Novel Phospholipid-modified-polyethylenimine
(PLPEI)-based-Nanocarriers for siRNA Delivery


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PROJECT OVERVIEW AND SPECIFIC AIMS

This project was designed with a clear aim of developing effective, novel, non-viral siRNA nanocarriers for further application in the treatment of Cancer. Micelle-like nanoparticles (MNPs) based on phospholipid-modified polyethylenimine (PLPEI) have been previously reported by a group in our lab for delivery of plasmid DNA in an in vivo tumor model. The first aim of this project was to evaluate if these MNPs could be utilized for siRNA delivery and gene silencing in vitro. The possibilities of two other structurally different PLPEI-based nanocarriers for their siRNA delivery and gene silencing potential were explored, followed by studies to elucidate their mechanism of action and thus, explain why some PLPEI nanocarriers performed better in vitro with respect to siRNA delivery and silencing over the other PLPEI-based nanocarriers. Dioleoyl phosphoethanolamine-PEI (DOPE-PEI) was found to be best PLPEI nanocarrier in vitro for intracellular siRNA and downregulation of a model protein (Green fluorescent protein or GFP). Further, DOPE-PEI was chosen for downregulating the MDR1 gene that encodes for P-glycoprotein (P-gp) in human breast cancer cells in vitro. The aim was to exhibit that the combination therapy of siRNA and drug can overcome multidrug resistance (MDR) in cancer therapy by downregulating P-gp.

In vivo studies with the PLPEI nanocarriers have been branched into two sections. First, the biodistribution profile of the PLPEI-based nanocarriers was studied in an in vivo subcutaneous (s.c.) murine tumor model closely mimicking human breast cancer tumor xenograft, in female Balb/c mice. In an aim to overcome MDR in vivo, therapeutic efficacy and P-gp downregulation studies were then performed in s.c. human breast cancer tumor xenograft model (MCF7/ADR) in nude, female mice using the combination treatment of siRNA and doxorubicin hydrochloride (dox).
Preliminary data has also been presented with transferrin conjugated micelles that have been formulated based on PLPEI, loaded with siRNA, with the aim of developing a targeted siRNA delivery system for prospective improved tumor targeting in vivo.

**Specific Aims for the project**

1. To formulate and characterize PLPEI based *Micelle-like-Nanoparticles (MNPs)* specifically, 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine-PEI (PAzPC-PEI or PC-PEI) - based MNPs for in vitro siRNA delivery and gene downregulation.

2. To develop and characterize phosphoethanolamine-modified PEI (PE-PEI)-based nanocarriers for siRNA delivery and compare their effectiveness with regards to intracellular delivery and gene silencing in vitro against PAzPC-PEI- based nanocarriers via studies to elucidate their mechanism of action.

3. To develop and characterize PE-PEI-based, siRNA loaded MNPs, targeting the MDR1 gene (siMDR1) and evaluate the efficacy of the combination therapy of siMDR1 loaded MNPs and doxorubicin in overcoming MDR in human breast cancer cells.

4. To evaluate the biodistribution of DOPE-PEI/siRNA-based nanocarriers and the role of PEGylation in the organ distribution profile and blood circulation time.

5. To evaluate the therapeutic efficacy and MDR1 gene downregulation capacity of the DOPE-PEI/siRNA and doxorubicin combination therapy.

6. To formulate DOPE-PEI based transferrin (Tf)-conjugated micelles for prospective utilization as targeted siRNA delivery system in vitro.
RESOURCES AVAILABLE FOR THIS PROJECT

Laboratory space

The lab is formally known as the ‘Center for Pharmaceutical Biotechnology and Nanomedicine’ (CPBN) and the PI is Dr. Vladimir Torchilin, University Distinguished Professor in the Pharmaceutical Sciences department, Bouve College of Health Sciences, at Northeastern University. The lab is located on the second level in the new section at 140, The Fenway. The instruments employed in this project are listed below

- E-Gel electrophoresis system (Invitrogen Life Technologies)
- 96-well plate reader (Multiscan MCC/340, Fisher Scientific Co)
- Zeta Plus Particle Analyzer (Brookhaven Instruments Corp, Santa Barbara, CA)
- Malvern Zetasizer Nano ZS90 (Malvern Instruments, Westborough, MA)
- Becton-Dickinson FACSort™ flow cytometer (Franklin Lakes, NJ)
- Nikon Eclipse E400 fluorescent microscope
- Varian 400 MHz spectroscopy (Varian Inc.)
- Agilent 5500 AFM/SPM microscope (Agilent Technologies)
- Confocal Zeiss LSM 700 microscope (Carl Zeiss)
- LightCycler® 480 System (Roche applied sciences)
- JEOL 100X Transmission Electron Microscope (Peabody, MA)
- Miscellaneous: Centrifuges, freeze dryer, vortex mixer, rocking shaker, shaking incubator, UV light chamber, weighing balances, pH meters
**Animals:** Female Balb/c mice 6-8 weeks, obtained from Charles River Laboratory (CRL Wilmington, MA) were used in the biodistribution experiments. Female nude mice (6-8 weeks) from CRL were used in the therapeutic efficacy studies

**Radioisotopes:** N/A

**Recombinant DNA:** N/A

**Human Subjects:** N/A

**Laboratory Safety:** Received necessary Laboratory Safety and Chemical Hygiene training

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**BIOGRAPHICAL SKETCH**

**Education**

- High School: Father Agnel Junior college, Vashi, Navi Mumbai, India (June 2003)
- College: Bombay College of Pharmacy, Kalina, Mumbai, India (Major: Pharmacy, June 2007)
- Post-baccalaureate: Northeastern University, Boston, USA (MS in Pharmaceutical Sciences, May 2009)

**Awards and/or honors**

- Academic Research Award (*first place*) at the AAPS NERDG meeting in CT, April 2012
- Member of the Rho Chi honors society at Northeastern University (since June 2010)
LIST ANY PUBLICATIONS OR ABSTRACTS WITH YOU AS AN AUTHOR:

Peer-reviewed scientific publications


Book chapters

- Essex, S. and Torchilin VP. (Accepted and anticipated in 2013). “Liposomal formulations for Focal and Targeted Drug Delivery” in Abraham J. Domb, and Wahid Khan (Eds.), Advances in Delivery Science and Technology- Focal Drug Therapy. Springer business + media

## THESIS COMMITTEE

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GENERAL INTRODUCTION

Small interfering RNA (siRNA) is a 21-23 nucleotide RNA duplex that participates in the RNAi phenomena by paring with specific sequences of messenger RNA (mRNA), triggering its degradation and thus, prevents protein production [1]. Theoretically, the potential applicability of siRNA is unlimited, since siRNA could be designed for any gene of interest based on its mRNA sequence. In most siRNA-based cancer therapies, the aim is to silence the genes involved in apoptotic or proliferative cellular pathways as adjuvant therapies to treat non-resectable tumors or tumors that are resistant to conventional chemotherapy or radiotherapy [2]. To date, RNAi technology has achieved some promising results in cell culture and preclinical animal models. However, only a few products are undergoing clinical trials or are in the market [3]. One major challenge for siRNA- based therapies is the effective delivery of siRNA to target cells. Upon injection, naked siRNA has a very short half-life, low in vivo stability due to rapid enzymatic degradation, rapid excretion by kidney filtration, poor transfection efficiency and poor tissue penetration [4, 5]. Despite its small size and a molecular mass of \( \approx 13 \text{ kDa} \), siRNA does not cross the cellular membrane. The negative charge of siRNA impairs its interaction with the negatively charged cellular surface. The hydrophilic nature of siRNA also makes its free diffusion through cellular lipidic bilayers difficult. Even if siRNA enters the cells, it is entrapped by endosomes and lysosomes where enzymatic degradation significantly decreases its entry into the cytoplasm [6].

Apart from delivery difficulties, siRNA is associated with adverse effects including saturation of RNAi machinery by the exogenous siRNA that affects the normal function of RNAi, undesirable activation of the immune system, off-targets effects and non-specific silencing. Thus, it could be concluded that comprehensive applications of RNAi therapeutics for the clinic are mainly hampered by
the low stability of siRNA \textit{in vivo}, incomplete suppression of target genes and safety-related problems such as the activation of the immune system or non-specific gene silencing [7].

