td>
<td>2.660377</td>
<td>0.063653</td>
</tr>
<tr>
<td>15% mol ratio GNC-P</td>
<td>72.2</td>
<td>39.1</td>
<td>3.40566</td>
<td>0.061331</td>
</tr>
<tr>
<td>30% mol ratio GNC-P</td>
<td>59.2</td>
<td>33.8</td>
<td>2.792453</td>
<td>0.06466</td>
</tr>
</tbody>
</table>

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>9.3</td>
<td>6.37</td>
<td>1</td>
<td>0.107527</td>
</tr>
<tr>
<td>GNC plain</td>
<td>23.7</td>
<td>11.5</td>
<td>2.548387</td>
<td>0.076175</td>
</tr>
<tr>
<td>S6S-GNC</td>
<td>25.3</td>
<td>12.8</td>
<td>2.72043</td>
<td>0.079424</td>
</tr>
<tr>
<td>7.5% mol ratio GNC-P</td>
<td>23.9</td>
<td>11</td>
<td>2.569892</td>
<td>0.072253</td>
</tr>
<tr>
<td>10% mol ratio GNC-P</td>
<td>24</td>
<td>11</td>
<td>2.580645</td>
<td>0.071952</td>
</tr>
<tr>
<td>15% mol ratio GNC-P</td>
<td>22.1</td>
<td>10.7</td>
<td>2.376344</td>
<td>0.076007</td>
</tr>
<tr>
<td>30% mol ratio GNC-P</td>
<td>24.6</td>
<td>12.3</td>
<td>2.645161</td>
<td>0.078493</td>
</tr>
</tbody>
</table>
## Western Blot STAT6 Expression

### Trial 1

<table>
<thead>
<tr>
<th></th>
<th>fluor</th>
<th>stdev</th>
<th>relative fluor</th>
<th>rel stdev</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>11.3</td>
<td>8.15</td>
<td>1</td>
<td>0.088496</td>
</tr>
<tr>
<td>GNC plain</td>
<td>17.8</td>
<td>8.86</td>
<td>1.575221</td>
<td>0.080923</td>
</tr>
<tr>
<td>S6S-GNC</td>
<td>12.6</td>
<td>8.31</td>
<td>1.115044</td>
<td>0.057283</td>
</tr>
<tr>
<td>7.5% mol r</td>
<td>14.4</td>
<td>7.71</td>
<td>1.274336</td>
<td>0.065695</td>
</tr>
<tr>
<td>15% mol r</td>
<td>11.7</td>
<td>6.45</td>
<td>1.035398</td>
<td>0.067642</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>fluor</th>
<th>stdev</th>
<th>relative fluor</th>
<th>rel stdev</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>6</td>
<td>3.4</td>
<td>1</td>
<td>0.166667</td>
</tr>
<tr>
<td>GNC plain</td>
<td>67.9</td>
<td>39.1</td>
<td>11.31667</td>
<td>0.169367</td>
</tr>
<tr>
<td>S6S-GNC</td>
<td>67.4</td>
<td>38.4</td>
<td>11.23333</td>
<td>0.167569</td>
</tr>
<tr>
<td>7.5% mol r</td>
<td>123</td>
<td>56</td>
<td>20.5</td>
<td>0.133907</td>
</tr>
<tr>
<td>15% mol r</td>
<td>119</td>
<td>53.6</td>
<td>19.83333</td>
<td>0.132477</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>fluor</th>
<th>stdev</th>
<th>relative fluor</th>
<th>rel stdev</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>10.1</td>
<td>6.88</td>
<td>1</td>
<td>0.09901</td>
</tr>
<tr>
<td>GNC plain</td>
<td>59.8</td>
<td>31.2</td>
<td>5.920792</td>
<td>0.075834</td>
</tr>
<tr>
<td>S6S-GNC</td>
<td>61.6</td>
<td>30.8</td>
<td>6.09901</td>
<td>0.072674</td>
</tr>
<tr>
<td>7.5% mol r</td>
<td>41</td>
<td>20.4</td>
<td>4.059406</td>
<td>0.07232</td>
</tr>
<tr>
<td>15% mol r</td>
<td>72</td>
<td>34.1</td>
<td>7.128713</td>
<td>0.068839</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>fluor</th>
<th>stdev</th>
<th>relative fluor</th>
<th>rel stdev</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>21.2</td>
<td>8.83</td>
<td>1</td>
<td>0.04717</td>
</tr>
<tr>
<td>GNC plain</td>
<td>40.3</td>
<td>22.3</td>
<td>1.900943</td>
<td>0.062667</td>
</tr>
<tr>
<td>S6S-GNC</td>
<td>78.2</td>
<td>36.6</td>
<td>3.688679</td>
<td>0.053005</td>
</tr>
<tr>
<td>7.5% mol r</td>
<td>44.6</td>
<td>26.6</td>
<td>2.103774</td>
<td>0.067544</td>
</tr>
<tr>
<td>15% mol r</td>
<td>72.2</td>
<td>39.1</td>
<td>3.40566</td>
<td>0.061331</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>fluor</th>
<th>stdev</th>
<th>relative fluor</th>
<th>rel stdev</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>9.3</td>
<td>6.37</td>
<td>1</td>
<td>0.107527</td>
</tr>
<tr>
<td>GNC plain</td>
<td>23.7</td>
<td>11.5</td>
<td>2.548387</td>
<td>0.076175</td>
</tr>
<tr>
<td>S6S-GNC</td>
<td>25.3</td>
<td>12.8</td>
<td>2.72043</td>
<td>0.079424</td>
</tr>
<tr>
<td>7.5% mol r</td>
<td>23.9</td>
<td>11</td>
<td>2.569892</td>
<td>0.072253</td>
</tr>
<tr>
<td>15% mol r</td>
<td>22.1</td>
<td>10.7</td>
<td>2.376344</td>
<td>0.076007</td>
</tr>
</tbody>
</table>
72 hr Dose dependency Western Blot
1 Medium
2 Scrambled siRNA-GNC (200 nM)
3 S6S-GNC (25 nM)
4 S6S-GNC (50 nM)
5 S6S-GNC (100 nM)
6 S6S-GNC (150 nM)
7 S6S-GNC (200 nM)
8 Scrambled siRNA-GNC-P (200 nM)
9 S6S-GNC-P (25 nM)
10 S6S-GNC-P (50 nM)
11 S6S-GNC-P (100 nM)
12 S6S-GNC-P (150 nM)
13 S6S-GNC-P (200 nM)
72 hr Dose dependency Western Blot
1 Medium
2 Scrambled siRNA-GNC (200 nM)
3 S6S-GNC (25 nM)
4 S6S-GNC (50 nM)
5 S6S-GNC (100 nM)
6 S6S-GNC (150 nM)
7 S6S-GNC (200 nM)
8 Scrambled siRNA-GNC-P (200 nM)
9 S6S-GNC-P (25 nM)
10 S6S-GNC-P (50 nM)
11 S6S-GNC-P (100 nM)
12 S6S-GNC-P (150 nM)
13 S6S-GNC-P (200 nM)

STAT6
STAT6
STAT6
Beta
Beta
Beta
STAT6/BetaSTAT6/BetaSTAT6/BetaAverage SD
norm
5262.882 5055.64 5256.468 23490.43 25676.26 23837.31 0.224044 0.196899 0.220514 0.213819 0.014759
8484.539 8278.246 8137.589 23971.14 25997.07 24097.82 0.353948 0.31843 0.33769 0.336689 0.01778
1
7240.054 7185.004 7247.004 25124.72 27103.55 25389.07 0.288165 0.265095 0.285438 0.279566 0.012606 0.830337
8438.589 8179.175 8202.225 25681.45 28718.94 26032.87 0.328587 0.284801 0.315072 0.309487 0.022421 0.919205
9458.832 9217.539 9181.953 27140.84 30817.79 27959.35 0.348509 0.299098 0.328404 0.325337 0.024848 0.966282
9008.882 8463.69 8784.054 25919.18 29622.37 26920.4 0.347576 0.28572 0.326297 0.319864 0.031426 0.950028
8008.225 7691.397 7896.225 24747.44 31757.24 24930.37 0.323598 0.242193 0.316731 0.294174 0.045147 0.873726
9076.539 10137.3 9643.761 24458.62 31192.62 24668.09 0.371098 0.32499 0.390941 0.362343 0.033836
1
8178.225 7799.933 7994.225 21558.56 30237.43 22821.03 0.379349 0.257956 0.350301 0.329202 0.063387 0.908537
9646.811 9621.175 9564.225 23044.08 28540.78 23895.3 0.418624 0.337103 0.400255 0.385328 0.042762 1.063433
7509.518 6642.033 6918.154 23394.32 25676.73 22582.54 0.320997 0.258679 0.30635 0.295342 0.032585 0.81509
5323.569 5282.397 5395.397 21336.62 25073.25 22114.92 0.249504 0.210679 0.243971 0.234718 0.021002 0.647778
6294.104 6534.882 6641.882 21538.18 26105.51 22716.47 0.29223 0.250326 0.292382 0.278313 0.024237 0.768092
STAT6
STAT6
STAT6
Beta
Beta
Beta
STAT6/BetaSTAT6/BetaSTAT6/BetaAverage SD
norm
6517.125 6480.711 6408.004 23490.43 25676.26 23837.31 0.277437 0.252401 0.268822 0.26622 0.012719
10850.56 10819.68 10750.27 23971.14 25997.07 24097.82 0.452651 0.416189 0.446109 0.438316 0.01944
1
10578.27 10295.32 10423.15 25124.72 27103.55 25389.07 0.42103 0.379851 0.410537 0.403806 0.021399 0.92026
9023.217 9001.217 8863.095 25681.45 28718.94 26032.87 0.351352 0.313424 0.340458 0.335078 0.019528 0.763171
6567.439 6610.439 6605.853 27140.84 30817.79 27959.35 0.241976 0.214501 0.236266 0.230914 0.014499 0.526821
7623.974 7657.388 7344.681 25919.18 29622.37 26920.4 0.294144
0.2585 0.27283 0.275158 0.017936 0.627761
7812.731 7815.731 7862.853 24747.44 31757.24 24930.37 0.315699 0.246109 0.315393
0.2924 0.04009 0.667098
11758.63 11852.63 11504.92 21336.62 25073.25 22114.92 0.551101 0.47272 0.520234 0.514685 0.039484
1
9097.459 9260.874 9050.338 24458.62 31192.62 24668.09 0.371953 0.296893 0.366884 0.345244 0.041949 0.670786
6902.267 7142.803 6873.731 21558.56 30237.43 22821.03 0.320164 0.236224 0.301202 0.285863 0.044022 0.555414
7197.267 7279.267 7017.853 23044.08 28540.78 23895.3 0.312326 0.255048 0.293692 0.287022 0.029216 0.557665
8857.681 8950.388 8615.56 23394.32 25676.73 22582.54 0.378625 0.34858 0.381514 0.369573 0.018238 0.718057
8649.368 8417.246 8479.66 21538.18 26105.51 22716.47 0.401583 0.322432 0.373282 0.365766 0.040107 0.71066

72 hr Dose dependency Western Blot
1 Medium
2 Scrambled siRNA-GNC (200 nM)
3 S6S-GNC (25 nM)
4 S6S-GNC (50 nM)
5 S6S-GNC (100 nM)
6 S6S-GNC (150 nM)
7 S6S-GNC (200 nM)
8 Scrambled siRNA-GNC-P (200 nM)
9 S6S-GNC-P (25 nM)
10 S6S-GNC-P (50 nM)
11 S6S-GNC-P (100 nM)
12 S6S-GNC-P (150 nM)
13 S6S-GNC-P (200 nM)

