DESIGN, DEVELOPMENT AND EVALUATION OF TARGETED DELIVERY SYSTEM
FOR THE TREATMENT OF LUNG CANCER

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DEDICATION

This dissertation is dedicated to my mother, father and my sister,

for their constant support throughout my journey to of finishing Ph.D.
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Abstract

Lung cancer is the leading cause of cancer-related deaths in the world. Lung cancer alone causes more deaths than pancreas, colon, prostate, and breast cancer deaths combined. Only 15% of lung cancer patients survive for 5 or more years after diagnosis. Lung cancer is further divided into small cell lung cancer and non-small cell lung cancer (NSCLC). The NSCLC accounts for 85% of all lung cancer cases. The current mainstay of treatment of lung cancer is multi-drug therapy. Chemotherapy is the preferred option for the treatment of lung cancer. However, severe side effects caused by chemotherapy demands developing novel methods for the treatment. The Luteinizing hormone-releasing hormone (LHRH) is overexpressed in non-small cell lung cancer (NSCLC). This thesis is focused on investigating the ability of poly (amino ether) (PAE) polymer based formulation of small-interfering RNA (siRNA) to silence the mammalian target of Rapamycin (mTOR) in NSCLC cell lines in vitro.

In the first part of this thesis, we modified and developed a bio-reducible polymer by introducing a sulfhydryl group (-SH) to PAE polymer. The modified PAE polymer (mPAE) showed decreased cytotoxicity and improved buffering capacity compared to the widely used transfection polymer poly-ethyleneimine (PEI).

In the second part, cationic bio-reducible polymer modified Poly (amino-ether) was used to formulate bio-reducible nanoparticles. The mPAE was used to deliver mTOR siRNA to the non-small cell lung cancer cell lines (A549 and H460) and access their potential as a siRNA deliver carrier for lung cancer therapy. The mPAE and mTOR siRNA formed stable, bio-reducible nanoparticles (NPs) at a polymer to siRNA weight ratio of 45:1, with average diameter 114 nm and surface charge of around +27mV. The mTOR siRNA showed increase release in the presence of 10mM GSH. By optimizing the concentration of the mPAE polymer, we were able
to fabricate polymeric NPs capable of efficient gene knockdown (60% and 64%) in A549 and H460 cells, respectively without significant cytotoxicity at 30µg/ml concentrations. The MS-MP-NPs showed improved cell growth inhibition (31% and 32%) in A549 and H460 cells in vitro, respectively. The MS-MP-NPs also showed time-dependent cellular uptake as determined by FACS for up to 24 hours. The results demonstrate that the mPAE polymer based NPs show strong potential for future modification with Poly (ethylene glycol) and targeting ligand to improve the gene delivery and achieve higher lung cancer growth inhibition in vitro and in vivo.

In the third part, PEGylated-targeted NPs (MS-MP-PG-LR) were prepared. To attach PEG on the surface of the nanoparticles, first, the PEG was conjugated with AMAS and then allowed to attach on the MS-MP NP surface. Further, cysteine-terminated LHRH was subsequently conjugated on the surface of the PEG group through a maleimide reaction with the cysteine group. We found that PEGylation of the mPAE based nanoparticles allows increased delivery of the siRNA. 22% PEGylated prevented the cytotoxic effect of 60 µg/ml concentrations of the nanoparticle system. Western blot results confirmed the overexpression of the LHRH receptor on the non-tumor (Breast adipocytes) and the tumor cells (SKOV-3). As shown before, PEGylation also provided serum stability to the NP system. The optimal particle size of the MS-MP-PG and MS-MP-PG-LR20 nanoparticles was found to be 124 ± 5.9and 132 ± 6.8 respectively. The zeta potential of the respective nanoparticles was positive (19.5 ± 4.6 and 20.2 ± 5.4). The FACS analysis showed that the targeted MS-MP-PG-LR20 nanoparticle system selectively internalizes in the LHRH-R overexpressed A549 and H460 cells compared to the SKOV-3 cells (significantly lower LHRH-R expression). The mTOR siRNA encapsulated, targeted MS-MP-PG-LR20 NPs showed significantly increased cell growth inhibition and mTOR gene silencing compared to the SS-MP-PG-LR20 nanoparticle system. The caspase
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**Keywords**- Bioreducible, mTOR, siRNA, nanoparticles, drug delivery, lung cancer, nanomedicine.
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Figure 4.19: Cell binding and uptake analysis of FS-MP-PG NPs and FS-MP-PG-LR NPs (1,10,20 and 40µM) by FACS. Targeted FS-MP-PG-LR NPs were formed at a weight ratio of 45:1 siRNA. FITC labelled siRNA was used to prepare the NPs to detect them in flow cytometry. Non-targeted PS-MP-PG NPs were also prepared to compare against the targeted NPs. Naked siRNA treated cells were used as control for the experiment. After 4h the cells were washed trypsinized and collected to perform the flow cytometry experiment. The fluorescence intensity of FS-MP-PG-NPs and FS-MP-PG-LR-NPs (1,10,20 and 40µM) in (A and B) A549, (C and D) H460, (E and F) SKOV-3 cells (5 × 10^3 cell/well). (C) Bar graph representing the mean percentages of cellular uptake. The results are presented as mean ± SD (n=3). Analysis of cellular uptake was performed by using Flowjo 7.6.1 single cell analysis software. (*** p < 0.001)

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Figure 4.21: Competitive binding studies of T-NPS (FS-MP-PG-LR20 NPs) in (A) A549 and (B)H460 cells. Block of A549 and H460 cells with 1,10,30 and 50 µM LHRH-R peptide; The cells were pre-incubated with 1, 10, 30 and 50 µM LHRH analog peptide for 1 h, respectively. The peptide was removed after 1 hour and the cells were washed thrice with ice cold PBS. After washing, the T-NPs were incubated with the cells for a period of 1h in RPMI medium. The results are presented as mean ± SD (n=3). Analysis of cellular uptake was performed by using Flowjo 7.6.1 single cell analysis software. (* p < 0.05, **p<0.01).

Figure 4.22: The A549 cells were treated with 60 µg/ml of the targeted NPs(MS-MP-PG-LR20). The NPs were imaged at various time points using 40 X non-confocal imaging using Operetta high content Imaging system. The experiments were repeated at least thrice. The best representative images are shown here. (The red arrow shows the co-localization of the NPs and the endo/lysosomes. The Green arrow shows the presence of siRNA inside the cytoplasm)
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Figure 4.24: Cell proliferation assay: Cell viability (%) of placebo MP-PG-LR-NPs, control (SS-MP-PG-LR20 NPs) and mTOR siRNA encapsulated targeted (MS-MP-PG-LR20 NPs) in (A) A549 cells and H460 cells seeded at a density of (5 × 10^3 cell/well and 6 x 10^4 cells / well for A549 and (B) H460 cells respectively) at 48h incubation. The NPs were prepared at 45:1 weight ratios, were incubated with cells at a concentration of 30 µg/ml and 60 µg/ml of mPAE polymer to achieve 50nM and 100nM siRNA concentration, respectively (siRNA 133 and 266ng per well) Figure (A) and (C) indicates the cell viability (%) of the A549 and H460 cells after transfection with the formulations. The results are presented as mean ± SD (n = 3). (B) and (D) represent the 20X pictures of A549 and H460 cells taken after 48 h incubation with control (SS-MP-PG-LR20 NPs) and mTOR siRNA encapsulated targeted (MS-MP-PG-LR20 NPs), respectively, using Moticam Pro 252 A camera. A value of p less than 0.05 was accepted to be significant (**p<0.01) and the statistics were performed with Student’s t test.

Figure 4.25: Determination of Caspase-3 activity. After infection for 48h, A549 and H460 cells were harvested and then protein was extracted. The caspase activity (%) was determined using a commercial kit (EnzChek® Caspase-3 Assay Kit #1, Z-DEVD-AMC substrate) whole cell extracts were analyzed by enzyme-linked immunosorbent assay. Data represent means ±S.D. (n = 3).
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>5-FU</td>
<td>Fluorouracil</td>
</tr>
<tr>
<td>AMAS</td>
<td>(N- maleimidoacet-oxysuccinimide ester)</td>
</tr>
<tr>
<td>AGO2</td>
<td>argonaute 2</td>
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<tr>
<td>BCA</td>
<td>bicinchoninic acid assay</td>
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<tr>
<td>BSC</td>
<td>best supportive care</td>
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<tr>
<td>CDKs</td>
<td>Cyclin-dependent kinases</td>
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<tr>
<td>ddH₂O</td>
<td>Double Distilled water</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5'-dithiobis-(2-nitrobenzoic) acid</td>
</tr>
<tr>
<td>dsRNA’s</td>
<td>double-stranded ribonucleic acids</td>
</tr>
<tr>
<td>EGFR</td>
<td>endothelial growth factor receptor</td>
</tr>
<tr>
<td>EPR</td>
<td>enhanced permeability and retention</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Glutathione</td>
<td>(GSH, c-glutamyl–cysteinyl–glycine)</td>
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<tr>
<td>GSSG</td>
<td>glutathione disulfide</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>LLF</td>
<td>lung lining fluid</td>
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<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>miRNA</td>
<td>microRNA</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MPS</td>
<td>mononuclear phagocyte system</td>
</tr>
<tr>
<td>mPAE</td>
<td>Modified poly (amino ether)</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
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<tr>
<td>NSCLC</td>
<td>non-small cell lung cancer</td>
</tr>
<tr>
<td>PAE</td>
<td>poly (amino ether)</td>
</tr>
<tr>
<td>PAMAM</td>
<td>Poly (amidoamine)</td>
</tr>
<tr>
<td>PBAEs</td>
<td>poly (amido amine) poly (β-amino ester)</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>RES</td>
<td>reticuloendothelial system</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>(RISC)</td>
<td>RNA-induced silencing complex</td>
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<tr>
<td>RIPA</td>
<td>radio immune precipitation assay</td>
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<tr>
<td>SCLC</td>
<td>small cell lung cancer</td>
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<tr>
<td>siRNA</td>
<td>Short interfering RNA</td>
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<tr>
<td>SRB</td>
<td>Sulforhodamine B</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetic acid-EDTA</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TLRs</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>TNB</td>
<td>5-thio-2-nitrobenzoic acid.</td>
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<td>VEGF</td>
<td>vascular endothelial growth factors</td>
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Chapter 1

INTRODUCTION:

Cancer is the leading cause of death worldwide, accounting for 7.6 million deaths (around 13% of all deaths) in 2015; worldwide this figure is projected to rise to over 13.1 million in 2030. Lung (1.6 million, 12.7% of the total for men and women), breast (1.4 million, 10.9% of the total for women), colorectal (1.2 million, 9.7% of the total for men and women) and stomach cancers (1 million, 7.8% of the total for men and women) were the most common, accounting for more than 40% of all cases. About 70% of all cancer deaths are reported in low and middle-income countries [1, 2].

1.1 Introduction Lung Cancer:

Lung cancer is the leading cause of cancer-related deaths in the world, comprised of a group of aggressive malignant tumors of the lower respiratory tract[3]. According to a recent statistics published by the American Cancer Society, lung cancer alone causes more deaths than pancreas, colon, prostate, and breast cancer deaths combined (Fig. 1.1). Furthermore, the survival rate of patients suffering from lung cancer is also extremely poor in both developed and developing parts of the world[4]. It has been reported that only 15% of lung cancer patients survive 5 or more years after being diagnosed with the disease[5]. The primary reason for this lower survival rate in patients is the spread of the tumor cells to visceral organs also called as lung cancer metastasis [6]. The metastasis usually occurs very early and rapidly in the disease progression. This rapid disease progression makes lung cancer one of the most difficult diseases to treat [7]. Moreover, about 90-95 % of lung cancers originate in the epithelial cells lining the larger and smaller airways of the lung [8]. The key signs and symptoms of lung cancer viz. a cough, dyspnea, wheezing, and hemoptysis, are nonspecific and absent early in lung cancer.
Hemoptysis, any change in the pattern of a cough, unexplained chest pain, recurrent pneumonia, unintentional weight loss with anorexia, or hoarseness in a two-pack per day smoker should prompt a thorough history and physical examination with a chest radiograph[9]

Figure 1.1 Estimated cancer deaths comparison between lung cancer and other cancers. Lung cancer alone causes more deaths (around 160,000) compared to the combined deaths due to the pancreas (40,000), colon (55,000), prostate (25,000) and breast (40,000) cancer. *(Source American Cancer Society, 2015)*
1.2. Etiology of Lung Cancer

The main cause of lung cancer is attributed to cigarette smoking and in only a small fraction (12%) of cases it occurs in non-smokers [10]. It has been reported that around 80% of lung cancer cases are caused by smoking, while other cases are caused by exposure to second-hand smoke [11]. Although smoking has been linked to most lung cancers, other exposures such as heavy metals (arsenic, cadmium, chromium, or beryllium) or asbestos and radon also contribute to lung cancer cases [12]. However, not all smokers develop lung cancer since genetic factors also play an important role in the formation of the disease [13].

There are certain genes in the cells known as oncogenes, which contain instructions to control the cell growth. The cells also contain some genes that slow down cell division or cause cell death called as *tumor suppressor genes* [14]. It has been reported that lung cancer can also be caused by DNA changes that either turn on oncogenes or turn off tumor suppressor genes [15, 16]. These DNA mutations can be of two subtypes, namely inherited and acquired genetic changes [17]. Specifically inherited DNA mutations, or germline mutations, are passed from parents to their children and are present within nearly every cell in the body [18]. On the other hand, acquired, or somatic, gene mutations occur during a person’s life because of environmental factors and/or mistakes made as DNA replication occurs during cell division in certain cells [19, 20]. In particular, acquired genetic mutations are the most common genetic change that leads to lung cancer. Not all lung cancers share the same gene changes, so there are undoubtedly changes in other genes that have not yet been found.

1.3 Types of lung cancer

Lung cancer is categorized into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC).
1.3.1 Small cell lung cancer: The name SCLC is based on the small size of lung cancer cells. This type of lung cancer accounts for approximately 10-15% of total lung cancer cases[21]. SCLC differs from other types of lung cancers in its propensity for an early systemic spread and its aggressive clinical course if left untreated. The SCLC is also referred to as oat-cell carcinoma and it usually occurs in the central areas of the lung [22]. This is an anaplastic or embryonic type of tumor, and therefore shows a high incidence of metastasis [23]. Furthermore, the SCLC is usually always caused by smoking.

1.3.2 Non-small cell lung cancer: The NSCLC accounts for approximately 80-85% of all lung cancers and is composed of a heterogeneous aggregate of at least three histological subtypes including squamous cell, adenocarcinoma, and large cell carcinoma (Fig. 1.2). The Squamous cell tumors are usually located in the bronchi [24], while the adenocarcinoma usually occurs in the periphery of lung tissue, including the terminal bronchioles and the alveoli[25]. On the other hand, large-cell carcinoma is highly anaplastic and is mainly associated with rapid metastasis, often occurring peripherally and spreading centrally in the lungs [26].

![Lung Cancer Histology](image)
Figure 1.2: Types of Lung Cancer based on histology. Two main types of lung cancer, viz., small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC is further divided into Adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. As can be seen from the figure, the adenocarcinoma makes up to 50% of lung cancer cases.

1.4 Treatment of lung cancer

The mainstay of treatment for lung cancer is multi-drug chemotherapy. There are other modalities used to treat lung cancer including radiotherapy, surgical interventions, and the combination of chemotherapy and radiotherapy[27]. Chemotherapy is the most important and established mode of treatment for lung cancer[28]. A combination of drugs, including either cisplatin or carboplatin along with etoposide or gemcitabine is used for the treatment [29]. Small cell lung cancer usually responds well to these treatments. In the case of severe and relapse cases, combinations used are - CAV (cyclophosphamide, doxorubicin, and vincristine), CAVE (CAV plus etoposide), and ACE (doxorubicin, cyclophosphamide, and etoposide) [30-32] (Current guidelines for the management of small cell lung cancer, 1999).

Approximately 180,000 Americans have diagnosed with NSCLC annually, and more than half have advanced (Stage III/IV) disease [33]. Historically, survival for these patients has been poor (e.g. 1-year survival ≈23%) compared to other common cancers [33]. For patients who have a good performance status (PS), chemotherapy has been shown to produce longer survival, palliate disease-related symptoms, and produce a better quality of life than with best supportive care (BSC)[34, 35]. Many patients benefit from initial treatment with chemotherapy, although all patients eventually experience disease progression, generally within a median of 3–6 months of initiating chemotherapy [36-38]. While it is difficult to estimate the proportion of patients who receive second-line treatment, approximately 40%–50% of patients did so in recent first-line trials [39]. Patients who appear more likely to receive second-line therapy are those with a good
PS, female patients, and those with non-squamous histology [36, 40]. Many patients who maintain a good PS and tolerate therapy without significant toxicities will receive third-line therapy. In a recent phase III trial of second-line therapies, approximately 40% of the patients subsequently received third-line therapy [41]. The characteristics of this patient population have not been well studied.

This worldwide study collected data about lung cancer on more than 81,000 patients from 19 countries [42]. Lung cancer is divided into 4 stages (Stage I – IV). Stage I non-small cell lung cancer is divided into 2 stages, stage 1A, and 1B. The survival rates in Stage IA are 58% to 73% and for stage IB NSCLC is 43% to 58%. Stage 2 NSCLC cancers are also divided into stage IIA and IIB. The survival rates of patients with Stage IA and stage IIB is found to be 36% to 46% and 25% to 36% respectively. Surgery is recommended for patients with stage I or II non–small cell lung cancer (NSCLC) and may provide the best possibility for a cure[43]. Surgery (radiation if the patient is not a surgical candidate), with or without adjuvant chemotherapy based on risk factors, for stages IB and II is generally appropriate. Adjuvant chemotherapy after surgical resection provides an absolute increase in 5-y survival of approximately 5% [44]; median 5-y overall survival rates range from 45-70%. No benefit has been shown for adjuvant chemotherapy after surgery for stage I disease; the benefit of adding adjuvant chemotherapy increases as disease stage increases [44].

Stage III NSCLC is also divided into stage IIIA and stage IIIB. The survival rates in Stage IIIA are 19% to 24% and in the stage, IIIB is 7% to 9%. Treatment recommendations for patients diagnosed with stage IIIA or stage IIIB include the use of concurrent chemotherapy and radiation, or chemotherapy and radiation can be given sequentially if necessary. Selected patients (predominantly those with stage IIIa) may be surgical candidates; these patients may receive
chemotherapy alone or chemotherapy with radiation before surgical resection. Stage IIIa and IIIb disease are typically treated with a combination of chemotherapy and radiation if the patient is not a surgical candidate. Chemotherapy and radiation therapy are preferably given concurrently, but in patients with poor performance status, these therapies may be given sequentially; the decision to treat the patient with concurrent chemoradiation rather than surgery, radiation or chemotherapy individually should be made by a multidisciplinary tumor board (including a medical oncologist, radiation therapist, and thoracic surgeon)[45, 46].

The survival rates for patients in stage IV is very low (2% to 13%) compared to previous stages. Patients with metastatic disease (stage IV) or recurrent disease after primary therapy (eg, surgery and/or radiation) should be considered for chemotherapy in order to improve the quality of life, palliate symptoms, and improve overall survival[45, 46]. The goal is to treat for four to six cycles unless otherwise specified. Second-line chemotherapy is given for advanced or recurrent disease after disease progression following first-line therapy. Some of the drugs that are used in second-line therapy include Nivolumab, Pembrolizumab, Docetaxel, Pemetrexed, Erlotinib, Afatinib. Third-line chemotherapy is given for advanced or recurrent non–small cell lung cancer (NSCLC) after disease progression following first-line and second-line therapy. Options include erlotinib, ramucirumab, and nivolumab. Erlotinib is indicated for patients with endothelial growth factor receptor (EGFR) mutation or gene amplification. It is given in a dosage of 150 mg PO daily until disease progression [47]. Ramucirumab is indicated for metastatic NSCLC with disease progression on or after platinum-based chemotherapy. Patients with EGFR or ALK genomic tumor aberrations should have disease progression on FDA-approved therapy for these aberrations prior to receiving ramucirumab[48]. Nivolumab is indicated for metastatic squamous and nonsquamous (including adenomas) NSCLC with
Pembrolizumab is indicated for metastatic NSCLC in patients whose tumors express PD-L1 who also have disease progression on or after platinum-containing chemotherapy.

The major problem associated with chemotherapeutic agents is their lack of specificity and selectivity. This is mainly due to the presence of very subtle metabolic differences between the tumor cells and the normal cells. Hence, very large doses of chemotherapeutic drugs are required for successful chemotherapy. Furthermore, cancer chemotherapy leads to severe adverse effects, such as hair loss, damages to the liver, kidney, and bone marrow, as the normal cells are also exposed to these cytotoxic agents. A lot of research efforts have therefore been directed to improve the specificity of anticancer agents. One of the widely used approaches is to selectively administer the drugs to the tissue/organ involved, as in the case of intra-arterial infusions. This selective drug delivery has been reported for hepatic carcinomas, oral cancers and renal cancers.

There are various other strategies used to specifically deliver drugs to the cancer cells, which relate to the unique pathophysiological characteristics of the tumor cells. Passive targeting is one such strategy which exploits the leaky tumor vasculature present in the vicinity of cancer cells. Quick formation of new blood vessels in rapidly growing cancer cells is a common phenomenon. This vasculature formation is inherently defective in nature, which in turn, allows large molecules and lipids to easily enter the extravascular space in tumors. In addition, due to faster development of cancerous cells, lymphatic drainage is undeveloped, leading to retention of large molecules and lipids in the extravascular space of the tumors. This phenomenon is termed as enhanced permeability and retention (EPR) effect. Another approach that is widely used to deliver drugs is the active targeting approach which is based on the targeting of drugs to...
the overexpressed receptors on cancer cells [60] [61, 62]. Tumor cells require various nutrients and vitamins for rapid development, and hence they overexpress many tumor-specific receptors, which can be used as targets to deliver cytotoxic agents into tumors[63]. Various ligands or active targeting agents used are antibodies, growth factors (vascular endothelial growth factor), receptors (such as transferrin, epidermal growth factor receptor), ligands (including peptides and small synthetic organic molecules), nutrient transporters; enzymes (such as matrix metalloproteinase), vascular cell adhesion molecules and vitamins (folic acid, biotin).

1.5 Challenges in delivery of therapeutic agents in lung cancer

Conventional chemotherapeutic agents are usually administered via the intravenous (i.v.) route. However, the major drawbacks with i.v. administration of therapeutic agents is their widespread distribution throughout the body via the bloodstream, affecting both malignant and rapidly dividing normal cells in the bone marrow [64]. This leads to increased side effects, higher patient risks, decreased biodistribution of drugs to the tumor site and development of multidrug resistance against the chemotherapeutic drugs by the cancer cells[65]. Chemotherapeutics are also rapidly cleared by the reticuloendothelial system (RES), which results in a reduced amount of drugs reaching the target site leading to decreased efficacy [66]. Most of the anticancer drugs are hydrophobic and due to their toxicity to normal and cancer cells, they have to be developed in a formulation to be delivered via i.v. route [67].

The landing of the particle at the site of interest is the primary determinant of the increased therapeutic efficiency. The retention of drug in the lung and the ability to overcome extracellular and cellular lung barriers is also a significant factor [68]. The mucociliary clearance mechanism designed to clear inhaled and possibly noxious material limits the amount of residence time of the deposited formulation in the lungs. Thus the amount of drug reaching the
target site is also reduced [69]. The drug permanence in situ and duration of effect is also affected by the nature and extent of the interactions of the drug with lung lining fluid (LLF), airway macrophages and lung epithelial cells. Nucleic acids including DNA and antisense oligonucleotide have been widely studied for the treatment of lung cancer. Short interfering RNA (siRNA) is the most popular candidate being studied in RNAi therapy, amongst the different types of nucleic acids that are involved in the RNAi[70].

The biggest hurdle in front of the scientific community is to deliver these nucleic acids to lung tumor tissues in increased amounts. These hydrophilic macromolecules are highly susceptible to nuclease degradation and susceptible to cross the biological membrane on their own due to the negative charge. This renders them ineffective in reaching the site of action [71, 72]. Thus, in order to protect the therapeutic nucleic acids from nuclease-mediated degradation, enhancing cellular uptake and facilitating release at the site of action, a delivery vector is required[71].

1.6 Parenteral Delivery Systems

The parenteral delivery systems include delivery via several routes such as intravenous, intramuscular, subcutaneous, etc. The liposomal formulation “DOXIL” is the only marketed formulation currently available [73]. DOXIL is a doxorubicin HCl liposomes injection used for the treatment of leukemia, breast cancer, bone cancer, lung cancer, and brain cancer [74]. The liposomal systems delivered through the parenteral delivery route have the potential of improving delivery of drugs through nasal and sublingual routes and avoid first pass metabolism [75]. Parenteral delivery also allows easy access to sites which are difficult to reach such as ocular, brain and intraocular cavities enabling higher concentrations and improved therapeutic efficiency due to enhanced delivery at the site[76].
One of the most successful approaches for the treatment of cancer has been the development of a prodrug, where the parent drug (capecitabine) is enzymatically converted to the active metabolite in the target tissues [77]. Several chemotherapeutic agents such as 5-Fluorouracil (5-FU) and several others have been delivered systematically for the treatment of cancer [78, 79]. However, they are not successful due to their less plasma circulation lifetime and reduced therapeutic activity at the prescribed dose, thus exhibiting increased toxicities [80]. In a recent study, the liposomal formulation of 5-FU was successfully delivered to lung cancer by parenteral administration [81]. In another recent study, a novel drug loading method utilizing transition metal complexation properties of selected drug for a liposomal formulation extended plasma circulation lifetime and enhanced therapeutic activity at dose exhibiting acceptable toxicities [82]. These studies have shown that a liposomal formulation can be a potential drug delivery system to encapsulate drugs and prevent their leakage, thus enhancing the therapeutic efficiency. The use of liposomes has also been applied to deliver nucleic acid for the treatment of lung cancer [83, 84].