These results strongly suggest that the association of siRNA with suitable carriers that could potentially protect the siRNA from premature degradation and efficiently transfer the siRNA to target cells is crucial for wider application in cancer therapy. Off-target effects and immunogenicity problems can be solved with an optimal design of the siRNA sequence, size and structure [8]. The low \textit{in vivo} availability of siRNA can be overcome by the modification of the siRNA backbone or by conjugating one of the siRNA strands with lipids or peptides to improve nuclease stability [9] or by siRNA association with viral or non-viral carriers to improve transfection and tumor specificity. siRNA delivery systems could be classified into two groups: \textit{viral vectors} and \textit{non-viral vectors}. Though viral vectors, in general, display high transfection efficacy, the use of viruses in patients is limited by difficulties in scale up of production and safety issues [10, 11]. Non-viral or synthetic vectors are attractive alternatives due to their inherent attributes of improved safety, greater flexibility and easier manufacturing. These are usually comprised of polycations of a lipdic or polymeric nature that electrostatically interact with the negatively charged siRNA molecules. In this way, siRNA is condensed into compact complexes, referred as polyplexes or lipoplexes depending on the nature of the polycation, with sizes of 100-200 nm that prevent the anchoring of nuclease enzymes and the degradation of the siRNA.

One major approach to non-viral siRNA delivery is based on the use of cationic polymers such as chitosan, poly-L-lysine (PLL), polyethylenimine (PEI), or PAMAM dendrimers [12, 13]. The presence of the amine groups in the structure of these polymers permits the spontaneous formation of complexes when mixed with siRNA due to electrostatic interactions between positively charged
amine groups and negatively charged phosphate groups in siRNA. The excess of positive charge within
the complexes permits the interaction with cellular membranes and enhances the siRNA cellular
uptake. Within the cells, the buffering capacity of the polymer amines has been reported to facilitate
the escape of the complexes from endosomes [14]. Among synthetic polycations, polyethylenimine,
PEI, has been a popular candidate which has been used successfully for nucleic acid delivery under
both in vitro and in vivo conditions [15 - 19]. PEI can be synthesized in different lengths, different
structure (branched or linear) and is capable of protonation of amino group at every third position.
This latter feature gives PEI a high positive charge density at physiological pH and permits the
condensation of negatively charged nucleotides (DNA, siRNA, ODN) into dense particles by
electrostatic interactions. PEI also has an intrinsic endosomal escape mechanism known as the ‘proton-
sponge’ effect, which causes osmotic swelling and rupture of the endosomal membrane that triggers
the release of PEI complexes into the cytosol; a phenomenon known as ‘endosomal escape’ [14,
20]. However, unless cell-binding ligands are present, an overall positive charge is essential for the
uptake of the PEI complexes by non-specific endocytosis promoted by electrostatic interaction with
the negatively charged proteoglycans on the cell surface, most likely by a combination of clathrin and
caveolin pathways [21, 22]. This requirement of an overall positive charge for an effective transfection
is usually linked to high toxicity and represents a major problem for systemic application [21, 23].
Positively charged complexes are prone to interact non- specifically with a variety of components in
the blood and other biological fluids or with non-target cells. These non-specific interactions cause
toxic side-effects, destabilization of the complexes and their removal from circulation by the organs of
the reticuloendothelial or RES system [24] and thus, decrease the bioavailability of the nucleic acid
molecules at the target site.
HYPOTHESIS#1 ‘MNPs that have been previously reported to deliver plasmid DNA to a distal tumor, could be harnessed to serve as a suitable carrier for siRNA delivery in vitro.’

SPECIFIC AIM#1 To formulate and characterize PLPEI based Micelle-like-Nanoparticles (MNPs) specifically, 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine-PEI (PAzPC-PEI or PC-PEI) - based MNPs for in vitro siRNA delivery and gene downregulation.

1.1 BACKGROUND FOR SPECIFIC AIM#1

In order to improve siRNA delivery and reduce the toxicity of PEI-based complexes, several approaches have been investigated such as coupling PEI to hydrophobic moieties, including fatty acid residues [17, 24-26] and degradable acrylates [17, 25, 27] or grafting hydrophilic polyethylene glycol (PEG) to PEI [18, 28, 29]. Lipidation of PEI has been reported in several studies to formulate micellar siRNA delivery systems, the rationale being, reducing the inherent polycation toxicity and improving transfection efficacy. Recently, a group in our lab reported a novel gene carrier, the micelle-like nanoparticles (MNPs), based on the combination of a covalent conjugate between phospholipid and polyethylenimine (PLPEI) with PEG and lipids. These MNPs combined the favorable properties of the low molecular weight PEI 1.8 kDa (nucleic acid condensation and protection, low cytotoxicity, endosomal escape) with those of PEG stabilized liposomes (in vivo stability, prolonged blood circulation) that resulted in an effective transfection of plasmid DNA in a distal tumor cell when the long-circulating MNPs were administered intravenously [30]. We hypothesized that these MNPs could also be used to deliver siRNA into the cells and downregulate a target model gene.
1.2 MATERIALS

All materials were purchased from Sigma-Aldrich unless otherwise stated. Branched polyethyleneimine (b-PEI) [31, 32] with a molecular weight of 1.8 and with a molecular weight 25 kDa were purchased from Polysciences, Inc (Warrington, PA). 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2- distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethyleneglycol)-2000] (PEG-PE), cholesterol, and oxidized phospholipid, 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine (PAzPC) were purchased from Avanti Polar Lipids (Alabaster, AL). All siRNA duplexes were purchased from Dharmaco (Lafayette, CO), namely, siRNA targeting Green Fluorescent Protein (GFP-siRNA): 5’- AUGAACUUCAGGGUCACGUdTdT-3’ (sense) [33], a non-targeting control duplex, (Negative-siRNA): 5’-AGUACUGCUUACGAUCGGdtdTdT-3’ (sense) and 6-Carboxyfluorescein (FAM)-labeled siRNA (siGLO® siRNA). RNase III was purchased from Ambion (Austin, TX). The CellTiter-Blue® Cell Viability Assay and Hoechst 33342 and Lysotracker® Red were purchased from Promega (Madison, WI). Nuclease-free water was purchased from Qiagen (Germantown, MD).

1.2.1 Cell culture

The cell lines B16F10 (mouse melanoma), NIH/3T3 (mouse fibroblast), and C166-GFP cells from the C166 cell line (mouse yolk sac embryo) stably transfected with a plasmid reporter vector, pEGFP-N1, encoding for the enhanced green fluorescent protein, GFP, were obtained from the American Type Culture Collection (Manassas, VA). B16F10 and NIH/3T3 cells were grown at 37°C under 5% CO2 in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin (100 units/ml) and streptomycin (100 μg/ml). For C166-GFP cells, DMEM media was supplemented with 10% FBS and 0.2 mg/ml of Geneticin (G-418, Invitrogen, CA). Cell culture
media and penicillin/streptomycin stock solutions were purchased from Cellgro (Herndon, VA). Heat-inactivated fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA).

1.3 METHODS
1.3.1 Synthesis and characterization of the phospholipid-polyethylenimine conjugate (PLPEI)
The phospholipid–polyethylenimine conjugate (PLPEI) was synthesized from PEI 1.8 kDa and oxidized phospholipid (PAzPC) as previously described [30]. Briefly, 12 mg of the branched PEI (7µmol) were dissolved in 0.5 ml of chloroform and mixed with 5 mg of the oxidized PC (PAzPC, 7µmol) dissolved in 1 ml of chloroform. Using the knowledge that branched PEI has a 1:2:1 molar ratio of primary: secondary: tertiary amines, the reaction mixture corresponds to an acid-to-primary amine molar ratio of 1:10. Carbonyldiimidazole (CDI, 0.5mg, 3µmol) was added to the above solution for the activation of the acid by formation of an imidazole derivative. The mixture was incubated with 10 µL of TEA (triethylamine) at room temperature for 24 h with stirring. The chloroform was evaporated under a stream of nitrogen gas and the residue was suspended in 2 ml of distilled water. The products were purified by dialysis (MWCO 2000 Da) against dH2O and lyophilized. The PLPEI conjugate was dissolved in water to a concentration of 1 µg/µL (0.7µg/µL as PEI).

The synthesized conjugate was characterized by $^1$H nuclear magnetic resonance (NMR) spectroscopy. NMR spectroscopy was performed using Varian 400 MHz spectrocope. The conjugates were dissolved in d-CDCl3, and a 5-10 mg/mL solution was used. The characteristic peaks noted at different PPM values are as follows (where singlet, doublet, triplets, multiplet, broad singlets are noted as s, d, t, m, bs respectively): For PLPEI, $^1$H-NMR in CDCl3. δ ppm 0.87 (t), 1.10 (s), 1.25-1.29 (m), 1.41 (s), 2.21-2.31 (m), 2.35-3.10 (m), 3.25-3.50 (m), 3.65-3.66 (d), 3.82-4.32 (m), 5.21
1.3.2 Preparation of PLPEI/siRNA complexes

The PLPEI/siRNA complexes were prepared by mixing a fixed amount of siRNA and varying amounts of PLPEI that were separately diluted in equal volumes of PBS buffer (pH 7.4, nuclease-free buffer). The siRNA solution was transferred to the polymer solution, mixed by vigorous pipetting and incubated for 15 min. The polymer/siRNA ratio was expressed as the nitrogen/phosphate (N/P) ratio, and calculated as 43 g/mol corresponds to each repeating unit of PEI [NCH₂CH₂] containing one amine and 316 g/mol corresponds to each repeating unit of siRNA nucleotide containing one phosphate.