Medium
Scrambled siRNA
STAT6 siRNA
Scram-GNC
S6S-GNC
Scram-GNC-P
S6S-GNC-P

STAT6
STAT6
STAT6
Beta
Beta
Beta
STAT6/BetaSTAT6/BetaSTAT6/BetaAverage SD
norm
5262.882 5055.64 5256.468 23490.43 25676.26 23837.31 0.224044 0.196899 0.220514 0.213819 0.014759
8484.539 8278.246 8137.589 23971.14 25997.07 24097.82 0.353948 0.31843 0.33769 0.336689 0.01778
1
7240.054 7185.004 7247.004 25124.72 27103.55 25389.07 0.288165 0.265095 0.285438 0.279566 0.012606 0.830337
8438.589 8179.175 8202.225 25681.45 28718.94 26032.87 0.328587 0.284801 0.315072 0.309487 0.022421 0.919205
9458.832 9217.539 9181.953 27140.84 30817.79 27959.35 0.348509 0.299098 0.328404 0.325337 0.024848 0.966282
9008.882 8463.69 8784.054 25919.18 29622.37 26920.4 0.347576 0.28572 0.326297 0.319864 0.031426 0.950028
8008.225 7691.397 7896.225 24747.44 31757.24 24930.37 0.323598 0.242193 0.316731 0.294174 0.045147 0.873726
6517.125 6480.711 6408.004 23490.43 25676.26 23837.31 0.277437 0.252401 0.268822 0.26622 0.012719
10850.56 10819.68 10750.27 23971.14 25997.07 24097.82 0.452651 0.416189 0.446109 0.438316 0.01944
1
10578.27 10295.32 10423.15 25124.72 27103.55 25389.07 0.42103 0.379851 0.410537 0.403806 0.021399 0.92026
9023.217 9001.217 8863.095 25681.45 28718.94 26032.87 0.351352 0.313424 0.340458 0.335078 0.019528 0.763171
6567.439 6610.439 6605.853 27140.84 30817.79 27959.35 0.241976 0.214501 0.236266 0.230914 0.014499 0.526821
7623.974 7657.388 7344.681 25919.18 29622.37 26920.4 0.294144
0.2585 0.27283 0.275158 0.017936 0.627761
7812.731 7815.731 7862.853 24747.44 31757.24 24930.37 0.315699 0.246109 0.315393
0.2924 0.04009 0.667098

STAT6
STAT6
STAT6
beta
beta
beta
STAT6/betaSTAT6/betaSTAT6/betaave
stdev
norm
stdev
8141.418 8202.418 8256.125 14053.15 13907.46 14663.97 0.579331 0.589786 0.563021 0.584558 0.007393
1 0.012647
7958.832 8030.539 8004.539 11122.27 11317.15 11221.56 0.715576 0.70959 0.713318 0.712583 0.004233 1.219012 0.007241
4867.468 4945.296 4966.589 10509.73 10809.9 10829.73 0.463139 0.457478 0.458607 0.460309 0.004003 0.787447 0.006848
9406.024 9551.146 9574.853 11340.9 10525.66 11609.49 0.829389 0.907415 0.824744 0.868402 0.055173 1.485571 0.094384
7556.782 7744.61 7780.317 11070.32 9739.782 11215.73 0.682617 0.795152 0.693697 0.738884 0.079575 1.264005 0.136128
10984.85 11112.68 10587.42 12231.37 11736.54 12384.66 0.898089 0.946845 0.854882 0.922467 0.034476 1.578058 0.058978
4250.933 4283.347 4281.347 14790.9 14614.73 14425.95 0.287402 0.293084 0.296781 0.290243 0.004018 0.496517 0.006874

RT-PCR STAT6

340


<table>
<thead>
<tr>
<th>Stat6</th>
<th>Ct</th>
<th>dCt</th>
<th>ddCt</th>
<th>Ave</th>
<th>Stdev</th>
<th>Norm ave</th>
<th>stdev</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>25.92</td>
<td>25.93</td>
<td>25.69</td>
<td>25.68</td>
<td>9.345</td>
<td>9.355</td>
<td>9.115</td>
</tr>
<tr>
<td>Scrambled siRNA</td>
<td>26.48</td>
<td>26.35</td>
<td>26.45</td>
<td>26.43</td>
<td>9.405</td>
<td>9.275</td>
<td>9.375</td>
</tr>
<tr>
<td>STAT6 siRNA</td>
<td>26.08</td>
<td>26.18</td>
<td>27.04</td>
<td>26</td>
<td>9.3875</td>
<td>9.3875</td>
<td>10.2475</td>
</tr>
<tr>
<td>Scram-GNC</td>
<td>27.03</td>
<td>26.61</td>
<td>26.82</td>
<td>26.77</td>
<td>9.12</td>
<td>8.7</td>
<td>8.91</td>
</tr>
<tr>
<td>S6S-GNC</td>
<td>26.28</td>
<td>26.45</td>
<td>26.17</td>
<td>26.17</td>
<td>8.745</td>
<td>8.915</td>
<td>8.635</td>
</tr>
<tr>
<td>Scram-GNC-P</td>
<td>28.35</td>
<td>27.87</td>
<td>28.02</td>
<td>28.22</td>
<td>9.6975</td>
<td>9.2175</td>
<td>9.3675</td>
</tr>
<tr>
<td>S6S-GNC-P</td>
<td>28.01</td>
<td>27.15</td>
<td>28.96</td>
<td>28.12</td>
<td>10.7975</td>
<td>9.8775</td>
<td>11.6875</td>
</tr>
<tr>
<td>IL4 peptide</td>
<td>28.26</td>
<td>27.32</td>
<td>27.67</td>
<td>27.38</td>
<td>9.84</td>
<td>8.9</td>
<td>9.25</td>
</tr>
<tr>
<td>neg</td>
<td>36.41</td>
<td>37.34</td>
<td>33.69</td>
<td>35.71</td>
<td>9.1575</td>
<td>2.8875</td>
<td>-0.7625</td>
</tr>
<tr>
<td>Gata3</td>
<td>Ct</td>
<td>dCt</td>
<td>ddCt</td>
<td>Ave</td>
<td>Stdev</td>
<td>Norm ave</td>
<td>stdev</td>
</tr>
<tr>
<td>-------</td>
<td>----</td>
<td>-----</td>
<td>------</td>
<td>-----</td>
<td>-------</td>
<td>----------</td>
<td>-------</td>
</tr>
<tr>
<td>Medium</td>
<td>23.55</td>
<td>24.02</td>
<td>23.79</td>
<td>23.02</td>
<td>5.13</td>
<td>5.6</td>
<td>5.37</td>
</tr>
<tr>
<td>Scrambled siRNA</td>
<td>23.52</td>
<td>22.97</td>
<td>23.67</td>
<td>23.53</td>
<td>6.3475</td>
<td>5.6975</td>
<td>6.3875</td>
</tr>
<tr>
<td>STAT6 siRNA</td>
<td>23.17</td>
<td>23.59</td>
<td>24</td>
<td>23.68</td>
<td>4.5175</td>
<td>4.9375</td>
<td>5.3475</td>
</tr>
<tr>
<td>Scram-GNC</td>
<td>22.11</td>
<td>22.13</td>
<td>22.07</td>
<td>22.25</td>
<td>4.575</td>
<td>4.595</td>
<td>4.535</td>
</tr>
<tr>
<td>S6S-GNC</td>
<td>22.73</td>
<td>22.87</td>
<td>22.19</td>
<td>22.2</td>
<td>4.82</td>
<td>4.96</td>
<td>4.28</td>
</tr>
<tr>
<td>Scram-GNC-P</td>
<td>21.44</td>
<td>21.33</td>
<td>21.34</td>
<td>21.25</td>
<td>4.6475</td>
<td>4.3375</td>
<td>4.5475</td>
</tr>
<tr>
<td>S6S-GNC-P</td>
<td>22.31</td>
<td>21.88</td>
<td>21.72</td>
<td>21.91</td>
<td>5.235</td>
<td>4.805</td>
<td>4.645</td>
</tr>
<tr>
<td>IL4 peptide</td>
<td>21.07</td>
<td>21.07</td>
<td>21.03</td>
<td>20.95</td>
<td>4.495</td>
<td>4.495</td>
<td>4.435</td>
</tr>
<tr>
<td>neg</td>
<td>39.58</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>5.1275</td>
<td>RVALUE</td>
<td>RVALUE</td>
</tr>
</tbody>
</table>
Appendix D: Data for Objective 3

Data for Gel type B article (Gem-GNCb)

Taguchi experiment

Constants: 10 ml batch size (gel solution 1% w/v type B HMW)
600 rpm stir rate
35 deg C
EtOH addition rate is 1 ml/min
Genipin dissolved in ethanol, 2 min after ethanol addition, 0.2 ml/min
pH-->7.4+/-.3

<table>
<thead>
<tr>
<th>Batch</th>
<th>ps 1</th>
<th>ps 2</th>
<th>ps 3</th>
<th>average</th>
<th>stdev</th>
<th>zeta (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>142.3</td>
<td>202.3</td>
<td>121</td>
<td>155.2</td>
<td>42.15721</td>
<td>-8.8</td>
</tr>
<tr>
<td>2</td>
<td>184.1</td>
<td>142.3</td>
<td>174.7</td>
<td>167.0</td>
<td>21.92928</td>
<td>-10.2</td>
</tr>
<tr>
<td>3</td>
<td>385.5</td>
<td>907.1</td>
<td>386.4</td>
<td>559.7</td>
<td>300.8864</td>
<td>-5.2</td>
</tr>
<tr>
<td>4</td>
<td>209.7</td>
<td>323.5</td>
<td>345</td>
<td>292.7</td>
<td>72.70807</td>
<td>-8.6</td>
</tr>
<tr>
<td>5</td>
<td>592.7</td>
<td>200.9</td>
<td>272.5</td>
<td>355.4</td>
<td>208.6312</td>
<td>-5.9</td>
</tr>
<tr>
<td>6</td>
<td>242.9</td>
<td>383.5</td>
<td>446.8</td>
<td>357.7</td>
<td>104.3635</td>
<td>-6.8</td>
</tr>
<tr>
<td>7</td>
<td>207.4</td>
<td>184</td>
<td>78.9</td>
<td>156.8</td>
<td>68.44197</td>
<td>-5.1</td>
</tr>
<tr>
<td>8</td>
<td>210.7</td>
<td>363.4</td>
<td>177.7</td>
<td>250.6</td>
<td>99.07134</td>
<td>-8.3</td>
</tr>
<tr>
<td>9</td>
<td>199.9</td>
<td>251.3</td>
<td>645.3</td>
<td>365.5</td>
<td>243.673</td>
<td>-8.3</td>
</tr>
</tbody>
</table>

Table. Formulation parameters and their levels.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Formulation Parameter</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Volume ratio v:v of 90% v/v aqueous ethanol</td>
<td>70 80 90</td>
</tr>
<tr>
<td>B</td>
<td>Genipin (% w/w)</td>
<td>0.2 0.6 1.0</td>
</tr>
<tr>
<td>C</td>
<td>Gelatin (% w/v)</td>
<td>0.5 1.0 1.5</td>
</tr>
</tbody>
</table>
### Table. Taguchi Orthogonal array $L_9(3^3)$

<table>
<thead>
<tr>
<th>Trial no.</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

### Table 3. Experimental matrix and responses from the $L_9$ orthogonal array and the S/N ratios of experimental results for particle size

<table>
<thead>
<tr>
<th>Trial no.</th>
<th>Formulation parameter level</th>
<th>Measured particle size</th>
<th>Average particle size, n=3</th>
<th>Standard deviation of particle size, n=3</th>
<th>S/N ($\eta_i$, $i=1-9$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>v:v 90% Ethanol addition</td>
<td>Genipin (% w/w)</td>
<td>Gelatin (% w/v)</td>
<td>[nm]</td>
<td>[nm]</td>
<td>[nm]</td>
</tr>
<tr>
<td>1</td>
<td>70 0.2 0.5</td>
<td>142.3 102.3 121</td>
<td>155.20</td>
<td>42.16</td>
<td>-43.72</td>
</tr>
<tr>
<td>2</td>
<td>70 0.6 1</td>
<td>184.1 142.3 174.7</td>
<td>167.03</td>
<td>21.93</td>
<td>-46.89</td>
</tr>
<tr>
<td>3</td>
<td>70 1 1.5</td>
<td>385.5 907.1 386.4</td>
<td>559.67</td>
<td>300.89</td>
<td>-53.64</td>
</tr>
<tr>
<td>4</td>
<td>80 0.2 1</td>
<td>209.7 323.5 345</td>
<td>292.73</td>
<td>72.71</td>
<td>-47.43</td>
</tr>
<tr>
<td>5</td>
<td>80 0.6 1.5</td>
<td>592.7 200.9 272.5</td>
<td>355.37</td>
<td>208.63</td>
<td>-51.60</td>
</tr>
<tr>
<td>6</td>
<td>80 1 0.5</td>
<td>242.9 383.5 446.8</td>
<td>357.73</td>
<td>104.36</td>
<td>-53.70</td>
</tr>
<tr>
<td>7</td>
<td>90 0.2 1.5</td>
<td>207.4 184 78.9</td>
<td>156.77</td>
<td>68.44</td>
<td>-46.81</td>
</tr>
<tr>
<td>8</td>
<td>90 0.6 0.5</td>
<td>210.7 363.4 177.7</td>
<td>250.60</td>
<td>99.07</td>
<td>-46.33</td>
</tr>
<tr>
<td>9</td>
<td>90 1 1</td>
<td>199.9 251.3 645.3</td>
<td>365.50</td>
<td>243.67</td>
<td>-52.08</td>
</tr>
</tbody>
</table>

Total average 295.62 -49.13

Particle size was determined with PBS pH 7.4 at 25°C by dynamic light scattering as described within the methods section.