1.7 RNA interference (RNAi)

Over the last decade, RNA molecular biology has made significant strides. One of the most important advances has been the discovery of the small (20-30 nt) non-coding RNAs that perform the function of regulating genes and genomes[85]. RNAi is a natural mechanism occurring in most eukaryotic cells in which the double-stranded ribonucleic acids (dsRNA’s) undertake the function of regulating gene expression[86]. It is a specific regulatory mechanism, which helps in regulating various biological pathways and protecting the body against various pathogens [87, 88]. RNAi represents a novel way of treating diseases, which would not have been possible with the conventional medicines[89]. The RNAi-based medicine involves delivery
of nucleic acids (siRNA, miRNA, shRNA etc.) to the diseased cells [86]. The RNAi sequences can be easily designed to target the specific genes. One of the important uses of RNAi-based medicine is to target some of the proteins which are involved in certain diseases and cannot be targeted using conventional molecules, due to the lack of enzymatic function or inaccessibility. Such non-druggable targets have been easily targeted using small RNAs[86]. These small RNAs usually function as the inhibitors of the gene expression and subsume the corresponding regulatory mechanisms. Although various categories of RNA have been recognized, including siRNA, microRNA(miRNA) and piwi-interacting RNAs, most of the research is focused on siRNA delivery due to its specificity in targeting and knocking down the particular gene[90, 91].

The siRNAs are 19-23 nt long chemically synthesized duplex with a 3’ two-nt overhang[92]. The overhang allows them to be easily recognized by the enzyme DICER and undergoes further processing in the cytoplasm (Fig. 1.3). The siRNA interacts with and activates the RNA-induced silencing complex (RISC). The duplex siRNAs are then unwound by helicase activity of endonuclease Argonaute 2(AGO2) component attached to the RISC assembly. As a result, the guide strand (antisense strand) remains attached to the RISC while the passenger strand (sense strand) is detached from the complex and is degraded by exonucleases[93]. The guide strand – RISC complex targets the mRNA for the cleavage[86]. The guide strand only binds to the complementary mRNA which causes specific gene silencing [94, 95].
**Fig 1.3: RNA interference mechanism: siRNA** - The siRNA pathway begins with cleavage of dsRNA by enzyme DICER resulting in siRNA in the cytoplasm of the cell [89, 96]. The siRNA then binds to Argonaute (AGO2) protein and RNA-inducing silencing complex (RISC)[97]. One strand of the siRNA duplex (the passenger strand) is removed by AGO2 resulting in RISC containing guide strand [98]. The activated RISC-siRNA binds to the complementary sequences on the mRNA and results in its cleavage and degradation [99]. Figure adapted with permission from [100].
1.7.1 siRNA as therapeutic agents:

The main advantage of using siRNA as the therapeutic agent is the ability to downregulate the expression of virtually all genes and their mRNA transcripts[101]. On contrast, the traditional small molecules and protein-based drugs can only target certain classes of proteins and they can target only limited cell-surface based receptors or circulating proteins, respectively[102]. There are various diseases, including cancer, which result from the over or under-expression of certain normal genes[103]. The discovery of siRNA has allowed targeting those genes and possibly advancing the treatment options for such diseases.

siRNA induces the gene specific cleavage through its complementary pairing with mRNA and resulting in degradation of mRNA[104]. siRNA has the ability to knock down genes and overcome the cellular pathways and help treat diseases caused by aberrant gene expression [105, 106]. Simultaneously inhibiting multiple targets using siRNAs of a different nature and origin is also an effective approach to treating cancer [107].

Gene therapy involves risk of mutation and teratogenicity [108]. Cancer treatment using siRNA, however, provides the highest degree of safety as it targets the post-translational stage of gene expression and does not interact with the cellular DNA[109]. Also, siRNA is more suitable compared to conventional drugs as it does not require genomic integration to be efficacious. Furthermore, siRNA can be designed rationally to inhibit endogenous and heterologous gene expression, which can modulate any disease-related gene expression. This property can be used in the treatment of cancer, which is usually caused by oncogene overexpression or gene mutation. Also, there has been a steady increase in the number of drugs entering clinical trials based on siRNA[110]. The clinical trials administering siRNAs are mostly based on local delivery via either intravitreal or intranasal routes. However, for certain diseases such as cancer
siRNAs need to be administered systematically by i.v injection and certain delivery systems will be needed to deliver the siRNA.

**1.7.2 siRNA targeting Cancer genes:**

siRNA-mediated silencing can be successfully used to target cancer genes that regulate uncontrolled cell proliferation[111]. Some of the targets such as cyclin-dependent kinases (CDKs), vascular endothelial growth factors (VEGF) and anti-apoptotic factors can be successfully targeted by siRNA. Cyclins play an important role in regulating checkpoints in the cell cycle and its overexpression could disrupt the cell cycle leading to cancer[112]. The abnormal expression of Cyclin B1 has been linked to various cancers including prostate adenocarcinoma, breast cancer, and renal cancer. siRNAs have been used in *in vivo* studies to silence the expression of cyclin B1 for the treatment of lung and prostate cancer[113]. However, there are certain challenges in the delivery of siRNA as described below.

**1.7.3 Problems with *in vivo* delivery of siRNA**

**1.7.3.1 Biological instability**

siRNAs are unstable under the physiological conditions which is one of the major obstacles towards their successful application as therapeutic agents [114, 115]. The first biological barrier for the injected siRNA is the presence of nuclease activity in the plasma and tissues. The unmodified/unprotected siRNA gets degraded by the 30 exonucleases and is cleared from the bloodstream. The reported half-life of the unmodified siRNA ranges from minutes to 1 h. [116-118]. Various strategies such as chemical modifications of the backbone, glycation, nucleic acid locking etc., have been investigated to improve their stability under biosystems [115, 116]. However, aforementioned motifs of attaining biological stability have its own allied limitations [119, 120], and hence the successful use of siRNA in cancer therapy demands...
alternative approaches such as delivery formulations including lipid and polymer systems. These delivery systems can protect siRNA from the adverse environment such as nucleases by encapsulating it as a cargo inside NPs (NPs) and can also deliver to the target cancer site[121].

In addition to elimination by the nucleases, kidney also plays an important role in the clearance of siRNA from the biological system[122]. Several pre-clinical animal studies have reported that after i.v. administration of siRNA, it is found in very high concentrations in the kidney [123]. siRNA can also be cleared from the biological system by a natural defense mechanism called reticuloendothelial system (RES)[124]. The RES is mainly composed of circulating monocytes and tissue macrophages. Their main function is to remove the foreign pathogens and to clear cellular debris and apoptotic cells from the body. They also prevent the siRNA from reaching the tumor site and perform the necessary function [125]. Certain organs such as liver contain higher percentages of this tissue macrophage (kupffer cells), which results in accumulation of siRNA in these organs. Other organs(spleen, bone marrow) and tissues receive a high blood flow and exhibit the fenestrated vasculature, which is responsible for the higher accumulation of siRNA in these organs following systemic administration[126]. The pharmacokinetics of siRNA is also affected due to interaction with the various components of blood including serum proteins. This can lead to the formation of aggregates that can get opsonized easily[127].

1.7.3.2 Stimulation of Innate Immune system

siRNA also has the ability to trigger sequence-specific innate immune system which primarily involves the activation of interferon (IFN) system [128, 129]. In several studies, siRNA was reported to induce IFN responses by binding to dsRNA-activated protein kinase (PKR), 2′,5′-oligoadenylate synthetase- RNase L system retinoic acid-inducible gene I (RIG-I) or several
Toll-like receptors (TLRs); which are mostly aimed at combating viral pathogens[92, 130, 131]. These outcomes, direct the need to explore a delivery system that can protect the exposure of such codes and prevent initiation of immune-responsive elements within the body (i.e. to avoid ‘off-target effect’). At the same time, it must be noted that such a delivery system must be capable of concomitantly delivering these bioactive at the desired site of action.

1.7.3.3 Off-target effects

Although originally thought to be highly specific, but similar to miRNA, siRNA also has the ability to regulate a large number of transcripts.[132, 133]. The off-target effects are generally prominent when there is a match between the seed region of siRNAs (positions 2–7) and sequences in the 3’UTR of the off-target gene. There are several reported modifications of siRNA that have shown to eliminate off-target effects such as phosphorothioate or boranophosphate introduction, modification of the 2’- position, etc.[134] Thus, in order to minimize the off-target effects of siRNA several factors such as dose, backbone design, and structural modification must also be taken into consideration [135].

1.8 Nanotechnology-based approaches to delivering RNAi-based combinations

Nanotechnology is a multidisciplinary field covering various areas of biology, engineering, chemistry, and physics [136, 137]. Nanotechnology-based therapeutics typically includes nanosized particles composed of different entities such as lipids, polymers, inorganic materials, etc. [138, 139]. The term nano-assembly is usually being given to the architect that ranges from 10 – 200 nm in diameter in size [140]. Various properties of NPs have been validated and used in the delivery of siRNA [141]. Various methods of association of siRNA into NPs, including, complexation, encapsulation have also been reported[111]. NPs are an essential
carrier for delivery of siRNA because their surface charge allows the delivery of anionic and hydrophilic double-stranded siRNA, which is not readily taken up by cells[141].

In order to grow, the tumor tissues employ new vasculature or engulf existing blood vessels to ensure the adequate supply of nutrients and oxygen[142]. This results in the formation of the abnormal network of vasculature within tumor tissues. The vascular network can be dilated, tortuous with saccular channels and heterogeneous in size, shape, tortuosity, and permeability [143, 144]. The tumor tissues can have large fenestrations and intercellular gaps which can be as wide as a few microns [145]. This leads to an increased permeability in the tumor tissues which allows the delivery systems to extravasate relatively easily. In addition, compared to the normal tissues the interstitial fluid is not efficiently drained from the tumor tissues due to the poorly functioning lymphatic vessels[146]. This poor lymphatic drainage further enables the NPs to get accumulated in the interstitium of the tumor tissues, this phenomenon is known as EPR effect [147].

The EPR effect is a property of the tumor tissue, which allows nanoscale molecules or particles to preferentially accumulate in the tumor tissues. Typically for the successful employment of the prolonged circulatory lifetime and EPR effect, NPs of 20-150 nm size are recommended for delivery of siRNA[148-150]. NPs of < 20 nm size usually undergo clearance via hepatic and renal routes of elimination, while > 150 nm do not efficiently enter the tumor tissue site[151]. In addition, the surface charge of NPs is also an important factor which determines the stability and biodistribution of the NPs inside the body [152]. For example, it has been reported that cationic and anionic liposomes activate the complement system through different pathways [153]. Recently, Xiao et al. have reported that a slight negative charged nanoparticle (NP) (around -8.5mV) helped in reducing the liver uptake, prevent aggregation in
the blood and deliver anti-cancer drugs more efficiently to the tumor cells compared to the positive and negative counterparts [152]. The variable results might be due to the inconsistent particle sizes, different types of NPs and the varying nature of the surface charges. These studies suggest that the NP surface property needs to be optimized for the surface charge to achieve an enhanced intratumoral delivery.

The RES comprises of the liver, spleen and other organs which are responsible for clearing the NPs from the system [65]. The rapid clearance of the NPs by the mononuclear phagocyte system is initiated by the adsorption of the serum proteins on the surface of NPs[154]. The serum proteins, especially the components of the complement system interact with the receptors on phagocytes resulting in clearance of the NPs[154, 155]. To minimize protein adsorption and to enable efficient escape from the RES system, the NP surface can be functionalized with hydrophilic polymers such as polyethylene glycol (PEG) [156]. The attachment of PEG to the surface significantly reduces the RES uptake of NPs and increases the circulation lifetime in comparison to the non-PEGylated NPs[157]. Pegylation also helps in reducing the aggregation of the NPs by avoiding interaction with the serum and tissue proteins. [158]. The PEGylated siRNA delivery systems have been widely reported for minimizing protein adsorption and reducing nonspecific uptake to create long-circulatory systems[155].

The potential advantages of the nanotherapeutic strategy include: (a) higher delivery of loaded therapeutic agents, (b) ease of delivery through various routes of administrations, including oral and inhalation, and (c) can be used to deliver both hydrophilic and hydrophobic therapeutic moieties. The intravascular deliverable nano-vectors represent the major class of nanotechnology- based systems used to deliver therapeutic agents for cancer therapy. Various carriers such as Liposomes [159], polymers poly (D,L-lactide-co-glycolide) [160, 161],
polylactic acid [162, 163], polycaprolactone [164-166], dendrimers[167, 168], and silica [169-171] have been used to deliver the siRNA-based combinations to treat cancer. Various types of NPs have been described in brief below.

### 1.8.1 Non-viral vectors

The non-viral vectors predominantly include carriers formulated from polymers of natural or synthetic origin. These carriers are designed to deliver the gene of interest (siRNA, DNA, miRNA) to the target cancer cells [172]. These carriers are not prone to elicit an immune response and are non-immunogenic to the human body [173]. In addition, the surface of the NPs (prepared using the non-viral carriers) can be functionalized by attaching a cell-specific functionality (ligand) in the design of chemical or biological vectors. The non-viral vectors can also be modified by tagging a cell-specific functionality on the surface to improve cell or tissue specificity and efficiency. They also provide the ease of production compared to viral vectors. Recent developments suggest that the gene delivery by non-viral vectors have reached clinically meaningful efficiency for cancer treatment. In addition, recent advances in improved efficiency, specificity and gene expression duration, led to an increased number of non-viral vectors entering clinical trials [174]. The Clinical Trials of non-viral vectors have also increased in between 2004 to 2013 while that of viral vector there is a significant decrease. Various types of non-viral vectors are described in the following section.

#### 1.8.1.1 Inorganic NPs based siRNA systems

Inorganic NPs represent an efficient alternative to deliver siRNA to the cancer cells. Their high efficiency is mainly due to lower toxicity [175] and ease of modification to produce controlled release NPs [176]. In perspective of drug delivery, bioactive can be incorporated into inorganic Nanoparticulate systems without their chemical modifications [177]. Currently,
various inorganic NPs have been used for delivery of siRNA/DNA including silica, calcium, gold, magnesium, strontium, quantum dots etc.[178]. One of the main reasons of widespread use of inorganic NPs is their versatile property, suitable for the cellular delivery of siRNA. In addition, the inorganic NPs are also biocompatible, can produce the controlled release of therapeutics agents, and achieve targeted drug delivery[179]. Furthermore, the inorganic NPs can be used for various routes of administration, including nasal, parenteral, intra-ocular, etc.[180]. All these advantages make inorganic NPs a very efficient non-viral gene vector for delivery of siRNA to the cancer cells.

1.8.1.2 Natural Chitosan polymeric NP-based siRNA NPs

Chitosan is a semi-synthetically modified, natural carbohydrate polymer prepared by the partial N-deacetylation of chitin. Chitin is a natural biopolymer derived from crustacean shells such as crabs, shrimps, and lobsters[181]. The chitosan-based NPs have gained more attention as drug delivery carriers because of their stability, low toxicity, biocompatibility [182]. However, it is found that the capacity of chitosan to enhance the absorption and permeation of drugs at GI mucosal sites is compromised due to deprotonation at physiological pH [183]. Also, chitosan gets easily degraded in the lysozyme in the serum [184, 185]. Ma Guang-hui et al. developed a partially quaternized derivative of CS N-((2-hydroxy-3-trimethylammonium) propyl) chitosan chloride to deliver poorly water soluble drugs by the oral route which has helped in the successful delivery of siRNA.

1.8.1.3 Dendrimers based siRNA combinations

Dendrimers are monodisperse highly branched macromolecules discovered in early 1980’s by Donald Tomalia and coworkers [186, 187]. Dendrimers are monodisperse, nanoscale-sized materials, which share their size property with the naturally occurring biomolecules [188].
The tunable properties of dendrimers allow control of their size and molecular mass for the formulation of NPs. In addition, their solubility characteristics can also be varied based upon the nature of surface groups to produce an efficient siRNA-based NP system[189]. Further, dendrimer surfaces may be functionally designed to enhance or resist trans-cellular, epithelial or vascular permeability[190]. Mathematically defined numbers of terminal surface groups (Z) present on dendrimers are suitable for conjugation of drugs, signaling groups and targeting moieties[191]. 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[191]. Dendrimers are routinely synthesized as tunable nanostructures that may be further designed and regulated as a function of their shape, size, surface chemistry and interior void space to efficiently deliver siRNA-based NPs [203].

Several polyamine polymers (dendrimers) have been explored as carriers for siRNA delivery, including poly (amidoamine) (PAMAM) dendrimers [192, 193]. The PAMAM dendrimers, also known as starburst dendrimers were the first class of dendrimers to be investigated for delivery of siRNA[194]. These cationic dendrimers have been used as non-viral delivery vectors for efficient siRNA delivery [195]. Recently, Minko et al. developed tumor-targeted delivery system using surface–engineered poly (propylene imine) dendrimers with siRNA caged inside the dendrimers (Fig. 4). PEGylation and caging modification stabilized the system and extended its systemic circulatory lifetime, indicating dendrimers are a potential siRNA delivery system [195]. Several other dendrimer systems have been successfully explored for the delivery of siRNA to the cancer cells[196].

1.8.1.4 Lipid-based NPs / Liposomes
Liposomes represent another class of non-viral vectors designed to deliver siRNA to the cancer cells. They are spherical structures comprised of an inner aqueous layer, covered by the outer lipid bilayers [197]. Liposomes are biocompatible and can be used to deliver both hydrophilic and hydrophobic drug [198]. The periphery of liposomes can be modified to render them long circulatory lifetime properties as well as site specific delivery to tumor tissues[199]. Liposomes are especially effective in treating diseases that affect the phagocytes of the immune system because they tend to accumulate in the phagocytes which recognize them as foreign invaders[200]. Liposomes size, charge, and other characteristics can be altered according to the drug and the desired site of action [200]. Liposomes provide a great opportunity to deliver therapeutic agents for cancer therapy and have been widely used for this purpose[198].

1.8.1.5 Polyethyleneimines(PEI) co-blocks based siRNA combinations

Positively charged cationic polymers have been widely studied as vectors to efficiently deliver siRNA to the cancer cells [201]. PEI is one such cationic polymer that has been extensively studied as a non-viral vector for efficient gene delivery [202, 203]. It has been proven that PEI polymer is responsible for the proton sponge effect inside the endosome resulting in the rupturing of the endosomal membrane and helping DNA/siRNA – PEI complex to release in the cytoplasm [204, 205]. However, the major disadvantage with PEI is its cytotoxicity to the normal cells. The cytotoxicity of PEI polymer has been reduced by coating with human serum albumin [206] and PEGylation [207, 208]. Boussif et al. explored the use of PEI for in vivo siRNA delivery and found that the positively charged PEI-siRNA complex protected the siRNA from degradation. The PEI-siRNA complex also facilitated the subsequent siRNA release from endosomes mainly due to the proton sponge effect [209]. Chen et al. used the PEI complexes to formulate PEI-siRNA (VEGFR2 and EGFR) complexes and evaluated in
vivo antitumor effects of combination with Cisplatin (CIS) in murine A549 NSCLC tumor xenograft models. The combination of VEGFR2 siRNA + EGFR siRNA + CIS resulted in significant downregulation of VEGFR2 and EGFR mRNA levels compared to siRNA’s administered individually[210]. Further Studies

1.8.1.6 Polymeric NPs based siRNA combinations

Polymeric NPs have unique physicochemical properties such as ultra-small and controllable size, larger surface area to mass ratio and functionalize structure[211]. The polymeric NPs have been shown to alter and improve the pharmacokinetic and pharmacodynamic properties of various bioactive molecules[212]. The above-mentioned properties of polymeric NPs can be applied to overcome some of the limitations in traditional drug delivery approaches [213]. Polymeric NPs have been used in vivo to protect the drug in the systemic circulation and to deliver the drug at a controlled rate to the site of action while minimizes undesirable side effects [214]. Following section mainly describing various polymer based NPs used to co-deliver siRNA and chemotherapeutic agents.

1.8.1.7 Polymersomes based siRNA combinations

Polymersomes constitute a type of polymeric vesicles that undergoes self-assembly in hydrophilic solutions. They have been widely studied as a potential drug delivery candidate since last one decade [215, 216]. It has been reported that polymersomes can conjugate biologically active ligands, such as avidin, antibodies, and biotin, to their surface and, thus, provide targeted therapy and imaging strategy[217]. Furthermore, polymersome can encapsulate relatively higher amount of drugs and could also be used in the controlled release of drugs due to the EPR effect[218]. Various studies have shown effective encapsulation of drugs by polymersome for delivery of the cancer cells. In one such study, polymersome encapsulating DOX and/or PTX
was researched as a treatment for cancer. Several other studies have been reported that have been reviewed elsewhere [219]. Overall, polymersomes have a great delivery potential owing to their advantages, such as robust and larger shell enhancing drug loading and stability, and the possibility of enhanced drug targeting and prolonged circulatory lifetime [220].

1.9 PEGylation:

The ideal circulation time for therapeutic NPs should ideally by longer (ranging from hours to days) to allow repeated exposure to affected area (tumor cells). Unfortunately, this can also expose healthy organ systems of the drug and cause adverse side effects. To avoid such adverse effects targeted NPs have been developed [221]. These targeted NP systems preferentially accumulate in the diseased area [158]. However, Aggregation of NPs can be induced by solvents of high (>100 mM) ionic strength (shielding of solvent from NP), highly concentrated solutions of NPs (less distance between the NPs), time from synthesis, or NP preparations with a very neutral (~±5 mV) zeta potential [30]. PEG is one such polymer that is widely used to decrease the amount of attraction between NPs. The PEG molecules increase the steric distance between NPs and further increases the hydrophilicity via ether repeats forming hydrogen bonds with solvent. Apart from stability, PEGylation can also be used to modify the size of the particle.

NPs are administered via intravenous injection. Following the administration of NPs, they are rapidly cleared from the body via a natural defense system of opsonization and recognition by the mononuclear phagocyte system (MPS) [222, 223]. The administered NPs mainly accumulate in organs such as the liver and spleen, which prevents them from reaching the tumor site [224]. Several attempts have been made to reduce the uptake of the NPs from the MPS system by reducing their size, introducing hydrophilicity, modifying surface functionality and
surface charge [225, 226]. The Pharmacokinetics and biodistribution of the NPs are usually
governed by their surface properties. Studies have shown that NPs with a diameter of less than
200 nm and a hydrophilic surface functionality exhibited prolonged blood circulation[227].
Thus, it is essential to coat the NPs surface with a hydrophilic and biocompatible moiety in order
to achieve successful intravenous administration. Various natural polymers such as
polysaccharides[228], poly-amino acids[229], etc. and synthetic polymers[230] have been used
as surface coatings to provide a hydrophilic surface. One such synthetic polymer, PEG have been
widely used to coat NP surface owing to its high hydrophilicity, lower protein or cellular
adsorption and non-toxicity[231, 232].

The Effectiveness of PEGylation of NPs is highly dependent on the PEG surface density
coating, molecular weight and polymer chain architecture [233]. Studies have shown that the
stealth properties of NPs can usually be achieved by surface coating with high-density PEG
chains with MW ranging from 2K to 10K[234]. The PEGylation can usually be achieved by any
of the techniques including covalent attachment of PEG on the surface, or using diblock PEG
derivatives or adsorbing PEG chains on the surface of an NP[235-237]. Several PEG-based
therapeutics have been approved by FDA for general use. One of the most widely accepted
mechanisms by which PEG increases circulation time and improve biodistribution is that PEG
provides a steric barrier which prevents the NP opsonization and delays the clearance from the
circulation by the MPS[238]. In order to formulate a PEGylated targeted delivery system, an
active ligand is usually attached to the NP surface. One such receptor-based targeting is
discussed in the following section.
1.10 Luteinizing hormone-releasing hormone (LHRH) receptor targeting

Targeting cancer by the NPs is usually achieved by conjugating a targeting ligand, which targets receptors specifically expressed or over-expressed on cancer cells [239]. There are various targeting moieties that have been used to achieve targeted drug delivery, including receptor ligands [240-243], lectins [244-246], sugars [247-250] and antibodies [251-253]. Previously it has been demonstrated that LHRH peptide has been used as a targeting ligand on DDS to enhance uptake and reduce adverse effects on various types of cancers, including breast, ovarian and prostate cancer cells [241, 254, 255]. Specifically, an apoptosis-inducing agent (camptothecin) carrying polymeric nanocarrier system was successfully targeted to A2780, MCF-7 and PC-3 prostate cancer cells [254]. The targeted DDS helped in significantly enhancing the antitumor efficacy of the drug and substantially reduced the adverse side effects on healthy tissues. It was further demonstrated that treating with LHRH-targeted carrier–drug conjugates in vivo (in mice bearing xenografts of human ovarian carcinoma) led to a dramatic reduction in the tumor size [256].

It has been reported that the receptors for the LHRH are found to be over-expressed in the NSCLC whereas only small detectable expression of LHRH receptors has been observed in most of the visceral organs [257-260]. Various studies have targeted the over-expressed LHRH receptor by attaching a peptide (LHRH ligand) to the nanocarrier system [257]. These studies provide a basis for modification of the drug delivery system with the targeting ligand LHRH to help facilitate delivery of therapeutic agents to the human lung cancer cells [224]. The functional importance of this was highlighted in a study showing that the LHRH peptide conjugated to silica nanocarrier (NC) loaded with siRNA (Multidrug resistance protein -1 & BCL2) and drug, has selectively delivered NC to lung cancer cells [254, 257].
There are two main advantages of using LHRH as the targeting ligand on the nanocarrier system. First, the LHRH can direct the nanocarrier system directly to the tumor and thus will prevent its accumulation in healthy tissues [261]. Second, the LRHR peptide on the nanocarrier system can interact with the receptor on the plasma membrane and enforce the internalization of the assembly via receptor-mediated endocytosis [261]. This provides a basis for modification of the siRNA delivery system with the targeting ligand LHRH to help facilitate delivery of therapeutic agents to the human lung cancer cells [224].