1.3.3 Gel retardation studies

For gel retardation studies, complexes containing 750 ng of siRNA with varying amounts of PLPEI or PEI 1.8 kDa in PBS were electrophoresed through a 0.8% agarose gel, using the E-Gel electrophoresis system (Invitrogen Life Technologies) and evaluated under UV-light.

1.3.4 Ethidium bromide exclusion assay

The binding of PLPEI to siRNA was examined by the fluorescence quenching method based on ethidium bromide (EtBr). The experiments were carried out by measuring the fluorescence intensity of complexes prepared from siRNA (5μg/ml) with EtBr intercalated at a molar ratio of 2:1 (siRNA:EtBr) as increasing amounts of PLPEI or PEI 1.8 kDa were added. Fluorescence was measured using a 96-well plate reader (Multiscan MCC/340, Fisher Scientific Co) at the excitation and
emission wavelengths of 540 and 580 nm, respectively. The relative fluorescence values were
determined as follows: Fr = (Fm – Fe) x100/ (Fo – Fe), were Fr is the relative fluorescence, Fm is the
measured fluorescence, Fe is the fluorescence of EtBr in the absence of siRNA and Fo is the initial
fluorescence in the absence of the polycation.

1.3.5 Serum stability of the complexes

The protection of siRNA within PLPEI complexes against serum degradation was evaluated by
incubating PLPEI and PEI complexes at N/P ratio of 10 and naked siRNA in the presence of 50% FBS
for 1 and 24 h. The complexes were disrupted with heparin (50U/µg siRNA), loaded onto 0.8 %
agarose gel (1µg siRNA per well) and evaluated under UV-light.

1.3.6 Biophysical characterization of the PLPEI/siRNA complexes

The PLPEI/siRNA complexes were characterized by Dynamic light scattering (DLS). The particle size
and zeta potential of the formulations was measured by using a Zeta Plus Particle Analyzer
(Brookhaven Instruments Corp, Santa Barbara, CA). Scattered light was detected at 25°C at an angle of
90°. Samples (50 µL) of PLPEI/siRNA complexes were diluted in 1.7 ml of nuclease-free water
and measured immediately after preparation.

1.3.7 Preparation of MNPs

The MNPs were formulated with PLPEI: POPC: Cholesterol: PEG-PE (4:3:3:0.3 mol/mol) and siRNA.
First, 18.6 µg PLPEI (13.6 µg as PEI) and 10 µg siRNA corresponding to an N/P ratio of 10 were
separately diluted in PBS and mixed to a final volume of 100 µL. A dry lipid film was prepared from
the mixture of POPC, cholesterol and PEG-PE (4.3 µg, 2.2 µg, 1.6 µg, 3:3:0.3 mol/mol). The lipid film was hydrated with preformed PLPEI complexes and incubated for 1 h at room temperature with intermittent vortexing.

1.3.8 Biophysical characterization of the MNPs

Samples (50 µL) of the MNPs were diluted in 1.7 ml of nuclease-free water and size and zeta potential was measured immediately after preparation using the Zeta Plus Particle Analyzer (Brookhaven Instruments Corp, Santa Barbara, CA).

1.3.9 Stability of siRNA in MNPs against RNase digestion

Nuclease resistance of the siRNA in the MNPs was determined by the treatment of the samples with 1U of RNase III/µg siRNA for 30 min at 37°C. MNPs were disrupted using 0.1% Triton-X 100 and Heparin (50U/µL) and analyzed by agarose gel electrophoresis. The integrity of siRNA in MNPs was compared with that of naked siRNA.

1.3.10 Cytotoxicity assays

For cytotoxicity studies, NIH/3T3, B16F10 and C166-GFP cells were seeded in 96-well plates (10 cells/well). After 24 h, the medium was replaced with 100 µL/well of serial dilutions containing equivalent concentrations of PEI for each formulation. After 4 hours of incubation, the cells were washed twice with PBS and returned to complete media (100 µL). After 24-48 h incubation, 20 µL of CellTiter Blue (Promega) was added to each well and the plates re-incubated for 2 h. The fluorescence was measure at excitation and emission wavelength of 560 nm and 590 nm, respectively.
1.3.11 Study of the MNPs uptake

To assess the ability of MNPs to transfer siRNA into cells, carboxyfluorescein (FAM) labeled siRNA was formulated in MNPs at the N/P ratio of 10 as described above. B16F10 melanoma cells were seeded into 6-well plates (10 cells/well) and incubated 24 h prior transfection. Cells were treated with MNPs or free siRNA at a concentration of 250 nM. After incubation for 4 h, the cells were incubated for another 24 h, washed with PBS and trypsinized. The uptake of FAM-labeled siRNA was detected with a Becton- Dickinson FACSort™ flow cytometer (Franklin Lakes, NJ) and data analyzed with CellQuest™ software (Becton-Dickinson). Additionally, the intracellular delivery and distribution of siRNA loaded in the MNPs was studied using fluorescence microscopy. B16F10 cells were seeded in a four-chamber well slide (10 cells/well) and allowed to attach overnight. Cells were then treated for 1, 4 and 24 h with free FAM-siRNA or loaded in MNPs at a concentration of 100 nM. At the end of the incubation period, the cells were washed with PBS, incubated with Hoechst 33342 and Lysotracker® Red for 15 min and 1 hour respectively and visualized using a Nikon Eclipse E400 fluorescent microscope.

1.3.12 In vitro gene silencing

In vitro gene silencing experiments were performed in stably transfected C166-GFPcells using GFP targeted-siRNA (GFP-siRNA). A non-targeting control duplex (Negative-siRNA) was used as a non-specific control siRNA. Briefly, 24 h before transfection, cells were seeded in 12-well plates at a density of \(5 \times 10^4\) per well. siRNA complexes with PLPEI or PEI 1.8 kDa polymers and MNPs were prepared at N/P of 10 as previously described. Complexes (50 μL) were added to cells to yield a final
concentration of 100 nM. After 4h of incubation, complexes were removed, fresh media was added and the cells were further incubated for 48 h. Thereafter, cells were washed, trypsinized and GFP downregulation was analyzed using flow cytometry.

1.3.13 Statistical Analysis

Results are presented as mean ± SD, and statistical significance of differences was evaluated by variance analysis (one-way ANOVA); p-values smaller than 0.05 were considered to be significant, * p<0.05.

1.4 RESULTS

1.4.1 Characterization of the PLPEI

PLPEI conjugates were constructed by following the previously established method by Ko et al. 2009, grafting the PAzPC phospholipid fatty acid tails to the PEI backbone. After synthesis followed by purification, the polymer were characterized by $^1$H-NMR spectroscopy. The NMR spectra of the polymer had detectable characteristic peaks for PEI and the lipid used for conjugation. The broad multiplet signal at ~δ ppm 2.20-2.30 in the spectra was from the ethylene (-CH2-CH2-) protons of PEI. The proton signal as triplet at 0.87 ppm was from the terminal -CH3 group of the lipid chain, present in the polymer. The characteristic proton signals from terminal CH3-groups of the -N(CH3)3 in PAzPC- PEI was at δ ppm 1.10 and 1.41. The presence of characteristic peaks of PEI and the lipids in the NMR spectra of the polymers indicates successful conjugation of the lipids with PEI (fig. 1.1).
Fig. 1.1 NMR spectra of PLPEI with detectable characteristic peaks for PEI (blue) and the lipid (red) used for conjugation

1.4.2 Nitrogen/phosphate ratio determination for polymer/siRNA complexes

The condensation of the siRNA within the PLPEI complexes is a prerequisite for preparation of MNPs. In this sense, the lipid-modified PEI is expected to possess enough positive charge so as to condense the siRNA and protect it from enzymatic degradation. Therefore, we evaluated the binding stability of PLPEI to siRNA by gel retardation and by a dye exclusion assay. PLPEI had a similar capacity to retain siRNA during gel electrophoresis when compared to non-modified PEI. The N/P ratio of 3 resulted in electrophoretic immobilization of siRNA for modified and unmodified PEIs (fig. 1.2A).

When the EtBr displacement assay was performed, further differences between modified and non-modified PEI were observed. As the N/P ratio of PEI complexes increased, the relative
fluorescence decreased to a maximum binding degree at an N/P of 4. No differences in relative fluorescence were observed at higher ratios. The same behavior was observed in the PLPEI complexes; however, the fluorescence plateau was reached at an N/P of 10 (fig. 1.2B). Since the benefits of using N/P ratios higher than those just adequate to provide condensation of DNA or siRNA when PEI is used as transfection reagent have been repeatedly shown [27, 34, 35] based on this data and gene silencing data (to be elucidated further), an N/P ratio of 10, when siRNA is totally condensed, was chosen for all further steps.