### Table. Mean S/N ratios [dB] of control factors for particle size.

<table>
<thead>
<tr>
<th>Control factors</th>
<th>Levels</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>$\Delta$ (max-min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td>-48.09</td>
<td>-50.91</td>
<td>-48.41</td>
<td>2.82</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td>-45.99</td>
<td>-48.28</td>
<td>-53.14</td>
<td>7.15</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td>-47.92</td>
<td>-48.80</td>
<td>-50.69</td>
<td>2.77</td>
</tr>
</tbody>
</table>
Regression Analysis: PMEAN3 versus 90% Ethanol, Genipin (% w, w, v/v)

The regression equation is
PMEAN3 = 169 - 1.82 90% Ethanol (% v/v) + 283 Genipin (% w/w)
+ 103 Gelatin (% v/v)

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Coef</th>
<th>SE Coef</th>
<th>T</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>168.69</td>
<td>48.71</td>
<td>3.46</td>
<td>0.001</td>
</tr>
<tr>
<td>90% Ethanol (% v/v)</td>
<td>-1.8172</td>
<td>0.5784</td>
<td>-3.14</td>
<td>0.002</td>
</tr>
<tr>
<td>Genipin (% w/w)</td>
<td>282.58</td>
<td>14.46</td>
<td>19.54</td>
<td>0.000</td>
</tr>
<tr>
<td>Gelatin (% v/v)</td>
<td>102.76</td>
<td>11.57</td>
<td>8.88</td>
<td>0.000</td>
</tr>
</tbody>
</table>

S = 42.5043    R-Sq = 85.9%    R-Sq(adj) = 85.4%

Analysis of Variance

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression</td>
<td>3</td>
<td>850308</td>
<td>283436</td>
<td>156.89</td>
<td>0.000</td>
</tr>
<tr>
<td>Residual Error</td>
<td>77</td>
<td>139110</td>
<td>1807</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>80</td>
<td>989417</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Stability at Different pH – Particle Size

<table>
<thead>
<tr>
<th>pH 5.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pH 6.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>127.5</td>
</tr>
<tr>
<td>128.0</td>
</tr>
<tr>
<td>101.5</td>
</tr>
<tr>
<td>99.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pH 7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>115.3</td>
</tr>
<tr>
<td>125.6</td>
</tr>
<tr>
<td>95.8</td>
</tr>
<tr>
<td>97.3</td>
</tr>
</tbody>
</table>

Table Analyzed

Two-way RM ANOVA

Matching by cols

Source of Variation
- Interaction: 0.66, P value 0.8647
- Time: 72.53, P<0.0001
- pH: 2.91, 0.0038
- Subjects (matching): 4.9112, 0.7533

Source of Variation

P value summary

<table>
<thead>
<tr>
<th>Interaction</th>
<th>% of total variation</th>
<th>P value</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>0.66</td>
<td>0.8647</td>
<td>No</td>
</tr>
<tr>
<td>Time</td>
<td>72.53</td>
<td>P&lt;0.0001</td>
<td>Yes</td>
</tr>
<tr>
<td>pH</td>
<td>2.91</td>
<td>0.0038</td>
<td>Yes</td>
</tr>
<tr>
<td>Subjects (matching)</td>
<td>4.9112</td>
<td>0.7533</td>
<td>No</td>
</tr>
</tbody>
</table>

Source of Variation

<table>
<thead>
<tr>
<th>Interaction</th>
<th>Df</th>
<th>Sum-of-squares</th>
<th>Mean square</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>6</td>
<td>100.2</td>
<td>16.70</td>
<td>0.4181</td>
</tr>
<tr>
<td>Time</td>
<td>3</td>
<td>10980</td>
<td>3660</td>
<td>91.66</td>
</tr>
<tr>
<td>pH</td>
<td>2</td>
<td>440.1</td>
<td>220.0</td>
<td>7.102</td>
</tr>
<tr>
<td>Subjects (matching)</td>
<td>24</td>
<td>743.6</td>
<td>30.98</td>
<td>0.7758</td>
</tr>
<tr>
<td>Residual</td>
<td>72</td>
<td>2875</td>
<td>39.94</td>
<td></td>
</tr>
</tbody>
</table>

Number of missing values: 0

Bonferroni posttests

<table>
<thead>
<tr>
<th>pH 5.4 vs pH 6.4</th>
<th>pH 5.4</th>
<th>pH 6.4</th>
<th>Difference</th>
<th>95% CI of diff.</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 5.4</td>
<td>116.0</td>
<td>120.5</td>
<td>4.433</td>
<td>-4.063 to 12.93</td>
</tr>
<tr>
<td>pH 5.4</td>
<td>114.7</td>
<td>120.8</td>
<td>6.144</td>
<td>-2.352 to 14.64</td>
</tr>
<tr>
<td>pH 5.4</td>
<td>97.81</td>
<td>103.0</td>
<td>5.178</td>
<td>-3.319 to 13.67</td>
</tr>
<tr>
<td>pH 5.4</td>
<td>95.43</td>
<td>99.12</td>
<td>3.689</td>
<td>-4.808 to 12.19</td>
</tr>
</tbody>
</table>
pH Difference  t  P value  Summary
0          4.433   1.532  P > 0.05  ns
1          6.144   2.123  P > 0.05  ns
2          5.178   1.789  P > 0.05  ns
3          3.689   1.275  P > 0.05  ns

pH 5.4 vs pH 7.4

pH  5.4  pH 7.4  Difference  95% CI of diff.
0         116.0  121.6  5.567   -2.930 to 14.06
1         114.7  118.8  4.111   -4.385 to 12.61
2          97.81  98.43  0.6222 -7.874 to 9.119
3          95.43  97.99  2.556   -5.941 to 11.05

pH 6.4 vs pH 7.4

pH  6.4  pH 7.4  Difference  95% CI of diff.
0         120.5  121.6  1.133  -7.363 to 9.630
1         120.8  118.8  -2.033  -10.53 to 6.463
2          103.0  98.43  -4.556  -13.05 to 3.941
3          99.12  97.99  -1.133  -9.630 to 7.363

Source of Variation  DF  Sum of Squares  Mean Square
Interaction          6.0  100.2       16.70
Time                3.0  10980      3660
pH                   2.0  440.1      220.0
Subjects (matching) 24.0  743.6      30.98
Residual (Error)    72.0  2875      39.94
Total                107.0 15140

Does Time have the same effect at all values of pH?
Interaction accounts for 0.66% of the total variance.
F = 0.42.  DFn=6  DFd=72
The P value = 0.8647
If there is no interaction overall, there is a 86% chance of randomly observing so much
interaction in an experiment of this size. The interaction is considered not significant.

Does pH affect the result? (Are the curves different?)
pH accounts for 2.91% of the total variance (after adjusting for matching).
F = 7.10.  DFn=2 DFd=72
The P value = 0.0038
If pH has no effect overall, there is a 0.38% chance of randomly observing an
effect this big (or bigger) in an experiment of this size. The effect is considered very significant.

Does Time affect the result? (Are the curves horizontal?)
Time accounts for 72.53% of the total variance (after adjusting for matching).
F = 91.66.  DFn=3 DFd=72
The P value is <0.0001
If Time has no effect overall, there is a less than 0.01% chance of randomly observing an
effect this big (or bigger) in an experiment of this size. The effect is considered extremely
significant.

Was the matching effective?
F = 0.78.  DFn=24 DFd=72
The P value = 0.7533
If matching were not effective overall, there is a 7.5% chance of randomly observing an
effect this big (or bigger) in an experiment of this size. The effect is considered not significant.

**Stability at Different pH – Polydispersity Index (PDI)**

<table>
<thead>
<tr>
<th>pH</th>
<th>0</th>
<th>0.598</th>
<th>0.558</th>
<th>0.657</th>
<th>0.549</th>
<th>0.539</th>
<th>0.558</th>
<th>0.575</th>
<th>0.566</th>
<th>0.530</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.518</td>
<td>0.529</td>
<td>0.542</td>
<td>0.516</td>
<td>0.615</td>
<td>0.602</td>
<td>0.551</td>
<td>0.548</td>
<td>0.560</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.540</td>
<td>0.582</td>
<td>0.549</td>
<td>0.570</td>
<td>0.527</td>
<td>0.542</td>
<td>0.516</td>
<td>0.510</td>
<td>0.497</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.545</td>
<td>0.578</td>
<td>0.500</td>
<td>0.540</td>
<td>0.567</td>
<td>0.546</td>
<td>0.511</td>
<td>0.426</td>
<td>0.520</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pH 6.4</th>
<th>0.484</th>
<th>0.542</th>
<th>0.520</th>
<th>0.523</th>
<th>0.610</th>
<th>0.557</th>
<th>0.506</th>
<th>0.613</th>
<th>0.692</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.601</td>
<td>0.518</td>
<td>0.582</td>
<td>0.560</td>
<td>0.533</td>
<td>0.506</td>
<td>0.508</td>
<td>0.554</td>
<td>0.536</td>
<td></td>
</tr>
<tr>
<td>0.564</td>
<td>0.534</td>
<td>0.596</td>
<td>0.516</td>
<td>0.521</td>
<td>0.496</td>
<td>0.632</td>
<td>0.570</td>
<td>0.573</td>
<td></td>
</tr>
<tr>
<td>0.596</td>
<td>0.573</td>
<td>0.551</td>
<td>0.545</td>
<td>0.552</td>
<td>0.545</td>
<td>0.594</td>
<td>0.524</td>
<td>0.514</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pH 7.4</th>
<th>0.433</th>
<th>0.575</th>
<th>0.550</th>
<th>0.524</th>
<th>0.517</th>
<th>0.549</th>
<th>0.597</th>
<th>0.530</th>
<th>0.545</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.539</td>
<td>0.503</td>
<td>0.545</td>
<td>0.624</td>
<td>0.605</td>
<td>0.605</td>
<td>0.572</td>
<td>0.566</td>
<td>0.523</td>
<td>0.584</td>
</tr>
<tr>
<td>0.554</td>
<td>0.540</td>
<td>0.572</td>
<td>0.585</td>
<td>0.581</td>
<td>0.545</td>
<td>0.584</td>
<td>0.623</td>
<td>0.659</td>
<td></td>
</tr>
<tr>
<td>0.510</td>
<td>0.560</td>
<td>0.594</td>
<td>0.536</td>
<td>0.581</td>
<td>0.579</td>
<td>0.551</td>
<td>0.565</td>
<td>0.512</td>
<td></td>
</tr>
</tbody>
</table>

Table Analyzed

**Two-way RM ANOVA**

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>% of total variation</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>10.48</td>
<td>0.1033</td>
</tr>
<tr>
<td>Time</td>
<td>1.49</td>
<td>0.6670</td>
</tr>
<tr>
<td>pH</td>
<td>1.48</td>
<td>0.3915</td>
</tr>
<tr>
<td>Subjects (matching)</td>
<td>18.2028</td>
<td>0.7259</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>P value summary</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>ns</td>
<td>No</td>
</tr>
<tr>
<td>Time</td>
<td>ns</td>
<td>No</td>
</tr>
<tr>
<td>Source of Variation</td>
<td>Df</td>
<td>Sum-of-squares</td>
</tr>
<tr>
<td>--------------------</td>
<td>----</td>
<td>----------------</td>
</tr>
<tr>
<td>Interaction</td>
<td>6</td>
<td>0.01890</td>
</tr>
<tr>
<td>Time</td>
<td>3</td>
<td>0.002692</td>
</tr>
<tr>
<td>pH</td>
<td>2</td>
<td>0.002668</td>
</tr>
<tr>
<td>Subjects (matching)</td>
<td>24</td>
<td>0.03282</td>
</tr>
<tr>
<td>Residual</td>
<td>72</td>
<td>0.1232</td>
</tr>
</tbody>
</table>

Number of missing values

Bonferroni posttests

### pH 5.4 vs pH 6.4

<table>
<thead>
<tr>
<th>pH</th>
<th>pH 5.4</th>
<th>pH 6.4</th>
<th>Difference</th>
<th>95% CI of diff.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.5700</td>
<td>0.5608</td>
<td>-0.009222</td>
<td>-0.06502 to 0.04657</td>
</tr>
<tr>
<td>1</td>
<td>0.5534</td>
<td>0.5442</td>
<td>-0.009222</td>
<td>-0.06502 to 0.04657</td>
</tr>
<tr>
<td>2</td>
<td>0.5370</td>
<td>0.5558</td>
<td>0.01878</td>
<td>-0.03702 to 0.07457</td>
</tr>
<tr>
<td>3</td>
<td>0.5259</td>
<td>0.5549</td>
<td>0.02900</td>
<td>-0.02679 to 0.08480</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pH</th>
<th>Difference</th>
<th>t</th>
<th>P value</th>
<th>Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-0.009222</td>
<td>0.4852</td>
<td>P &gt; 0.05</td>
<td>ns</td>
</tr>
<tr>
<td>1</td>
<td>-0.009222</td>
<td>0.4852</td>
<td>P &gt; 0.05</td>
<td>ns</td>
</tr>
<tr>
<td>2</td>
<td>0.01878</td>
<td>0.9879</td>
<td>P &gt; 0.05</td>
<td>ns</td>
</tr>
<tr>
<td>3</td>
<td>0.02900</td>
<td>1.526</td>
<td>P &gt; 0.05</td>
<td>ns</td>
</tr>
</tbody>
</table>