1.11 Extracellular Barriers

The administered NPs must go through various barriers before reaching the tumor site. One such barrier which prevents the NPs from reaching the site of action is the extracellular barriers[262]. Viral vectors have shown improved transfection efficiencies to deliver genes by avoiding the extracellular barriers[263]. However, they pose a serious threat of causing carcinogenicity and immunogenicity [264-266]. On the other hand, non-viral vectors do not cause the serious threat and could increase the chances of developing an ideal non-biological vector. In order to truly translate the gene delivery carriers from lab to commercial scale, there is a need to design, safe, effective and controllable delivery systems[267]. The synthetic cationic vectors represent a promising class of non-viral vectors to deliver the gene to the cancer cells. They have been widely used as gene delivery systems and have also been reported to overcome the potential extra and intracellular hurdles. However, one of the major challenges in improving gene delivery to the tumor cells using cationic vectors is various extracellular barriers [268, 269].

Upon entering the blood circulation, one of the major barriers for the NPs to reach the targeted tumor site is the non-specific binding with negatively charged components such as blood cells and proteins. The positively charged cationic vectors can interact with negatively
charged albumin, immunoglobulins, complements and blood cells. This interaction can result in aggregation and dissociation of the NPs. The unstable NPs thus can get rapidly cleared by RES, which can hamper the ability of the NPs to reach the tumor site [172]. Furthermore, colloidal stability of NPs is another obstacle in successful delivery to the tumor cells. The nucleic acid/cationic vector complex possesses different characteristics than the external environment in the biological fluids which can lead to colloidal instability followed by aggregation of the complexes. Body’s natural defense mechanism and the activation of the immune system by the NPs would be another extracellular issue. The mononuclear phagocytic system specializes in opsonizing and removes the foreign hydrophobic particles, thus resulting in reduced efficiency. Some cationic vectors have also shown to activate complement and induce an inflammatory response [112, 113].

1.12 Intracellular Barriers

The properly designed NPs must avoid the extracellular barriers and also reach the tumor site through EPR effect. Various studies of cells were grown in a culture medium, allows the internalization of >95% of particular vectors, while only a fraction <50% actually expresses the transgene[270]. Upon reaching the tumor site the NPs can get internalized via endocytosis. The targeted NPs with an attached targeting ligand usually get internalized via receptor-mediated endocytosis. Upon internalization, the NPs enter the endocytic pathway and are further trafficked to the acidic lysosomes [271]. In order to achieve the siRNA therapeutic effect, the NPs must escape the endosomes/lysosomes and release the content into the cytoplasm of the cell.

1.13 Proton sponge effect

Several protonable residues containing polymers such as PAMAM dendrimers and lipopolyamines were reported to successfully achieve high gene transfection efficiencies [272].
Further studies revealed that the buffering capacity of the polymers resulted in higher transfection efficiencies [273]. In contrast, other cationic polymers such as Polyllysine failed to demonstrate desirable transfection efficiency, mainly due to insufficient buffering capacity. This pH buffering capacity was thus confirmed as one of the most important features of cationic polymers to induce endosomal disruption and the escape of the nucleic acid[274] [275]. Thus, it can be assumed that the high buffering capacity of the cationic polymers results in high efficiency of nucleic acid delivery[275]. ‘Proton Sponge’ polymers including PAMAM dendrimers and PEI polymers usually exhibit pKa values between physiological and lysosomal pH [209, 276].

PEI is a polyamine cationic polymer, which has been widely used to transfect the nucleic acids(including pDNA, siRNA)[277]. The potential of PEI as a transfection reagent was recently tested by Boussif et al. by condensing pDNA with PEI and delivering to the cancer cell lines. They found that the polymer effectively transferred the luciferase reporter gene into the different cell lines including the primary cells [209]. It has also been reported that the extraordinary ability of PEI to deliver nucleic acid effectively is mainly attributed to the proton sponge effect (high buffering capacity of PEI). Especially, the extensive buffering capacity was responsible for inhibiting the activity of lysosomal nucleases and to change the osmolarity of acidic vesicles.

The membrane of the endolysosomes contains an ATPase pump. This pump is mainly responsible for maintaining the pH of the vesicles Fig. 1.4. The pump maintains an acidic pH inside the vesicles by translocating the protons from the cytosol into the endosomes and activating the hydrolytic enzymes. The proton sponge polymer based NPs upon entering the endosomes gets extensively protonated at this stage due to their high buffering capacity which results in reduced acidification of the endocytic vesicles[209]. As a result, there is more
accumulation of protons inside the vesicles required to maintain lower endosomal pH. This is followed by passive entry of the counter ion (chloride Cl-) inside the vesicles, which results in increased ion concentration and water influx[276]. This ultimately results in the osmotic swelling and rupture of the endocytic vesicle membrane, thus releasing the NC’s into the cytosol.

The theory of Proton sponge effect was recently tested by Sonawane et al.[278]. They studied the effect of internalization of polyplexes composed of pDNA, PLL, PEI and PAMAM on the concentrations of chloride ions, pH and volume of endosomes. They found a significant chloride accumulation; volume expansion and membrane lysis following internalization of PEI and PAMAM based pDNA complexes. However, no significant changes in chloride ion accumulation and volume have been detected following the internalization of the PLL. They concluded that the proton sponge effect was responsible for the internalization and escape of the polyplexes, which further provides a rationale for the design and use of polymer-based nucleic acid delivery vectors. Although, there is still some ongoing debate about the exact mechanism of the escape of cationic polymers following the proton sponge effect, there are strong indications that cationic polymers which possess good buffering capacity between pH 5 and pH 7.4 correlate positively with good endosomal escape properties.
Figure 1.4: The ‘proton sponge effect’ (pH-buffering effect). (A) The NPs enter cells via endocytosis. After internalization, they get trapped in the acidic vesicles (endosomes/lysosomes). The membrane bound ATPase proton pump transports proton into the endosomes. The cationic polymers become protonated and resist the acidification of the endosomes. Hence, more protons get pumped into the endosomes continuously to lower the pH. This is followed by the passive entry of chloride ions into the endosomes resulting in increased ionic concentrations and water influx. This ultimately results in osmotic pressure causing the swelling and rupture of the endosomes, releasing the content into the cytosol. Adapted with permission from reference [279]
CHAPTER 2. HYPOTHESIS, OBJECTIVES, AND RATIONALE

The central hypothesis - The targeted mammalian target of rapamycin (mTOR) siRNA loaded mPAE NPs will selectively deliver mTOR siRNA to NSCLC cells (A549 and H460) and silence mTOR mRNA and protein expression thereby inhibiting the transcription involved in the initiation and progression of NSCLC. The overall objective of this work was to modify, formulate and characterize LHRH receptor targeted PAE polymer based NPs for the targeted delivery of mTOR siRNA to NSCLC cells. The targeted NPs will be further evaluated in vitro in NSCLC cancer cells (A549 and H460). The development and evaluation of LHRH-R targeted NPs represents the first critical step in the pre-clinical development of a novel nanotherapeutics for cancer therapy.

Objective 1. Synthesis and Characterization of Bioreducible Poly (amino ether) (PAE) polymer

Working Hypothesis: The modification of PAE polymer by introducing a thiol group will provide disulfide bond upon cross-linking and thereby release siRNA inside the cytoplasm.

Rationale: Bioreducible polymers have a long tradition in the delivery of nucleic acids for the treatment of cancer [280]. In particular, one of the main attractive features of bioreducible polymers is the ease of encapsulation of the nucleic acids to form NPs. This enables protection and facilitation of siRNA transport across the cancer cell membranes [281, 282]. The bioreducible polymers have been shown to form disulfide bonds upon oxidation and encapsulate the nucleic acid (siRNA, miRNA, DNA). They further impart stability to the NPs and protect the siRNA against nuclease degradation[283]. Some of the examples of this class of polymers include the thiol group-containing bioreducible polymers which easily form disulfide linkages.
They can be further degraded in response to the redox potential present inside the cells and release the nucleic acid inside the cytoplasm[284].

Most of the intracellular compartments in the cancer cells maintain an abundant reducing environment [285]. There are various redox couples that participate in maintaining the intracellular reduced state for various cellular functions such as stabilization of protein structures, redox cycles etc.[285, 286]. In particular, Glutathione (GSH, c-glutamyl–cysteinyl–glycine) is one of the most abundant intracellular reducing molecules which forms a major reducing coupling inside the cells in association with glutathione disulfide (GSSG) [287]. This is aided by the increased intracellular levels of GSH concentration (0.5–10 mM) and GSH/GSSG ratio (30: 1–100:1) compared to the lower extracellular levels (GSH: 2–20 µM)[288]. Thus, polymeric siRNA delivery bioreducible carriers containing disulfide bonds can be degraded inside the cytoplasm via thiol-disulfide reaction due to the presence of high intracellular GSH and release the siRNA efficiently. Apart from the increased siRNA release, other advantages of using bioreducible polymers for gene delivery include reduced cytotoxicity due to less accumulation of higher molecular weight cationic polymers and a controlled intracellular release of the siRNA [289, 290]. These properties make the bio reducible polymers very attractive to deliver siRNA to the cancer cells.

Recently, Barua et. al., generated a cationic polymer library by addition polymerization reaction between diglycidyl ethers and amines[291]. The generated polymers were screened for their DNA binding properties. The group found that a number of polymers demonstrated significant DNA-binding properties. In particular, 1,4- cyclohexanedimethanol diglycidyl ether (1,4C) and 1,4-bis (3- amino propyl) piperazine (1,4Bis) based polymers/polyplexes were found to be more effective transfection agent compared to the cationic polymer polyethylene-imine-
25kD (PEI-25) (the current standard for polymer-mediated gene delivery). These polymers exhibited higher transfection efficiencies in both human prostate cancer cells and murine osteoblasts compared to PEI-25. Further, these polymers also exhibited less cytotoxicity compared to the PEI-25 polymer. The same group further used the (1,4C) (1,4Bis) based polymers to functionalize the gold nanorods which also showed improved transfection efficiency[292].

From the studies, they identified one such polymer that specifically showed very high transfection efficiency. The polymer (PAE) was synthesized by the reaction of neomycin with glycidyl ether at a molar ratio of 1:2 [292]. The group explored the use of PAE by functionalizing gold nanorods on the surface to produce PAE-functionalized GNR (PAE-GNR) assemblies. This assembly was generated by depositing polyelectrolyte multilayers on cetyltrimethyl ammonium bromide based gold nanorods (CTAB-GNRs). The PAE-GNR nano-assemblies demonstrated significantly higher transgene expression efficacy and lower or comparable cytotoxicity compared to PEI-25 functionalized GNR’s(PEI-GNRs).

Thus, for objective 1 we have selected PAE polymer to formulate and deliver mTOR siRNA to the NSCLC cells. We are proposing the introduction of the thiol group in PAE to exploit the intracellular reducing environment, which would result in a faster release of the payload. The thiol group will be introduced by using a simple and fast one-step reaction with Iminothiolane Hydrochloride (Traut’s reagent) while retaining the net positive charge due to -NH₂ group introduction. In addition, the thiol group present in the polymer would easily form a disulfide group under oxidative conditions and result in stable NP formulation. This NP will be further reduced inside the cells by GSH and release the siRNA payload.
Objective 2: Formulation and Characterization of Bioreducible NPs for mTOR siRNA delivery to the Non-small cell Lung cancer cells.

Working Hypothesis: The development of modified polymer (mPAE) based NPs of mTOR siRNA will provide biostability and deliver the mTOR siRNA to the tumor cells and thereby will exert a significant anticancer activity.

Rationale: Extensive efforts have been made to design new drugs that selectively target precise pathways driving the development of NSCLC. But these new drugs have been rendered ineffective as many attractive cancer targets remain undruggable [293]. Recently, it was shown that abnormal activation of the Akt/mTOR pathway is commonly observed in the NSCLC cases [294]. The deregulation of the Akt/PI3K/mTOR pathway is known to have contributed to the development and maintenance of NSCLC. Various studies have shown that the downstream effector, eIF-4E coupling with the mTOR pathway plays a major role in lung carcinogenesis[295]. Furthermore, PTEN and LKB1 negatively regulate the mTOR signaling by dephosphorylating PIP3 to PIP2. This limits the AKT's ability to bind to the membrane LKB1-mediated activation of AMP-activated protein kinase (AMPK) and AMPK inhibition of mTOR through TSC2, respectively [296]. These negative regulators act as tumor suppressors and have been found to be mutated in NSCLC, suggesting a role of the mTOR pathway in lung cancer [297]. Various mTOR inhibitors (temsirolimus, everolimus, and deforolimus) have been evaluated to target mTOR and treat NSCLC, however, they cause adverse effects due to the unwanted effect on normal cells[298, 299]. These mTOR inhibitors cause various adverse side effects such as liver damage, anemia and non-infectious pneumonitis [298, 300]. Thus, the newer type of drugs such as siRNA targeting mTOR is needed to treat NSCLC to possibly avoid these
side effects. Furthermore, mTOR siRNA has been used recently to successfully inhibit cell growth in NSCLC cells[301].

**Figure 2.1:** mTOR-signaling network. Activated growth factor receptors trigger activation of the PI3K/Akt pathways and the Ras/MEK/Erk pathway. Activated Akt leads to increased mTOR activity through signaling by means of the TSC1/2 complex. mTOR phosphorylates S6K1 and 4E-BP1, resulting in increased gene transcription, cell growth, and cell proliferation. PI3-K, phosphatidylinositol 3-kinase; AMPK, adenosine mono-phosphate-activated protein kinase; LKB1, liver kinase B1; PTEN, phosphatase and tensin homologue; STRAD, Ste20-like adaptor protein; TSC, tuberous sclerosis complex; EGFR, epidermal growth factor receptor; mTOR, mammalian target of rapamycin; m, signaling proteins frequently mutated in lung cancer; bm, potential as biomarker; Ras, ras2 Kirsten rat sarcoma viral oncogene homolog; MEK, MAPK/ERK kinase; Erk, extracellular signal-regulated kinase. Adapted with permission from reference [302].
RNAi-induced gene knockdown is an exciting, naturally occurring cellular pathway that can be used to target mTOR mRNA transcripts [303]. This pathway can be exploited to target mTOR gene and alter cellular behavior with the delivery of siRNA complementary to the mTOR mRNA transcript [304]. Furthermore, mTOR siRNA has been used recently to successfully inhibit cell growth in NSCLC cells [301]. In particular, siRNA-based therapies are a promising alternative approach because they can theoretically silence expression of any oncogene [305-307]. However, the therapeutic potential of siRNA cannot be realized without efficient delivery to its site of action in the cytoplasm [82, 93, 308, 309]. There are various carrier systems that can be employed to deliver sustained dosages of siRNA, however, unpredictability and subsequent toxic dosages in vivo limit their use [310]. Furthermore, unlike pDNA, synthetic siRNA does not need to enter and rely on the host nucleus for subsequent function [311].

Non-viral gene delivery has gained significant attention as a carrier for nucleic acid delivery [173, 312-315]. These gene delivery carriers have been successfully used to deliver siRNA into the cells and prevent its degradation in the acidic vesicles. These polymeric delivery vehicles usually include poly (β-amino ester) s (PBAEs), polylactic acid (PLA) etc. [316]. Other such polymers which are widely used to deliver siRNA include cationic polymers (PEI) with high buffering capacities [317, 318]. In particular, PEI encapsulates siRNA by promoting nucleic acid compaction [319]. In addition, PEI polymers are known to result from significantly more efficient transfection and protection of nucleic acids (including DNA, siRNA, miRNA etc.) against nuclease degradation [319]. PEI polymer has become one of the gold standards for the non-viral cationic gene delivery, however, the density of positive charges in PEI results in a high toxicity. [209]. Consequently, the toxicity and the fact that these polymers are not biodegradable, are limiting factors for it’s in vivo use [28, 31]. Another such polymer with better transfection
ability, better toxicity profile and buffering capacity than PEI, is poly-amino ether (PAE)[320]. The PAE polymer has shown promise in gene transfection studies[320]. In particular, the appropriate density of amino groups on PAE confers significant buffering capacity to the polymer over a wide pH range. This property, known as the "proton sponge effect" is probably one of the most important factors explaining higher transfection efficiencies obtained with these polymers[321].

There are several cellular barriers which contribute to the poor transfection efficiency of the non-viral gene vectors [322, 323]. One of the major barriers for siRNA encapsulated mPAE based NPs to gain entry into the cells include traversing the cellular membrane [324-326]. The cellular membrane is usually composed of a lipid bilayer and various integral proteins which act as a gatekeeper. These proteins only allow entry of selected foreign materials inside the cells [327, 328]. In addition, the cell membranes also consist of various constituents, including glycoproteins, proteoglycans, and glycerophosphates, which contribute to the negative charge of the cell membrane[329]. This negative charge property of the cell membrane, however, has been exploited by many cationic polymer NPs to interact with the cancer cells and enter via endocytosis [330, 331]. This process of cellular uptake of endocytosis includes a number of distinct uptake routes. In particular, the NPs are usually taken up inside the cells by pinocytosis [332, 333]. Pinocytosis includes various routes, including macropinocytosis (for molecules up to a diameter >1 μM), clathrin-mediated endocytosis (≤150 nm), and caveolin-mediated endocytosis (≤120 nm)[334]. The cationic polymer based NPs usually enter the cells through either of the following mechanism, including adsorptive endocytosis, clathrin-coated pit mechanism or fluid phase endocytosis [335-337]. In all the above processes, the siRNA
encapsulating NPs are transferred into intracellular acidic organelles such as endosomes or caveosomes before being released into the cytoplasm or degraded in the lysosomes[338].

**Figure 2.2** shows the endocytosis pathway of a GSH responsive cationic polymer based nano-assembly system. Following internalization, the NPs should ideally release the siRNA into the cytoplasm. However, the NPs end up in the acidic organelles (i.e. the NPs follow the path of the endolysosomal compartment, beginning from early endosomes to late endosomes and ultimately end up in the lysosomal compartment) and get degraded by the acidic environment. In the lysosomes stage, the lysosomal vesicles fuse and assemble in the perinuclear region [339]. Here the majority of the NP remain and get degraded without significant changes in the distribution patterns [340]. In order to be effective in the delivery of siRNA into the cytoplasm, the NPs must escape the lysosomal compartment. The endosomal escape seems to be one of the key steps in limiting the delivery of siRNA encapsulated NPs into the cytoplasm. As demonstrated in the last chapter, the cationic mPAE has a strong buffering capacity compared to PEI polymer. Several studies have shown the effective endolysosomal escape of the PEI polymer based NPs [341, 342]. The mPAE is predicted to have a similar proton sponge effect based release of the NPs in the cytoplasm.
Figure 2.2 - Schematic illustration of the GSH responsive release of siRNA from the NPs following the endocytosis of the drug delivery system. On reaching the tumor site, bio-reducible siRNA NPs gets internalized by endocytosis. The NPs escape from the endosomes/lysosomes due to the proton sponge effect of the cationic polymer (mPAE). The disulfide bond in the GSH responsive NPs gets cleaved in the cytoplasm by excess GSH (2-10mM) to release the siRNA. This enables RNA interference due to the released siRNA which affects the levels of the target protein. Figure adapted with permission from reference [343].

Disulfide bonds can be reductively degraded in the human body via GSH. This reducing molecule is predominantly present in the cytosol of human tissues at concentrations ranging from 2-10 mM (three orders of magnitude greater than the concentration in blood serum (2-20 μM)[344]. This property can be harnessed to target siRNA release to its site of action in the cytoplasm by employing disulfide-containing polymers as vectors. The use of bio reducible NPs to deliver siRNA have met with great success in the past. Linear low molecular weight PEI segments with disulfide linkage were shown to be effective with less cytotoxic effects. The NPs
prepared from bio reducible PEI and siRNA were successful in 50% knockdown of the fluorescent marker gene in Chinese hamster ovary cells (CHO-K1) using [345]. In another study with bio reducible cationic polymer based NPs, disulfide-containing poly (amido amine) s have shown successful \textit{in vitro} siRNA delivery in non-small cell lung carcinoma (H1299)[346], human head and neck carcinoma cells (UM-SCC-14C), [347] and human prostate cancer cells. Especially in human prostate cancer cells, \raisebox{1pt}{\textasciitilde}80% knockdown was achieved with 30 nM siRNA.[293, 348]. Several peptides based bio reducible polymer have also been used to deliver siRNA. In a recent study, cysteine modified KALA peptide (a 30-residue peptide containing 3 Lys-Ala-Leu-Ala repeats)[349] was cross-linked to form a bio reducible carrier. This carrier was further used to deliver PEGylated siRNA to MDA-MB-435 cells[350]. The 60 nM siRNA containing bio reducible system achieved 50% gene knockdown \textit{in vitro} in 10% serum containing media. PBAE containing disulfide bond in the polymer achieved around 70% knockdown in human umbilical vein cells \textit{in vitro} in 2% serum containing media using 60nM siRNA[351]. Thus in objective 2, we explored the use of mTOR siRNA encapsulated mPAE bio reducible polymer to target NSCLC cells.

\textbf{Objective 3: LHRH Receptor -Targeted Bioreducible NPs for mTOR siRNA Delivery to Lung Cancer cells}

\textbf{Working Hypothesis:} The working hypothesis of this study was that the targeted NPs MS-MP-PG-LR will effectively deliver mTOR siRNA to A549 and H460 cells to downregulate mTOR siRNA gene expression. The proposed MS-MP-PG-LR will specifically target NSCLC cells by means of surface anchored PEGylated LHRH targeting peptide.
Rationale: Development of targeted drug delivery system has been studied extensively over the past two decades mainly due to their potential promise of solving key therapeutic issues associated with the non-targeted delivery system such as low treatment efficacy and significant side effects to normal healthy tissues [370, 397, 398].

Upon administration by the intravenous route, the NPs are usually rapidly cleared from the circulation and accumulate mainly in the liver and spleen [139, 204]. The accumulation is the result of the opsonization and recognition by the MPS [143, 205, 399]. Various attempts have been made to reduce the NP uptake by the MPS by controlling characteristics such as size, surface charge, hydrophilicity and surface functionality [400, 401]. Studies have shown that NPs with a mean diameter of less than 20 nm or less and an extended hydrophilic polymer surface exhibit prolonged blood circulation [216, 401].

Hydrophilic polymers such as PEG have been used extensively on a variety of cationic polymers based NP systems to increase surface hydrophilicity and improve circulation half-life [233, 236, 237, 352-356]. PEG can be attached to the surface by various techniques including covalent attachment, entrapping or adsorbing PEG chains onto the surface of NPs [224, 236]. The stealth properties of PEG molecule are highly dependent on its molecular weight (MW) and surface density of PEG coating. The PEG molecule attached NP usually shows stealth properties of PEG coating with a high density and MW ranges from 2,000 to 10,000[234, 357]. The PEG molecule usually gets attached to the NP surface and forms two main types of conformations viz, brush and mushroom [358, 359]. In a study by Sundaram et al. deslorelin (LHRH-R agonist)-docetaxel (D-D) conjugate was prepared and tested for it’s in vitro and in vivo efficacy against H1299 cells. The D-D conjugated resulted in 13-fold increase in in vitro docetaxel efficacy compared to docetaxel alone after 24 h [219]. The conjugate also exhibited 82- and 15-fold
tumor growth inhibition *in vivo* when delivered in repeated weekly i.v. injections and a single intratumoral (i.t.) injection, respectively in combination with RGD-Flt23k-NP. The central problem in using the chemotherapeutic agents for the treatment of cancer includes severe adverse effects on healthy tissues. To minimize the toxic adverse effects various strategies, have been employed, including reduction of dose, treatment delay and even discontinuing the therapy altogether. Dharap et al investigated the potential of LHRH targeted cancer chemotherapeutic agents on various cancer cell lines including Breast and ovarian carcinoma to overcome the adverse side effects [234]. The results showed that targeting the LHRH receptors by using the LHRH peptide-based delivery system substantially enhanced the efficacy of the chemotherapy. The LHRH targeted delivery system also did not show *in vivo* pituitary toxicity in the human ovarian carcinoma xenografts. Fig. 2.3 shows endocytosis pathway of an LHRH-R mediated, GSH responsive cationic polymer based nano-assembly system.