Fig. 1.2 PLPEI/siRNA complex formation. (A) Gel retardation and (B) relative binding affinity measured by ethidium bromide displacement assay of PLPEI and PEI complexes with siRNA at varying N/P ratios.
1.4.3 Characterization of the PLPEI complexes

At N/P 10, DLS showed the size of PLPEI/siRNA complexes as 167 ± 24 nm, PDI was 0.093. The zeta potential was shown to be 31 ± 2 mV.

1.4.4 Preparation of MNPs

A mixture of free lipids comprising POPC, cholesterol, PEG-PE (3:3:0.3 mol/mol) was hydrated with PLPEI/siRNA complex solution and kept for 1 h at RT. These ratios were decided based on findings from previous studies and earlier work by another group in our lab [30, 36]. The formation of the MNPs was suggested by DLS and zeta potential measurement data in addition to the previously reported cryo- Transmission electron microscopy (cryo-TEM) data [30]. The hydrophobic interactions between the lipid portion of PLPEI and the free lipids led to the formation of MNPs (see fig. 1.3) as suggested by the small particle sizes and a neutral zeta potential. The incorporation of lipids slightly increased the size of polymer/siRNA complexes from 167 ± 24 nm to 213 ± 35 nm in MNPs, while the zeta potential decreased dramatically from a very positive 31 ± 2 mV to a neutral surface charge of 0 ± 3 mV.

![Diagram showing the self-assembly process of MNPs](image)

**Fig. 1.3** Schematic representation suggesting the self-assembly process of MNPs
1.4.5 Nuclease stability of siRNA in polymer/siRNA complex and MNPs

To investigate whether the MNPs could protect siRNA against digestion by nucleases, the stability of siRNA within the PLPEI complexes was first evaluated. Naked siRNA and PLPEI/siRNA complexes were incubated in the presence of 50 % FBS for 1 and 24 h. It was found that the naked siRNA was completely degraded after 24 h of incubation with serum, while degradation of siRNA was prevented in the PLPEI complex (fig. 1.4A). Similarly, the nuclease stability of the siRNA within the MNPs was demonstrated in the presence of ds-siRNA specific RNase III. Even after 20 min of incubation with RNase III, naked siRNA was totally digested, and no siRNA band was detected on the agarose gel. On the contrary, siRNA bands with the intensity similar to controls were detected for MNPs incubated in the presence or in the absence of enzyme, suggesting that siRNA formulated in MNPs was totally protected from enzymatic degradation (fig. 1.4B).

![Fig. 1.4 Protection of siRNA within the PLPEI complexes (A) and MNPs (B) against serum and RNase III degradation](image-url)
1.4.6 Cytotoxicity of MNPs

To demonstrate the safety profile of the PLPEI complexes and MNPs in different cell lines, cytotoxicity assays were performed. It was shown that MNPs exhibited no toxicity towards NIH/3T3 and B16F10 cells over the concentration range of 1-to-7 µg/ml used in our in vitro experiments (figs. 1.5A and 1.5B). Moreover, the viability of cells was higher than 80% even at high concentrations (up to 50-100 µg/ml). PLPEI complexes showed a similar profile when compared to that of MNPs, although an increase in toxicity was detected at the highest concentration for 16F10 cells. In the case of non-modified PEI, PEI 1.8 kDa complexes were non-toxic over the concentration range tested, in sharp contrast to those formulated with PEI 25 kDa (positive control) that were highly toxic at a concentration of 15 µg/ml.

Fig. 1.5 Cytotoxicity of MNPs, PLPEI complexes, PEI 1.8 kDa complexes, and PEI 25 kDa complexes towards (A) NIH/3T3 cells (B) B16F10 cells at different PEI concentrations. Relative cell viability was expressed as a percentage of control cells.
1.4.7 Cellular uptake of MNPs

The cellular uptake of MNPs, PLPEI complexes and PEI complexes was studied in B16F10 cells with fluorescein-labeled siRNA (FAM-siRNA). Cells were treated for 4h with the fluorescently-labeled formulations and analyzed by FACS after 24 h. The flow cytometric histogram of the different formulations is shown (fig. 1.6A). It was shown that all formulations generated a significant increase in the mean fluorescence of cells as compared to non-treated cells or those treated with free siRNA. As shown in (fig. 1.6B), more than 70% of the cells were transformed after MNPs treatment. PLPEI complexes showed a 3-fold higher uptake levels than PEI complexes, whereas the increase for MNPs was 2.5-fold higher. The influence of different incubation times on MNPs uptake is shown in (fig. 1.7).
Fig. 1.6 Cellular uptake of FAM-labeled siRNA in various complexes and MNPs. (A) Changes in FACS histograms indicative of siRNA-positive cells after 24 hours of incubation following 4 hours of treatment with different formulations. (B) Bars represent quantitative analysis of FACS histograms in (A) to obtain the percentage of cells positive for FAM-siRNA. Data are expressed as the mean ± SD (n=3) (* p < 0.05 vs free siRNA and PEI 1.8 complexes).

1.4.8 siRNA internalization and intracellular distribution

After 1 h of incubation of MNPs with cells, green fluorescence of siRNA was detected at the surface of the cells. After 4 h, the internalized siRNA was located in the cytoplasm. When B16F10 cells were incubated with MNPs for one day, the siRNA was detected in the nuclei of cells. At the same time point (24 h), naked siRNA gave almost no detectable fluorescence in cells (fig. 1.7d). It is important to note that nuclear localization of the fluorescence is not due to the translocation properties of the carrier but due to the chemical modification of the siRNA itself. The FAM- siRNA used in this experiment, siGLO transfection indicator (Dharmacon, Lafayette, CO) is modified to localize finally in the nucleus after 24 h as an unmistakable signal of uptake and transfection.
**Fig. 1.7** Intracellular trafficking of MNPs (a-c) and free FAM- siRNA (d) after different incubation times with B16 cells. The nuclei (blue) were stained with Hoechst dye. The internalized FAM-siRNA appears green, and the cytoplasmic boundaries (red) were marked with Lysotracker Red

1.4.9 *Green Fluorescent Protein down-regulation*

To evaluate the knockdown efficacy of MNPs, the downregulation of GFP was measured by the decrease in the mean fluorescence of stably GFP-expressing C166 cells (C166-GFP) after treatment with MNPs loaded with GFP-siRNA using flow cytometry (FACS). The cells treated with MNPs produced an almost 20 % reduction (**fig. 1.8A**) of GFP fluorescence (p<0.05). This fluorescence shift was not observed when cells were treated with negative-siRNA loaded MNPs, suggesting that the decrease in the fluorescence of cells was due to the GFP knockdown and not due to the toxicity of the formulation. Cytotoxicity studies were also performed at the same experimental conditions as the FACS downregulation experiments and the absence of MNPs toxicity was confirmed in C166-GFP cells. MNPs and PLPEI were non-toxic to the cells PEI 25 kDa complexes decreased GFP fluorescence more than MNPs but also showed significantly higher toxicity to the cells (**fig. 1.8B**).
Fig. 1.8 (A) siRNA mediated down-regulation of the target gene and (B) cytotoxicity profile of treatments. Experiments were performed in stably transfected C166-GFPcells using GFP-siRNA. Cells were treated with MNPs for 4 h, and the GFP fluorescence was analyzed by FACS after 48 h incubation. A non-targeting control duplex (Negative-siRNA) was used as a non-specific control siRNA. PEI 25 kDa was used as positive control. Data are expressed as the mean ± SD (n=3). (* p < 0.05 vs non-treated control cells)

1.5 DISCUSSION

Our lab has previously reported a new gene delivery system consisting of micelle-like nanoparticles, MNPs, prepared by condensing plasmid DNA with lipid-modified PEI (PLPEI) and enveloping the new complexes with a PEG/lipid layer [24, 26, 30, 36]. These MNPs protected the loaded DNA from enzymatic degradation, exhibited decreased cytotoxicity and demonstrated improved in vivo stability as compared to plain PEI complexes. As in the case of DNA, the in vivo application of siRNA is hampered by its rapid degradation in the plasma and fast renal clearance and inefficient uptake by tissue cells. Taking all this into account, we hypothesized that siRNA delivery technology could be enhanced by employing MNPs as gene carriers. In this study, we prepared MNPs loaded with siRNA and characterized them for their biophysical properties, cytotoxicity, cellular uptake and in vitro knockdown efficacy.