### pH 5.4 vs pH 7.4

<table>
<thead>
<tr>
<th>pH</th>
<th>pH 5.4</th>
<th>pH 7.4</th>
<th>Difference</th>
<th>95% CI of diff.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.5700</td>
<td>0.5356</td>
<td>-0.03444</td>
<td>-0.09024 to 0.02135</td>
</tr>
<tr>
<td>1</td>
<td>0.5534</td>
<td>0.5623</td>
<td>0.008889</td>
<td>-0.04691 to 0.06468</td>
</tr>
<tr>
<td>2</td>
<td>0.5370</td>
<td>0.5826</td>
<td>0.04556</td>
<td>-0.01024 to 0.1014</td>
</tr>
<tr>
<td>3</td>
<td>0.5259</td>
<td>0.5542</td>
<td>0.02833</td>
<td>-0.02746 to 0.08413</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pH</th>
<th>Difference</th>
<th>t</th>
<th>P value</th>
<th>Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-0.03444</td>
<td>1.812</td>
<td>P &gt; 0.05</td>
<td>ns</td>
</tr>
<tr>
<td>1</td>
<td>0.008889</td>
<td>0.4677</td>
<td>P &gt; 0.05</td>
<td>ns</td>
</tr>
<tr>
<td>2</td>
<td>0.04556</td>
<td>2.397</td>
<td>P &gt; 0.05</td>
<td>ns</td>
</tr>
<tr>
<td>3</td>
<td>0.02833</td>
<td>1.491</td>
<td>P &gt; 0.05</td>
<td>ns</td>
</tr>
</tbody>
</table>

### pH 6.4 vs pH 7.4

<table>
<thead>
<tr>
<th>pH</th>
<th>pH 6.4</th>
<th>pH 7.4</th>
<th>Difference</th>
<th>95% CI of diff.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.5608</td>
<td>0.5356</td>
<td>-0.02522</td>
<td>-0.08102 to 0.03057</td>
</tr>
<tr>
<td>1</td>
<td>0.5442</td>
<td>0.5623</td>
<td>0.01811</td>
<td>-0.03768 to 0.07391</td>
</tr>
<tr>
<td>2</td>
<td>0.5558</td>
<td>0.5826</td>
<td>-0.02678</td>
<td>-0.02902 to 0.08257</td>
</tr>
<tr>
<td>3</td>
<td>0.5549</td>
<td>0.5542</td>
<td>0.0006667</td>
<td>-0.05646 to 0.05513</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pH</th>
<th>Difference</th>
<th>t</th>
<th>P value</th>
<th>Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-0.02522</td>
<td>1.327</td>
<td>P &gt; 0.05</td>
<td>ns</td>
</tr>
<tr>
<td>1</td>
<td>0.01811</td>
<td>0.9529</td>
<td>P &gt; 0.05</td>
<td>ns</td>
</tr>
<tr>
<td>2</td>
<td>0.02678</td>
<td>1.409</td>
<td>P &gt; 0.05</td>
<td>ns</td>
</tr>
<tr>
<td>3</td>
<td>-0.0006667</td>
<td>0.03508</td>
<td>P &gt; 0.05</td>
<td>ns</td>
</tr>
</tbody>
</table>
Source of Variation  |  DF  |  Sum of Squares  |  Mean Square
---|---|---|---
Interaction          |  6.0  |  0.01890         |  0.003150
Time                 |  3.0  |  0.002692        |  0.0008974
pH                   |  2.0  |  0.002668        |  0.001334
Subjects (matching)  |  24.0 |  0.03282         |  0.001368
Residual (Error)     |  72.0 |  0.1232          |  0.001712
Total                |  107.0 |  0.1803          |  

Does Time have the same effect at all values of pH?
Interaction accounts for 10.48% of the total variance.
F = 1.84.  DFn=6 DFd=72
The P value = 0.1033
If there is no interaction overall, there is a 10% chance of randomly observing so much interaction in an experiment of this size. The interaction is considered not significant.

Does pH affect the result? (Are the curves different?)
pH accounts for 1.48% of the total variance (after adjusting for matching).
F = 0.98.  DFn=2 DFd=72
The P value = 0.3915
If pH has no effect overall, there is a 39% chance of randomly observing an effect this big (or bigger) in an experiment of this size. The effect is considered not significant.

Does Time affect the result? (Are the curves horizontal?)
Time accounts for 1.49% of the total variance (after adjusting for matching).
F = 0.52.  DFn=3 DFd=72
The P value = 0.6670
If Time has no effect overall, there is a 67% chance of randomly observing an effect this big (or bigger) in an experiment of this size. The effect is considered not significant.

Was the matching effective?
F = 0.80.  DFn=24 DFd=72
The P value = 0.7259
If matching were not effective overall, there is a 73% chance of randomly observing an effect this big (or bigger) in an experiment of this size. The effect is considered not significant.

**In vitro Release of Gem from Gem-GNC**

<table>
<thead>
<tr>
<th>Time</th>
<th>Gem Solution in DPBS</th>
<th></th>
<th>Gem Solution in SLF</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>0.00</td>
<td>3</td>
<td>0.00</td>
</tr>
<tr>
<td>0.25</td>
<td>17.34518</td>
<td>3</td>
<td>8.410273</td>
</tr>
<tr>
<td>0.50</td>
<td>3.6562</td>
<td>3</td>
<td>26.21</td>
</tr>
<tr>
<td>1.00</td>
<td>36.5436</td>
<td>3</td>
<td>74.54</td>
</tr>
<tr>
<td>2.00</td>
<td>5.464</td>
<td>3</td>
<td>5.464</td>
</tr>
<tr>
<td>3.00</td>
<td>5.4631</td>
<td>3</td>
<td>5.4631</td>
</tr>
<tr>
<td>4.00</td>
<td>5.234</td>
<td>3</td>
<td>5.234</td>
</tr>
<tr>
<td>5.00</td>
<td>100.00</td>
<td>3</td>
<td>100.00</td>
</tr>
<tr>
<td>24.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>48.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>72.00</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
In vitro Release Kinetic Analysis

Gem-GNC release in DPBS

<table>
<thead>
<tr>
<th>time</th>
<th>OBS</th>
<th>PRED</th>
<th>RMSE</th>
<th>AIC</th>
<th>BIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00000000000000E-010</td>
<td>1.00000000000000E-010</td>
<td>6.36320929929233E+000</td>
<td>6.63761263094000E+001</td>
<td>6.7179168549968E+001</td>
<td></td>
</tr>
<tr>
<td>2.50000000000000E-001</td>
<td>1.86667000000000E-001</td>
<td>6.5834743456982E+000</td>
<td>6.63761263094000E+001</td>
<td>6.7179168549968E+001</td>
<td></td>
</tr>
<tr>
<td>5.00000000000000E-001</td>
<td>1.26000000000000E+000</td>
<td>6.8037393218941E+000</td>
<td>6.83761263094000E+001</td>
<td>6.8879168549968E+001</td>
<td></td>
</tr>
<tr>
<td>1.00000000000000E+000</td>
<td>4.35100000000000E+000</td>
<td>7.24426948517459E+000</td>
<td>7.28761263094000E+001</td>
<td>7.3479168549968E+001</td>
<td></td>
</tr>
<tr>
<td>2.00000000000000E+000</td>
<td>8.15000000000000E+000</td>
<td>8.1253296714495E+000</td>
<td>8.15761263094000E+001</td>
<td>8.2179168549968E+001</td>
<td></td>
</tr>
<tr>
<td>3.00000000000000E+000</td>
<td>1.24500000000000E+000</td>
<td>9.00638985711531E+000</td>
<td>9.03861263094000E+001</td>
<td>9.1089168549968E+001</td>
<td></td>
</tr>
<tr>
<td>4.00000000000000E+000</td>
<td>1.62000000000000E+000</td>
<td>9.8874504308568E+000</td>
<td>9.92961263094000E+001</td>
<td>1.0009168549968E+001</td>
<td></td>
</tr>
<tr>
<td>5.00000000000000E+000</td>
<td>1.82400000000000E+000</td>
<td>1.0768512290560E+001</td>
<td>1.08816126309400E+001</td>
<td>1.10029168549968E+001</td>
<td></td>
</tr>
<tr>
<td>2.40000000000000E+000</td>
<td>3.85200000000000E+000</td>
<td>2.75086537624929E+001</td>
<td>2.76316126309400E+001</td>
<td>2.77549168549968E+001</td>
<td></td>
</tr>
<tr>
<td>4.80000000000000E+000</td>
<td>5.05400000000000E+000</td>
<td>4.86540982257816E+001</td>
<td>4.87786126309400E+001</td>
<td>4.88919168549968E+001</td>
<td></td>
</tr>
<tr>
<td>7.20000000000000E+000</td>
<td>6.55150000000000E+000</td>
<td>6.97995426890703E+001</td>
<td>7.00236126309400E+001</td>
<td>7.01469168549968E+001</td>
<td></td>
</tr>
</tbody>
</table>

Model order is 0
Start point is 1.00000000000000E-010 Point no = 1
End point is 7.20000000000000E+001 Point no = 11
a = 8.81060185970363E-001
b = 6.36320929929233E+000
r = 9.39441633613809E-001
standard error for a = 7.45652530043843E-002
standard error for b = 2.02581499638538E+000
percent standard error for a = 8.46312819393391E+000
percent standard error for b = 3.18363722004040E+001
Model independent description
DE = 4.09713946770834E+001
MDT = 2.69730532435320E+001
No of timepoints = 11

--------------------------------------------
Model order is 1
Start point is 1.00000000000000E-010  Point no = 1
End point is 7.20000000000000E+001  Point no = 11
a = 1.08294208257828E-001  b = -1.66975938070666E+000
r = 1.11981647439502E-001
standard error for a = 1.01653308997369E-001
standard error for b = 2.76175282053904E+000
percent standard error for a = 9.38677244450148E+001
percent standard error for b = 1.65398251535514E+002

Fitting results

<table>
<thead>
<tr>
<th>time</th>
<th>OBS</th>
<th>PRED</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00000000000000E-010</td>
<td>1.00000000000000E-010</td>
<td>1.88292366966661E-001</td>
</tr>
<tr>
<td>2.50000000000000E-001</td>
<td>1.86667000000000E-001</td>
<td>1.93459744165867E-001</td>
</tr>
<tr>
<td>5.00000000000000E-001</td>
<td>1.26000000000000E+000</td>
<td>1.98768931614496E-001</td>
</tr>
<tr>
<td>1.00000000000000E+000</td>
<td>4.35100000000000E+000</td>
<td>2.09828411061354E-001</td>
</tr>
<tr>
<td>2.00000000000000E+000</td>
<td>8.15000000000000E+000</td>
<td>2.33827652168210E-001</td>
</tr>
<tr>
<td>3.00000000000000E+000</td>
<td>1.24500000000000E+001</td>
<td>2.60571819811905E-001</td>
</tr>
<tr>
<td>4.00000000000000E+000</td>
<td>1.62000000000000E+001</td>
<td>2.9037486490314E-001</td>
</tr>
<tr>
<td>5.00000000000000E+000</td>
<td>1.82400000000000E+001</td>
<td>3.23586653192709E-001</td>
</tr>
<tr>
<td>2.40000000000000E+001</td>
<td>3.38520000000000E+001</td>
<td>2.53273973865903E+000</td>
</tr>
<tr>
<td>4.80000000000000E+001</td>
<td>5.05400000000000E+001</td>
<td>3.40681392835637E+001</td>
</tr>
<tr>
<td>7.20000000000000E+001</td>
<td>6.55150000000000E+001</td>
<td>4.58253999228045E+002</td>
</tr>
</tbody>
</table>

RMSE = 1.19201819986943E+002
AIC = 1.35554844503736E+002
BIC = 1.36350635049332E+002

--------------------------------------------
Model is Higuchi y=K*t^(1/2)
Which is equal to ln(y) = 1/2*ln(t)+b
K = exp(b) = 1.3657308119032984E+000
Start point is 1.00000000000000E-010  Point no = 1
End point is 7.20000000000000E+001  Point no = 11
a = 5.00000000000000E-010  b = 3.11689678700797E-001
r = -2.30136314705878E-001