Herein, we report the PEG-LHRH peptide conjugated MS-MP NPs for the targeted delivery of mTOR siRNA to lung tumor cells, utilizing bioreducible cationic polymer mPAE based NPs as the delivery carrier. Towards this aim, MS-MP NPs was synthesized using the nanoprecipitation method to encapsulate the mTOR siRNA and form MS-MP NPS. The MS-MP NPs was then conjugated with PEG and PEG-LHRH to form non-targeted (NT-NPs) and targeted (T-NPs) gene delivery system for the treatment of lung cancer. The NT-NPs and the T-NPs of uniform size, zeta potential were produced. The PEG and PEG-LHRH were conjugated on the NP surface by covalent conjugation using Maleimide – PEG – NHS chemistry.
Figure 2.3: Schematic illustration of the GSH responsive release of siRNA from the NPs following the receptor-mediated endocytosis of the drug delivery system. On reaching the tumor site, bio-reducible siRNA NPs first get internalized by endocytosis. The NPs escape from the endosomes/lysosomes due to the proton sponge effect of the cationic polymer (mPAE). The disulfide bond in the GSH responsive NPs gets cleaved in the cytoplasm to release the siRNA. This enables RNA interference due to the released siRNA which affects the levels of the target protein (Figure adapted with permission from ref [360]).
Chapter 3 Materials and Methods

3.1 Objective 1: Synthesis and Characterization of Bioreducible PAE polymer

3.1.1 Materials

PAE was obtained from Dr. Kaushal Rege’s lab (SEMTE) in Arizona State University. All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

3.1.2 Experimental Methods

3.1.2.1 Synthesis of the bioreducible modified-PAE

The obtained PAE polymer was synthesized by reacting neomycin with glycidyl ether at a molar ratio of 1:2 as reported in detail by Rege et al., previously [361]. The modified bioreducible PAE (mPAE) was synthesized by a simple one-step reaction with Traut’s reagent as described previously (Fig. 3.1) [362]. Briefly, 10 g of PAE was dissolved in 1 ml of ddH₂O (Double distilled water) and was allowed to react with 1.85 g (5-times molar excess) of 2-iminothiolane hydrochloride (IH) at room temperature for 15 h [363]. The product was dialyzed using a dialysis membrane (MWCO = 1,000 Da) against 5 mM HCl for 24 h followed by dialysis against 1 mM HCl solution for 24 h. The purified mPAE was lyophilized and stored at -20°C for future use [364].
Figure 3.1: Scheme for modification of Polyamino-ether (PAE): The PAE polymer was dissolved in water and allowed to react overnight with Iminothiolane hydrochloride. The mPAE was collected after extensive dialysis and freeze dried for future use. The modification of amino
groups (-NH₂) is shown as representative scheme. Figure reused with permission, from reference [365].

3.1.2.2 Thiol group estimation

Ellman’s reagent test was performed to determine the substitution of the thiol group in PAE and mPAE. The assay was performed according to the manufacturer’s protocol as described before [366]. Briefly, a set of test tubes, each containing 50 μl of Ellman’s Reagent Solution and 2.5 mL of Reaction Buffer (0.1M sodium phosphate, pH 8.0, containing 1mM EDTA) were prepared. To the above test tubes, 250 μl of unknown (PAE and mPAE) are added and incubated for 15 minutes at room temperature. The absorbance is measured spectrophotometrically using UV-1800 Spectrophotometer (Shimadzu) at 412nm. The absorbance between PAE and mPAE was compared and the data is represented. All data were presented as the mean ± SD (Standard Deviation).

3.1.2.3 Sulforhodamine B (SRB) cytotoxicity assay

In vitro cytotoxicity assay was performed to evaluate the cytotoxicity of mPAE and PEI polymers in A549 and H460 cells. For this experiment, the A549 and H460 cells were plated in 96-well plates at a density 5×10³ and 6×10³ cells per well, respectively and allowed to grow overnight. The cells were then incubated with 2.5, 5, 10, 25, 50, 100 and 200 µg/ml of polymers mPAE and PEI for 48 h in RPMI medium (supplemented with 10% FBS). After the incubation period, the polymer containing media was removed and replaced with fresh 200 µl of RPMI media. The cell viability (%) was evaluated using a modified SRB assay as described before[367]. Briefly, 100 µl of cold 10% w/v Trichloroacetic acid (TCA) was added to each well and the 96 well plate was incubated at 4°C for 1 h. After incubation, the plates were washed 4-5 times with ddH₂O and excess water was removed using paper towels. The plates were allowed to
air-dry at room temperature (20-25°C). After fixing step, 100 µl of 0.057 % (w/v in 1% acetic acid) SRB solution was added to each well. After 30 min, the plate was quickly rinsed 4 times with 1% (v/v) acetic acid to remove the unbound dye. The plates were allowed to dry at room temperature. After the drying step, 200 µl of 10 mM Tris base solution (pH 10.5) was added to each well and was allowed to incubate for 5 min in the gyratory shaker to dissolve the dye. The absorbance of SRB was measured at 564 nm using a Synergy plate reader.

The cell viability (%) was calculated according to the following formula. All data were presented as the mean ± SD (Standard Deviation).

\[
\text{Cell viability (\%)} = \frac{\text{absorption test}}{\text{absorption control}} \times 100\%
\]

3.1.2.4 Acid base titration

The acid-base titration was performed to evaluate the buffering capacity of the mPAE and PEI polymers according to the procedure described previously [342, 368]. The polymer (PAE and PEI) solutions were prepared in DEPC treated RNase free water with a concentration of 1.5mM of protonatable nitrogens in 150 mM NaCl. The pH of the polymer solutions (10 ml) was adjusted to 4.5 using 0.5 M HCl. This solution was titrated with 0.01M NaOH to pH 7.5 using a pH meter (Mettler Toledo Seven Multi). The solution of 150 mM NaCl solution was used as a reference for this procedure.

3.2 Objective 2: Formulation and Characterization of Bioreducible NPs for mTOR siRNA delivery to the Non-small cell Lung cancer cells.

3.2.1 Materials

SMARTpool: ON-TARGETplus mTOR siRNA, DHARMAfect 1 Transfection Reagent, and ON-TARGETplus non-targeting pool were obtained from Dharmacon (GE Dharmacon USA). The SMARTpool mTOR siRNA was obtained with the following target sequences:
GGCCAUAGCUAGCCUCAUA, CAAAGGACUUCGCCCAUAA, GCAGAAUUGUCAAGGGGAUA, and CCAAAGCACUACACUACAA. An additional scrambled (SS) negative control siRNA was purchased from Santa Cruz Biotechnology (Dallas Tx), AccuBlue high sensitivity dsDNA quantification kit was obtained from Biotium, Inc (Fremont, CA), mTOR (7C10) Rabbit mAb and β-Actin (13E5) Rabbit mAb were obtained from Cell Signaling Technology. Goat anti-Rabbit IgG (H&L) Secondary Antibody, DYLight 800 4X was obtained from Thermofisher Scientific USA. Immobilon-FL PVDF, 0.45 µm membrane was obtained from EMD Millipore USA. Other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

3.2.2 Experimental Methods:

3.2.2.1 Formulation of mTOR-siRNA-mPAE NPs (MS-MP-NPs)

The mTOR siRNA encapsulated NPs were prepared by a modified nanoprecipitation method as shown in Fig. 3.2. Briefly, 1 mg of the mPAE polymer and mTOR siRNA (2 µg) was dissolved in the DEPC treated RNAse free water for 1 h. The above solution was added dropwise (0.2 ml/min) to 1 ml of 70% v/v acetone to obtain MS-MP NP suspension. The NP suspension was allowed to stir overnight to evaporate the acetone. To the above suspension, 2 µl of 40% glyoxal was added to cross-link the sulfhydryl groups and form stable disulfide cross-linked NPs. The MS-MP NPs were dialyzed extensively against DEPC treated RNAse free water using dialysis membrane (50 kDa) to remove any unreacted glyoxal and obtain the final MS-MP-NPs for future use.
Figure 3.2: Scheme for the formation of disulfide cross-linked MS-MP-NPs - The first step involves the formation of mTOR siRNA encapsulated NPs by the nanoprecipitation method. In the second step, the NPs with thiol group are oxidized to allow the formation of MS-MP-NPs. The final formulation (MS-MP NPs) was collected by centrifugation for future studies.

3.2.2.2. Agarose gel retardation assay

3.2.2.2.1 mTOR siRNA encapsulation ability

Gel Retardation assay was performed to determine the optimum concentration of mPAE, required to fully encapsulate mTOR siRNA. For this experiment, MS-MP NPs were formed by the nanoprecipitation method as described above with mPAE polymer to mTOR siRNA weight (w/w) ratios ranging from 300:1 to 1:1 based on siRNA (weight 500 ng) in DEPC treated RNase free water. In brief, mPAE polymer (150 mg, 100 mg, 50 mg, 12.5 mg, 5 mg and 0.5 mg) was dissolved in 15 ml, 10 ml, 5 ml, 12.5 ml, 5 ml, 0.5 ml and 0.05 ml of DEPC treated nuclease free water, respectively. To above polymer solutions, mTOR siRNA (500 ng) was added and stirred for 1 h. To the above solutions, acetone (1.5 ml, 1 ml, 0.5 ml, 0.125 ml, 0.05 ml and 0.05 ml) was added dropwise to formulate the NPs with different weight ratios. The NPs were collected following centrifugation of the NPs suspension. The gel retardation assay was performed by
loading 20 μl aliquot of the sample together with 4 μl of loading dye (6X) New England Biolabs on a 1% agarose gel, prepared in 1X Tris-acetic acid-EDTA (TAE) buffer. The Electrophoresis was carried out in TAE buffer (40 mM Tris(hydroxymethyl) aminomethane, 20 mM acetic acid, 10 mM EDTA, pH 8.0) supplemented with 1.25 μM ethidium bromide at 100V for 1 h using Biorad PowerPac 300 Electrophoresis instrument. After electrophoresis, pictures were taken on a Biorad multi-image ChemiDoc XRS+ UV illumination and analyzed using Biorad Image lab software version 3.0.

3.2.2.2. Serum protection assay and GSH mediated release

Gel retardation assay was performed to evaluate the mTOR siRNA protection ability of MS-MP NPs and the enhanced GSH mediated release. For this experiment, mTOR siRNA loaded mPAE NPs were prepared with mPAE: mTOR siRNA w/w ratio of 45:1 as described above. The ratio for the formulation of NP was selected based on their smaller particle size, net positive charge, and high entrapment efficiency. A volume of 200 μl of siRNA-loaded mPAE NPs (containing 5 μg of mTOR siRNA) was incubated at 37 ⁰C with an equal volume of Roswell Park Memorial Institute (RPMI) medium supplemented with 10% FBS and GSH 10 mM (together and separately) for a period of 4 h at 37 ⁰C incubator, respectively. Naked siRNA acted as a control, was treated in the same manner. After each time interval, 40 μL of the mixture was removed and incubated in a batch incubator at 60 ⁰C for 3 min to terminate the serum activity. The samples were subjected to agarose gel electrophoresis as described in the above procedure. After electrophoresis, pictures were taken on a Biorad multi-image ChemiDoc XRS+ UV illumination and analyzed using Biorad Image lab software version 3.0.
3.2.2.3 Dynamic light scattering (Particle Size) and zeta potential measurement

The Particle size distribution and the zeta potential of the MS-MP NPs prepared at weight ratios of 30:1, 45:1 and 60:1 (mPAE: mTOR siRNA) was measured using NICOMP370 particle sizer (Nicomp Particle Sizing Inc., Santa Barbara, CA) in the intensity mode. The MS-MP NPs were formed in DEPC treated RNAse free water by nanoprecipitation method as described above. For particle size measurement, The NP suspension was diluted using distilled water to a final volume of 600 µl. For zeta potential measurement, the NP suspension (200 µl) was diluted to 2 ml and the measurement was performed using NICOMP 380ZLS. The channel width of the digital autocorrelator in the Nicomp is reported in terms of microseconds, or μsec. In most cases, the system should be allowed to set the channel width automatically. Ideally, the channel width will be adjusted so that the number of decays in the autocorrelation function lies between 1.7 and 2.7. For NPs in the range of 100 – 150 nm, the channel width was set at 12 – 15 μ sec.

3.2.2.4 Entrapment efficiency (%) and loading capacity (%)

The entrapment efficiency of mTOR siRNA in MS-MP NPs was measured using AccuBlue high sensitivity dsDNA quantification kit (Biotium). Based on the results obtained from the gel retardation assay of different weight ratios of mPAE and mTOR siRNA NPs, three different weight ratios (30:1, 45:1, and 60:1) were selected to determine the entrapment efficiency (%) and loading capacity (%). Briefly, the MS-MP NPs suspension was subjected to centrifugation (13,000 x g at 10°C for 30 min) to separate the mTOR siRNA. The free siRNA in the supernatant obtained from centrifugation was quantified by using the AccuBlue high sensitivity dsDNA quantification kit (Biotium) as described in the manufacturer’s protocol by measuring the excitation/emission wavelength of 485/530 nm with Synergy plate reader[369].
The unknown concentration of siRNA in the supernatant was calculated based on the standard curve of siRNA at different concentrations of 0.05, 0.5, 1, 2.5 and 10 µM (equivalent to 0.65, 6.5, 13, 26, 65 and 130 ng/µl of mTOR siRNA). The entrapment efficiency (%) and loading capacity (%) was calculated by using the following equations.

\[
\text{Entrapment Efficiency} = \frac{\text{Total amount of entrapped siRNA}}{\text{Total amount of siRNA used in preparation of NPs}} \times 100
\]

\[
\text{Loading Capacity} = \frac{\text{Total amount of entrapped siRNA}}{\text{Total amount of NPs}} \times 100
\]

**3.2.2.5 In vitro mTOR siRNA release study:**

To evaluate the mTOR release kinetics, *in vitro* release study was performed. In brief, MS-MP NPs (containing 1 µg equivalent mTOR siRNA) was incubated in 0.1 M PBS solution (pH 7.4) containing 10 mM and 10 µM GSH, respectively at 37 °C for a period of 7 d. After incubation for certain time period interval (1, 2, 3, 4, 5, 6 and 7 d), 100 µl of the media was taken out. The released siRNA was separated from the sample by centrifugation at 13,000 x g for 15 min using a Vivaspin filter membrane (50kDa) at 37 °C. The released siRNA was measured using the AccuBlue high sensitivity dsDNA quantification kit (Biotium). As determined previously, a standard curve of mTOR siRNA was plotted to determine the unknown concentration in the supernatant. The cumulative release of mTOR siRNA (%) was calculated as described previously [370].

**3.2.2.6 Cell Viability assay:**

To evaluate the cell viability effects mTOR siRNA in NSCLC cell lines, MS-MP-NPs were incubated in A549 and H460 cell lines and cell viability (%) was calculated. For this experiment, the A549 and H460 cells were plated in 96-well plates at a density 5×10³ and 6×10³
cells per well, respectively and allowed to grow overnight. The cells were then transfected with mTOR siRNA or scrambled siRNA (SS) using Dharmafect (Dharmacon, GE life sciences, USA) (siRNA concentration 50 nM) according to the manufacturer’s protocol for a period of 48 h. The cells were also transfected with mTOR siRNA and SS siRNA (control) containing mPAE NPs (siRNA concentration 50 and 100 nM) for 48 h. The NPs prepared at a weight ratio of 45:1 (20 µl and 10 µl) was mixed with 180 µl and 190 µl of RPMI (supplemented with 10% FBS) and allowed to incubate with the A549 and H460 cells for a period of 48 h. The final concentration of mPAE polymer (30µg/ml and 60µg/ml) NPs was used to incubate 50 nM and 100 nM siRNA concentration, respectively.

The cell viability (%) of the mTOR siRNA was evaluated using a modified SRB assay according to the protocol as described before[367]. Briefly, 100 µl of cold 10% w/v Trichloroacetic acid (TCA) was added to each well and the 96 well plate was incubated at 4 °C for 1 h. After incubation, the plates were washed 4-5 times with double distilled water and excess water was removed using paper towels. The plates were allowed to air-dry at room temperature (20 – 25 °C). After fixing step, 100 µl of 0.057% (w/v in 1% acetic acid) SRB solution was added to each well. After 30 min, the plate was quickly rinsed 4 times with 1% (v/v) acetic acid to remove the unbound dye. The plates were allowed to dry at room temperature. After the drying step, 200 µl of 10 mM Tris base solution (pH 10.5) was added to each well and was allowed to incubate for 5 min in the gyratory shaker to dissolve the dye. The absorbance of SRB was measured at 564 nm using Synergy plate reader. The cell viability (%) was calculated according to the following formula. All data were presented as the mean ± SD (Standard Deviation).

\[
\text{Cell viability (\%)} = \frac{\text{absorption test}}{\text{absorption control}} \times 100\% 
\]
3.2.2.7 Cellular Uptake of MS-MP-NPs by Fluorescence-activated cell sorting (FACS) analysis

To investigate the cellular uptake of MS-MP-NPs prepared at the weight ratio of 45:1 (mPAE: siRNA), flow cytometry analysis was performed. To impart fluorescence to the NPs, fluorescein isothiocyanate (FITC) was attached (MS-MP-FITC NPs) according to the procedure discussed before[371, 372]. In brief, 100 µg/ml of MS-MP-NPs were allowed to react with 2 times molar excess of FITC solution (1 mg/ml in DMSO) overnight in 1 ml of ddH2O. The unreacted FITC was removed by dialyzing against the membrane (MWCO 15kDa) against 50 ml of double distilled water and MS-MP-FITC-NPs were collected for further experiments.

The cellular uptake behavior was assessed using a BD ACCURI C6 PLUS flow cytometer at a minimum of 10 x 10^3 cells gated per sample. A549 and H460 cells (2 x 10^5 cells/well) were seeded in 6-well plate in RPMI medium containing 10% FBS and incubated for 48 h. When the confluence reached ~ 70- 80%, the media in each well were removed and the cells were incubated with MS-MP-FITC-NPs 200 µl and 1800 µl of RPMI media (supplemented with 10% FBS). After certain time intervals (30 min, 2 h, and 24 h), the cells were washed three times with PBS and trypsinized for further processing. The cells were collected by centrifugation for 4 min at 3000 x g. The collected cells were suspended in 100 µl of PBS and measured for cellular uptake by BD ACCURI C6 PLUS flow cytometer. Analysis of cellular uptake was performed by using Flowjo 7.6.1 single cell analysis software. At least 10,000 cells were analyzed for each experiment. The flow cytometric data were presented as frequency distribution histograms. The uptake of maximum fluorescence NPs was set at 100%, and the relative uptake
of different treatments was presented as a percentage. The mean cell fluorescence was normalized to that of the untreated cells. All experiments were repeated at least three times.

3.2.2.8 mTOR gene silencing efficiency by western blot analysis

To evaluate the mTOR gene silencing efficiency of the mTOR siRNA loaded MS-MP-NPs in A549 and H460 cells, western blot analysis was performed. To perform the experiment, A549 and H460 cells were plated in 6-well plates at 2×10⁵ cells per well and allowed to grow overnight. The cells were then transfected with mTOR siRNA or scrambled siRNA (SS) using Dharmafect (Dharmacon, GE life sciences USA) (siRNA concentration 50 nM) for 48 h. The cells were also transfected with mTOR siRNA and SS siRNA containing MP-NPs (siRNA concentration 50 nM) for 48 h. The MS-MP NPs were prepared by dissolving mTOR siRNA (45 µg) and mPAE (2 mg) in ddH2O. The solution was then added to the 2 ml of 70% v/v acetone. After nanoprecipitation, 10 µl of glyoxal was added and was allowed to react overnight. The NPs were then separated using centrifugation (15 kDa MWCO). The prepared NPs suspension (200 µl) at the weight ratio of 45:1 were mixed with 1800 µl of RPMI (supplemented with 10% FBS) and allowed to incubate with the cells for a period of 48 h to achieve a final concentration of 30 µg/ml.

The cells were collected and washed thrice with ice-cold PBS. Cells were washed and lysed by the cocktail containing radio immunoprecipitation assay (RIPA) buffer (Cell Signaling Technology, Danvers, USA), protease inhibitor and phosphatase inhibitor using ultrasonication method (Qsonica Sonicators USA) for 3 seconds. The lysates were then centrifuged at 13,000 x g at 4 °C for 10 min. The supernatant was collected and the total protein concentration was determined using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). To confirm the mTOR protein(289kDa) levels in A549 and H460 cell lines, twenty-µg of protein was mixed
with equal volumes of loading buffer (2X Laemlli sample buffer, 5% β-mercaptoethanol) and loaded in each lane and further separated by an 8% SDS-polyacrylamide gel electrophoresis. The Electrophoresis were conducted in the running buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS). For the first 45 min, the run was conducted under 90 V then it was increased to 130 V for 90 min. The proteins were then transferred to a PVDF membrane using Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad) at 300 constant mA. The PVDF membrane was then blocked with 5% milk in Tris-buffered saline with 0.1% Tween 20(TBS-T) for 1 h at room temperature.

The PVDF membrane was then incubated with primary mTOR (7C10) Rabbit antibody (1:1000 dilution Cell Signaling Technology) in 5% BSA in TBST at 4°C overnight. The membrane was washed three times with TBST, followed by incubation with anti-Rabbit antibody [1: 10,000 Goat anti-Rabbit IgG (H&L)] Secondary Antibody and imaged with Odyssey imaging system (LI-COR Biosciences). The membrane was washed three times with TBST and was incubated with β-Actin (13E5) Rabbit antibody (1:1000 dilution, Cell Signaling Technology) prepared in 5% BSA in TBST for 3-4 h. The membrane was then washed three times followed by incubation with anti-Rabbit antibody (1: 10,000) Goat anti-Rabbit IgG (H&L) Secondary Antibody) and imaged using Odyssey imaging (LI-COR Biosciences). The quantitative amount of mTOR gene silencing was calculated using the densitometric analysis (Image J software) with β-Actin as the loading control. The results are presented as mean ± SD (n=3).

3.2.2.9 Statistical Analysis:

Results are expressed as the mean ± standard error. Significant differences between two groups were determined using Student’s t-test and differences among multiple groups were determined using Analysis of Variance (ANOVA). We performed statistical analysis with
GraphPad Prism Version 5 software. The differences were considered significant at a p-value <0.05 and p<0.01 in all cases.

3.3 Objective 3: LHRH Receptor -Targeted Bioreducible NPs for mTOR siRNA Delivery to Lung Cancer cells

3.3.1 Materials:
SMARTpool: ON-TARGETplus mTOR siRNA, DHARMAfect 1 Transfection Reagent, and ON-TARGETplus non-targeting pool were obtained from Dharmacon (GE Dharmacon USA). AccuBlue high sensitivity dsDNA quantification kit was obtained from Biotium Inc (Fremont, CA) mTOR (7C10) Rabbit mAb, β-Actin (13E5) Rabbit mAb and GAPDH Rabbit mAb were obtained from Cell Signaling Technology. LHRH primary antibody was obtained from (Lab Vision Corporation, Fremont, CA, USA). Goat anti-Rabbit IgG (H&L) Secondary Antibody, DYLight 800 4X was obtained from Thermofisher Scientific USA. Immobilon-FL PVDF, 0.45 µm membrane was obtained from EMD Millipore USA. Other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

3.3.2 Experimental Methods
3.3.2.1 Formulation of mTORsiRNA-mPAE-PEG (MS-MP-PG) and targeted mTORsiRNA-mPAE-PEG-LHRH(MS-MP-PG-LR) NPs:

The mTOR siRNA encapsulated PEGylated and targeted (PEG-LHRH) NPs were prepared by a modified nanoprecipitation method as described in Fig. 4.1. Briefly, 2 mg of the mPAE polymer and mTOR siRNA (45 µg) was dissolved in the 1 ml of DEPC treated RNAse free water for 1 h. The above solution was added dropwise (0.2 ml/min) to 2 ml of 70% v/v acetone to obtain MS-MP NP suspension. The NP suspension was allowed to stir overnight. To the above suspension, 2 µl of 40% glyoxal was added to cross-link the sulfhydryl groups and
form stable disulfide cross-linked NPs. The NPs were dialyzed extensively against DEPC treated RNAse free water using dialysis membrane (50kDa) to remove any unreacted glyoxal and obtain the MS-MP-NPs.

The surface modified NPs with PEG and LHRH targeted moiety (MS-MP-PG-LR), was synthesized by a two-step reaction. In the first step, the NH$_2$-PEG5K-COOH was conjugated with AMAS (N-maleimidoacet-oxysuccinimide ester). Briefly, 5 mg of NH$_2$-PEG5K-COOH was dissolved in 1 ml Phosphate buffered saline (PBS, pH 7.2). The cross-linker AMAS solution prepared in DMSO was added to the above mixture in 10-fold molar excess at 1 mM final concentration. The reaction mixture was incubated for 2 h at room temperature. The cross-linker was removed via dialysis against 500 ml DMSO and water mixture (1:10) using a dialysis membrane (MWCO 1kDa). The mPAE-PEG5K NPs were prepared by adding a fixed molar ratio of PEG5K-AMAS to the MS-MP-NPs prepared in the above step. To achieve a target PEG% of 20-25% on the surface of MS-MP-NPs, 2 times excess of PEG-AMAS (4 mg) was allowed to react with 10 mg of MS-MP-NPs (equivalent to 10 mg of mPAE polymers).

To formulate LHRH-R targeted NPs; in first step PEGylated peptide (PEG-LHRH) was prepared. Five mg of PEG5K-AMAS was prepared as described in the above step in 1 ml Phosphate buffered saline (PBS, pH 7.2). To this 1 ml of cysteine-terminated LHRH solution (3 mg LHRH peptide solution prepared in PBS pH 7.2 buffer) was allowed to react for a period of 2 h. After, the time period, the unreacted peptide was removed by dialyzing extensively overnight against double distilled water using a dialyzing membrane (MWCO 5kDa). The PEG-LHRH peptide was then allowed to react with MS-MP-NPs for a period of 4-5 hours and the unreacted PEG-LHRH peptide was removed using the dialysis membrane (15 kDa MWCO). The targeted NPs were freeze dried for future use using 5% lactose as a cryoprotectant.
To formulate different peptide concentrations of targeted formulations (MS-MP-PG-LR1, MS-MP-PG-LR10, MS-MP-PG-LR20, and MS-MP-PG-LR20) different amounts of LHRH (6 µg, 12 µg, 24 µg and 48 µg) peptide was attached to the surface of 150 µg of MS-MP-PG NPs. The procedure was performed as described above and the NPs were stored for future use.