The first step for the preparation of MNPs was condensation of the siRNA with the lipid grafted PEI. The lipid-PEI conjugates (PLPEI) were prepared by coupling low molecular weight PEI (1.8 kDa) with the tail modified PAzPC phospholipid and was characterized successfully using NMR. PEI 1.8 kDa, is known to be less toxic than its high molecular weight counterparts (PEI 25kDa, 800
kDa) due to the smaller number of primary amines present on its surface but also is less efficient in the transfection of nucleic acids to the cells [29, 37]. In addition, the incorporation of the lipid to the backbone of the polymer decreases the number of available positive groups that can interact with the siRNA. Therefore, it was important to optimize the amount of PLPEI needed for the complete binding and protection of siRNA. An N/P ratio of 10 was found to be sufficient for the total condensation of the siRNA as small complexes (ca. 160 nm) and for its protection from serum degradation (figs. 1.2 and 1.4A).

Lipid modification of PEI has been reported to improve the stability and uptake of the PEI complexes [30]. However, with MNPs, the lipid moiety of PLPEI plays the additional role of anchor that facilitates the envelopment of PLPEI/siRNA complexes with the lipids and PEG-PE without additional procedures (such as extrusion, sonication) usually required to prepare systems similar to MNPs [16, 27]. Just one hour of incubation of PLPEI complexes with a mixture of lipids was sufficient to permit the self-assembly of MNPs driven by the hydrophobic interactions between the exposed lipid moieties of the complexes and the free lipids. The finding of somewhat increased sizes for MNPs vs PLPEI complexes (167 ± 24 nm to 213 ± 35 nm) supports the notion that the lipids and PEG-lipid conjugates formed a shell around the complexes, which is also suggested by the zeta potential data. The positive surface charge of PLPEI decreased from 31 ± 2 mV to a neutral surface charge 0 ± 3 mV probably due to because of the shielding effects of the PEG and lipids. In addition, the presence and protective effect of the MNPS envelope was further demonstrated by the protection of the loaded siRNA from RNase III digestion, that specifically cleaves double-stranded siRNA, a more accurate and stringent assay than the incubation in serum performed with PLPEI complexes (fig. 1.4B). Several studies carried out with plain, PEGylated or liposomal-PEI formulations have revealed that, for a given carrier, DNA and siRNA transfection efficacy do not always correlate
[16, 24, 27, 29, 38]. Thus, the ability of MNPs to deliver siRNA into cells, already demonstrated for plasmid DNA, was evaluated in B16F10 cells with a FAM- labeled siRNA and compared to that of PEI 1.8 kDa and PLPEI (fig. 1.6). On one hand, the lipid modification of PEI dramatically improved the uptake of siRNA with respect to unmodified PEI, most probably because the lipid residues provided a better interaction with the cellular membrane. Early studies demonstrated that the conjugation of cholesterol to PEI 1.8 kDa increased the DNA transfection levels compared to PEI 1.8 and 25 kDa.

The benefits of lipid-PEI conjugation were later confirmed for siRNA delivery [24, 27, 38]. On the other hand, the MNPs had slightly lower cellular uptake than PLPEI complexes suggesting that the presence of PEG in MNPs may hinder the interactions with the cells [27, 30]. Still, the MNPs effectively delivered FAM-siRNA to B16F10 cells. More than 70% of the cells were siRNA-positive after the treatment with siRNA-loaded MNPs. The internalization of FAM-siRNA mediated by MNPs was confirmed by fluorescence microscopy (green dots in fig. 1.7b). Once inside the cytosol, the loaded siRNA maintained its biological activity since MNPs down- regulated GFP expression significantly compared to the control and to MNPs loaded with negative- siRNA, respectively (fig. 1.8A). Taken together, the data obtained previously indicated that the stability of MNPs was important for the final level of downregulation and often an increase in the efficacy of delivery would be accompanied by an increased downregulation. Studies with PEG-grafted PEI suggested that the intracellular complex stability, rather than its cellular uptake, was a major determinant of the complex bioactivity [39]. In the case of MNPs, the presence of PEG and lipids did not influence their cellular uptake but provided additional stability to PLPEI complexes that could interfere with the siRNA release process in the cytoplasm.
Finally, PEI 25 kDa, used as a positive control, showed a decrease in GFP fluorescence 1.5-fold greater than the MNPs but also had significantly higher toxicity (Figure 1.8B). The lack of MNPs toxicity represents one of the major advantages over non-modified PEIs or PLPEI. MNPs were non-toxic irrespective of the dose or the cell line employed for testing (figs. 1.5 and 1.8B).

1.6 CONCLUSIONS

In this study, the suitability of MNPs based on PAzPC-PEI for siRNA delivery and gene silencing was probed. MNPs based on a dense PLPEI/siRNA core enveloped by a lipid/PEG layer exhibited good capacity to complex and protect siRNA from serum degradation as that of PEI, but with improved biocompatibility properties including a neutral surface charge and thus, an absence of in vitro cytotoxicity. These findings together with our previous in vivo data (long circulation time, absence of acute in vivo toxicity) [30] suggest a promise for the application of MNPs for systemic delivery of siRNA.

2. HYPOTHESIS#2 ‘PE-PEI will be able to complex, protect, deliver siRNA intracellularly and cause gene downregulation in vitro.’
SPECIFIC AIM#2 To develop and characterize phosphoethanolamine-modified PEI (PE-PEI)-based nanocarriers for siRNA delivery and compare their effectiveness with regards to intracellular delivery and gene silencing in vitro against PAzPC-PEI- based nanocarriers via studies to elucidate their mechanism of action.

2.1 BACKGROUND FOR SPECIFIC AIM#2

It was shown via previous studies that the PAzPC-PEI based siRNA complexes and MNPs encapsulating these complexes were able to successfully downregulate GFP protein in a stably GFP expressing cell line. As it is with any delivery system, there is always scope for improvement and continuing to build up from our previous PAzPC modified PEI, we decided to further synthesize phosphoethanolamine modified PEI designated as PE-PEI. We chose dioleoyl phosphoethanolamine (DOPE) as one phospholipid to form DOPE-PEI since DOPE is frequently used in gene delivery as a helper lipid and it was hypothesized that this trait could be exploited to possibly promote the fusion of the carrier with the cell membrane and once in the cell PEI would facilitate the disruption of endosomes after the uptake of the complexes [17, 40-42].

The other phospholipid chosen was dipalmitoyl phosphoethanolamine (DPPE) which is structurally very similar to DOPE. The potential of two different PE-modified PEIs was evaluated for stable condensation and protection of siRNA, the ability to deliver siRNA and down-regulate gene expression and their performance was compared against PAzPC modified PEI (described previously in the text). In particular, we wanted to evaluate the effect of the phospholipid components and the conjugate/siRNA ratio on the biophysical characteristics and the in vitro performance of such amphiphiles for application in future work.
2.2 MATERIALS

All materials were purchased from Sigma-Aldrich unless otherwise stated. Branched polyethyleneimine (PEI) with molecular weights of 1.8 kDa and 25 kDa were purchased from Polysciences, Inc (Warrington, PA). Glutaryl head-modified phospholipids 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(glutaryl) (NG-DOPE) and dipamitoyl-sn-glycero-3-phosphoethanolamine-N-(glutaryl) (NG-DPPE), the oxidized phospholipid 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine (Az PC Ester) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethyleneglycol)-2000] (PEG-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). A siRNA duplex targeting Green Fluorescent Protein (GFP-siRNA): 5’-AUGAACUUCAGGGUCACGUdTdT-3’ (sense), a non-targeting control duplex, (Negative-siRNA): 5’-AGUACUGCUUACGAUACGGdTdT-3’ (sense) and a DY547- labeled siRNA were purchased from Dharmaco (Lafayette, CO). RNase III was purchased from Ambion/Life Technologies (Carlsbad, CA). The CellTiter-Blue® Cell Viability Assay and Hoechst 33342 were purchased from Promega (Madison, WI). Nuclease-free water was purchased from Qiagen (Germantown, MD).

2.2.1 Cell Culture

The cell lines C166 (mouse yolk sac embryo) and C166-GFP (C166 cells stably transfected with a plasmid reporter vector, pEGFP-N1, encoding for the enhanced green fluorescent protein, GFP) were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown at 37°C under 5% CO2 in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL) and streptomycin (100 μg/mL). For C166-GFP cells,
DMEM media was supplemented with 10% FBS and 0.2 mg/mL of Geneticin (G-418, Life Technologies, Carlsbad, CA). Cell culture media and penicillin/streptomycin stock solutions were purchased from Cellgro (Herndon, VA). Heat-inactivated fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA).

2.3 METHODS

2.3.1 Synthesis of phospholipid-polyethylenimine conjugates

The DOPE-PEI and DPPE-PEI conjugates were synthesized from PEI 1.8 kDa and the glutaryl head-modified phospholipids: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(glutaryl) (NG-DOPE) and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(glutaryl) (NG-DPPE) as described in [43]. Briefly, NG-DOPE or NG-DPPE (5.5 µM) in chloroform (1mL) were activated with N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide HCl (EDCI) and N-hydroxysuccinimide (NHS) followed by addition of PEI 1.8kDa (5.5 µM). The mixture was incubated with 4 µL of triethylamine (TEA) at room temperature for 24 h with stirring. The chloroform was removed under nitrogen gas and the residue was suspended with 1 mL of dH₂O. The products were purified by dialysis (MWCO 2000 Da) against dH₂O and lyophilized.