------------------------------------------------------------------
standard error for a =  1.77257262522395E-001
standard error for b =  1.28953086035837E+000
percent standard error for a =  3.54514525044789E+001
percent standard error for b =  4.1372269846967E+002

Fitting results

<table>
<thead>
<tr>
<th>time</th>
<th>OBS</th>
<th>PRED</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00000000000000E-010</td>
<td>1.00000000000000E-010</td>
<td>1.36573081190330E-005</td>
</tr>
<tr>
<td>2.50000000000000E-001</td>
<td>1.86667000000000E-001</td>
<td>6.82865405951649E-001</td>
</tr>
<tr>
<td>5.00000000000000E-001</td>
<td>1.26000000000000E+000</td>
<td>9.6571518372232E-001</td>
</tr>
<tr>
<td>1.00000000000000E+000</td>
<td>4.35100000000000E+000</td>
<td>1.36573081190330E+000</td>
</tr>
<tr>
<td>2.00000000000000E+000</td>
<td>8.15000000000000E+000</td>
<td>1.93143503674446E+000</td>
</tr>
<tr>
<td>3.00000000000000E+000</td>
<td>1.24500000000000E+001</td>
<td>2.36551503674446E+000</td>
</tr>
<tr>
<td>4.00000000000000E+000</td>
<td>1.62000000000000E+001</td>
<td>2.73146162380660E+000</td>
</tr>
<tr>
<td>5.00000000000000E+000</td>
<td>1.82400000000000E+001</td>
<td>3.05386693438175E+000</td>
</tr>
<tr>
<td>2.40000000000000E+001</td>
<td>3.38520000000000E+001</td>
<td>6.69068723032014E+000</td>
</tr>
<tr>
<td>4.80000000000000E+001</td>
<td>5.05400000000000E+001</td>
<td>9.4620606271523E+000</td>
</tr>
<tr>
<td>7.20000000000000E+001</td>
<td>6.55150000000000E+001</td>
<td>1.15886102204668E+001</td>
</tr>
</tbody>
</table>

RMSE =  2.31493603385577E+001
AIC =  9.95001252784891E+001
BIC =  1.00295915824086E+002

Baker-Lonsdale 3/2[1-(1-y)^2/3]-y=Kt
Start point is  1.00000000000000E-010  Point no =  1
End point is  7.20000000000000E+001  Point no =  11
a =  1.3984455843843E-003
b =  -1.99992920778640E-003
r =  9.79450647162727E-001

standard error for a =  6.7519948912768E-005
standard error for b =  1.83440569905413E-003
percent standard error for a =  4.82821433450451E+000
percent standard error for b =  9.17235316086272E+001

Fitting results

<table>
<thead>
<tr>
<th>time</th>
<th>OBS</th>
<th>PRED</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00000000000000E-010</td>
<td>1.00000000000000E-010</td>
<td>0.00000000000000E+000</td>
</tr>
<tr>
<td>2.50000000000000E-001</td>
<td>1.86667000000000E-001</td>
<td>0.00000000000000E+000</td>
</tr>
<tr>
<td>5.00000000000000E-001</td>
<td>1.26000000000000E+000</td>
<td>0.00000000000000E+000</td>
</tr>
<tr>
<td>1.00000000000000E+000</td>
<td>4.35100000000000E+000</td>
<td>0.00000000000000E+000</td>
</tr>
<tr>
<td>2.00000000000000E+000</td>
<td>8.15000000000000E+000</td>
<td>0.00000000000000E+000</td>
</tr>
<tr>
<td>3.00000000000000E+000</td>
<td>1.24500000000000E+001</td>
<td>0.00000000000000E+000</td>
</tr>
<tr>
<td>4.00000000000000E+000</td>
<td>1.62000000000000E+001</td>
<td>0.00000000000000E+000</td>
</tr>
<tr>
<td>5.00000000000000E+000</td>
<td>1.82400000000000E+001</td>
<td>0.00000000000000E+000</td>
</tr>
</tbody>
</table>

362
Model is Weibull \( y = 100 \times (1 - \exp[-(t-\text{lag})^A]/K] \)

\[ K = 4.1917562583986491 \times 10^01 \]
\[ A = 1.03332395427528 \times 10^00 \]

\( \text{lag} = 0 \)

Start point is \( 1.00000000000000 \times 10^{-010} \)
Point no. = 1
End point is \( 7.20000000000000 \times 10^01 \)
Point no. = 11

\[ a = 1.03332395427528 \times 10^00 \]
\[ b = -3.73570489385636 \times 10^{-001} \]
\[ r = 9.93860129601386 \times 10^{-001} \]

standard error for \( a = 2.70727398672744 \times 10^{-002} \)
standard error for \( b = 1.96951780911623 \times 10^{-001} \)

percent standard error for \( a = 2.61996634794573 \times 10^000 \)
percent standard error for \( b = 5.27214505716241 \times 10^000 \)

Fitting results

time OBS PRED
\[ 1.00000000000000 \times 10^{-010} \] \[ 1.00000000000000 \times 10^{-010} \] \[ 1.10755274309454 \times 10^{-010} \]
\[ 2.50000000000000 \times 10^{-001} \] \[ 1.86667000000000 \times 10^{-001} \] \[ 5.67864784574267 \times 10^{-001} \]
\[ 5.00000000000000 \times 10^{-001} \] \[ 1.26000000000000 \times 10^{-001} \] \[ 1.15881445642651 \times 10^{-001} \]
\[ 1.00000000000000 \times 10^{-000} \] \[ 4.35100000000000 \times 10^{-000} \] \[ 2.35740356979768 \times 10^{-000} \]
\[ 2.00000000000000 \times 10^{-000} \] \[ 8.15000000000000 \times 10^{-000} \] \[ 4.76547114644570 \times 10^{-000} \]
\[ 3.00000000000000 \times 10^{-000} \] \[ 1.24500000000000 \times 10^{-000} \] \[ 7.15490715102139 \times 10^{-000} \]
\[ 4.00000000000000 \times 10^{-000} \] \[ 1.62000000000000 \times 10^{-000} \] \[ 9.51057172718429 \times 10^{-000} \]
\[ 5.00000000000000 \times 10^{-000} \] \[ 1.82400000000000 \times 10^{-000} \] \[ 1.18256275802051 \times 10^{-001} \]
\[ 2.40000000000000 \times 10^{-001} \] \[ 3.38520000000000 \times 10^{-001} \] \[ 4.70867278776388 \times 10^{-001} \]
\[ 4.80000000000000 \times 10^{-001} \] \[ 5.05400000000000 \times 10^{-001} \] \[ 7.28224580691815 \times 10^{-001} \]
\[ 7.20000000000000 \times 10^{-001} \] \[ 6.55150000000000 \times 10^{-001} \] \[ 8.62034573809497 \times 10^{-001} \]

RMSE = \[ 1.05709459168925 \times 10^{-001} \]
AIC = \[ 8.22552523059027 \times 10^{-001} \]
BIC = \[ 8.30510428514995 \times 10^{-001} \]

Korsmeyer-Peppas model
Model is \( \ln(y) = a \times \ln(x) + b \)
Which is equal to \( y = K \times x^A \)
\[ K = 2.0956850132510391 \times 10^{-000} \]
\[ A = 1.02381691960188 \times 10^{-000} \]
Start point is 1.00000000000000E-010  Point no =  1
End point is 7.20000000000000E+001  Point no =  11
a =  1.02381691960188E+000
b =  7.39880475228153E-001
r =  9.92052731909335E-001
standard error for a =  3.05451738162640E-002
standard error for b =  2.22213429850901E-001
percent standard error for a =  2.98346054176772E+000
percent standard error for b =  3.00336929126799E+001

Fitting results

time  OBS  PRED
1.00000000000000E-010  1.00000000000000E-010  1.21103532574109E-010
2.50000000000000E-001  1.86667000000000E-001  5.06905264826975E-001
5.00000000000000E-001  1.26000000000000E+000  1.03068606599285E+000
1.00000000000000E+000  4.35100000000000E+000  2.09568501325104E+000
2.00000000000000E+000  8.15000000000000E+000  4.26113810952301E+000
3.00000000000000E+000  1.24500000000000E+001  6.45373042733799E+000
4.00000000000000E+000  1.62000000000000E+001  8.66413505421296E+000
5.00000000000000E+000  1.82400000000000E+001  1.08878799678410E+001
2.40000000000000E+001  3.38520000000000E+001  5.42512350455204E+001
4.80000000000000E+001  5.05400000000000E+001  1.10308564368185E+002
7.20000000000000E+001  6.55150000000000E+001  1.67068497805715E+002

RMSE = 3.62675365079586E+001
AIC =  1.09377154662114E+002
BIC =  1.10172945207711E+002

----------------------------------------------
Gem-GMC Release in SLF
----------------------------------------------
Model order is 0
Start point is 1.00000000000000E-010  Point no =  1
End point is 7.20000000000000E+001  Point no =  11
a =  1.11616531756337E+000
b =  4.70269004720362E+000
r =  9.70848576476384E-001
standard error for a =  6.44705541817893E-002
standard error for b =  1.75155867142388E+000
percent standard error for a =  5.77607544991865E+000
percent standard error for b =  3.72458880734744E+001

Fitting results

time  OBS  PRED
1.00000000000000E-010  1.00000000000000E-010  4.70269004731523E+000
<table>
<thead>
<tr>
<th>time</th>
<th>OBS</th>
<th>PRED</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00000000000000E-010</td>
<td>1.00000000000000E-010</td>
<td>1.59404679617344E-001</td>
</tr>
<tr>
<td>2.50000000000000E-001</td>
<td>1.35000000000000E-001</td>
<td>1.64033540117875E-001</td>
</tr>
<tr>
<td>5.00000000000000E-001</td>
<td>1.16400000000000E+000</td>
<td>1.68796815429144E-001</td>
</tr>
<tr>
<td>1.00000000000000E+000</td>
<td>4.21500000000000E+000</td>
<td>1.7874236597291E-001</td>
</tr>
<tr>
<td>2.00000000000000E+000</td>
<td>6.54600000000000E+000</td>
<td>2.00425878144351E-001</td>
</tr>
<tr>
<td>3.00000000000000E+000</td>
<td>8.95100000000000E+000</td>
<td>2.24739887564742E-001</td>
</tr>
<tr>
<td>4.00000000000000E+000</td>
<td>1.24560000000000E+001</td>
<td>2.52003471459088E-001</td>
</tr>
<tr>
<td>5.00000000000000E+000</td>
<td>1.56700000000000E+001</td>
<td>2.8257447800847E-001</td>
</tr>
<tr>
<td>2.40000000000000E+001</td>
<td>4.13000000000000E+001</td>
<td>2.48847800653731E+000</td>
</tr>
</tbody>
</table>
Model is Higuchi $y=Kt^{1/2}$
Which is equal to $\ln(y) = \frac{1}{2}\ln(t)+b$

$K = \exp(b) = 1.265228941143278E+0000$

Start point is $1.00000000000000E-010$  Point no =  1
End point is $7.20000000000000E+001$  Point no =  11

$a = 5.00000000000000E-001$
$b = 2.35253086945300E-001$
$r = -2.09593456320514E-001$

standard error for $a = 1.77145816931397E+001$
standard error for $b = 1.28872010357020E+000$
percent standard error for $a = 3.54291633862793E+001$
percent standard error for $b = 5.47801569919397E+002$

Fitting results

<table>
<thead>
<tr>
<th>time</th>
<th>OBS</th>
<th>PRED</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.000000000000000E-010</td>
<td>1.000000000000000E-010</td>
<td>1.265228941143278E-005</td>
</tr>
<tr>
<td>2.500000000000000E-001</td>
<td>1.350000000000000E-001</td>
<td>6.32614470571637E-001</td>
</tr>
<tr>
<td>5.000000000000000E-001</td>
<td>1.164000000000000E+000</td>
<td>8.94651964035884E-001</td>
</tr>
<tr>
<td>1.000000000000000E+000</td>
<td>4.215000000000000E+000</td>
<td>1.265228941143278E+000</td>
</tr>
<tr>
<td>2.000000000000000E+000</td>
<td>6.546000000000000E+000</td>
<td>1.78930392807177E+000</td>
</tr>
<tr>
<td>3.000000000000000E+000</td>
<td>8.951000000000000E+000</td>
<td>2.19144080926672E+000</td>
</tr>
<tr>
<td>4.000000000000000E+000</td>
<td>1.245600000000000E+001</td>
<td>2.53045788228655E+000</td>
</tr>
<tr>
<td>5.000000000000000E+000</td>
<td>1.567000000000000E+001</td>
<td>2.82913791949644E+000</td>
</tr>
<tr>
<td>2.400000000000000E+001</td>
<td>4.130000000000000E+001</td>
<td>6.19833062720574E+000</td>
</tr>
<tr>
<td>4.800000000000000E+001</td>
<td>5.840000000000000E+001</td>
<td>8.7657632370689E+000</td>
</tr>
<tr>
<td>7.200000000000000E+001</td>
<td>8.120000000000000E+001</td>
<td>1.07358235684306E+001</td>
</tr>
</tbody>
</table>