To formulation fluorescent tagged siRNA (FS) based NPs, the similar procedure as above is used. For the formulation of FS-MP NPs, 2 mg of the mPAE polymer and FS (45 µg) was dissolved in the 1 ml of DEPC treated RNAse free water for 1 h. The above solution was added dropwise (0.2 ml/min) to 2 ml of 70% v/v acetone to obtain FS-MP NP suspension. The NP suspension was allowed to stir overnight. To the above suspension, 2 µl of 40% glyoxal was added to cross-link the sulfhydryl groups and form stable disulfide cross-linked NPs. The NPs were dialyzed extensively against DEPC treated RNAse free water using dialysis membrane (50kDa) to remove any unreacted glyoxal and obtain the FS-MP-NPs. The formulation of FS-MP-PG and FS-MP-PG-LR20 were prepared as discussed above for mTOR siRNA-based NPs.
Figure 3.3: Scheme for the formation of disulfide cross-linked MS-MP-PG-LR NPs - The first step involves the formation of mTOR siRNA encapsulated NPs by nanoprecipitation method. In the second step, the NPs with thiol group is oxidized to allow the formation of MS-MP-NPs. In the third step, the PEG-LHRH was conjugated to the amino group of the MS-MP NPs to give the final formulation of MS-MP-PG-LR NPs. The final formulation (MS-MP-PG-LR NPs) was collected by centrifugation for future studies.

3.3.2.2 In vitro cytotoxicity assay of MP-NP, MP-PG-NP, and MP-PG-LR-NP

To evaluate the in vitro cytotoxicity of MP-NP, MP-PG-NP and MP-PG-LR-NP in A549 and H460 cells lines, cells (200 µl suspension) were plated into 96-well plates at a density $5 \times 10^3$ and $6 \times 10^3$ cells per well respectively and allowed to grow overnight. The cells were then incubated with MP-NP (15 µg/ml, 30 µg/ml, and 60 µg/ml equivalent of MP-NP), MP-PG-NP (15 µg/ml, 30 µg/ml and 60 µg/ml equivalent of MP-NP) and MP-PG-LR10-NP (15 µg/ml, 30 µg/ml and 60 µg/ml equivalent of MP-NP) for a period of 48 h. The three different types of
formulations were prepared without mTOR siRNA. For the formulation of MP-NP, mPAE (2mg) was dissolved in 2 ml of ddH2O. The solution was added dropwise to 2 ml of 70% v/v acetone. The acetone was evaporated by overnight stirring. For MP-PG NPs and MP-PG-LR NPs, the 2 mg of MP-NP NPs was allowed to react with 4 mg of PEG-AMAS and 4.1 mg of PEG-AMAS-LHRH. The reaction was stirred overnight and the NPs were separated by dialysis membrane (15 kDa MWCO).

The prepared NPs (MP NP, MP-PG NP, and MP-PG-LR10 NP) 20 µl were mixed with 180 µl of RPMI (supplemented with 10% FBS) and was allowed to incubate with the cells in separate wells for a period of 48 h. The cytotoxicity of the formulations was evaluated using a modified SRB assay according to the protocol[367]. Briefly, after 48 h treatment, 100 µl of cold 10% w/v Trichloroacetic acid (TCA) was added to each well and the 96 well plate was incubated at 4 °C for 1 h. After incubation, the plates were washed 4-5 times with double distilled water and excess water was removed using paper towels. The plates were allowed to air-dry at room temperature (20-25 °C). After fixing step, 100 µl of 0.057 % (w/v in 1% acetic acid) SRB solution was added to each well. After 30 min, the plate was quickly rinsed 4 times with 1% (v/v) acetic acid to remove the unbound dye. The plates were allowed to dry at room temperature. After the drying step, 200 µl of 10 mM Tris base solution (pH 10.5) was added to each well and was allowed to incubate for 5 min in the gyratory shaker to dissolve the dye. The absorbance of SRB was measured at 564 nm using Synergy plate reader. The cell viability was calculated according to the following formula. All data were presented as the mean ± SD (Standard Deviation).

\[
\text{Cell viability} (\%) = \frac{\text{absorption test}}{\text{absorption control}} \times 100\% \quad \text{Eq1}
\]

3.3.2.3 In vitro mTOR gene silencing efficiency of MP-NP, MP-PG-NP, and MP-PG-LR-NP
A549 and H460 cells (2 ml cell suspension) were plated into 6-well plates at $2 \times 10^5$ and $2.5 \times 10^5$ cells per well respectively and allowed to grow for a period of 24 h. After seeding, the medium was changed and the cells were incubated with MP-NP (15 µg/ml, 30 µg/ml, and 60 µg/ml), MP-PG-NP (15 µg/ml, 30 µg/ml, and 60 µg/ml) and MP-PG-LR-NP (15 µg/ml, 30 µg/ml and 60 µg/ml) for a period of 48 h. The three concentrations (15 µg/ml, 30 µg/ml, and 60 µg/ml) refer to the actual concentration of polymer mPAE in the wells. The NPs were prepared as described in the above section. The NP suspension (200 µl) was mixed with 1800 µl of RPMI (supplemented with 10% FBS) and was allowed to incubate with the cells in separate wells for a period of 48 h.

After the incubation period, the cells were collected and washed thrice with ice-cold PBS. Cells were washed and lysed by the cocktail containing RIPA buffer (Cell Signaling Technology, Danvers, USA), protease inhibitor and phosphatase inhibitor using ultrasonication method (Qsonica Sonicators USA) for 3 seconds. The lysates were then centrifuged at 13,000 x g at 4 °C for 10 min. The supernatant was collected and the total protein concentration was determined using BCA assay (Pierce, Rockford, IL). To confirm the mTOR protein(289kDa) levels in A549 and H460 cell lines, twenty-µg of protein was mixed with equal volumes of loading buffer (2X Laemmli sample buffer, 5% β-mercaptoethanol) and loaded in each lane and separated by an 8% SDS-polyacrylamide gel electrophoresis as described above. The electrophoresis was conducted in the running buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS). For the first 45 min, the run was conducted under 90 V then it was increased to 130 V for 90 min. The proteins were then transferred to a PVDF membrane using Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad) at 300 constant mA. The PVDF membrane was then blocked with 5% milk in Tris-
buffered saline with 0.1% Tween 20(TBS-T) for 1 h at room temperature by stirring in a gyratory shaker to prevent nonspecific binding of the antibodies.

The PVDF membrane was then incubated with primary mTOR (7C10) Rabbit antibody (1:1000 dilution Cell Signaling Technology) in 5% BSA in TBST at 4 °C overnight. The membrane was washed three times with TBST, followed by incubation with the anti-Rabbit antibody (1: 10,000 Goat anti-Rabbit IgG (H&L) Secondary Antibody) and imaged with Odyssey imaging system (LI-COR Biosciences). The membrane was washed three times with TBST and was incubated with β-Actin (13E5) Rabbit antibody (1:1000 dilution, Cell Signaling Technology) prepared in 5% BSA in TBST for 3-4 h. The membrane was then washed three times with followed by incubation with the anti-Rabbit antibody (1: 10,000 Goat anti-Rabbit IgG (H&L) Secondary Antibody) and imaged using Odyssey imaging (LI-COR Biosciences). The amount of mTOR protein silencing was calculated using the densitometric analysis (Image J software) with β-Actin as the loading control.

3.3.2.4 Serum Stability assay:

To determine the serum stability, mTOR siRNA loaded MS-MP-PG NPs were prepared by the nanoprecipitation method as described in the previous section. A volume of 800 µL of MS-MP-PG NPs (containing 2 µg of mTOR siRNA) was incubated with 800 µl volume of RPMI (10% FBS), phosphate buffered saline (PBS) (10% FBS) and Water (10% FBS). At each time interval (0 d, 1 d, 2 d, 3 d, 4 d, 5 d and 6 d), 200 µl of the mixture was removed and mixed with equal volume of water. The mixture was then used to analyze the particle size of the MS-MP-PG NPs at each separate time intervals using the Nicomp 380 ZLS particle sizer. The results are presented as mean ± SD (n=3).
3.3.2.5 NC nano-molarity and PEG surface density

The number of MS-MP-PG NPs in the 1 ml formulation was calculated using the relative viscosity measured by Ostwald viscometer according to the manufacture-supplied protocol. Briefly, 1 ml of MS-MP-PG NPs (prepared at the weight ratio of 45:1) formulation was allowed to pass through the viscometer from point A to point B and the time was recorded. Further dilutions of the NPs were made and the same procedure was repeated. The time for the NCs to pass through the viscometer is recorded as same. The unreacted PEG was separated from the PEGylated NPs by Vivaspin (cut-off molecular weight: 100 kDa) and its concentration was measured using the Iodine/barium method as described previously[373]. Briefly, the PEG containing sample and water was mixed with barium salt solution (5% w/v barium chloride in 1.0 M of hydrochloric acid) and the Iodine solution (1.27 g of iodine in 100 mL of 2% w/v potassium iodine) at the volume ratio of 8:2:1 in this sequential order specifically. The absorbance at 535 nm was recorded by the plate reader. The PEG surface density was calculated using the following equation (Equation 3).

\[
PEG \text{ surface density} = \frac{[Total \ PEG] - [Unreacted \ PEG]}{[Nanoparticle]}
\]

where brackets here denoted the molar concentration.

3.3.2.6 Expression of LHRH receptors in A549, H460, Breast adipocytes and SKOV-3 cells by western blot analysis:

The expression of LHRH receptors in A549, H460, SKOV-3 cells and breast adipocytes was determined using western blot analysis. To perform this experiment, A549, H460 and SKOV-3 cells and breast adipocytes were seeded in 6 well plates at a density of 2 x 10^5 cells/well respectively. After 72 h the cells were washed 3 times with ice-cold PBS and then trypsinized to
collect the cells. The cell pellet was then solubilized in RIPA buffer and Protease inhibitor (PI), Phosphatase inhibitor (PhI) cocktail and lysed using ultrasonication for 3 seconds in ice. The protein was collected and the total amount was calculated using BCA assay. Protein samples (40 µg/lane) was loaded into each well and electrophoresed on a 10% polyacrylamide gel. Proteins were then electroblotted onto a poly-vinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA, USA) which was probed (overnight, 4 °C) with the mouse monoclonal anti-GnRH-R antibody (Lab Vision Corporation, Fremont, CA, USA) (1:40) in TBS containing 0.02% Tween 20 (TBS-T) and 5% BSA (blocking buffer). The blot was then overlaid with the LICOR secondary antibody (Vector, Burlingame, CA, USA; 1: 10,000) for 60 min in blocking buffer at room temperature. The protein bands were detected using an enhanced chemiluminescence system (ECL, Amersham, Buckinghamshire. Membranes were reprobed with an anti-GAPDH mAB (cell signaling 1: 1000) as an internal control for protein loading. The signals were quantitated by densitometry using Image J software. The units of the protein of interest were then corrected for the densitometric units of GAPDH. The specific protein/GAPDH ratio from each treated sample was divided by the value obtained under control conditions to obtain the fold change in GnRH-R level. The data was presented as mean ±SD (n = 3).

3.3.2.7 Cellular Uptake of FS-MP-NPs by Fluorescence-activated cell sorting (FACS) analysis

To investigate the cellular uptake of FS-MP-PG-LR NPs prepared at the weight ratio of 45:1(mPAE: siRNA) and with different concentrations of surface LHRH-R peptide (1, 10, 20, and 40 µM), flow cytometry analysis was performed. To impart fluorescence to the NPs, FITC attached siRNA (FS) was used to prepare the non-targeted (FS-MP-PG-NPs) and targeted NPs (FS-MP-PG-LR-NPs) according to the procedure discussed before [371, 372]. The cellular
uptake behavior was assessed using a BD ACCURI C6 PLUS flow cytometer at a minimum of 10 x 10³ cells gated per sample. A549, H460 cells, and SKOV-3 (5 x 10³ cells/well) were seeded into 6-well plate in RPMI medium containing 10% FBS and incubated for 48 h. The FS-MP-PG-LR NPs at 4 different surface concentrations to obtain 1 µM(FS-MP-PG-LR1), 10 µM (FS-MP-PG-LR10), 20 µM (FS-MP-PG-LR20) and 40 µM (FS-MP-PG-LR) concentrations in treatment were formed according to the procedure described in above section.

When the confluence reached ~ 70-80%, the media in each well was removed and the cells were incubated with 50 µl of 4 different concentrations of NPs described above and 450 µl of RPMI media (supplemented with 10% FBS). After 4 h, the cells were washed three times with PBS and trypsinized for further processing. The cells were collected by centrifugation for 4 min at 3000 x g. The collected cells were suspended in 100 µl of PBS and measured for cellular uptake by BD ACCURI C6 PLUS flow cytometer. Analysis of cellular uptake was performed by using Flowjo 7.6.1 single cell analysis software. At least 10,000 cells were analyzed for each experiment. The flow cytometric data were presented as frequency distribution histograms. The uptake of maximum fluorescence NCs was set as 100%, and the relative uptake of different treatments was presented as a percentage. The mean cell fluorescence was normalized to that of that of naked FITC-siRNA treated cells. All experiments were repeated at least three times.

3.3.2.8 Cellular Uptake of FS-MP-NPs by Fluorescence-activated cell sorting (FACS) analysis at different time intervals (0.5 h, 2 h, and 4 h):

To investigate the effect of incubation time on cellular uptake of non-targeted and targeted NPs prepared at the weight ratio of 45:1(mPAE: siRNA) flow cytometry analysis was performed. To impart fluorescence to the NPs, FITC attached siRNA (FS) was used to prepare the non-targeted (FS-MP-PG-NPs) and targeted NPs (FS-MP-PG-LR-NPs) according to the
procedure discussed before [371, 372]. The cellular uptake behavior was assessed using a BD ACCURI C6 PLUS flow cytometer at a minimum of 10,000 cells gated per sample. A549, H460 cells, and SKOV-3 (5 × 10^3 cells/well) were seeded into 6-well plate in RPMI medium containing 10% FBS and incubated for 48 h. The non-targeted FS-MP-PG NPs and targeted FS-MP-PG-LR NPs at 20 µM (FS-MP-PG-LR20) concentrations in treatment was formed according to the procedure above and the cells were collected after the treatment as described in the previous procedures. When the confluence reached ~ 70-80%, the media in each well was removed and the cells were incubated with 50 µl of 4 different concentrations of NPs described above and 450 µl of RPMI media (supplemented with 10% FBS). After time intervals (0.5 h, 2 h, and 6 h) the cells were washed three times with PBS and trypsinized for further processing. The cells were collected by centrifugation for 4 min at 3000 x g. The collected cells were suspended in 100 µl of PBS and measured for cellular uptake by BD ACCURI C6 PLUS flow cytometer. Analysis of cellular uptake was performed by using Flowjo 7.6.1 single cell analysis software. At least 10,000 cells were analyzed for each experiment. The flow cytometric data were presented as frequency distribution histograms. The uptake of maximum fluorescence NCs was set as 100%, and the relative uptake of different treatments was presented as a percentage. The mean cell fluorescence was normalized to that of naked FITC-siRNA treated cells. All experiments were repeated at least three times.

3.3.2.9 Competitive binding inhibition Studies

A549, H460 cells were seeded at a density of 1 × 10^5 and 1.2 × 10^5 cells/well, respectively, in a 12 well plate and allowed to attach overnight. The Cells were co-incubated with 1, 10, 30 and 50 µM LHRH peptide (sequence: pGlu-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Gly-NH2), respectively for 1 h. The excess LHRH peptide was removed after 1 hour and the cells were
incubated with Fluorescent labeled siRNA-based targeted NPs (FS-MP-PG-LR20) at 60µg/ml for 1 h. After incubation time period, the cells were washed three times with PBS and trypsinized to collect the cells. The surface-bound fluorescence was quenched using 0.25 Trypan blue in PBS and washed with PBS. The cells were then analyzed for internalization using flow cytometry (BD accuri C6, BD Biosciences) as described in the previous section. Data were analyzed using Flowjo 7.6 software. The data is presented as mean ±SD (n=3).

3.3.2.10 Intracellular Trafficking of Targeted NPs

To monitor the intracellular trafficking, MS-MP-PG-LR20 NPs were incubated with A549, H460, and SKOV-3 cells for various time periods (0.5 h, 2 h, and 6h) in a CellCarrier-96 plate (PerkinElmer). A549, H460, and SKOV-3 cells were seeded at a density of 5 x 10⁵ cells per well in a CellCarrier-96 plate. After 24 h incubation, the RPMI media was removed from the cells and cells were washed several times with extracellular media. The nuclei of the cells and the lysosomes were first stained using Hoescht 33342(Thermofisher Scientific Waltham, MA USA) and with LysoTracker Red DND-99((Thermofisher Scientific Waltham, MA USA) at a concentration of 1mM and 10mM solutions prepared in extracellular fluid (10mM glucose, 10mM calcium, and 1mM magnesium). After 1 h incubation, the dye containing extracellular fluid was removed and the cells were incubated with the FS-MP-PG-LR20 NPs dispersed in an extracellular fluid. The 96-well plate was then placed inside the Operetta high content imaging system. The confocal microscopy of the live cells was recorded using Operetta high content imaging system at 40X resolution. The internal temperature of the live cell chamber was regulated and fixed to 37 °C for the duration of the experiment.
3.3.2.11 mTOR gene silencing efficiency of MS-MP-PG-LR20 NPs by western blot analysis

To evaluate the mTOR gene silencing efficiency of the mTOR siRNA loaded MS-MP-PG-LR20 NPs in A549 and H460 cells, western blot analysis was performed. To perform the experiment, A549 and H460 cells were plated into 6-well plates at $2 \times 10^5$ and $2 \times 10^5$ cells per well, respectively and allowed to grow overnight. The cells were then transfected with mTOR siRNA or scrambled siRNA (SS) using Dharmafect (Dharmacon, GE lifesciences USA) (siRNA concentration 50 nM) for 48 h. The cells were also transfected with mTOR siRNA and SS siRNA containing MP-PG-LR20 NPs (siRNA concentration 50 nM and 100 nM) for 48h. The NPs (200 µl) prepared at a weight ratio of 45:1 was mixed with 1800 µl of RPMI (supplemented with 10% FBS) and allowed to incubate with the cells for a period of 48h to achieve a final concentration of 30 µg/ml and 60 µg/ml.

The cells were collected and washed thrice with ice-cold PBS. Cells were washed and lysed by the cocktail containing RIPA buffer (Cell Signaling Technology, Denvers, USA), protease inhibitor and phosphatase inhibitor using ultrasonication method (Qsonica Sonicators USA) for 3 seconds. The western blot analysis was performed as described in the previous sections. The quantitative amount of mTOR gene silencing was calculated using the densitometric analysis (Image J software) with β-Actin as the loading control. The results are presented as mean ± SD (n = 3).

3.3.2.12 Cell Proliferation assay of targeted MS-MP-PG-LR NPs

To evaluate the mTOR siRNA effects on cell proliferation, MS-MP-PG-LR20 NPs were incubated in A549 and H460 cell lines and cell viability (%) was calculated. For this experiment, the A549 and H460 cells were plated into 96-well plates at a density $5 \times 10^3$ and $6 \times 10^3$ cells per well, respectively and allowed to grow overnight. The cells were transfected with mTOR siRNA
and SS siRNA (control) containing mPAE NPs (siRNA concentration 50 and 100 nM) for 48 h. The cells were also transfected with placebo NPs (MP-PG-LR20). The NPs (20 µl and 10 µl) prepared at a weight ratio of 45:1, was mixed with 180 µl of RPMI (supplemented with 10% FBS) and allowed to incubate with the A549 and H460 cells for a period of 48 h. The final concentration of mPAE polymer achieved on incubation of MS-MP-NPs was 30 µg/ml and 60 µg/ml to incubate 50 nM and 100 nM siRNA concentration respectively.

The cell viability (%) of the mTOR siRNA was evaluated using a modified SRB assay according to the protocol as described before[367]. The absorbance of SRB was measured at 564 nm using Synergy plate reader. The cell viability (%) was calculated according to the following formula. All data were presented as the mean ± SD (Standard Deviation) (n=3).

\[
\text{Cell viability} (\%) = \frac{\text{absorption test}}{\text{absorption control}} \times 100\%
\]

3.3.2.13 Caspase-3 activation

A549 cells (2×10^5) were seeded in 6-well plates and treated with MS-MP-PG-LR20, SS-MP-PG-LR20 and mTOR siRNA alone for 48 h. The cells were lysed with a radioimmunoprecipitation assay (RIPA) buffer that was supplemented with a phosphatase inhibitor cocktail (Promega, Madison, WI). Protein concentrations were determined using the BCA assay kit (Pierce Biotechnology) following the manufacturer’s recommendations. Forty µg protein of each sample was used to detect caspase-3 activity of the cell or tumor lysates according to the manufacturer’s instructions (EnzChek® Caspase-3 Assay Kit #1, Z-DEVD-AMC substrate, Thermofisher scientific, USA). Reactions were carried out at room temperature, and fluorescence was measured in a fluorescence microplate reader using excitation at 360 ± 20 nm with emission detection at 460 ± 20 nm after the indicated amount of time. The results are presented as mean ± SD (n = 3).
3.3.2.14 Statistical Analysis:

Results are expressed as means ± standard errors. Significant differences between two groups were determined using Student’s t-test and differences among multiple groups were determined using Analysis of Variance (ANOVA). We performed statistical analysis with GraphPad Prism Version 5 software. The differences were considered significant at a p-value <0.05 and p<0.01 was considered statistically significant in all cases.
Chapter 4: Results

Objective 1: Synthesis and Characterization of Bioreducible Poly (amino ether) polymer

4.1 Results

4.1.1 Synthesis and Characterization of thiolated polymer (mPAE)

The reaction of PAE with Traut’s reagent resulted in the successful introduction of a thiol group, to produce mPAE[364, 374]. The introduction of thiol group was confirmed by using Ellman’s reagent. The Ellman’s reagent based test is based on the reaction of the chromogenic reagent with free thiols group present in the polymer to produce one molecule of 5-thio-2-nitrobenzoic acid. (TNB) as shown in Fig 4.1 [375]. The quantitative estimation of the free thiol group in the mPAE polymer was further estimated. The concentration of thiol group was found to be equivalent to 0.5mM (cysteine thiol group) compared to the 0.045 mM in PAE. In this case, the reaction of the mPAE free thiol with the highly oxidizing disulfide bond of DTNB resulted in the release of one molecule of TNB. In particular, for the presence of one free thiol group, there is a release of one TNB group, which can be detected spectrophotometrically. Thus, in conclusion, the results suggest that the reaction of PAE with the excess of Traut’s reagent resulted in the successful formation of modified PAE(mPAE) with significantly higher(***P<0.001) thiol groups Fig 4.2 than PAE polymer alone. Furthermore, the mPAE can easily form NPs (NPs) encapsulating the siRNA and get oxidized on the surface to form a disulfide cross-linked delivery system, which can release the contents inside the cytoplasm upon degradation by excess GSH.
Figure 4.1: Schematic of Ellman’s reagent reaction. The Reaction of DTNB (Ellman’s reagent) with the free thiols group resulting in the formation of 5-thio-2-nitrobenzoic acid (TNB) which can be detected spectrophotometrically at 412 nm. (Adopted with permission from reference[376]).

Figure 4.2: Comparison of thiol group concentration in polymers PAE and mPAE. Estimation of total thiol groups in PAE and mPAE was performed using the Ellman’s reagent. The absorbance was measured spectrophotometrically at 412 nm using a UV spectrophotometer. All data were presented as the mean (n=3) ± SD (Standard Deviation). (***p<0.001).
4.1.2 Cytotoxicity of Polymers

A549 and H460 cells were seeded and transfected with mPAE and PEI polymers as described in the experiment Section. Cytotoxicity is an important parameter to evaluate the delivery vehicle for safe gene delivery[377]. To ensure the safety of the polymeric vehicles being used in this study, the cytotoxicity of mPAE and 25kDa PEI was investigated in A549 and H460 cells using SRB assay. As shown in Fig. 4.3 mPAE and PEI exhibited cytotoxicity for both A549 and H460 lung cancer cells in a dose-dependent way. Fundamentally, with the increase of mPAE and PEI concentration, the cytotoxicity increased accordingly in all tested cell lines. Compared with 25-kD PEI, 5, 10 and 25 µg/ml, mPAE had no apparent cytotoxicity on A549 and H460 cell lines. The lethal dose 50 (LD50) of the two polymers are summarized in Table 4.1. The Lethal Dose 50 (LD50) of the PEI was around 30 ± 5.4 µg/ml and 25 ±5.2 µg/ml for A549 and H460 cells respectively which was lower (2.33 times and 2.6 times) than the LD50 for the mPAE polymer (75 ± 6.2 µg/ml and 70 ± 3.4 µg/ml for A549 and H460 cells respectively). This suggests that on a weight basis the mPAE polymer possesses less cytotoxicity compared to the PEI polymer which can be exploited to safely deliver genes to lung cancer cells.
Figure 4.3: Cytotoxicity analysis of mPAE and PEI polymers. (A and B) Cell viability (%) of the “mPAE and PEI” polymers at various log concentrations (µg/ml) in A549 and H460 cell lines (C and D) Cell viability (%) after treatment of mPAE and PEI at concentrations (2.5, 5, 10, 25, 50, 100 and 200 µg/ml) in A549 and H460 cell lines. The cytotoxicity was determined using SRB assay after 48 h polymer exposure (n=3, error bars represent standard deviation). (**p<0.01, *p<0.05).

Table 4.1 – LD₅₀ Values of PEI and mPAE polymers in A549 and H460 cells (n=3, ± represent standard deviation).

<table>
<thead>
<tr>
<th>polymers</th>
<th>A549 cells – LD5₀ (µg/ml)</th>
<th>H460 cells – LD 5₀ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEI</td>
<td>30 ± 5.4</td>
<td>25 ± 5.2</td>
</tr>
<tr>
<td>mPAE</td>
<td>70 ± 6.2</td>
<td>65 ± 3.4</td>
</tr>
</tbody>
</table>
Figure 4.4: Determination of the buffering capacity of mPAE, PEI (Mw =25,000 g/mol) and NaCl by acid-base titration.