The PAzPC-PEI or PC-PEI conjugate was synthesized from PEI 1.8 kDa and the oxidized phospholipid 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine (Az PC Ester) as described in the text previously (section 1.3.1). Briefly, 12 mg of the branched PEI (7µmol) were dissolved in 0.5 mL of chloroform and mixed with 5 mg of the oxidized PC (AzPC Ester, 7µmol) dissolved in 1 mL of chloroform. Carbonyldiimidazole (CDI, 0.5mg, 3µmol) was added to the above solution for the activation of acid by formation of an imidazole derivative. The mixture was incubated with 10 µL of
TEA at room temperature for 24 h with stirring. The chloroform was removed under nitrogen gas and the residue was suspended with 2 mL of dH2O. The products were purified by dialysis (MWCO 2000 Da) against dH2O and lyophilized.

2.3.2 NMR

The synthesized conjugates were characterized by 1H nuclear magnetic resonance (NMR) spectroscopy. NMR spectroscopy was performed using Varian 400 MHz spectroscope. The conjugates were dissolved in d-CDCl3, and a 5-10 mg/mL solution was used. The characteristic peaks noted at different PPM values are as follows (where singlet, doublet, triplet, multiplet, broad singlets are noted as s, d, t, m, bs respectively): For DOPE-PEI, 1H-NMR in CDC13. δ ppm 0.87 (t, 6H), 1.26-1.33 (d), 1.99-2.02 (m) and 2.22-2.30 (m), 2.40-3.00 (m), 3.31-3.41 (m), 3.93 (bs), 4.10-4.12 (m), 4.37-4.40 (d), 5.20 (bs) and 5.32-5.35 (t). For DPPE-PEI, 1H-NMR in CDC13. δ ppm 0.87 (t, 6H), 1.26-1.28 (d), 1.75-1.94 (m), 2.22-2.28 (m), 2.25-2.30 (m), 3.33-3.41 (m), 3.91 (bs), 4.10-4.12 (m), 4.36-4.39 (d), 5.19 (bs). For PC-PEI, 1H- NMR in CDC13. δ ppm 0.87 (t), 1.10 (s), 1.25-1.29 (m), 1.41 (s), 2.21-2.31 (m), 2.35-3.10 (m), 3.25-3.50 (m), 3.65-3.66 (d), 3.82-4.32 (m), 5.21 (bs).

2.3.3 Determination of Critical Micelle Concentration (CMC) of the conjugates

CMC was estimated by the pyrene method [44]. Briefly, phospholipid-PEI conjugates were serially diluted with dH2O to obtain 10^{-7} to 10^{-4} M solutions. Then, 1mL of each dilution was added to pyrene- coated glass tubes containing 0.5 mg of dry pyrene crystals. The mixtures were kept overnight in a shaking incubator at 150 rpm at 25 °C. Free pyrene was removed by filtration through
polycarbonate membranes with 0.2 μm pore size. The filtered samples were transferred to a 96-well plate and the fluorescence was measured using a plate reader (Multiscan MCC/340, Fisher Scientific) at excitation and emission wavelengths of \( \lambda_{\text{ex}} \) 360/40 and \( \lambda_{\text{em}} \) 460/40 nm, respectively. CMC values were determined from the pyrene fluorescence in solution as a function of conjugate concentration, and corresponded to concentrations at which a sharp increase in the solution fluorescence occurred. The amphiphile PEG-PE was used as a reference.

2.3.4 Atomic Force Microscopy

The various phospholipid-modified PEI were characterized by Atomic Force Microscopy (AFM) as a visual tool for the polymer structure. Briefly, AFM images were obtained on an Agilent 5500 AFM/SPM microscope under acoustic AC mode using Si probes operating at a resonant frequency of 154 kHz. All measurements were carried out at room temperature and acquired images had a resolution of 512 x 512 pixels collected at a speed of 1 line/minute. Freshly cleaved mica surface was used as the substrate for imaging. To acquire images, about 10-50 μl of the prepared sample was pipetted on to the mica surface and allowed to interact with the surface for 1-5 minutes. Following this, excess solution was dried under a gentle stream of air. Post image processing of AFM images was done using Pico Image software provided with the instrument. The images were subjected to standard image processing techniques that included line correction, form removal, leveling and threshold adjusting. In all the AFM measurements, topography, phase and amplitude images were obtained. For clarity, only the phase images were compared and presented. Additionally, line profile images, i.e., a plot of height across a line on the XY plane from topography image were also plotted.
2.3.5 Cytotoxicity of phospholipid-PEI conjugates

For cytotoxicity studies, C166-GFP cells were seeded in 96-well plates (10^4 cells/well). After 24 h, the media was replaced with 100 µL/well serial dilutions of PL-PEI conjugates and non-modified 1.8 kDa PEI. Additionally, PEI 25kDa was used as a control. After 4 hours of incubation, the cells were washed twice with fresh media and returned to complete media (100 µL). After 24 h incubation, 20 µL of CellTiter Blue (Promega) was added to each well and the plates re-incubated for 2 h. The fluorescence was measured at excitation and emission wavelengths of 560 nm and 590 nm, respectively. When possible, sigmoidal curves of cell viability vs log (concentration) were transformed into the pseudo-Hill plots, and the polymer concentrations at 50% cell death (IC_{50}) were calculated.

2.3.6 Preparation of phospholipid-PEI/siRNA complexes

Complexes were prepared by mixing a fixed amount of siRNA and varying amounts of phospholipid-PEI conjugates that were diluted separately in equal volumes (50 µL) of buffered HEPES glucose (BHG, pH 7.4, nuclease-free buffer). The siRNA solution was transferred to the polymer solution, mixed by smooth pipetting and incubated for 15-20 min. The polymer/siRNA ratio was expressed as the nitrogen/phosphate (N/P) ratio and calculated assuming that 43 g/mol corresponded to each repeating unit of PEI containing one amine and 316 g/mol corresponded to each repeating unit of siRNA containing one phosphate. The molecular weight of the grafted lipids was also factored in to calculate the amount of polymer in the complex.

Complex formation was studied by gel retardation. Complexes containing 750 ng of siRNA with varying amounts of phospholipid-PEI were electrophoresed through a 0.8% agarose gel, using the E-Gel...
electrophoresis system (Life Technologies) and evaluated under UV-light.

Nuclease resistance of the siRNA in phospholipid-PEI complexes was evaluated by incubation of phospholipid-PEI/siRNA complexes at an N/P ratio of 16 with 1U of RNase III/µg siRNA for 2h at 37°C. The progression of the enzymatic degradation was stopped by the incubation of each sample with 30mM EDTA for 5 min. Complexes were then disassembled by adding heparin (10U/µg siRNA) and analyzed by agarose gel electrophoresis. The integrity of siRNA in complexes in the presence or the absence of enzyme was compared with that of naked siRNA under the same experimental conditions.

The particle size and zeta potential of the formulations were measured using a Zeta Plus Particle Analyzer (Brookhaven Instruments Corp, Santa Barbara, CA). Scattered light intensity (DLS) was detected at 25°C at an angle of 90°. Samples (100 µL) of complexes were diluted in 1.7 mL of nuclease-free water and measured immediately after preparation. The final siRNA concentration in the samples was 5-10 µg/mL.

2.3.7 GFP silencing

In vitro GFP silencing experiments were performed in stably transfected c166 GFP cells using GFP-siRNA. A non-targeting control duplex (Negative-siRNA, scramble siRNA) was used as a non-specific control siRNA. In a typical experiment, cells were seeded 24 h prior to transfection in 12-well plates at a density of 5 x 10⁴ per well and complete medium was replaced with fresh serum-free medium. Phospholipid-PEI complexes at varying N/P ratios were added to cells to yield a final siRNA concentration of 100 nM. After 4h of incubation, the complexes were removed and fresh complete media was added. The cells were further incubated for 48 h. Thereafter, the cells were washed, detached by trypsinization, and GFP down-regulation analyzed by flow cytometry. In selected experiments, c166 GFP cells were pre-incubated for 1h with chloroquine (50 µM) or bafilomycin A1 (175 nM) before
siRNA transfection.

2.3.8 Intracellular delivery of phospholipid-PEI/siRNA complexes

The ability of phospholipid-PEI complexes to deliver siRNA into the cells was studied by confocal microscopy and flow cytometry using a fluorescence-labeled siRNA. The condensation of fluorescent siRNA with the different conjugates did not affect the intrinsic fluorescence of siRNA. Briefly, cells were seeded into 12-well plates (5 x10^4 cells/well) and incubated 24 h prior to transfection. Cells were treated with complexes prepared with a siRNA concentration of 100 nM at various N/P ratios. After incubation for 1 h or 4 h with complexes, the cells were washed with PBS and trypsinized. The fluorescence from siRNA inside the cells was detected with a Becton-Dickinson FACSortTM flow cytometer (Franklin Lakes, NJ). For the distinction between internalized and surface-bound siRNA, trypan blue (1.2 mg/ml) was used to quench surface-bound fluorescence. Data was analyzed with CellQuestTM software (Becton-Dickinson).