RMSE = 2.86064647065461E+001
AIC = 1.04156768082195E+002
BIC = 1.04952558627792E+002

Baker-Lonsdale 3/2[1-(1-y)^2/3] - y=Kt

Start point is $1.00000000000000E-010$  Point no =  1
End point is $7.20000000000000E+001$  Point no =  11

$a = 2.44289848541378E-003$
$b = -6.31029232746742E-003$
$r = 9.44195768171728E-001$

standard error for $a = 1.97964222168891E-004$
Fitting results

<table>
<thead>
<tr>
<th>time</th>
<th>OBS</th>
<th>PRED</th>
<th>Pred - Obs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00000000000000E-010</td>
<td>1.00000000000000E-010</td>
<td>0.00000000000000E+000</td>
<td></td>
</tr>
<tr>
<td>2.50000000000000E-001</td>
<td>1.35000000000000E-001</td>
<td>0.00000000000000E+000</td>
<td></td>
</tr>
<tr>
<td>5.00000000000000E-001</td>
<td>1.16400000000000E+000</td>
<td>0.00000000000000E+000</td>
<td></td>
</tr>
<tr>
<td>1.00000000000000E+000</td>
<td>4.21500000000000E+000</td>
<td>0.00000000000000E+000</td>
<td></td>
</tr>
<tr>
<td>2.00000000000000E+000</td>
<td>6.54600000000000E+000</td>
<td>0.00000000000000E+000</td>
<td></td>
</tr>
<tr>
<td>3.00000000000000E+000</td>
<td>8.95100000000000E+000</td>
<td>0.00000000000000E+000</td>
<td></td>
</tr>
<tr>
<td>4.00000000000000E+000</td>
<td>1.24560000000000E+001</td>
<td>0.00000000000000E+000</td>
<td></td>
</tr>
<tr>
<td>5.00000000000000E+000</td>
<td>1.56700000000000E+001</td>
<td>0.00000000000000E+000</td>
<td></td>
</tr>
<tr>
<td>2.40000000000000E+001</td>
<td>4.13000000000000E+001</td>
<td>0.00000000000000E+000</td>
<td></td>
</tr>
<tr>
<td>4.80000000000000E+001</td>
<td>5.84000000000000E+001</td>
<td>0.00000000000000E+000</td>
<td></td>
</tr>
<tr>
<td>7.20000000000000E+001</td>
<td>8.12000000000000E+001</td>
<td>0.00000000000000E+000</td>
<td></td>
</tr>
</tbody>
</table>

RMSE = 3.33745966254139E+001
AIC = 1.07548338672166E+002
BIC = 1.08344129217763E+002

Model is Weibull \( y = 100 \times (1 - \exp[-(t - \text{lag})^A]/K] \)

\[ K = 4.3872546370578973E+000 \]

\[ A = 1.03757970377739E+000 \]

\[ \text{lag} = 0 \]

Fitting results

<table>
<thead>
<tr>
<th>time</th>
<th>OBS</th>
<th>PRED</th>
<th>Pred - Obs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00000000000000E-010</td>
<td>1.00000000000000E-010</td>
<td>0.00000000000000E+000</td>
<td></td>
</tr>
<tr>
<td>2.50000000000000E-001</td>
<td>1.35000000000000E-001</td>
<td>0.00000000000000E+000</td>
<td></td>
</tr>
<tr>
<td>5.00000000000000E-001</td>
<td>1.16400000000000E+000</td>
<td>0.00000000000000E+000</td>
<td></td>
</tr>
<tr>
<td>1.00000000000000E+000</td>
<td>4.21500000000000E+000</td>
<td>0.00000000000000E+000</td>
<td></td>
</tr>
<tr>
<td>2.00000000000000E+000</td>
<td>6.54600000000000E+000</td>
<td>0.00000000000000E+000</td>
<td></td>
</tr>
<tr>
<td>3.00000000000000E+000</td>
<td>8.95100000000000E+000</td>
<td>0.00000000000000E+000</td>
<td></td>
</tr>
<tr>
<td>4.00000000000000E+000</td>
<td>1.24560000000000E+001</td>
<td>0.00000000000000E+000</td>
<td></td>
</tr>
<tr>
<td>5.00000000000000E+000</td>
<td>1.56700000000000E+001</td>
<td>0.00000000000000E+000</td>
<td></td>
</tr>
<tr>
<td>2.40000000000000E+001</td>
<td>4.13000000000000E+001</td>
<td>0.00000000000000E+000</td>
<td></td>
</tr>
<tr>
<td>4.80000000000000E+001</td>
<td>5.84000000000000E+001</td>
<td>0.00000000000000E+000</td>
<td></td>
</tr>
<tr>
<td>7.20000000000000E+001</td>
<td>8.12000000000000E+001</td>
<td>0.00000000000000E+000</td>
<td></td>
</tr>
</tbody>
</table>

standard error for b = 5.37836155392547E-003
percent standard error for a = 8.10366142313769E+000
percent standard error for b = 8.52315752554689E+001
Korsmeyer-Peppas model
Model is \( \ln(y) = a \ln(x) + b \)
Which is equal to \( y = K x^A \)
\( K = 1.9436859015071059E+0000 \)
\( A = 1.02521424681931E+000 \)
Start point is \( 1.00000000000000E-010 \)  Point no = 1
End point is \( 7.20000000000000E+001 \)  Point no = 11
\( a = 1.02521424681931E+000 \)
\( b = 6.64586119685062E-001 \)
\( r = 9.93782611307516E-001 \)
standard error for \( a = 2.70303513452925E-002 \)
standard error for \( b = 1.96643408174490E-001 \)
percent standard error for \( a = 2.6365635191893E+000 \)
percent standard error for \( b = 2.9588527235080E+001 \)
Fitting results
\begin{array}{ccc}
\text{time} & \text{OBS} & \text{PRED} \\
1.00000000000000E-010 & 1.00000000000000E-010 & 1.08763610602269E-010 \\
2.50000000000000E-001 & 1.35000000000000E-001 & 4.69229819819258E-001 \\
5.00000000000000E-001 & 1.16400000000000E+000 & 9.55005437340234E-001 \\
1.00000000000000E+000 & 4.21500000000000E+000 & 1.94368590150711E+000 \\
2.00000000000000E+000 & 6.54600000000000E+000 & 3.9559092924370E+000 \\
3.00000000000000E+000 & 8.95100000000000E+000 & 5.9948399430368E+000 \\
4.00000000000000E+000 & 1.24560000000000E+001 & 8.05131030498595E+000 \\
5.00000000000000E+000 & 1.56700000000000E+001 & 1.01209223082318E+001 \\
2.40000000000000E+001 & 4.13000000000000E+001 & 5.05403579132364E+001 \\
4.80000000000000E+001 & 5.84000000000000E+001 & 1.02862850086667E+002 \\
7.20000000000000E+001 & 8.12000000000000E+001 & 1.55879793197145E+002 \\
\end{array}
RMSE = 2.64752989971785E+001
AIC = 1.02453516070571E+002
BIC = 1.03249306616168E+002
## MTT Assay

### A549 48 hr

<table>
<thead>
<tr>
<th>Gemcitabine concentration (uM)</th>
<th>log (M)</th>
<th>Abs 1</th>
<th>abs 2</th>
<th>abs 3</th>
<th>abs average</th>
<th>abs corrected viability</th>
<th>kill</th>
<th>stdev</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-12</td>
<td>0.224</td>
<td>0.221</td>
<td>0.225</td>
<td>0.22333333</td>
<td>100</td>
<td>0</td>
<td>0.02082</td>
</tr>
<tr>
<td>0.01</td>
<td>-8</td>
<td>0.215</td>
<td>0.227</td>
<td>0.196</td>
<td>0.21266667</td>
<td>95.22388</td>
<td>4.776119</td>
<td>0.015631</td>
</tr>
<tr>
<td>0.1</td>
<td>-7</td>
<td>0.22</td>
<td>0.208</td>
<td>0.178</td>
<td>0.202</td>
<td>90.44776</td>
<td>9.552239</td>
<td>0.021633</td>
</tr>
<tr>
<td>1</td>
<td>-6</td>
<td>0.187</td>
<td>0.141</td>
<td>0.208</td>
<td>0.17866667</td>
<td>80</td>
<td>20</td>
<td>0.034269</td>
</tr>
<tr>
<td>10</td>
<td>-5</td>
<td>0.187</td>
<td>0.173</td>
<td>0.168</td>
<td>0.176</td>
<td>78.80597</td>
<td>21.19403</td>
<td>0.009849</td>
</tr>
<tr>
<td>100</td>
<td>-4</td>
<td>0.165</td>
<td>0.184</td>
<td>0.177</td>
<td>0.17533333</td>
<td>78.50746</td>
<td>21.49254</td>
<td>0.009609</td>
</tr>
<tr>
<td>1000</td>
<td>-3</td>
<td>0.195</td>
<td>0.163</td>
<td>0.164</td>
<td>0.174</td>
<td>77.91045</td>
<td>22.08955</td>
<td>0.018193</td>
</tr>
</tbody>
</table>

### Gem GNC (mg/mL)

<table>
<thead>
<tr>
<th>Abs 1</th>
<th>abs 2</th>
<th>abs 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.224</td>
<td>0.221</td>
<td>0.225</td>
</tr>
</tbody>
</table>

### Abs average

<table>
<thead>
<tr>
<th>Abs average</th>
<th>abs corrected viability</th>
<th>kill</th>
<th>stdev</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.22333333</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

### Gem GNC (mg/mL)

<table>
<thead>
<tr>
<th>Abs 1</th>
<th>abs 2</th>
<th>abs 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.206</td>
<td>0.198</td>
<td>0.208</td>
</tr>
</tbody>
</table>

### Abs average

<table>
<thead>
<tr>
<th>Abs average</th>
<th>abs corrected viability</th>
<th>kill</th>
<th>stdev</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.204</td>
<td>91.34328</td>
<td>8.656716</td>
<td>5.804608</td>
</tr>
</tbody>
</table>

### Gem GNC uM

<table>
<thead>
<tr>
<th>Abs 1</th>
<th>abs 2</th>
<th>abs 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.148</td>
<td>0.185</td>
<td>0.183</td>
</tr>
</tbody>
</table>

### Abs average

<table>
<thead>
<tr>
<th>Abs average</th>
<th>abs corrected viability</th>
<th>kill</th>
<th>ave</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.172</td>
<td>77.01493</td>
<td>25.98507</td>
<td>80.29851</td>
</tr>
</tbody>
</table>

### Gem GNC uM

<table>
<thead>
<tr>
<th>Abs 1</th>
<th>abs 2</th>
<th>abs 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.139</td>
<td>0.145</td>
<td>0.151</td>
</tr>
</tbody>
</table>

### Abs average

<table>
<thead>
<tr>
<th>Abs average</th>
<th>abs corrected viability</th>
<th>kill</th>
<th>ave</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.145</td>
<td>64.92537</td>
<td>35.07463</td>
<td>64.25373</td>
</tr>
</tbody>
</table>

## A549 72 hr

<table>
<thead>
<tr>
<th>Gemcitabine concentration (uM)</th>
<th>log (M)</th>
<th>Abs 1</th>
<th>abs 2</th>
<th>abs 3</th>
<th>abs average</th>
<th>abs corrected viability</th>
<th>kill</th>
<th>stdev</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-12</td>
<td>0.224</td>
<td>0.221</td>
<td>0.225</td>
<td>0.22333333</td>
<td>100</td>
<td>0</td>
<td>0.02082</td>
</tr>
<tr>
<td>0.01</td>
<td>-8</td>
<td>0.215</td>
<td>0.227</td>
<td>0.196</td>
<td>0.21266667</td>
<td>95.22388</td>
<td>4.776119</td>
<td>0.015631</td>
</tr>
<tr>
<td>0.1</td>
<td>-7</td>
<td>0.22</td>
<td>0.208</td>
<td>0.178</td>
<td>0.202</td>
<td>90.44776</td>
<td>9.552239</td>
<td>0.021633</td>
</tr>
<tr>
<td>1</td>
<td>-6</td>
<td>0.187</td>
<td>0.141</td>
<td>0.208</td>
<td>0.17866667</td>
<td>80</td>
<td>20</td>
<td>0.034269</td>
</tr>
<tr>
<td>10</td>
<td>-5</td>
<td>0.187</td>
<td>0.173</td>
<td>0.168</td>
<td>0.176</td>
<td>78.80597</td>
<td>21.19403</td>
<td>0.009849</td>
</tr>
<tr>
<td>100</td>
<td>-4</td>
<td>0.165</td>
<td>0.184</td>
<td>0.177</td>
<td>0.17533333</td>
<td>78.50746</td>
<td>21.49254</td>
<td>0.009609</td>
</tr>
<tr>
<td>1000</td>
<td>-3</td>
<td>0.195</td>
<td>0.163</td>
<td>0.164</td>
<td>0.174</td>
<td>77.91045</td>
<td>22.08955</td>
<td>0.018193</td>
</tr>
</tbody>
</table>