Note: The solution containing the polymer were dissolved at a concentration of 1.5 mM of protonable nitrogens in 150 mM NaCl, was adjusted to pH 4.5 using HCl, and then titrated with 0.01M NaOH from 4.5 to 7.5(n=3). The buffering capacity of mPAE was shown to be superior to PEI 25K alone.

**Abbreviations:** mPAE- modified polyaminoether, PEI, Polyethyleneimine and NaCl, Sodium Chloride.

### 4.1.3 Buffering Capacity

The buffering capacity of the cationic polymer has been shown to be responsible for the release of siRNA encapsulated NPs from the endosome. To evaluate the potential of mPAE polymer to escape endosome via proton sponge effect, buffering capacity of mPAE and PEI polymers was assessed by measuring the change in pH of the suspension upon addition of 0.01M NaOH. From the titration graph **Fig. 4.4**, it was observed that there was a small change in pH in mPAE curve compared to PEI curve on the addition of the same amount of 0.01M NaOH to the solutions. A high amount of proton required to change the pH of the mPAE solution compared to PEI solution indicates that the mPAE polymer had the strong buffering capacity and high
tendency to be protonated compared to PEI[378]. A polymer with high buffering capacity would undergo a small change in pH when the same amount of NaOH was added to the polymer solution during titration. This further demonstrates that the mPAE would be able to undergo proton sponge effect upon internalization in the acidic compartments of the cells and release the siRNA in the cytoplasm.

Objective 2: Formulation and Characterization of Bioreducible NPs for mTOR siRNA delivery to the Non-small cell Lung cancer cells.

4.2 Results:

4.2.1 MS-MP-NP encapsulated mTOR siRNA at polymer: siRNA weight ratios of 25:1 and higher

We investigated the ability of the mPAE polymer to form NPs with mTOR siRNA as a function of weight ratio (w/w) of polymer weight to mTOR siRNA by gel electrophoresis method. The NPs were prepared by the nanoprecipitation method as described in the above section. We performed a gel retention assay at weight ratios of 1:1 – 300:1 of mPAE polymer to mTOR siRNA. As shown in Fig. 4.5 (A) Lane 4, the mTOR siRNA band is seen to disappear in the gel as the polymer to mTOR siRNA w/w ratio increases from 25:1 and higher, indicating that the mTOR siRNA was largely encapsulated within the MP-NP and was unable to flow through the pores of the gel (compare lanes 5–7: MS-MP-NP formed at w/w ratio of 100:1–300:1, with lane 2-4: MS-MP-NP formed at w/w ratio of 1:1–25:1). The entrapment efficiency calculated by image J software of the mPAE NPs to mTOR siRNA is seen to be below (< 60%) of the polymer to siRNA w/w ratio of 25:1 Fig. 4.5(B). However, at weight ratios higher than 100:1, the mTOR siRNA is completely encapsulated in the NPs and 100% entrapment is achieved.
Figure 4.5: (A) Gel electrophoresis of mPAE-siRNA NPs at different w/w ratios. Notes: Lane 1: naked siRNA; Lane 2-7 mPAE to siRNA w/w ratio (1:1 – 300:1)
Samples were prepared at room temperature before being loaded onto a 1.0% w/v agarose gel (75 V, 45 min). (B) Densitometric analysis of the siRNA bands performed using Image J software.

**Abbreviations:** mPAE-siRNA, modified polyaminoether – mTOR siRNA NPs; w/w, the weight ratio between of mPAE polymer and mTOR siRNA.
The experiments were repeated at least thrice. The best representative images are shown. (n=3, error bars represent standard deviation).

4.2.2 MS-MP-NP protected mTOR siRNA from nuclease degradation as well as mediated enhanced release in GSH:

To achieve maximum delivery efficiency, the NP carrier must protect siRNA from degradation by nucleases present in the biological systems. To evaluate the serum protection property of mPAE polymer, serum stability test was carried out for MP-NP in 10% FBS Fig. 4.6(A). For this experiment, MS-MP NPs prepared at the weight ratio of 45:1 (mPAE: mTOR siRNA) was used. As can be seen in lane 2, the presence of RNAses in 10% FBS resulted in nuclease-mediated degradation of the siRNA and thus disappearance of the siRNA band. In
comparison to naked siRNA, lane 5 shows that MS-MP-NP effectively protected siRNA from nuclease degradation as a brighter and dense band was observed in the NP well. mTOR siRNA encapsulated in the crosslinked NPs remained intact upon the treatment of RNase compared with the naked siRNA, indicating that NPs was able to prevent nucleolytic degradation. The NPs was able to protect the siRNA from the RNAse degradation as can be seen in the lane 6 with lower intensity siRNA band. The GSH mediated cleavage of the siRNA resulted in the release of the protected siRNA from the MS-MP NPs. The MS-MP NP was incubated with GSH final concentration (10 mM) to verify intracellular release of mTOR siRNA by agarose gel electrophoresis. As shown in Fig. 4.6 (B) (Lane -7) around 55 ± 3.6 % of the siRNA release can be observed at elevated GSH concentrations of 10 mM. This result suggests that bio-reducible NPs can protect the siRNA against nuclease degradation as well as reductively disassemble by GSH to release the mTOR siRNA. The Lane 8 further shows that the MP-NP were able to protect the mTOR siRNA from degradation by nucleases and around 25% of mTOR siRNA was released from the NPs. The bands were not as intense as the non-treated free siRNA (control) because some siRNA might remain within the NPs (could not be stained by ethidium bromide) or be degraded in small quantity.
Figure 4.6: Serum Stability and GSH mediated siRNA release from MS-MP NPs using gel retardation assay. (A) Gel electrophoresis of MS-MP NPs prepared at weight ratio 45:1 with (mPAE: mTOR siRNA). The NPs were incubated with 10%FBS containing RNAse and GSH 10mM for a period of 4h at 37°C. Samples were prepared at room temperature before being loaded onto a 1% w/v agarose gel (75 V, 45 min). (B) Densitometric analysis of the siRNA band using Image J analysis software.

**Abbreviations:** RNAse – 10%FBS containing RNAse, GSH – Glutathione, MS-MP -NPs – mTOR siRNA – mPAE NPs.

The experiments were repeated at least thrice. The best representative images are shown. (n=3, error bars represent standard deviation).

### 4.2.3 MS-MP NPs particle size and zeta potential measurement

It is essential to optimize the physicochemical properties (particle size and surface charge) of MS-MP NPs to achieve parameters that ensure high efficiency for mTOR siRNA delivery to NSCLC cells. Therefore, particle size and zeta potential of the MS-MP-NPs were studied. Based on the gel retardation assay studies, three different weight ratios of mPAE to...
mTOR siRNA-based MS-MP-NPs were investigated at constant a siRNA concentration. As shown in Table 4.2, the sizes of the NPs were found to be between 112 nm and 122 nm at the various polymers to siRNA ratios ranging from 30:1 to 60:1. The size of the NPs decreased steadily from 112 nm to 122 nm with increasing ratio from 30:1 to 60:1. This result is in good agreement with previous reports [379-381]. In addition, the relative homogeneous size distributions of MS-MP NPs are unimodal with a polydispersity index of 0.35 for mPAE: mTOR siRNA ratio of 45:1 as shown in Fig. 4.7 (A). The surface charge of the NPs was also studied at various weight ratios (Table 4.2). The zeta potential for all the NPs remained in the range of +26.5 - +32.2 mV except for the ratio of 30:1, where stable NPs of mTOR siRNA and mPAE NPs were not formed completely.

The concentration of mPAE polymer in MS-MP NPs also influenced the surface charge of NPs (Table 3.1). Increasing the concentration of mPAE in NPs increased the zeta potential of NPs possibly because of the cationic charge of mPAE. The relative surface charge of the MS-MP-NPs (45:1 ratio) also showed a unimodal distribution as shown in Fig. 4.7. For enhanced cellular uptake of mTOR loaded mPAE NPs, positive surface charge and small particle size (<150 nm) is necessary for reaching and binding to anionic cells surface of the NSCLC cells. We believe that the nano-sized positively charged MS-MP-NPs has a potential as an efficient mTOR siRNA delivery carrier.
Table 4.2: Characterization of the MS-MP-NPs. Particle Size, Zeta Potential, Entrapment efficiency (%) and Loading capacity (%) of MS-MP NPs at different mPAE: siRNA weight ratio (30:1, 45:1 and 60:1). The results are presented as mean ± SD (n = 3).

<table>
<thead>
<tr>
<th>mPAE: siRNA ratio</th>
<th>Particle Size (nm)</th>
<th>Zeta Potential (mV)</th>
<th>Entrapment Efficiency (%)</th>
<th>Loading Capacity (%)</th>
<th>mTOR siRNA loading (µg/mg NPs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30:1</td>
<td>112.9 ± 8.2</td>
<td>-2.8 ± 1.5</td>
<td>55.5 ± 2.8</td>
<td>1.83 ± 0.86</td>
<td>18.3 ± 8.6</td>
</tr>
<tr>
<td>45:1</td>
<td>114.2 ± 7.4</td>
<td>+26.5 ± 5.3</td>
<td>99.9 ± 3.5</td>
<td>2.25 ± 0.34</td>
<td>22.2 ± 3.4</td>
</tr>
<tr>
<td>60:1</td>
<td>121.5 ± 6.5</td>
<td>+32.2 ± 4.3</td>
<td>100 ± 4.2</td>
<td>1.66 ± 0.54</td>
<td>16.7 ± 5.4</td>
</tr>
</tbody>
</table>
Figure 4.7: Physicochemical properties of mTOR siRNA -mPAE based NPs (MS-MP NPs). (A) Distribution of particle size and (B) Zeta potential (surface charge) of MS-MP-NPs at weight ratio 45:1 (mPAE:mTOR-siRNA). For particle size measurements, 200 µl of NP suspension was mixed with equal volume of water and size was measured using NICOMP particle sizer. For zeta potential measurement 200 µl of NP suspension was mixed with 1800 µl of water and surface charge was measured. The experiments were repeated at least thrice. The best representative images are shown. (n=3).
4.2.4 MS-MP NPs entrapment efficiency (%) and loading capacity (%)

Based on the results obtained from the gel retardation assay, three different w/w ratio of MS-MP-NPs were prepared (30:1, 45:1 and 60:1) and their entrapment efficiency (%) and loading capacity (%) was calculated. The free siRNA was separated from the NP suspension by centrifugation using vivaspin column (MWCO size 50kDa). The concentrations of siRNA were then measured using AccuBlue high sensitivity dsDNA quantification kit by plotting a standard curve of mTOR siRNA at 0.65, 6.5 13, 26, 65 and 130 ng/µl as shown in Fig. 4.8. The results of the entrapment efficiency (%) are shown in Table 4.2. For MS-MP -NPs, we achieved the mTOR- siRNA entrapment efficiency in the range of 55.5% and 100%. In general, the entrapment efficiency decreased with a decrease in polymer weight ratio from 30:1 to 60:1 as shown in Table 4.2. The higher concentrations of cationic polymer mPAE allowed better binding with the negatively charged mTOR siRNA resulting in almost 100% entrapment efficiencies for ratios 45:1 and higher. On the basis of entrapment efficiency loading capacity (%) was also calculated as shown in Table 4.2. The most optimum loading capacity was found for the polymer: mTOR siRNA weight ratio of 45:1 that indicates the entrapment of 2.2 µg of siRNA per 100 µg of polymer (2.2%). Thus, 45:1 polymer to mTOR siRNA weight ratio was selected for future experiments.
Figure 4.8: mTOR siRNA calibration curve. Calibration curve for gradient concentration of mTOR siRNA within the range of 0.65 – 130 ng/µl calculated using Accublue high sensitivity quantitation kit according to the manufacturer’s protocol. A typical linear relationship ($R^2 = 0.9979$) was found between peak height and concentration of mTOR siRNA. The results are presented as mean ± SD (n = 3).

4.2.5 Accelerated mTOR siRNA release in presence of excess of GSH

The *in vitro* release profiles of siRNA-loaded MS-MP-NPs were investigated for 7 d in PBS at pH 7.4 as a function of GSH concentration. GSH is an intracellular reducing agent that is present in higher concentration inside the cells(2 mM - 10 mM) and rarely present in the extracellular environment(2 µM – 20 µM)[82]. As can be seen from the Fig 4.9 (A), the release of siRNA can be divided into 2 stages on the basis of release rate, which was calculated as the slope of the release profile. In the first stage, siRNA-loaded MS-MP-NPs showed rapid release in the presence of excess GSH (10mM). Also at this stage, the siRNA was released at a slower rate in lower GSH concentration (10µM) at day 1 Fig. 4.9 (A). There was a significant increase in the release of siRNA due to the presence of excess GSH (10 mM) compared to lower GSH.
concentration (10 µM) (p < 0.001). The second stage can be seen of as similar in both GSH concentrations with a steady release of siRNA from the NPs Fig. 4.9 (B). The initial burst release of siRNA from MS-MP-NPs in PBS pH 7.4 10mM GSH was expected due to the cleavage of disulfide bonds present in the MS-MP-NPs compared to less or almost no cleavage of disulfide bonds in the MS-MP-NPs in presence of lower concentration of GSH (10 µM). In the second stage, siRNA was released at a sustained constant rate from MS-MP-NPs for up to 7 days. The sustained slow release of siRNA from the NPs at both conditions after day 1 can be attributed to the ionic interactions between the siRNA and the cationic polymer (mPAE) NPs.

**Figure 4.9:** *In vitro* release of siRNA from the NPs. Profile of siRNA release from MS-MP-NPs prepared at a weight ratio of 45:1. Initial burst release effect followed by sustained release can be seen for MS-MP NPs in presence of 10mM GSH. The results are presented as mean ± SD (n = 3). (***p<0.001)

### 4.2.6 MS-MP-NPs induced cell growth inhibition at 50nM concentration in NSCLC cell lines

It has been reported that mTOR siRNA-inhibition by siRNA induces cell apoptosis and inhibits cell proliferation [302, 382]. We performed cell growth inhibition studies to evaluate cell growth inhibitory property of the MS-MP-NPs in A549 and H460 cells. Fig. 4.10 (B) and (C) shows the cell viability (%) of MS-MP-NPs over the negative scrambled siRNA control based NPs (SS-MP NPs) and placebo NPs in both A549 and H460 cells. The cell viability (%) with 50 nM of siRNA concentrations of MS-MP-NPs was significantly lower (*p<0.05) compared to
both SS-MP-NPs and placebo NPs alone in A549 and H460 cells. The 100 nM siRNA concentration of MS-MP-NPs also induced significant cell growth inhibition compared to the controls. However, the placebo NPs and SS-MP NPs were cytotoxic to the cells and resulted in significant toxicity (*p<0.05) to the cells themselves. Compared to the cell growth inhibition caused by 50 nM of Dharmafect mTOR siRNA 36% and 42% (A549 and H460 cells respectively) [Fig 4.10 (A) and (D)], the 50 nM concentration MS-MP NPs resulted in cell growth inhibition of 31% and 32% after 48 h treatment in A549 and H460 cells, respectively. These results demonstrate that the MS-MP NPs induced inhibition was significantly larger than the inhibition observed in SS-MP NPs and placebo control treated A549 and H460 cells.

![Figure 4.10](image-url): Cell viability (%) of mTOR siRNA with commercial transfection reagent Dharmafect (DF) and mPAE based NPs in A549 cells (A and B) and H460 cells (C and D) seeded at density of (5 × 10^3 cell/well and 6 x 10^4 cells / well for A549 and H460 cells respectively) at 48 h incubation. MS-MP-NPs and SS-MP-NPs prepared at 45:1 weight ratios were incubated with cells at a concentration of 30 µg/ml and 60 µg/ml of mPAE polymer to achieve 50 nM and 100 nM siRNA concentrations, respectively. Figure (A) and (C) indicates the cell viability (%) of the A549 and H460 cells after transfection with scrambled siRNA and mTOR siRNA using DF reagent. Figure (B) and (D) indicates the cell viability (%) of the
scrambled siRNA and mTOR siRNA using mPAE NPs in A549 and H460 cells. The results are presented as mean ± SD (n = 3). A value of p less than 0.05 was accepted to be significant (**p<0.01, *p<0.05) and the statistics were performed with student’s t-test.

4.2.7 The MS-MP-NPs showed increased cellular uptake in A549 and H460 cells

Usually, gene delivery vehicles are encountered with low transfection efficiency, mainly due to the lower cellular uptake of the vehicles [41]. Hence, the efficacy of cellular uptake of the vehicles was determined by flow cytometry experiment. The MS-MP NPs were prepared by the nanoprecipitation method as described above at a weight ratio of 45:1 (mPAE: mTOR siRNA). The average size of the NPs was about 114 nm and zeta potential was around +26 ± 4.2 mV. The MS-MP-NPs was conjugated with FITC to verify the cellular uptake ability of the NPs after incubation with cells at various time periods (0.5 h, 2 h, and 24 h). As shown in Fig. 4.11 (A and B), the cellular uptake efficiency of MS-MP-NPs at a fixed mTOR siRNA concentration (equivalent to 50 nM mTOR siRNA) was time dependent in A549, and H460 cells. Fig. 4.11 C shows relative cellular uptake of the cells that have taken up NPs and is an indirect measure of the intracellular amount of siRNA. The mTOR siRNA encapsulated MS-MP NPs showed quantified cellular uptake (%) 34.18 ± 3.2(0.5 h), 49.6 ± 3.6(2 h), 95.44 ± 7.6(24 h) and 40.42 ± 45(0.5 h), 54.88 ± 2.98(2 h), 100.15 ± 4.77(24 h) in A549 and H460 cells, respectively. Nearly 50% of the NPs were inside the A549 and H460 cells after 2 h of transfection with NPs. As mentioned before, the MS-MP NPs are nano-sized and possess positive charge which enables the NPs to get entry into the cells. The NPs did not show any significant (p>0.05) increase in the cellular uptake upon further incubation at 36 h (result not shown here).
Figure 4.11: Cellular uptake analysis of MS-MP-NPs by FACs. MS-MP NPs were formed at a weight ratio of 45:1 siRNA (1.33 μg). FITC was conjugated to the surface of NPs using a one-step reaction before the flow cytometry experiment to form MS-MP-FITC-NPs. After certain time intervals (0.5 h, 2 h, and 24 h) the cells were washed trypsinized and collected to perform the flow cytometry experiment. The fluorescence intensity of MS-MP-FITC-NPs in (A) A549, (B) H460 cells (2 x 10^5 cell/well). (C) Bar graph representing the mean percentages of cellular uptake. The results are presented as mean ± SD (n=3). Analysis of cellular uptake was performed by using Flowjo 7.6.1 single cell analysis software.
4.2.8 MS-MP NPs demonstrate high mTOR gene silencing efficiency in human NSCLC cells

We investigated the mTOR silencing efficiency of MS-MP NP (using 50 nM siRNA) in human lung cancer cell lines A549 and H460 cells. The mTOR gene silencing ability was measured at the fixed weight ratio of 45:1 (mPAE: mTOR siRNA) The scrambled siRNA-based mPAE NPs was also prepared as the control. As shown in Fig. 4.12 (A and C) the negative control (SS-MP NPs) did not show gene silencing effects in the A549 and H460 cell lines. Meanwhile, the expression of the mTOR protein was significantly decreased after treatment with MS-MP-NPs in both A549 and H460 cells (**p<0.01) Fig. 4.12 (A) and (C). Representative bands show the expression of mTOR protein (upper band) β-actin (lower bands). As shown in Fig. 4.12 (B) and (D) the relative mTOR protein expression expressed as histograms (%) in MS-MP NPs treated A549 and H460 cell lines (normalized to the β-actin expression) was significantly less (~60% and 64% mTOR knockdown) than SS-MP NPs (*p<0.05). We found that the mTOR siRNA loaded NPs efficiently silenced the mTOR gene by using 50 nM siRNA concentrations compared to using Dharmafect (Transfection reagent) at 50 nM siRNA.
Figure 4.12: Silencing efficiency of MS-DF transfection reagent and MS-MP NPs at 50 nM concentration. Expression of mTOR protein determined using Western blot in A549 and H460 cells (2 × 10^5 cell/well). MS-MP NPs/mTOR siRNA and SS-MP NPS/scrambled siRNA prepared at weight ratio 45:1 (siRNA concentration 50 nM or 1.33µg per well) (A) and (C) Western blot showed a marked reduction in the mTOR protein levels after treatment with MS-MP NPS at 50nM concentrations. (B) and (D) mTOR protein silencing was calculated using the densitometric analysis and β-Actin as the loading control. The results are presented as mean ± SD (n=3). (* p < 0.05, ** p < 0.01)

Objective 3: LHRH Receptor -Targeted Bioreducible NPs for mTOR siRNA Delivery to Lung Cancer cells

4.3 Results

4.3.1 PEGylation reduced the cytotoxicity of the cationic mPAE based NPs in A549 and H460 cells

To evaluate the cytotoxic effects of the non-targeted MP-NP at three different concentrations (equivalent to transfect 25, 50 and 100 nM of mTOR siRNA), *in vitro*
cytotoxicity assay was performed in A549 and H460 cells. As shown in Fig. 4.13(A and B), the cell viability (%) of non-targeted MP-NP formulation in three different concentrations of 15 µg/ml, 30 µg/ml and 60 µg/ml (93.33 ± 7.6%, 83.7 ± 8.2%, and 65.4 ± 10.4%) and (94.33 ± 6.60%, 82.33 ± 13.60%, and 60.81 ± 8.59%) in A549 and H460 cells respectively. The data shows that at higher concentration of 60 µg/ml MP-NP resulted in significant cytotoxicity(**p<0.01) to the A549 and H460 cells Fig. 4.13(A and B) The cell viability (%) of the cells (compared to untreated control) after treatment with 60 µg/ml MP-NP reduced to 65.4 ±10.4% and 60.81 ± 8.59% in A549 and H460 cells respectively. Also to evaluate the effects of PEGylation on the NPs two different PEGylated and PEGylated targeted formulations were also prepared and their cytotoxic effects were evaluated in A549 and H460 cells. In contrast to MP-NP formulations, the non-targeted PEGylated (MP-PG-NP) and PEGylated targeted formulations (MP-PG-LR-NP) did not show significant reductions in the cytotoxicity at 60 µg/ml in both cell lines Fig. 4.2(A and B). The cell viability (%) of the cells after treatment with 60 µg/ml of MP-PG-NP and MP-PG-LR-NP was found to be (93.33 ± 9.6% and 91.44 ± 3.4%) and (92.5 ± 11.5% and 90.2 ± 8.0%) for A549 and H460 cells respectively indicating reduced toxicity with PEGylation of the NPs.
Figure 4.13: *In vitro* Cytotoxicity of MP-NP, MP-PG-NP and MP-PG-LR-NP treated A549 and H460 cells. Cell viability (%) of the three formulations at various concentrations (15μg/ml, 30 μg/ml, and 60 μg/ml) in A549 (A) and H460 (B) cell lines following incubation for a period of 48h. Following incubation, the cytotoxicity was determined using SRB assay. The MP-NP induces significant cytotoxicity at the concentration of (60 μg/ml) untreated control cells. The PEGylated and PEGylated targeted NPs did not show significant cytotoxicity at higher concentration (60 μg/ml). The results are presented as mean ± SD (n = 3). (**p<0.01) (ns = statistically non-significant).
4.3.2 The PEGylated MP-PG NPs and targeted MP-PG-LR NPs did not induce mTOR gene silencing at higher toxic concentrations

To evaluate the mTOR gene silencing efficiency of the non-targeted MP-NP, MP-PG-NP(PEGylated) and MP-PG-LR-NP (PEGylated-LHRH targeted) at different concentrations (15 µg/ml, 30 µg/ml, and 60 µg/ml) in A549 and H460 cells, western blot analysis was performed after a treatment for a period of 48 h. The different concentrations (15, 30 and 60 µg/ml) refer to the concentrations of bioreducible polymer mPAE in the wells. Western blot analysis showed that non-targeted NPs did not cause any significant reduction in the mTOR protein level in both A549 and H460 cells, Fig. 4.14 (A, B, C, and D) also shows that the PEGylated NPs(MP-PG-NP) and the PEGylated targeted NPs(MP-PG-LR-NP) also did not cause any significant reduction in the mTOR protein level in both NSCLC cell lines. These results confirm that the cytotoxic effects of the non-PEGylated mPAE polymer based NPs are through some other mechanisms.

Figure 4.14: Gene Silencing efficiency of MP-NP, MP-PG-NP, and MP-PG-LR-NP. (A) and (C) Expression of mTOR protein determined using Western blot in A549 and H460 cells (2× 10⁵
The 3 formulations were incubated with A549 and H460 cells at 3 different concentrations (15 µg/ml, 30 µg/ml, and 60µg/ml). After 48 hours, the cells were harvested and mTOR protein level was determined by Western blot analysis. Western blot showed no marked reduction in the mTOR protein levels after treatment with the three formulations at 3 different concentrations. (B) and (D) mTOR protein silencing was calculated using the densitometric analysis and β-Actin as the loading control. Data represent relative mTOR protein expression obtained after the normalization with β-actin protein expression. The results are presented as mean ± SD (n = 3). The acronym “ns” indicates statistically insignificant differences between the values compared to the control untreated cells.