For better visualization of the siRNA in microscopy experiments, non-GFP expressing c166 cells were employed. Briefly, cells were seeded into 6-well plates (3 x10^3 cells /well) and incubated 24 h prior transfection. Cells were treated with complexes for 4 h, washed with PBS and incubated with Hoechst 33342 for 15 min. The cells were washed several times with PBS. The cover slips were mounted with Fluoromount-G medium and examined with a Confocal Zeiss LSM 700 microscope. Zeiss ZEN2009 software was used for set up, and Image J software was used for image processing.

2.3.9 Statistical analysis

Results are presented as mean ± SD. Unless otherwise stated, comparisons between the groups were made using Student’s t test. p < 0.05 were considered to indicate a significant difference.
2.4 RESULTS

2.4.1 Synthesis of phospholipid-PEI conjugates

Water soluble polyethylenimine (PEI 1.8kDa) was hydrophobically modified with phosphoethanolamine (PE-PEI) or phosphocholine phospholipids (PC-PEI) to obtain different amphiphilic structures (fig. 2.1). In the case of PE-PEI conjugates, the head of DOPE or DPPE phospholipid was grafted to the PEI backbone. PC-PEI conjugates were constructed by grafting one of the phospholipid fatty acid tails to the PEI backbone.
**Fig. 2.1** Synthesis scheme of Phospholipid conjugation of PEI. 1 – NG-DOPE, 2-NG-DPPE, 3-PAzPC, 4-PEI 1.8kDa, 6-DOPE-PEI, 7-DPPE-PEI, 8-PAzPC-PEI or PC-PEI

### 2.4.2 NMR of PLPEI conjugates

After synthesis followed by purification, the polymers were characterized by $^1$H-NMR spectroscopy. The NMR spectra of all three polymers had detectable characteristic peaks for PEI and the lipid used for conjugation (**fig. 2.2**). The broad multiplet signal at $\sim$δ ppm 2.20-2.30 in all three spectra was from the ethylene (-CH2-CH2-) protons of PEI. The proton signal as triplet at 0.87 ppm was from the terminal - CH3 group of the lipid chain, present in all three polymers. The characteristic triplet proton signal at 5.33 ppm in PEI-DOPE was characteristic peak from the protons adjacent to the double bond in the fatty acid chain in DOPE, which was absent in PEI-DPPE and PC-PEI. The characteristic proton signals from terminal CH3-groups of the -N(CH3)3 in PC-PEI was at δ ppm 1.10 and 1.41. The presence of characteristic peaks of PEI and the lipids in the NMR spectra of the polymers indicates successful conjugation of lipids with PEI.
Fig. 2.2 NMR spectra of all three polymers with detectable characteristic peaks for PEI (blue) and the lipid (red) used for conjugation

2.4.3 *CMC determination of the PLPEI conjugates*

Due to their amphiphilic nature, and as suggested by the AFM images, the novel conjugates were expected to self-assemble into micellar structures. To determine the concentration at which the conjugate assembled into micellar structures, the fluorescence values of pyrene at increasing concentrations of the amphiphilic conjugates was measured. The fluorescence of pyrene increases as it is incorporated into the hydrophobic core of the micelles. The self-association of the polymers is shown in (fig. 2.3).

The critical micelle concentration (CMC) measured for PEG2000-DSPE used as reference amphiphile was $43 \mu g/mL \ (1.5 \times 10^{-5} \ M)$ which was in agreement with reported values [44]. Both DOPE-PEI and DPPE-PEI conjugates with a hydrophilic-hydrophobic di-block structure spontaneously assembled into micellar structures with CMC= 97 $\mu g/mL$ and 72 $\mu g/mL$, respectively. PC-PEI did not facilitate micellisation. The absence of a sharp increase in the fluorescence indicated the
minimal solubilization capacity of the PC-PEI conjugate similar to that of non-modified PEI. The hydrodynamic diameters of about 1µm measured by DLS suggested that the hydrophobic-hydrophylc-hydrophobic architecture of PC-PEI prevented the formation of organized structures and is prone to aggregation at higher concentrations.

**Fig. 2.3** Self-association of phospholipid-PEI conjugates. The critical micelle concentration (CMC) of conjugates was determined from fluorescence of the pyrene incorporated into the hydrophobic core of the micelles. The absence of a sharp increase in the fluorescence indicated the minimal solubilization capacity of the PC-PEI conjugate and non-modified PEI.

2.4.4 AFM of the PLPEI conjugates

The detailed structure of the amphiphiles assemblies was investigated by AFM (fig. 2.4). AFM images of phospholipid-PEI conjugates from solutions were prepared at concentration of 1mg/mL and 0.5 mg/mL in BHG buffer. For clarity, only the amplitude images are compared and presented. Height profiles i.e., a plot of height across a line (dashed line) on the XY plane from topography image are also
shown. All the three lipid-modified PEI samples showed a very distinct morphology and were different from each other.

In case of PC-PEI samples, large irregular shaped objects (notice the 500nm scale bar) were seen with an average size of 200 nm and height of 10 nm. However, when diluted to 0.5 mg/mL it induced disaggregation to smaller structures. The DPPE-PEI samples formed spherical structures with the majority of them less than 100 nm in size with a few structures slightly larger. When diluted by half, the sample retained the morphology and size. The DOPE-PEI structures displayed a morphology that was entirely different from the other samples with no individual or distinct structures but rather a continuous film-like structure with a bumpy texture. The separation between these bumps was slightly larger than 100 nm. The same morphology was retained upon a two-fold dilution (concentration closer to those used in in vitro experiments).
Fig. 2.4 Analysis of micelles morphology by atomic force microscopy from samples prepared at 1mg/mL and 0.5 mg/mL. The line profile images for 1mg/mL concentration is also shown.

2.4.5 Cytotoxicity of PL-PEI conjugates

The effect of phospholipid-modification of low MW PEI on the interaction of the polymer with cellular membranes was further investigated via cytotoxicity studies in C166-GFP cells. The main reason for PEI cytotoxicity is the strong interaction with anionic proteoglycans at the cell surface that
ultimately induces cell death by disruption of the cell membrane [45, 46]. Thus, it was proposed that if phospholipid conjugation could improve the association of PEI with cell membranes, then the differences between the toxicity profiles of the polymers could be detected. The effect of the different phospholipids grafted to PEI on cell viability is shown in (fig. 2.5A). Up to a concentration of 65.2µg/mL, phospholipid-modified and non-modified PEI 1.8 kDa had a remarkably low toxicity. The viability was close to the 100% viability of non-treated control cells.

It is worth mentioning that the highest concentration of PEI 1.8 kDa and phospholipid-PEI conjugates used in our in vitro experiments was 8µg/mL (final concentration per well) and therefore non-toxic for cells. By contrast, high molecular weight PEI (PEI 25 kDa) was very toxic. The IC₅₀ was 15.4µg/mL in agreement with other reported IC₅₀ values [47]. At high polymer concentrations (≥ 125 µg/mL), the toxicity of DOPE-PEI and DPPE-PE increased considerably. Cell viability dropped to 40% or lower (IC₅₀=123 µg/mL and 104 µg/mL, respectively) whereas PC-PEI conjugates and non-modified PEI 1.8 kDa remained non-toxic. The same concentration of the components themselves, free lipids (DOPE and DPPE) and PEI 1.8 kDa showed no toxicity (fig. 2.5B). Thus, the increased toxicity observed for PE-PEI conjugates can be specifically attributed to increased cell interaction of the phospholipid -PEI amphiphile.
Fig. 2.5 Cytotoxicity of free phospholipid-PEI conjugates (A) and free phospholipids (B) towards c166–GFP cells. Data are expressed as the mean ±SD (n=3)

2.4.6 Protection of siRNA against nucleases in the PLPEI conjugates

Phospholipid-grafting of PEI did not affect the siRNA condensation ability of PEI (Fig. 2.6A). Complexes prepared at N/P ratios of 3 or higher completely retarded the migration of siRNA in the agarose gel. All the conjugates (PC-PEI, DPPE-PEI and DOPE-PEI) neutralized the anionic charge of siRNA and spontaneously collapsed into small and positively charged particles with a mean diameter of 150-200 nm and a positive zeta potential of 30-35 mV (N/P 16) (Fig. 2.6B). The formation of complexes between siRNA and lipid-modified PEI was also confirmed by AFM. The images showed complex structures that were different from individual polymers or free uncondensed siRNA (Fig. 2.7).