### Gem GNC (mg/mL)

<table>
<thead>
<tr>
<th>Abs 1</th>
<th>abs 2</th>
<th>abs 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.001</td>
<td>0.206</td>
<td>0.198</td>
</tr>
</tbody>
</table>

### Abs average

<table>
<thead>
<tr>
<th>Abs average</th>
<th>abs corrected viability</th>
<th>kill</th>
<th>stdev</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.22333333</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

### Gem GNC (mg/mL)

<table>
<thead>
<tr>
<th>Abs 1</th>
<th>abs 2</th>
<th>abs 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.178</td>
<td>0.186</td>
<td>0.227</td>
</tr>
</tbody>
</table>

### Abs average

<table>
<thead>
<tr>
<th>Abs average</th>
<th>abs corrected viability</th>
<th>kill</th>
<th>stdev</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.197</td>
<td>88.20896</td>
<td>11.79104</td>
<td>10.5538</td>
</tr>
</tbody>
</table>

### Gem GNC uM

<table>
<thead>
<tr>
<th>Abs 1</th>
<th>abs 2</th>
<th>abs 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.148</td>
<td>0.185</td>
<td>0.183</td>
</tr>
</tbody>
</table>

### Abs average

<table>
<thead>
<tr>
<th>Abs average</th>
<th>abs corrected viability</th>
<th>kill</th>
<th>ave</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.172</td>
<td>77.01493</td>
<td>25.98507</td>
<td>80.29851</td>
</tr>
</tbody>
</table>

### Gem GNC uM

<table>
<thead>
<tr>
<th>Abs 1</th>
<th>abs 2</th>
<th>abs 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.139</td>
<td>0.145</td>
<td>0.151</td>
</tr>
</tbody>
</table>

### Abs average

<table>
<thead>
<tr>
<th>Abs average</th>
<th>abs corrected viability</th>
<th>kill</th>
<th>ave</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.145</td>
<td>64.92537</td>
<td>35.07463</td>
<td>64.25373</td>
</tr>
</tbody>
</table>

### Gem GNC uM

<table>
<thead>
<tr>
<th>Abs 1</th>
<th>abs 2</th>
<th>abs 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.154</td>
<td>0.159</td>
<td>0.149</td>
</tr>
</tbody>
</table>

### Abs average

<table>
<thead>
<tr>
<th>Abs average</th>
<th>abs corrected viability</th>
<th>kill</th>
<th>ave</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.154</td>
<td>68.95522</td>
<td>31.04478</td>
<td>71.49254</td>
</tr>
</tbody>
</table>

### Gem GNC uM

<table>
<thead>
<tr>
<th>Abs 1</th>
<th>abs 2</th>
<th>abs 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.145</td>
<td>0.135</td>
<td>0.146</td>
</tr>
</tbody>
</table>

### Abs average

<table>
<thead>
<tr>
<th>Abs average</th>
<th>abs corrected viability</th>
<th>kill</th>
<th>ave</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.142</td>
<td>63.58209</td>
<td>36.41791</td>
<td>64.25373</td>
</tr>
<tr>
<td>Gemcitabine concentration (uM)</td>
<td>log (M)</td>
<td>abs 1</td>
<td>abs 2</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>----------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>370</td>
<td>0</td>
<td>0.52</td>
<td>0.314</td>
</tr>
<tr>
<td>72</td>
<td>0.01</td>
<td>0.229</td>
<td>0.219</td>
</tr>
<tr>
<td>11</td>
<td>0.1</td>
<td>0.177</td>
<td>0.186</td>
</tr>
<tr>
<td>17</td>
<td>1</td>
<td>0.165</td>
<td>0.169</td>
</tr>
<tr>
<td>100</td>
<td>10</td>
<td>0.156</td>
<td>0.167</td>
</tr>
<tr>
<td>1000</td>
<td>100</td>
<td>0.163</td>
<td>0.166</td>
</tr>
<tr>
<td>1000</td>
<td>1000</td>
<td>0.137</td>
<td>0.127</td>
</tr>
<tr>
<td>Gem GNC (mg/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0.52</td>
<td>0.314</td>
</tr>
<tr>
<td>0.001</td>
<td>0.01</td>
<td>0.25</td>
<td>0.262</td>
</tr>
<tr>
<td>0.1</td>
<td>0.211</td>
<td>0.22</td>
<td>0.222</td>
</tr>
<tr>
<td>1</td>
<td>0.196</td>
<td>0.191</td>
<td>0.194</td>
</tr>
<tr>
<td>2</td>
<td>0.184</td>
<td>0.177</td>
<td>0.175</td>
</tr>
<tr>
<td></td>
<td>0.172</td>
<td>0.161</td>
<td>0.161</td>
</tr>
<tr>
<td></td>
<td>0.122</td>
<td>0.126</td>
<td>0.126</td>
</tr>
<tr>
<td>2</td>
<td>0.001</td>
<td>0.205</td>
<td>0.207</td>
</tr>
<tr>
<td>0.01</td>
<td>0.23</td>
<td>0.197</td>
<td>0.204</td>
</tr>
<tr>
<td>0.1</td>
<td>0.182</td>
<td>0.171</td>
<td>0.164</td>
</tr>
<tr>
<td>1</td>
<td>0.186</td>
<td>0.168</td>
<td>0.147</td>
</tr>
<tr>
<td>10</td>
<td>0.143</td>
<td>0.159</td>
<td>0.139</td>
</tr>
<tr>
<td>100</td>
<td>0.124</td>
<td>0.125</td>
<td>0.129</td>
</tr>
</tbody>
</table>

H460 48 hr
| Gemcita bine concentration (uM) | log (M) | Abs 1 | abs 2 | abs 3 | abs average | abs corrected | Cell viability | cell kill % | stdev |
|---|---|---|---|---|---|---|---|---|---|---|
| 0 | -12 | 0.412 | 0.373 | 0.32 | 0.368333 | 100 | 0 | 0.046177 |
| 0.01 | -8 | 0.336 | 0.343 | 0.328 | 0.335667 | 91.13122 | 8.868778 | 0.007506 |
| 0.1 | -7 | 0.294 | 0.239 | 0.361 | 0.298 | 80.90498 | 19.09502 | 0.061098 |
| 1 | -6 | 0.257 | 0.248 | 0.26 | 0.255 | 69.23077 | 30.76923 | 0.006245 |
| 10 | -5 | 0.224 | 0.235 | 0.217 | 0.225333 | 61.17647 | 38.82353 | 0.009074 |
| 100 | -4 | 0.106 | 0.107 | 0.112 | 0.108333 | 51.56 | 48.44 | 0.003215 |
| 1000 | -3 | 0.031 | 0.022 | 0.041 | 0.031333 | 40.61 | 59.39 | 0.009504 |

| Gem GNC (mg/mL) | Abs 1 | abs 2 | abs 3 | abs average | abs corrected | Cell viability | cell kill % | stdev |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 0.412 | 0.373 | 0.32 | 0.368333 | 100 | 0 | 0 |
| 0.001 | 0.314 | 0.337 | 0.316 | 0.322333 | 87.51131 | 12.48869 | 2.87962 |
| 0.01 | 0.275 | 0.28 | 0.253 | 0.269333 | 73.12217 | 26.87783 | 3.327516 |
| 0.1 | 0.206 | 0.192 | 0.212 | 0.203333 | 55.20362 | 44.79638 | 1.535798 |
| 1 | 0.194 | 0.181 | 0.183 | 0.186 | 50.97744 | 49.50226 | 0 |
| 10 | 0.103 | 0.107 | 0.109 | 0.106333 | 28.86878 | 71.13122 | 89.5822 |
| 100 | 0.039 | 0.022 | 0.038 | 0.033 | 8.959276 | 91.04072 | 0.383949 |

| Gem GNC (mg/mL) | Abs 1 | abs 2 | abs 3 | abs average | abs corrected | Cell viability | cell kill % |
|---|---|---|---|---|---|---|---|---|
| 0 | 0.412 | 0.373 | 0.32 | 0.368333 | 100 | 0 | 100 |
| 0.001 | 0.317 | 0.302 | 0.303 | 0.307333 | 83.43891 | 16.56109 | 85.47511 |
| 0.01 | 0.281 | 0.29 | 0.289 | 0.286667 | 77.82805 | 22.17195 | 75.47511 |
| 0.1 | 0.205 | 0.192 | 0.237 | 0.211333 | 57.37557 | 42.62443 | 56.28959 |
| 1 | 0.192 | 0.187 | 0.179 | 0.186 | 50.49774 | 49.50226 | 0.49774 |
| 10 | 0.092 | 0.108 | 0.105 | 0.101667 | 27.60181 | 72.39819 | 28.23529 |
| 100 | 0.029 | 0.036 | 0.028 | 0.031 | 8.41629 | 91.58371 | 8.687783 |