4.3.3 LHRH-R is overexpressed in NSCLC cell lines (A549 and H460) compared to the SKOV-3 and Breast adipocytes.

To compare the expression of LHRH receptors in NSCLC cell lines (A549 and H460 cells) and breast adipocytes, western blot analysis was performed. We also compared the expression of LHRH receptors in NSCLC cell lines (A549 and H460) and ovarian cancer cell line (SKOV-3) cells. The densitometry analysis shows that there is a nearly 5.5 fold and 6.5 fold times overexpression of LHRH receptor in A549 and H460 compared to the Breast Adipocytes Fig. 4.15. As shown in Fig. 4.16, the LHRH-R was found to be significantly (***p<0.001) overexpressed in A549 and H460 cells compared to the ovarian cancer cell line SKOV-3. The densitometry analysis shows that there is a nearly 4 fold and 4.5 fold times overexpression of LHRH-R in A549 and H460 cells compared to the SKOV-3 cells.
Figure 4.15: Western blot analysis of LHRH-R (A) Western blot analysis of LHRH-R in A549, H460 cells and Breast adipocytes (minimal LHRH-R expression). Expression of LHRH-R protein determined using Western blot performed on protein lysates of A549, H460 cells and Breast adipocytes (2×10^5 cell/well). (B) and (C) LHRH-R protein silencing was calculated using the densitometric analysis and GAPDH as the loading control. Western blot showed a marked reduced LHRH-R protein level in Breast Adipocytes compared to A549 and H460 cells. The results are presented as mean ± SD (n = 3). (***) p < 0.001.
Figure 4.16 Western blot analysis of LHRH-R (A) Western blot analysis of LHRH-R in A549, H460, and SKOV-3 (minimal LHRH-R expression). Expression of LHRH-R protein determined using Western blot performed on protein lysates of A549, H460 cells and SKOV-3 (2 × 10^5 cell/well). (B) and (C) LHRH-R protein silencing was calculated using the densitometry analysis and GAPDH as the loading control. Western blot showed a marked reduced LHRH-R protein level in SKOV-3 compared to A549 and H460 cells. The results are presented as mean ± SD (n = 3). (*** p < 0.001).

4.3.4 The PEGylated MS-MP NPs were found to be stable in serum conditions

The systemic administration of siRNA loaded NPs exposes them to physiological ionic strengths and anionically charged proteins (albumin, globulin etc.) which can induce aggregation and destabilization of the NPs[383]. To investigate whether PEGylation prevents NPs aggregation at physiological salt concentrations, as well as protein concentrations, pre-formed
MS-MP-PG-NPs were incubated in RPMI (10% FBS), PBS (10% FBS) and water (10% FBS) and size measurements were performed using dynamic light scattering. As shown in Fig. 4.17, we found that the 22.5% wt PEG attached to NPs (Table 4.3) resulted in the only moderate increase in the particle size of the NPs. In PBS (10% FBS) the particle size increased from 124 nm to around 220 nm over a period of 6 d. However, in the case of RPMI and water with 20%FBS, the particle size only increased steadily to around 190 nm indicating no aggregation in the NPs.

![Figure 4.17: Serum Stability of PEGylated MS-MP-PG NPs for 6 days.](image)

**Figure 4.17: Serum Stability of PEGylated MS-MP-PG NPs for 6 days.** The NPs were prepared at a weight ratio of 45:1 using nanoprecipitation method. For measurement, 300 µL of PEGylated NPs was incubated with 1200 µL of RPMI, PBS, and water supplemented with 20% FBS. After the time intervals (0-6 days) 200 µl of the suspension was withdrawn and mixed with equal amounts of water and particle size measurement was performed using Nicomp 380 ZLS particle sizer. (n = 3).
Table 4.3 – Physicochemical characteristics of the PEG surface coated mTOR-siRNA encapsulated mPAE NPs prepared at a weight ratio of 45:1 using nanoprecipitation method

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Observation</th>
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<tbody>
<tr>
<td>Molarity of NP</td>
<td>1.2 µM</td>
</tr>
<tr>
<td>PEG</td>
<td>700 µM</td>
</tr>
<tr>
<td>Peg per particle</td>
<td>4.7x10^4 nm²</td>
</tr>
<tr>
<td>NC surface area</td>
<td>7.3 x 10^4 nm²</td>
</tr>
<tr>
<td>PEG density</td>
<td>0.64/ nm²</td>
</tr>
<tr>
<td>Surface PEG content (wt%)</td>
<td>22.5</td>
</tr>
</tbody>
</table>

Recent studies have shown, coating the NPs with PEG to shield the surface can improve stability[384], prolong blood circulation half-life[385, 386] and reduce interactions with the contents in the biological system[387, 388]. Thus, we have attached the PEG (5 kDa) on the surface of the NPs to impart stability and reduce interactions. We aimed to coat the NPs with around 30% of the weight of the total mPAE polymer concentrations as observed effective in the literature for effective shielding of NPs and reduce cytotoxicity of the cationic polymers. As shown in Table 4.3, we were able to achieve high surface PEG density 0.64/nm² on the NPs surface which is essential to achieve brush configuration for NP stability. Also, the surface PEG content of 22.5% was achieved with the PEG coating. Thus our method of attaching PEG via a chemical cross-linking process allowed most of the PEG to coat on the surface and especially in a dense bush configuration. The characterization of the NPs is shown below in Table 4.4 and Fig. 4.18.

Table 4.4: Characterization of the NPs. Particle Size, Zeta Potential and PDI of MS-MP-PG and MS-MP-PG-LR20 NPs at polymer to siRNA weight ratio of (45:1)

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Particle size</th>
<th>Zeta Potential</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS-MP-PG</td>
<td>124 ± 5.9</td>
<td>19.5 ± 4.6</td>
<td>0.37</td>
</tr>
<tr>
<td>MS-MP-PG-LR20</td>
<td>132 ± 6.8</td>
<td>20.2 ± 5.4</td>
<td>0.32</td>
</tr>
</tbody>
</table>
4.3.5 The targeted MS-MP-PG-LR NPs selectively internalizes in A549 and H460 cells

The optimum amount of surface peptide determines the maximum cellular uptake of gene delivery vehicle. [41]. Hence, to determine the optimum concentration of peptide attached NPs required to obtain maximum cellular uptake, flow cytometry experiment was performed. The FS-MP-PG-NPs and FS-MP-PG-LR-NPs (1 µM, 10 µM, 20 µM and 40µM) were prepared by the nanoprecipitation method as described above at a weight ratio of 45:1 (mPAE: mTOR-siRNA). Cellular uptake of NPs was evaluated by using flow cytometry in A549, H460, and SKOV-3 cell lines. As shown in Fig. 4.19(A and B), the cellular uptake efficiency of FS-MP-PG-LR20 NPs was significantly higher than the non-targeted FS-MP-PG NPs in A549 cells ($p<0.001$) in A549 cells. Similar results were obtained for H460 cells, where FS-MP-PG-LR20 NPs showed significantly higher cellular uptake compared to the non-targeted FS-MP-PG NPs Fig. 4.19(C and D). We also found no significant difference between the cellular uptake in FS-MP-PG-LR20 NPs and FS-MP-PG-LR40 NPs, indicating 20 µM concentration of LHRH-R peptide is sufficient for optimum cellular uptake of NPs Fig. 4.19(A, B, C, and D).
significant difference in cellular uptake of FS-MP-PG NPs and FS-MP-PG-LR1 NPs, which indicates that 1 µM concentrations of LHRH-R peptide don’t facilitate in cellular uptake through endocytosis in A549 and H460 cells Fig 4.19 (A, B, C, and D).

As shown in Fig. 4.19 (E and F), we also compared the cellular uptake of non-targeted and targeted NPs in LHRH-R lower level expressing SKOV-3 cell lines. We found that there was no significant difference in cellular uptake of FS-MP-PGNPs and FS-MP-PG-LR20 NPs in the SKOV-3 cell lines. This also confirms that LHRH receptor is an essential property for the effective internalization of the targeted (FS-MP-PG-LR20) NPs.
Figure 4.19: Cell binding and uptake analysis of FS-MP-PG NPs and FS-MP-PG-LR NPs (1, 10, 20 and 40µM) by FACs. Targeted FS-MP-PG-LR NPs were formed at a weight ratio of 45:1 siRNA. FITC labeled siRNA was used to prepare the NPs to detect them in flow cytometry. Non-targeted PS-MP-PG NPs were also prepared to compare against the targeted NPs. Naked siRNA treated cells were used as the control for the experiment. After 4h the cells were washed trypsinized and collected to perform the flow cytometry experiment. The fluorescence intensity of FS-MP-PG-NPs and FS-MP-PG-LR-NPs (1, 10, 20 and 40µM) in (A and B) A549, (C and D) H460, (E and F) SKOV-3 cells (5 × 10^3 cell/well). (C) Bar graph representing the mean percentages of cellular uptake. The results are presented as mean ± SD (n=3). Analysis of cellular uptake was performed by using Flowjo 7.6.1 single cell analysis software. (*** p < 0.001)

To further investigate the endocytosis mediated internalization of the LHRH-R peptide conjugated FS-PG-LR20-NPs (T-NPs) in A549, H460, and SKOV-3 cells, flow cytometry experiment was performed. As shown in Fig. 4.20, at time 0.5 h there was a significant difference (*p<0.05) in the cellular uptake of non-targeted NPs (NT-NPs) and LHRH-R targeted NPs (T-NPs). Similar trends were observed with increased time intervals at 2 h and 6 h time point with significantly increased cellular uptake between NT-NPs and T-NPs in A549 cells and H460 cells Fig. 4.20. We found a 4-fold increase in cellular uptake between NT-NPs and T-NPs at time intervals 2 h and 4 h in A549 and H460 cells. In contrast, we found no significant difference in the cellular uptake of NT-NPs and T-NPs in SKOV-3 cells (low LHRH-expressing cell lines) at all the studied time intervals (0.5 h, 2 h, and 6 h) Fig. 4.20. The results further confirm the specificity of the T-NPs (FS-MP-PG-LR20 NPs) for NSCLC cell lines (A549 and H460) compared to the LHRH negative or low expressing cell lines (SKOV-3).
Figure 4.20: Cellular uptake of FS-MP-PG-NPs (NT-NPs) and FS-MP-PG-LR20-NPs (T-NPs). *In vitro* cellular uptake of different concentrations of FITC labeled siRNA loaded non-targeted (FS-MP-PG-NPs) or targeted (FS-MP-PG-LR20-NPs) for 0.5, 2 and 6 h incubation time in A549, H460 and SKOV-3 cells (5 × 10³ cell/well). The results are presented as mean ± SD (n=3). Analysis of cellular uptake was performed by using Flowjo 7.6.1 single cell analysis software. (* p < 0.05, **p<0.01).

4.3.6 The binding of targeted NPs (FS-MP-PG-LR20 NPs) was inhibited by blocking the LHRH receptors

The LHRH receptor-blocking experiment was performed to investigate the role of LHRH in the internalization of the MS-MP-PG-LR20 NPs by A549 and H460 Cells. The data suggests the blocking of receptors with 1-50 µM LHRH peptide analogue reduces the uptake of targeted NPs in a dose-dependent manner (Fig. 4.21 A and B). The cellular uptake of T-NPs (MS-MP-PG-LR20 NPs) reduced significantly after 10µM blocking of the receptors by around 75% and 85% respectively in A549 and H460 cells respectively. Similar results were obtained for receptor blocking with H460 cells after blocking with 30 and 50µM. Pre-incubation with 30 and 50 µM did no completely reduce the uptake of the targeted NPs due to the upregulation of the LHRH-R after endocytosis.
Figure 4.21: Competitive binding studies of T-NPS (FS-MP-PG-LR20 NPs) in (A) A549 and (B) H460 cells. Block of A549 and H460 cells with 1, 10, 30 and 50 µM LHRH-R peptide; The cells were pre-incubated with 1, 10, 30 and 50 µM LHRH analog peptide for 1 h, respectively. The peptide was removed after 1 hour and the cells were washed thrice with ice-cold PBS. After washing, the T-NPs were incubated with the cells for a period of 1 h in RPMI medium. The results are presented as mean ± SD (n=3). Analysis of cellular uptake was performed by using Flowjo 7.6.1 single cell analysis software. (* p < 0.05, **p<0.01).
4.3.7 The MS-MP-PG-LR NPs escapes endosomes/lysosomes in 6 h time period

The Cationic delivery system enables the NP to escape the endosome/lysosome resulting in the release of the siRNA from the NP delivery system. This is essential for the effective delivery of siRNA to perform its necessary gene silencing action. We examined the intracellular localization of the FL-siRNA-mPAE-PEG-LHRH NPs by tracking the bright green fluorescence of FITC tagged siRNA with 40X confocal imaging using Operetta high content imaging system Fig 4.22. The nuclei and the acidic late endosomes early lysosomes were stained with Hoescht 33342 and LysoTracker Red respectively. Maximum levels of colocalization could be observed after 2 h when almost all of the FITC tagged siRNA was located in the endosome/lysosome. By the end of 6 h, some of the Bio-reducible NPs escaped the endosome/lysosomes to reach the cytoplasm and released the siRNA due to the presence of excess GSH inside the cells releasing the siRNA load from the NPs. From the studies we further found that the amount of siRNA co-localized in the endo/lysosomes were about $5.75 \pm 1.2\%$, $45.7 \pm 8.5\%$ and $59.3 \pm 12.5\%$ at times 0.5 h, 2 h and 6 h, respectively (Appendix B).
**Figure 4.22:** The A549 cells were treated with 60 µg/ml of the targeted NPs (MS-MP-PG-LR20). The NPs were imaged at various time points using 40 X non-confocal imaging using Operetta high content imaging system. The experiments were repeated at least thrice. The best representative images are shown here. (The red arrow shows the co-localization of the NPs and the endo/lysosomes. The Green arrow shows the presence of siRNA inside the cytoplasm)
Table 4.5: The table shows the % of siRNA co-localized in acidic vesicles (endosomes/lysosomes) at a certain time period (0.5 h, 2 h, and 6 h)

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>% siRNA co-localized in endo/lysosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>5.75</td>
</tr>
<tr>
<td>2</td>
<td>45.7</td>
</tr>
<tr>
<td>6</td>
<td>59.3</td>
</tr>
</tbody>
</table>

4.3.8 MS-MP-PG-LR20 NPs demonstrate high mTOR gene silencing efficiency in human NSCLC cells

We investigated the mTOR silencing efficiency of MS-MP-PG-LR20 NPS (using 50 nM siRNA and 100 nM) in human lung cancer cell lines A549 and H460 cells. The mTOR gene silencing ability was measured by preparing NPs at the fixed weight ratio of 45:1 (mPAE: mTOR siRNA). The scrambled siRNA-based mPAE NPs was also prepared as the control. As shown in Fig. 4.23 (A and C) the negative control (SS-MP-PG-LR20 NPs) did not show gene silencing effects in the A549 and H460 cell lines at 50 nM and 100 nM concentrations. Meanwhile, the expression of the mTOR protein was significantly decreased after treatment with MS-MP-PG-LR20 NPs in both A549 and H460 cells at 50 nM and 100 nM concentrations (**p<0.01) Fig. 4.23 (A) and (C). As shown in Fig. 4.23 (B) and (D) the relative mTOR protein expression expressed as histograms (%) in MS-MP-PG-LR20 NPs treated A549 and H460 cell lines (normalized to the β-actin expression) was significantly less (~67% and 70% mTOR knockdown for A549 and ~70% and 75% mTOR knockdown for H460 cells) than SS-MP-PG-
LR20 NPs (\(**p<0.01\)). We found that the mTOR siRNA loaded NPs efficiently silenced the mTOR gene by using 50nM and 100nM siRNA concentrations.
Figure 4.23: Silencing efficiency of MS-DF transfection reagent and MS-MP-PG-LR20 NPs at 50nM and 100nM concentration. Expression of mTOR protein determined using Western blot in A549 and H460 cells (2× 10^5 cell/well). MS-MP-PG-LR20 NPs/mTOR siRNA and SS-MP-PG-LR20 NPS/scrambled siRNA prepared at weight ratio 45:1 (siRNA concentration 50nM or 1.33ug per well) (A) and (C) Western blot showed a marked reduction in the mTOR protein levels after treatment with MS-MP-PG-LR20 NPS at 50nM and 100nM concentrations. (B) and (D) mTOR protein silencing was calculated using the densitometric analysis and β-Actin as the loading control. The results are presented as mean ± SD (n=3). (** p < 0.01).

4.3.9 The targeted MS-MP-PG-LR20 NPs resulted in significant inhibition of cell proliferation in NSCLC cell lines

One of the major factors determining the efficacy of a drug is its ability to induce apoptosis of tumor cells. Therefore, we evaluated the effect of mTOR siRNA encapsulated targeted NPs on apoptosis in A549 and H460 cells, by performing cell proliferation inhibition studies using SRB assay[389]. As shown in Fig. 4.24 (A) and (C) cell viability (%) of MS-MP-PG-LR20 NPs were significantly less than the negative scrambled siRNA controls based NPs (SS-MP-PG-LR20 NPs) and placebo (MP-PG-LR20) NPs in both A549 and H460 cells(**p<0.01). The targeted MS-MP-PG-LR20 NPs produced around 30% and 35% inhibition of cell growth in A549 cells with 50 and 100nM mTOR siRNA concentrations respectively. The targeted NPs also resulted in around 38 and 42% inhibition of cell proliferation in H460 cells with 50nM and 100nM concentrations respectively. The inhibition of cell proliferation can be seen further from the images of the A549 and H460 cells treated with control (SS-MP-PG-LR20 NPs) and mTOR siRNA encapsulated (MS-MP-PG-LR) NPs as shown in Fig.4.24 (B) and (D), respectively.
Figure 4.24: Cell proliferation assay: Cell viability (%) of placebo MP-PG-LR-NPs, control (SS-MP-PG-LR20 NPs) and mTOR siRNA encapsulated targeted (MS-MP-PG-LR20 NPs) in
A549 cells and H460 cells seeded at a density of \((5 \times 10^3\) cell/well and \(6\times 10^4\) cells / well for A549 and (B) H460 cells respectively) at 48h incubation. The NPs were prepared at 45:1 weight ratios, were incubated with cells at a concentration of 30 µg/ml and 60 µg/ml of mPAE polymer to achieve 50nM and 100nM siRNA concentration, respectively (siRNA 133 and 266ng per well). Figure (A) and (C) indicates the cell viability (%) of the A549 and H460 cells after transfection with the formulations. The results are presented as mean ± SD (n = 3). (B) and (D) represent the 20X pictures of A549 and H460 cells taken after 48 h incubation with control (SS-MP-PG-LR20 NPs) and mTOR siRNA encapsulated targeted (MS-MP-PG-LR20 NPs), respectively, using Moticam Pro 252 A camera. A value of p less than 0.05 was accepted to be significant (**p<0.01) and the statistics were performed with Student’s t-test.

4.3.10 the targeted MS-MP-PG-LR20 NPs resulted in increased activity of Caspase-3 (protease)

We investigated whether loss of mTOR promoted apoptosis in the various formulations treated A549 and H460 cells. We found the significant increase in the caspase activity (%) (*p<0.01) in MS-MP-PG-LR20 treated A549 and H460 cells compared to SS-MP-PG-LR20 after 48 h treatment Fig (4.25).
**Figure 4.25: Determination of Caspase-3 activity.** After infection for 48h, A549 cells were harvested and then protein was extracted. The caspase activity (%) was determined using a commercial kit (EnzChek® Caspase-3 Assay Kit #1, Z-DEVD-AMC substrate) whole cell extracts were analyzed by enzyme-linked immunosorbent assay. Data represent means ±S.D. of three experiments in duplicate.
Chapter 5: Discussion

Bioresducible polymers have been widely investigated as siRNA delivery vectors for treatment of cancer because of their favorable toxicity profile and their propensity to preferentially release the drug load in the cytoplasm. However, it is still yet to be proven that bioreducible polymers result in improved transfection of siRNA. The goal of this study was to modify the biodegradable PAE polymer to make a bioreducible polymer and to systematically investigate its toxicity against the current gold standard for gene transfection (PEI). To our knowledge, this is the first study to demonstrate the modification of this newer PAE polymer to a bioreducible polymer.

In this study, we have successfully developed the thiolated PAE (mPAE) by using a one-step reaction with Traut’s reagent. In a similar method kommareddy et al. prepared thiolated gelatin with the 2-iminothiolane HCl reagent. They further used the thiolated biopolymer to prepare bio reducible NPs by desolvation method.[364, 390]. Bioreducible polymers have been extensively studied during past decades due to their attractive structural advantages. They possess unique features of high stability in extracellular physiological condition and immediate cleavage of disulfide linkages in the reductive intracellular environment. This property reduces cytotoxicity due to the avoidance of accumulation of the high molecular weight polycations and triggers a controlled delivery of genetic materials from the bioreducible polyplexes.

Cationic polymers like PEI have been extensively studied for their high gene transfection efficiency to deliver nucleic acids such as DNA[391-393], siRNA[394-397] miRNA[398, 399] both in vivo and in vitro. However, their use is limited due to a significant toxicity exhibited by polymers like PEI which causes significant cell damage to the normal cells due to various
cellular mechanisms. In a recent study by Moghimi et al., PEI-induced membrane damage and initiated apoptosis in different cell lines including Jurkat T cells, umbilical vein endothelial cells, and THLE3 hepatocyte-like cells[400]. Also in addition, recently a study by Beyerle et al., showed that PEI – 25kDa caused severe toxicity at concentrations of 50µg/ml and higher, rendering the cell viability levels below detection[377]. Thus, newer less cytotoxic, biodegradable polymers are needed, which possess high gene transfection efficiency as well as being relatively safer than polymers like PEI. In this study on lung cancer cells (A549 and H460) we found that the mPAE exhibited less cytotoxicity compared to the PEI polymer Fig. 4.3. This will allow formulating mPAE based NPs to deliver higher amounts of siRNA to the cancer cells and avoiding toxicity to the normal tissues. However, in order to demonstrate the safety of mPAE based NPs in normal cells, further cytotoxicity studies need to be conducted in liver, kidney and spleen cells.

We also found that the polymers containing primary amine groups such as native PEI and mPAE exhibited a considerable buffering capacity over a wide pH range of 4.5 to 7.5 due to a proton sponge effect[201]. The buffering capacity of the studied polymers mPAE and PEI was determined by the acid-base titration method and it was found that the mPAE polymer possesses a higher proton capturing tendency compared to PEI alone. This may be due to the presence of the higher amount of amine groups in the mPAE polymer compared to the PEI polymer per weight basis. In a similar study, Singh et al. concluded that the lower the amine groups present in the polymers, the lower the buffering capacity[368]. The presence of amine group along with other moieties contribute to the endo/lysosomal membrane destabilization [401]. Pinchon et al., clearly demonstrated in a study using confocal microscopy the protonated amine groups in PEI
helped in the membrane destabilization of the acidic components and deliver the DNA inside the cytosol.

**Objective 2:**

In this study, we have developed disulfide chemistry based multifunctional delivery systems which can deal with both intracellular and extracellular barriers. Various other researchers have performed similar studies with the bioreducible cationic polymer based NP systems[360]. Recently, Chang *et al.* reported that various GSH responsive nanocarriers such as micelles, NPs, dendrimers etc. possess enormous potential to facilitate the release of drugs/nucleic acid in the cytoplasm with the help of excess GSH[402]. We have developed mTOR encapsulated bioreducible mPAE polymer based NPs for the treatment of lung cancer. To our knowledge, this is the first study to demonstrate the GSH mediated enhanced release of mTOR siRNA from the bioreducible mPAE based NPs.

In this study, we found that the encapsulation of mTOR siRNA in the MS-MP-NP is via electrostatic binding and subsequently by the coating of the positively charged cationic mPAE to form NPs. Various studies have shown that at certain weight ratios the positively charged cationic polymer encapsulates the siRNA and form stable NPs[403-405]. In a recent study by Shen *et al.*, they found that Fe$_3$O$_4$@SiO$_2$ coated with cationic PEI resulted in the successful encapsulation of VEGF siRNA at weight ratios of 30:1 and higher [406]. In another study, Read *et al.* recently reported successful nucleic acid encapsulation by versatile reducible Polycation (consisting of histidine and polylysine residues) (RPC) based NPs at various weight ratios (4-7 % w/w)[407]. Agarose gel electrophoresis study confirmed that DNA was partially retained by RPC at (w/w) ratio of 2 and completely retained at w/w ratio of 4 and higher[82].
Similarly, in our study gel electrophoresis confirmed the successful encapsulation of mTOR siRNA in MS-MP NPs at weight ratio of 25:1 (mPAE: mTOR siRNA) and higher. Further, the % encapsulation efficiency experiment proved that at a weight ratio of 45:1 (mPAE: mTOR siRNA) and higher we were able to successfully encapsulate ~100% of the mTOR siRNA Table 3.1. Thus, the data indicates that mPAE polymer was capable of successfully binding and encapsulates mTOR siRNA and could be employed as a gene delivery carrier to NSCLC cells.

The systemic administration of siRNA is required to achieve the therapeutic effect of cancer treatment. However, several obstacles limit the systemic administration of therapeutic siRNA including the extremely low plasma half-life of siRNA due to degradation by serum nucleases [311]. The measurement of siRNA therapeutics stability in serum containing media systemic delivery is an important evaluation. Several studies have shown the potential of cationic polymer based NPs to successfully protect the therapeutic siRNA against serum nuclease-mediated degradation [405]. In a recent study by Shen et al., they showed that PEI based NPs successfully protected the siRNA from degradation by serum enzyme at a weight ratio of 15:1(Polymer: siRNA)[405]. In another study, by Mokhtarieh et al., cationic lipids based NPs successfully protected the siRNA against RNAse degradation for a period of 4 h[408]. In this study, we also showed that at an mPAE polymer to mTOR siRNA weight ratio of 45:1, the NPs successfully protect the mTOR siRNA against nuclease degradation. We found that at least 25% of the siRNA were released following the incubation with RNase and GSH for a period of 4 h each at 37°C.

The differential level of GSH provides a high-redox potential which provides a highly reducing environment inside the cells for disulfide bonds[409]. The high-redox potential gradient between the extracellular and intracellular environments provides the ability for disulfide
cleavage inside the cells, resulting in the fast siRNA release from the cationic – mPAE based siRNA NPs[410, 411]. In this study, we found that the mTOR siRNA release from MS-MP NPs was significantly higher in the presence of 10 mM GSH (Fig. 4.9A). This release can be attributed to the breaking of NP disulfide bonds by excess GSH present in the medium. Specifically, the disulfide-linked MS-MP-NPs resulted in the increased siRNA release at the end of day 1 due to cleavage of the disulfide bond in the presence of 10 mM GSH (Fig. 4.9 B). The release after a period of day 1(day 2 – day 7) was at a slow controlled release rate. This controlled release can be explained due to the interaction of the cationic polymer with siRNA and the slow release of the siRNA from the complex. This significant release can be beneficial for improved siRNA release on reaching the cytosol and thus improve the gene silencing efficiency. Several others have reported the similar mechanism of improved gene silencing efficiency with increased GSH mediated siRNA release in the cytoplasm from cationic polymeric NPs[412-415]. In a recent study by Breunig et al., disulfide-linked PEI-based siRNA delivery showed the improved release of siRNA in the cytosol and resulted in improved accessibility of siRNA for the gene silencing complex.

mTOR is known to promote cell growth and proliferation by regulating protein synthesis in NSCLC cell lines[302]. Therefore, it can be conceived that mTOR knockdown may control or alter cell proliferation to some extent[416]. In this study, we found suppression of cell proliferation in A549 and H460 cells after transfection with mTOR siRNA via MS-MP-NPs. Nearly 31% and 32% decrease in cell viability in cell number after MS-MP-NPs treatment is observed when compared to the control SS-MP-NPs transfections Fig. 4.10 (B) and (D). This data supports the hypothesis that mTOR gene knockdown was responsible for the tumor cell growth inhibition. Several studies have reported delivery of mTOR siRNA to different cell lines
(Fibroblast and PASMC) with the help of NPs which resulted in reduced cell proliferation [417, 418]. You et al., administered mTOR siRNA in a DNA nanotubes to the pulmonary artery smooth muscle cells and found that mTOR siRNA resulted in 47% reduction in cell viability after 48 h treatment[418]. In another study Takahashi et al., treated fibroblasts with mTOR siRNA loaded branched cationic polyethyleneimine (bPEI) NPs. The mTOR siRNA knockdown by the bPEI NPs resulted in 70% inhibition of fibroblast proliferation [417].

We also observed significant tumor growth inhibition with higher concentrations of MS-MP-NPs (mTOR siRNA 100 nM) compared to SS-MP-NPs (SC-siRNA 100 nm), however, the negative control based NPs also showed significant cytotoxicity to the cells(*p<0.05). It can also be seen from the Fig 4.10, that relatively higher concentrations of placebo NPs (60 µg/ml) and SS-MP-NPs (60 µg/ml) results in better cellular cell growth inhibition. Unfortunately, such higher concentrations of NPs also resulted in significant cytotoxicity in both A549 and H460 cells and are predicted to cause cytotoxicity to the normal cell as well. As indicated by the SRB assay results obtained using placebo NPs and SS-MP-NPs, concentrations higher than 30 µg/ml reduced the cell viability below 80% and are thus not suitable to load mTOR siRNA for further investigation.

Free siRNA cannot passively diffuse across the cell membrane due to its high molecular weight, hydrophilicity and an overall net negative charge[419]. In such cases positively charged cationic polymers based NPs can facilitate their enhanced association with the anionic cell membrane and subsequent internalization of siRNA by one of the several internalization mechanisms[419]. In this study, we found an increase in mTOR siRNA loaded MS-MP-FITC-NPs cellular uptake with increasing incubation time from 0.5 h - 24 h Fig. 4.11. The cellular uptake of siRNA represented an indirect measurement in the A549 and H460 cells. The direct
measurement of siRNA has not been reported as it is not feasible to quantify the amount of siRNA inside the cells at one particular time. Thus, indirect measurement methods for siRNA quantification have been used widely.

As mentioned above, the MS-MP-NPs prepared at a weight ratio of 45:1, (mPAE: mTOR siRNA) formed a nano-sized particle and has a positive surface charge. The small size (~114 nm) and a positive surface charge (~+27 mV) is a very important characteristic for the efficient siRNA delivery into the cells as observed in this study. Several studies reported that small sized and positively charged carriers can facilitate better uptake by the cell in comparison with both large sized and negatively charged carriers[420-423]. The nano-sized particles can preferentially accumulate at the tumor site via the EPR effect and tend to circulate for longer times when injected via intravenous injection [423, 424]. Various other studies demonstrated the ability of the cationic polymer to form NPs and increase the cellular uptake. In a recent study, PAM-ABP polymer formed compact NPs, which showed enhanced cellular uptake of 5 folds more than ABP at a weight ratio of 5:1 [425]. Generally, Cationic polymers are well known in assisting enhanced membrane permeability, facilitating nuclear localization, and adsorptive endocytosis [426, 427]. Our study further confirms that nano-sized positively charged cationic NPs can enhance cellular siRNA delivery.

Transfection of mTOR siRNA using MS-MP-NPs at a polymer concentration of 30 µg/ml (mTOR siRNA 50 nM) produced mTOR gene silencing as monitored by Western blot experiment two days’ post-transfection. We found that ~ 60% and 64% of mTOR gene was knockdown at 50 nM mTOR siRNA with MS-MP-NPs in A549 and H460 cells, respectively. Thus, these data confirm that MS-MP-NPs suppressed the targeted mTOR gene specifically.
through RNAi mechanism. For efficient silencing of the gene via mTOR siRNA into cancer cells, the delivery system must release the siRNA inside the cytoplasm. There may be two factors that might have contributed to the mTOR gene silencing efficiency observed in this study. First, the improved siRNA release due to the degradable disulfide linkage present in the MS-MP-NPs. Secondly, degradation of the biodegradable cationic polymer in the presence of enzymes, resulting in deprotonation and loss of siRNA binding ability and thus released into the cytoplasm [428].

The major advantage of cationic polymer based gene delivery systems is their high intracellular siRNA release with bio reducible properties as well as the proton sponge effects. Even though the siRNA encapsulated cationic polymer based therapeutics are designed to be toxic to cancer cells (anticancer treatment) they should not cause cytotoxicity to the normal cells (undesirable effects)[429]. In this study, we found that the non-targeted MP-NP at higher concentration of 60 µg/ml caused significant cytotoxicity (p<0.05) to the A549 and H460 cells Fig. 4.4. Also, as was shown before, the lower concentrations (15 µg/ml and 30 µg/ml) were not significantly cytotoxic to the cells.

Objective 3

As mentioned before, PEI is a very potent transfecting agent and is being widely used to deliver siRNA to the cancer cells[394]. However, PEI has been shown to cause significant cytotoxicity to the cells in in vitro and in vivo experiments. In particular, the PEI molecules can be released from the NPs internalized by cells and cause delayed cytotoxicity by interacting with cellular components [429, 430]. Also in a recent study, PEI was shown to cause membrane damage by binding to the outer plasma membrane proteoglycans of the cells at concentrations higher than 20 µg/ml[431, 432]. The membrane destabilization was shown to be accompanied by
rapid phospatidylserine movement from the inner membrane to the outer cell surface[431]. In another study, PEI was found to interact with glycoprotein-polysaccharide (Glycocalyx) on the cell surface resulting in the formation of large clusters and cause cytotoxicity [433]. Several modifications have been introduced in PEI including the grafting of PEG molecules on the surface to improve the biocompatibility [434, 435]. The attachment of PEG to the surface of the NPs provides a shielding effect of the high positive charges of the cationic polymer(PEI) and results in decreased cytotoxicity[436-438]. Recently Beyerle et al. developed different hydrophobic and hydrophilic PEGylated PEI-based nanocarriers and evaluated them in the lungs of mice by intratracheal administration[439]. They showed that hydrophilic PEG modifications resulted in less proinflammatory effect as well as prevented the depletion of the macrophages and disruption of the pulmonary epithelial/endothelial barriers. On the other hand, the hydrophobic modifications on PEI-based nanocarriers resulted in the severe inflammatory response. In a recent study by Wen et al., they found that PEGylated PEI enabled efficient transfection of siRNA (siROCK2) and exhibited low cytotoxicity compared to the non-PEGylated PEI toward U251 cells[435]. Similarly, in this study, we found that attachment of PEG (5K) on the surface of the NPs results in decreased cytotoxicity to the NSCLC cells, even at higher concentrations of 60 µg/ml of the cationic polymer. The PEGylation of the MP-NP led to the increase in cell viability (%) from 65.4 ± 10.4% and 60.81 ± 8.59% to 93.33 ± 9.6% and 92.5 ± 11.5% for A549 and H460 cells respectively Fig. 4.13.

Various siRNA encapsulated polymeric NPs cause significant nanotoxicity to the cells due to modification of one or several genes [429]. Certain cationic dendrimers such as polypropylenimine (PPI) have emerged as a powerful siRNA delivery carrier. The cytotoxicity of PPI dendrimer in A431 cells was found to be due to modification of several genes, including
CDK4, CD27 antigen, RPA3, DUSP1, HSPD1, ANXA2 proteasome 4, and cellular enzymes including ALOX5, GMPS and TH were all found altered using microarrays. Thus in order to evaluate if the cytotoxicity of the mPAE based NPs was due to mTOR gene silencing, we performed Western blot analysis. In this study, we found that the MP-NP caused significant (**P<0.01) toxicity to the two NSCLC cell lines (A549 and H460) at the highest concentration of 60 µg/ml (Fig. 4.13). We also found that at 60 µg/ml, the same formulation did not cause any significant reduction in the mTOR protein level in the cancer cells (Fig. 4.14). This indicates that the cytotoxic effects of the mPAE at the highest tested concentration might be the result of some other cellular death mechanism. Recently, Chenggang Li et al., showed that cationic polymer based polyamidoamine (PAMAM) NPs triggers autophagic cell death by deregulating the Akt-TSC2-mTOR signaling pathway in A549 cells[440]. They specifically found that treatment with PAMAM G3 based NPs resulted in inhibition of mTOR in A549 cells as determined via western blot analysis of the protein lysates. Also, the phosphorylation of mTOR substrate, S6 was downregulated by PAMAM G3 NPs, further confirming the inhibition of mTOR activity by the NPs in NSCLC cells. They further concluded that autophagy was involved in PAMAM NPs induced acute lung injury. On the other hand, In a recent study by Man et al., they found that higher concentrations of positively charged cationic lipids (N-[1-(2,3-Dioleoyloxy) propyl]-N, N, N-trimethylammonium methylsulfate) (DOTAP) induces autophagy by enhancing autophagosome formation[441, 442]. The cationic lipid, DOTAP did not reduce the phosphorylation level of mTOR and its substrates ribosomal S6 protein kinase (p70S6K) and eukaryotic initiation factor 4E-binding protein1 (4E-BP1). Unlike Rapamycin (mTOR inhibitor), DOTAP induced autophagy in an mTOR-independent manner. Thus, it can be concluded from
our study as well that the cytotoxic effects of the polymer mPAE might be in an mTOR-independent manner.

The presence of serum proteins, as well as extracellular matrix components in the biological system, can cause vector disassembly as well as induce aggregation of the assembly. In this study, we found that the NPs were stable in the presence of ionic conditions as well as serum proteins over a period of 6 d. We found that only in presence of PBS and 10% FBS the size of NPs increases to over 200 nm which may be due to the presence of ionic interactions between positively charged NPs and the salt (Fig. 4.17) [443]. Various cationic polymers based siRNA delivery systems have emphasized the importance of attaching PEG to improve serum stability[443]. Recently, Vader et al. showed that siRNA encapsulated polyamido(amines) based delivery system showed improved serum and salt stability along with reduced interactions with the erythrocytes[443]. Merkel OM further investigated PEGylated PEI-siRNA NPs for in vivo behavior. They found that the NPs were stable on delivery to the lung via the pulmonary route reaffirming the importance of surface PEG coating [444].

The LHRH receptor is found to be over-expressed in a variety of human tumors and is also a potential binding site for targeted lung cancer therapy. Various recent reports have shown that the LHRH-R, a member of the GPCR superfamily is expressed in various tissues, including normal lung [258, 445] and are overexpressed on the surface of lung cancers cells [259, 446]. In this study, we compared the expression of the LHRH receptor in NSCLC cell lines (A549 and H460) with breast adipocytes and ovarian cancer cell lines (SKOV-3). LHRH-R are not expressed at a significant level in most visceral organs [261]. We found that the expression of LHRH-R in A549 and H460 cells was significantly higher (*P<0.001) compared to both breast adipocytes and SKOV-3 cells (Fig 4.15 and 4.16). To our knowledge, this is the first study to
compare and confirm the expression of LHRH-R in A549, H460, SKOV-3 cells and breast adipocytes. Various studies have shown that SKOV-3 cells show very less expression of the LHRH-R compared to other cells [447-450]. Similarly, we found that the expression of LHRH-R in SKOV-3 was 4 fold and 4.5 fold less than A549 and H460 cells respectively. This provides a rationale to develop LHRH-R targeted NP drug delivery system to be delivered to the NSCLC cell lines. Further studies need to be performed using liver, kidney, and spleen cells to confirm the differential expression of LHRH-R in cancer cells and normal cells. The NPs are usually eliminated via the liver, kidney, and spleen and thus it is important to establish the over-expression of LHRH-R on NSCLC compared to these organs.

LHRH-R lung cancer targeting peptide was attached to the NPs via cysteine-maleimide reaction. Firstly, the PEG-AMAS group was attached to the surface of NPs using the NHS – amino group coupling reaction. Secondly, the cysteine-terminated LHRH peptide was conjugated to the maleimide group in the PEG-AMAS present on the surface of the NPs. The attachment of the LHRH-R targeting peptide was confirmed using the BCA protein assay. The LHRH peptide developed a strong and clear absorbance signal due to the biuret reaction (exhibited by proteins and peptides due to the well-known reduction of Cu$^{+2}$ to Cu$^{+1}$). Mainly, the primary amino acids (cysteine, tryptophan, and tyrosine) present in the peptide is responsible for the color detection in the LHRH-R peptide. The optimum concentration of peptide on the surface of NPs is essential in binding to the receptors and getting internalized via endocytosis [451, 452]. We determined the optimum concentration of LHRH-R peptide for MS-MP-NPs required attaining maximum cellular uptake efficiency. We found that the fluorescent-labeled siRNA encapsulated targeted NPs (FS-MP-PG-LR20), on incubation at 20 µM in both A549 and H460 cells resulted in significantly increased(*p<0.001) cellular uptake compared to non-targeted NPs(FS-MP-PG-
LR20). This effect can be explained by the improved binding and internalization of the targeted NPs with the LHRH-R on the surface of A549 and H460 NSCLC cells Fig. 4.19 (A, B, C, and D). However, a further increase in the concentration of surface LHRH-R peptide did not increase the cellular uptake of the NPs indicating saturation of the receptors in A549 and H460 cells Fig. 4.19 (A, B, C, and D). Similar results were obtained by Leuschner et al., when they attached LHRH peptide on the surface of magnetic ferrous oxide NPs[453]. They found that increase in the peptide concentrations (beyond 10 µm concentrations) did not result in significant increase in cellular uptake due to the oversaturation.

LHRH-receptor-targeting drug delivery systems, using targeting peptides and ligands have key advantages of facilitating rapid uptake into NSCLC cells and of preventing non-specific delivery into normal tissues[100]. In our study, receptor-targeted mTOR-siRNA-mPAE-PEG-LHRH NPs efficiently silenced the mTOR gene by 67% and 70% in A549 cells and 70% and 75% in H460 cells using the targeting peptide LHRH (Fig. 4.22). The peptide used in this study is a synthetic analogue of natural LHRH analogue, which has been used previously to show improved internalization of the nanocarrier systems [261]. We also found an increase in caspase-3 activity in the MS-MP-PG-LR20 treated A549 cells (Fig 4.24). The increase of cleaved caspase-3 activity is observed in the cells undergoing apoptosis [454].
Chapter 6: Summary and Future Directions

6.1 Dissertation Summary

In this study, we have developed and accessed the potential of the cationic polymer mPAE as an mTOR siRNA delivery carrier for targeted lung cancer therapy. The mPAE polymer formed nano-sized (114 nm) and positively charged (+27 mV) NPs with mTOR siRNA and the mTOR siRNA was prevented from degradation by the nuclease action. The release of mTOR siRNA was also accelerated in the presence of 10 mM GSH. The MS-MP-NPs showed inhibition of cell proliferation at a siRNA concentration of 50 nM. In addition, the MS-MP-NPs showed increase cellular uptake due to cationic nature of the polymer and effective mTOR gene silencing. Our results demonstrated that the newer cationic mPAE polymer consisting of disulfide bonds is an effective mTOR siRNA carrier for lung cancer therapy applications.

Further, in this study, we have developed and tested the feasibility of LHRH peptide conjugated bio-reducible polymer, mPAE-PEG-LHRH for lung tumor-targeted therapeutic mTOR gene delivery. The targeted MS-MP-PG-LR NPs efficiently delivered mTOR siRNA into LHRH-R expressing cancer cells, A549 and H460, with low cytotoxicity. Competition assay with free LHRH and cellular uptake assay confirmed LHRH-R mediated gene delivery efficiency of MS-MP-PG-LR20 into A549 and H460 cells. To confirm the anti-cancer therapeutic effect, the mTOR siRNA encapsulated MS-MP-PG-LR20 was delivered to the A549 and H460 cells. The targeted NPs showed 30%-45% decrease in cell viability in A549 and H460 cells. This was further confirmed using western blot analysis, wherein the MS-MP-PG-LR20 showed efficient mTOR gene knockdown (70%-85%). While our study results are exciting, it is to be noted that additional preclinical studies are warranted. Furthermore, mTOR gene knockdown alone did not
result in anticipated tumor growth inhibition of more than 50%. However, this targeted gene therapy system can be further used to deliver two different siRNA simultaneously and can produce synergistic anti-cancer activity in NSCLC cells.

To summarize, we have designed, synthesized and evaluated NSCLC targeted nanocarrier system as nucleic acid delivery systems. Various analytical methods were established to investigate the underlying mechanism of nucleic acid delivery. The novel NCs developed in this study are promising for the therapeutic delivery of mTOR siRNA drugs \textit{in vivo} for the treatment of lung cancer and other types of diseases. We hope the findings of this thesis will guide a rational design of better nucleic acid delivery systems and clinical translation of nanomedicines.

\textbf{6.2 Future Directions}

This dissertation provides insights into the mechanisms governing the efficient tumor penetration and delivery of mTOR siRNA by LHRH peptide conjugated targeted mPAE NPs. The effect of LHRH targeting ligand density was studied extensively and was found to have a pronounced impact on the cellular internalization of targeted MS-MP-PG-LR NPs through A549 and H460 cells. Given this framework, some future directions of this work could be to deliver two siRNA targeting different genes and determine a correlation between the tumoral distribution of siRNA and their anticancer efficacy or to improve the MP-PG-LR20 system such that it could be useful for \textit{in vivo} siRNA delivery for lung cancer therapy.

Further, the insights derived in this work regarding the optimum concentration of LHRH targeting ligand density required for efficient cellular uptake and transport of nanoscale materials can be applied to improve the tumor penetration abilities of other synthetic siRNA delivery systems.
Further, as mTOR siRNA would likely be used clinically in conjunction with a chemotherapy drug and/or siRNA, a co-treatment regimen of MS-MP-PG-LR20 to facilitate siRNA delivery along with a chemotherapy compound could be developed. This would improve the efficiency and the antitumor activity of this delivery system. Further studies need to be performed to determine the synergistic or additive effect of combination siRNA/drug using this targeted delivery system. Thus, a logical extension of this work would be to optimize this therapeutically-relevant treatment regimen for the treatment of NSCLC using MS-MP-PG-LR20 and to determine if this treatment regimen is able to retard the growth of NSCLC cells in a three-dimensional lung tumor model. Further, the pharmacokinetic studies will determine the extent of targeting and improvement in the biodistribution of mTOR siRNA with and without combination drug.
Appendix A

Theory of Particle Size and Zeta potential

**Particle Size:** The particle size is a vital property for characterization of NPs. It plays an important role in NP properties such as stability and distribution under biological fluids [98]. To develop next-generation nanomedicines (NMs) and in order to achieve superior anticancer activities, numerous studies have been performed to understand the correlation between the particle size of NMs and their interactions with the biological systems including tumor tissues [99]. Most of the currently approved anti-cancer NMs have been in the size ranges of 100-200 nm, however particles less than 100 nm exhibited enhanced performance in vivo, such as greater tissue penetration and enhanced tumor inhibition which makes it a mandatory property to characterize [100]. For particle size distribution, Dynamic Light Scattering (DLS), also called Quasi-Elastic Light Scattering or Photon-Correlation Spectroscopy, is a widely used, non-invasive technique to measure the size and size distribution of proteins, particles and other molecules in solution. The mechanism of particle size measurement is affected by the Brownian motion. The particles suspended in the liquid are buffeted by the solvent molecules which lead to a random motion of the molecules called Brownian motion. The larger the particles the slower the Brownian motion [101]. In a DLS measurement, the time-dependent fluctuations in the light scattered by the moving particles are measured. The particle size thus measured is given in terms of the hydrodynamic radius. The Stokes-Einstein equation is used to describe the hydrodynamic radius.
\[ D(H) = \frac{KT}{3\eta \pi D} \times 100 \]  

(1)

\( K \) = Boltzmann’s constant

\( \eta \) = Viscosity

\( D \) = velocity of Brownian motion

\( T \) = absolute temperature

**Zeta Potential:** The NPs are a type of colloidal systems and usually possess a charge. The NPs can either have a net positive, negative or neutral charge which can be a determining factor in predicting the colloidal stability in biological systems. Various techniques have been employed recently to quantitatively determine the surface charge or the zeta potential of the NPs in a suspension. Zeta potential is a measure of the magnitude of the electrostatic or charge repulsion or attraction between particles, and is one of the fundamental parameters known to affect the stability [102]. Electrophoretic mobility scattering which measures the zeta potential of the NPs is one such technique employed by the NICOMP 380 ZLS. The zeta potential of the nanocarriers will be measured using NICOMP 380ZLS (Port Richey, FL) [103].

**Autocorrelation function:** definition and motivation Let us consider the autocorrelation function of the net scattered light intensity \( I_s(t) \), which fluctuates in time as shown in Figures 4a, b, and c. The autocorrelation function, which we denote by \( C(t') \) is used to study the correlation, or similarity, between the value of \( I_s \) at a given time, \( t \), and the value of \( I_s \) at a given time, \( t \) and the value of \( I_s \) at an earlier time, \( t-t' \). This comparison is then made for many
different values of $t$ in order to obtain a good statistical average for $C(t')$ -- i.e. averaged over many "wiggles" of the fluctuating intensity $I_s$. $C(t')$ is evaluated according to,

$$C(t') = \langle I_s(t) \times I_s(t-t') \rangle$$

(2)

The bracket symbols $\langle \rangle$ are shorthand for a summation over many values of $t$. That is, one calculates a running sum of many products $I_s(t) \times I_s(t-t')$, all having the same separation in time, $t'$, for many different values of $t$. The ability of $C(t')$ to extract useful information from the fluctuating scattering intensity $I_s(t)$ can best be understood by considering a portion of a typical signal $I_s(t)$. We arbitrarily choose a particular time $t$ and record the value of $I_s$ at that time -- $I_s(t)$. We next consider a very small value of $t'$, equal to $t_1'$, and evaluate $I_s$ at this slightly earlier time, $t-t_1'$ -- $I_s(t-t_1')$. Because $t_1'$ is presumed to be small, $I_s(t-t_1')$ must be very similar to $I_s(t)$. The reason for this, of course, is that the particles have not been able to change their positions significantly (i.e. compared to $\lambda$) under diffusion in the (presumed) short time interval $t_1'$. 
Figure A1 Schematic illustration of intensity measurement and the corresponding autocorrelation function in dynamic light scattering. The figure illustrates dispersion composed of large and small particles. (a) Intensity fluctuation of scattered light with time, and (b) the variation of autocorrelation function with delay time. Figure adapted with permission from reference[455]
Appendix B

Supplementary Figures/Tables

Figure A1: (A) Expression levels of GNRH receptors in different tissues. (B) The expression level of GnRH-R protein in different tissues. Adapted from http://www.proteinatlas.org/ENSG00000109163 GNRHR /tissue #gene_information.
**Figure A2:** Calibration curve of PEG from concentrations ranging from 0.1 µg/ml to 10 µg/ml

**Figure A2:** Calibration curve of PEG from concentrations ranging from 0.1 µg/ml to 10 µg/ml
Appendix C

Nishant Gandhi’s Publications

Research Articles

1. SV Shirolkar, MG Tawar, **NS Gandhi***, NB Deore. Development and evaluation of floating microspheres of Pioglitazone hydrochloride using ethyl cellulose. Der Pharmacia Lettre 2 (5), 261-277
2. **NS Gandhi***, SV Shirolkar, MG Tawar Development and evaluation of microballoons of pioglitazone hydrochloride using eudragit S-100. International Journal of Pharmaceutical Sciences and Research 3 (1), 201
3. MG Tawar, SV Shirolkar, NB Deore, **NS Gandhi**. Development and Evaluation of Repaglinide Floating Matrix Tablet. Inventi Rapid: NDDS.
5. **Gandhi NS**, Yang R, Chougule MB” Pi0 loaded hybrid albumin nanoacarriers for the treatment of mesothelioma.” Drug development and Industrial Pharmacy. (Submitted for publication).

Invited Review Articles


Invited Book Chapters


References


[274] Ceresa B. Molecular Regulation of Endocytosis: INTECH.


Wheless JW. Use of the mTOR inhibitor everolimus in a patient with multiple manifestations of tuberous sclerosis complex including epilepsy. Epilepsy & Behavior Case Reports. 2015;4:63-6.


[455] Lim J, Yeap SP, Che HX, Low SC. Characterization of magnetic nanoparticle by dynamic light scattering. Nanoscale research letters. 2013;8:381-.