H460 72 hr
<table>
<thead>
<tr>
<th>Gemcitabine concentration (uM)</th>
<th>log (M)</th>
<th>Abs 1</th>
<th>abs 2</th>
<th>abs 3</th>
<th>abs average</th>
<th>abs corrected viability</th>
<th>kill</th>
<th>stdev</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-12</td>
<td>0.392</td>
<td>0.46</td>
<td>0.471</td>
<td>0.441</td>
<td>100</td>
<td>0</td>
<td>0.04279</td>
</tr>
<tr>
<td>0.01</td>
<td>-8</td>
<td>0.397</td>
<td>0.393</td>
<td>0.385</td>
<td>0.391667</td>
<td>88.8133</td>
<td>11.1867</td>
<td>0.00611</td>
</tr>
<tr>
<td>0.1</td>
<td>-7</td>
<td>0.353</td>
<td>0.321</td>
<td>0.318</td>
<td>0.330667</td>
<td>74.9811</td>
<td>25.0189</td>
<td>0.019399</td>
</tr>
<tr>
<td>1</td>
<td>-6</td>
<td>0.309</td>
<td>0.301</td>
<td>0.301</td>
<td>0.303667</td>
<td>68.85865</td>
<td>31.14135</td>
<td>0.004619</td>
</tr>
<tr>
<td>10</td>
<td>-5</td>
<td>0.203</td>
<td>0.187</td>
<td>0.191</td>
<td>0.193667</td>
<td>57.542</td>
<td>42.458</td>
<td>0.008327</td>
</tr>
<tr>
<td>100</td>
<td>-4</td>
<td>0.193</td>
<td>0.191</td>
<td>0.192</td>
<td>0.192</td>
<td>43.53741</td>
<td>56.46259</td>
<td>0.001</td>
</tr>
<tr>
<td>1000</td>
<td>-3</td>
<td>0.178</td>
<td>0.168</td>
<td>0.174</td>
<td>0.173333</td>
<td>39.30461</td>
<td>60.69539</td>
<td>0.005033</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gem GNC (mg/mL)</th>
<th>Abs 1</th>
<th>abs 2</th>
<th>abs 3</th>
<th>abs average</th>
<th>abs corrected viability</th>
<th>kill</th>
<th>avg</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.392</td>
<td>0.46</td>
<td>0.471</td>
<td>0.441</td>
<td>100</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>0.001</td>
<td>0.374</td>
<td>0.341</td>
<td>0.29</td>
<td>0.335</td>
<td>75.96372</td>
<td>24.03628</td>
<td>2.565467</td>
</tr>
<tr>
<td>0.01</td>
<td>0.266</td>
<td>0.301</td>
<td>0.262</td>
<td>0.276333</td>
<td>62.66062</td>
<td>37.33938</td>
<td>2.191336</td>
</tr>
<tr>
<td>0.1</td>
<td>0.307</td>
<td>0.292</td>
<td>0.261</td>
<td>0.286667</td>
<td>65.00378</td>
<td>34.99622</td>
<td>4.008542</td>
</tr>
<tr>
<td>1</td>
<td>0.177</td>
<td>0.191</td>
<td>0.188</td>
<td>0.185333</td>
<td>42.0257</td>
<td>57.9743</td>
<td>0.427578</td>
</tr>
<tr>
<td>10</td>
<td>0.172</td>
<td>0.171</td>
<td>0.12</td>
<td>0.154333</td>
<td>34.99622</td>
<td>65.00378</td>
<td>3.687858</td>
</tr>
<tr>
<td>100</td>
<td>0.016</td>
<td>0.022</td>
<td>0.02</td>
<td>0.019333</td>
<td>4.383976</td>
<td>95.61602</td>
<td>5.291275</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gem GNC (mg/mL)</th>
<th>Abs 1</th>
<th>abs 2</th>
<th>abs 3</th>
<th>abs average</th>
<th>abs corrected viability</th>
<th>kill</th>
<th>avg</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.392</td>
<td>0.46</td>
<td>0.471</td>
<td>0.441</td>
<td>100</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>0.001</td>
<td>0.296</td>
<td>0.336</td>
<td>0.325</td>
<td>0.319</td>
<td>72.3356</td>
<td>27.6644</td>
<td>74.14966</td>
</tr>
<tr>
<td>0.01</td>
<td>0.312</td>
<td>0.283</td>
<td>0.275</td>
<td>0.29</td>
<td>65.75964</td>
<td>34.24036</td>
<td>64.21013</td>
</tr>
<tr>
<td>0.1</td>
<td>0.239</td>
<td>0.261</td>
<td>0.285</td>
<td>0.261667</td>
<td>59.33485</td>
<td>40.66515</td>
<td>62.16931</td>
</tr>
<tr>
<td>1</td>
<td>0.194</td>
<td>0.173</td>
<td>0.181</td>
<td>0.182667</td>
<td>41.42101</td>
<td>58.57899</td>
<td>41.72336</td>
</tr>
<tr>
<td>10</td>
<td>0.116</td>
<td>0.13</td>
<td>0.148</td>
<td>0.131333</td>
<td>29.7808</td>
<td>70.2192</td>
<td>32.38851</td>
</tr>
<tr>
<td>100</td>
<td>0.048</td>
<td>0.051</td>
<td>0.058</td>
<td>0.052333</td>
<td>11.86697</td>
<td>88.13303</td>
<td>8.125472</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>48 hr Gem</th>
<th>SD</th>
<th>48 hr Gem-GNC</th>
<th>SD</th>
<th>72 hr Gem</th>
<th>SD</th>
<th>72 hr GNC</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0.002082</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0.10378</td>
<td>100</td>
</tr>
<tr>
<td>0.001</td>
<td>95.22388</td>
<td>0.015631</td>
<td>95.45</td>
<td>5.804608</td>
<td>52.64443</td>
<td>0.015275</td>
<td>55.77705</td>
</tr>
<tr>
<td>0.01</td>
<td>90.45</td>
<td>0.021633</td>
<td>88.28</td>
<td>0.105538</td>
<td>45.8096</td>
<td>0.01159</td>
<td>52.64443</td>
</tr>
<tr>
<td>0.1</td>
<td>80</td>
<td>0.034269</td>
<td>80.299</td>
<td>4.643686</td>
<td>43.61269</td>
<td>0.020306</td>
<td>44.67046</td>
</tr>
<tr>
<td>1</td>
<td>78.80597</td>
<td>0.009849</td>
<td>72.24</td>
<td>3.16615</td>
<td>40.43938</td>
<td>0.009074</td>
<td>42.18877</td>
</tr>
<tr>
<td>10</td>
<td>78.50746</td>
<td>0.009609</td>
<td>71.49</td>
<td>3.588303</td>
<td>41.65989</td>
<td>0.010786</td>
<td>38.03906</td>
</tr>
<tr>
<td>100</td>
<td>11.91045</td>
<td>0.018193</td>
<td>64.25</td>
<td>0.949845</td>
<td>33.44182</td>
<td>0.01</td>
<td>30.59398</td>
</tr>
</tbody>
</table>
### 8-STAGE NON-VIABLE IMPACTOR DATA REDUCTION

**Trial Name:** Gem-GNC 1  
**Test Date:** 08/01/06  
**Flow Rate:** 28.32 lpm  
**Calibrated:** 08/01/06  
**Sample Time:** 10 min

<table>
<thead>
<tr>
<th>STAGE NO.</th>
<th>Cut Diam. (µm)</th>
<th>Initial Weight, mg</th>
<th>Final Weight, mg</th>
<th>Net Weight, mg</th>
<th>Mass/Stage</th>
<th>Cum. %</th>
<th>% &gt;</th>
<th>% &lt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-seperator</td>
<td>10</td>
<td>0.00030000</td>
<td>9</td>
<td>0.00087660</td>
<td>14.78</td>
<td>14.78</td>
<td>85.22</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>5.8</td>
<td>0.00051740</td>
<td>6.50</td>
<td>21.28</td>
<td>78.72</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4.7</td>
<td>0.00041380</td>
<td>5.20</td>
<td>26.48</td>
<td>73.52</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3.3</td>
<td>0.00023260</td>
<td>2.92</td>
<td>29.40</td>
<td>70.60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2.1</td>
<td>0.00021840</td>
<td>2.74</td>
<td>32.15</td>
<td>67.85</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1.1</td>
<td>0.00213180</td>
<td>26.78</td>
<td>58.93</td>
<td>41.07</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.7</td>
<td>0.00037220</td>
<td>4.68</td>
<td>63.60</td>
<td>36.40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.4</td>
<td>0.00268700</td>
<td>33.76</td>
<td>97.36</td>
<td>2.64</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FILTER</td>
<td></td>
<td>0.00021000</td>
<td>2.64</td>
<td>100.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Sum:** 0.00795980 mg

**MMAD =** 1.93 µm  
**Geometric Std.Dev. =** 2.87

**Concentration =** 0.03 mg/m³
### 8-STAGE NON-VIABLE IMPACTOR DATA REDUCTION

**Trial Name:** Gem-GNC 2  
**Test Date:**  
**Flow Rate:** 28.32 lpm  
**Calibrated:**  
**Sample Time:** 10 min

<table>
<thead>
<tr>
<th>STAGE NO.</th>
<th>Cut Diam. (μm)</th>
<th>Initial Weight, mg</th>
<th>Final Weight, mg</th>
<th>Net Weight, mg</th>
<th>Mass/Stage (%)</th>
<th>Cum. % &gt;</th>
<th>Cum. % &lt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-seperator</td>
<td>10</td>
<td>0.0002810</td>
<td>0</td>
<td>9</td>
<td>0.0006442</td>
<td>12.72</td>
<td>12.72</td>
</tr>
<tr>
<td>1</td>
<td>5.8</td>
<td>0.0004266</td>
<td>5.86</td>
<td>18.58</td>
<td>81.42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4.7</td>
<td>0.0003554</td>
<td>4.89</td>
<td>23.47</td>
<td>76.53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3.3</td>
<td>0.0002284</td>
<td>3.14</td>
<td>26.61</td>
<td>73.39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2.1</td>
<td>0.0002676</td>
<td>3.68</td>
<td>30.29</td>
<td>69.71</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1.1</td>
<td>0.0023098</td>
<td>31.75</td>
<td>62.04</td>
<td>37.96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.7</td>
<td>0.0002854</td>
<td>3.92</td>
<td>65.96</td>
<td>34.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.4</td>
<td>0.0022958</td>
<td>31.56</td>
<td>97.53</td>
<td>2.47</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FILTER</td>
<td></td>
<td>0.001800</td>
<td>2.47</td>
<td>100.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sum</td>
<td></td>
<td>0.007274200</td>
<td>mg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**MMAD =** 1.88 μm  
**Geometric Std.Dev. =** 2.75  
**Concentration =** 0.03 mg/m³

---

Y-Point

Cumulative Probability Less Than Stated Size

Diameter, μm

100

10

1

0.1

0.1

0.1

0.1

0.1

0.1
8-STAGE NON-VIABLE IMPACTOR DATA REDUCTION

Trial Name: Gem-GNC 3   Test Date: 
Flow Rate: 15 lpm   Calibrated: 
Sample Time: 10 min

<table>
<thead>
<tr>
<th>STAGE NO.</th>
<th>Cut Diam.</th>
<th>Initial Weight, mg</th>
<th>Final Weight, mg</th>
<th>Net Weight, mg</th>
<th>% Cum.</th>
<th>% &gt;</th>
<th>% &lt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-seperator</td>
<td>10</td>
<td>0.000358</td>
<td>0</td>
<td>0.000358</td>
<td>10.91</td>
<td>10.91</td>
<td>89.09</td>
</tr>
<tr>
<td>0</td>
<td>9</td>
<td>0.000916</td>
<td>10.91</td>
<td>10.91</td>
<td>10.91</td>
<td>10.91</td>
<td>89.09</td>
</tr>
<tr>
<td>1</td>
<td>5.8</td>
<td>0.000828</td>
<td>7.09</td>
<td>18.01</td>
<td>67.51</td>
<td>67.51</td>
<td>32.49</td>
</tr>
<tr>
<td>2</td>
<td>4.7</td>
<td>0.000612</td>
<td>5.24</td>
<td>23.25</td>
<td>76.75</td>
<td>76.75</td>
<td>23.25</td>
</tr>
<tr>
<td>3</td>
<td>3.3</td>
<td>0.001079</td>
<td>9.25</td>
<td>32.49</td>
<td>67.51</td>
<td>67.51</td>
<td>32.49</td>
</tr>
<tr>
<td>4</td>
<td>2.1</td>
<td>0.001658</td>
<td>14.20</td>
<td>46.70</td>
<td>53.30</td>
<td>53.30</td>
<td>46.70</td>
</tr>
<tr>
<td>5</td>
<td>1.1</td>
<td>0.002347</td>
<td>20.11</td>
<td>66.80</td>
<td>33.20</td>
<td>33.20</td>
<td>66.80</td>
</tr>
<tr>
<td>6</td>
<td>0.7</td>
<td>0.000944</td>
<td>8.09</td>
<td>74.89</td>
<td>25.11</td>
<td>25.11</td>
<td>74.89</td>
</tr>
<tr>
<td>7</td>
<td>0.4</td>
<td>0.002790</td>
<td>23.90</td>
<td>98.79</td>
<td>1.21</td>
<td>1.21</td>
<td>98.79</td>
</tr>
<tr>
<td>FILTER</td>
<td></td>
<td>0.000141</td>
<td>1.21</td>
<td>100.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sum</td>
<td></td>
<td>0.011673</td>
<td>mg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cumulative Probability Less Than Stated Size

- MMAD = 2.17 μm
- Geometric Std.Dev. = 2.55
- Concentration = 0.0778 mg/m³
REFERENCES


94. Inactive Ingredient Search for Approved Drug Products, U.S. Food and Drug Administration: Silver Spring, MD.
148. Louis, J.A., et al., The use of the murine model of infection with Leishmania major to reveal the antagonistic effects that IL-4 can exert on T helper cell development and demonstrate that these opposite effects depend upon the nature of the cells targeted for IL-4 signaling. Pathologie Biologie, 2003. 51(2): p. 71-73.


272. Fireman, P. Understanding asthma pathophysiology. in Allergy and asthma proceedings. 2003. OceanSide Publications, Inc.

388


Bateman, E.D., et al., Tiotropium is noninferior to salmeterol in maintaining improved lung function in B16-Arg/Arg patients with asthma. Journal of Allergy and Clinical Immunology, 2011. 128(2): p. 315-322.


630. Xie, Y., et al., Targeted delivery of siRNA to activated T Cells via transferrin-polyethylenimine (Tf-PEI) as a potential therapy of asthma. Journal of Controlled Release.


Al Faraj, A., et al., Specific targeting and noninvasive magnetic resonance imaging of an asthma biomarker in the lung using polyethylene glycol functionalized magnetic nanocarriers. Contrast Media Mol Imaging, 2015.


414


419


426


429


Moghimi, S.M. and J. Szebeni, conformation and hydrodynamics. Lee, H., et al.,
Zuo, J., et al., loaded nanoparticles. Galindo
Chan, J.M., et al., International Symposium on Industrial Crystallization
Briancon, S., et al. according their phase feed rates to form emulsion, removing the solvent providing a continuous phase and continuously feeding dispersed phase and continuous phase
the "Ouzo region" for poly (lactide polylactide nanoparticles by nanoprecipitation. Beck
Thanoo, B.C. and J. Murtagh, Forming a dispersed phase comprising a drug and a polymer, providing a continuous phase and continuously feeding dispersed phase and continuous phase according their phase feed rates to form emulsion, removing the solvent, 1999, Google Patents.