The stability of the condensed siRNA against enzymatic degradation within the different formulations was tested by using RNase III. The integrity of siRNA was checked by agarose electrophoresis and quantified by Image J software (Fig. 2.6C). Control naked siRNA was completely
digested after the enzymatic treatment. By contrast, condensed siRNA was protected from nuclease degradation. Due to interference of the enzyme, the migration of siRNA was slightly retarded but the overall intensity of the siRNA bands before and after the enzymatic treatment was the same for all the formulations.

**Fig. 2.6** Analysis of complex formation (A) Gel retardation of complexes at varying N/P ratios. No migration of siRNA into the gel indicates the complex formation at N/P ≥ 3. (B) Particle size and zeta potential of complexes as at N/P ratio of 16. (C) Protection of siRNA within complexes against RNAse III degradation.

<table>
<thead>
<tr>
<th>Complex</th>
<th>Mean Size (nm)</th>
<th>Zeta Potential (mV)</th>
</tr>
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<tbody>
<tr>
<td>PC-PEI</td>
<td>184 ± 35</td>
<td>34.5 ± 7.25</td>
</tr>
<tr>
<td>DOPE-PEI</td>
<td>178 ± 34</td>
<td>35.9 ± 7.32</td>
</tr>
<tr>
<td>DPPE-PEI</td>
<td>222 ± 19</td>
<td>35.5 ± 3.72</td>
</tr>
<tr>
<td>PEI</td>
<td>191 ± 3</td>
<td>30.9 ± 6.02</td>
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Based on the gel retardation data and gene silencing experiments (elucidated later) an N/P ratio of 16 (a slight excess of positive charge) was chosen for all further studies. The benefits of using N/P ratios higher than those just adequate to provide condensation of DNA or siRNA when PEI is used as transfection reagent have been repeatedly shown [27, 34, 35].

2.4.7 AFM of PLPEI/siRNA complexes

Complexes were also analyzed and compared with free siRNA by AFM microscopy (fig. 2.7). Free siRNA (1:20 dilution) formed uniform spherical objects that are approximately 50 nm wide and 2 nm high. The DOPE-PEI/siRNA complexes showed several spherical structures with a very broad size distribution ranging from 50-500 nm. It is interesting to note that within these large structures, small structures are clearly seen which could be indicative of complexation of siRNA units within the larger lipid-PEI structures. In the cases of DPPE-PEI-siRNA complexes, uniform smaller spherical objects were seen with an average size of 100 nm and about 10 nm tall. These structures again are different from individual polymers or siRNA by themselves and indicate the formation of complexes between siRNA and lipid-modified PEI structures. The PC-PEI/siRNA complexes could not be imaged by this technique due to some anomalous interaction between the AFM instrument tip and the complex which showed up as aberrations (fig. not shown).

Note- All PLPEI/siRNA complexes were prepared at N/P 16.
siRNA (free form)  DOPE-PEI (free form)  DOPE-PEI/siRNA complex

(A) Free DPPE  (B) DPPE/siRNA

Height profile
Fig. 2.7 AFM images of (A) (a) free siRNA image and height profile (b) free DOPE-PEI image and height profile (c) DOPE-PEI/siRNA image and height profile (B) free DPPE-PEI and DPPE / siRNA

2.4.8 In vitro gene silencing

The silencing efficacy of PC-PEI, DPPE-PEI and DOPE-PEI was evaluated in c166 stably expressing GFP (c166-GFP) using GFP-targeted siRNA or scrambled siRNA. The silencing of GFP was measured by the decrease in the mean fluorescence of the cells after siRNA treatment. The complexes were prepared at a range of N/P ratios from 3 to 30. No detectable GFP suppression was observed with non-modified PEI (1.8 kDa) based-complexes regardless of the N/P employed (data not shown). At N/P ratios ≥16, DOPE-PEI complexes produced the highest protein suppression (60% reduction of GFP expression) followed by DPPE-PEI complexes (30% reduction). PC-PEI complexes were the least effective with less than a 5% of protein content reduction (fig. 2.8).
**Fig. 2.8** GFP silencing efficacy of phospholipid-PEI/siRNA complexes (PC-PEI, DPPE-PEI and DOPE-PEI) prepared at various N/P ratios. Data are expressed as the mean ± SD (n=3). (ANOVA, *# P< 0.05 vs scramble siRNA formulations).

2.4.9 *Cellular association and intracellular delivery of the PLPEI/siRNA complexes*

We asked whether the differences observed in the gene silencing of the conjugates could be explained by their different interaction with cell membranes. First, we studied the toxicity of the conjugates as a function of their concentration (**fig. 2.5A**). An increment of phospholipid-modified PEI interaction with cells should eventually translate into progressive membrane disruption and cell death. However, PC-PEI conjugates and non-modified PEI 1.8 kDa remained non-toxic for the cells regardless of the polymer concentration employed. DOPE-PEI and DPPE-PEI showed no toxic effect unless high concentrations (≥ 125 µg/mL) were reached. The IC$_{50}$ values were 123 µg/mL and 104 µg/mL respectively for DOPE-PEI, DPPE-PEI conjugates. In contrast, PEI 25kDa was highly toxic for cells. (IC$_{50}$=15.4 µg/mL). Interestingly, the same high concentrations of the conjugate components themselves, free lipids (DOPE and DPPE) and free non-modified PEI 1.8 kDa were non-toxic (**fig. 2.5B**) supporting the conclusion that the increase in the interaction of the conjugates with cells can be attributed specifically to the anchoring of DOPE and DPPE phospholipids to PEI. It is also worth mentioning that the highest concentration of phospholipid-PEI conjugates used for the formulation of siRNA in our *in vitro* experiments was 8 µg/mL (final concentration per well) and therefore non-toxic for cells.

Next, we studied and compared the cellular uptake of the different complexes prepared with fluorescently-labeled siRNA. Cells treated with DPPE-PEI and DOPE-PEI complexes showed increased
intracellular fluorescence compared to non-modified PEI and PC-PEI complexes (red dots in fig. 2.9). Images from selected planes at various depths within the cells (Z-stack images) confirmed the presence of siRNA associated both with the cell membrane and homogenously distributed within the cytoplasm (red dots in fig. 2.9B). The siRNA inside the cells was quantified by flow cytometry (fig. 2.9C). The membrane association and uptake of complexes was strongly influenced by the lipid grafted to PEI (PC, DPPE, DOPE) and by the amount of conjugate (N/P ratio). The mean fluorescence of cells treated with DPPE-PEI, DOPE-PEI and PC-PEI complexes was 23-, 16- and 3-fold higher, respectively, than that of non-modified PEI. DPPE-PEI and DOPE-PEI complexes were better taken up by cells. They showed at least a 3-fold increased cellular association and uptake compared to PC-PEI complexes. In addition, the comparison between the 1 h and 4 h fluorescence uptake values showed that 50-60% of the total siRNA is internalized during the first hour of incubation. This percentage rose to 80% and 100% respectively for DPPE-PEI and DOPE-PEI complexes prepared at the highest N/P ratio of 25, supporting the conclusion that an excess of conjugate in the complex not only promotes cellular association but accelerates the internalization of siRNA.
**Fig. 2.9** Intracellular delivery mediated by phospholipid–PEIs conjugates in c166 cells. (A) Cellular uptake of phospholipid-PEIs prepared at N/P 16 ratio after 4 h of incubation. The nuclei (blue) were stained with Hoechst dye. The internalized DY-547-siRNA appears in red. (B) Intracellular trafficking of DOPE-PEI/siRNA complexes after 4h of incubation. Selected images from sequentially numbered z-stacks are shown. C) Cellular uptake of the complexes prepared with fluorescence-labeled siRNA after 1h and 4 h of treatment with the complexes. The mean fluorescence intensity of cells after treatment with the complexes is shown. Data are expressed as the mean ± SD (n=3).
2.4.9 Effect of chloroquine and bafilomycin on the gene silencing mediated by DOPE-PEI and DPPE-PEI complexes

Although DPPE-PEI and DOPE-PEI carriers showed similar cell membrane interaction and siRNA uptake, DOPE-PEI displayed a more effective gene silencing. A possible reason for DOPE-PEI superiority may be the improved intracellular trafficking of siRNA complexes due to a greater escape from endosomes which is known to be a major barrier in gene delivery. In order to elucidate mechanistic differences in productive cytosolic entry of the conjugates, the silencing efficacy of DOPE-PEI and DPPE-PEI complexes was measured after pre-incubation of cells with chloroquine, (a low molecular drug which buffers lysosomes and is commonly used to improved non-viral transfection) and bafilomycin A1 (a vacuolar ATPase proton pump inhibitor). Akinc and co-workers showed that bafilomycin dramatically decreased the transfection activity of PEI 25 kDa by preventing acidification of endosomes and thus, the further protonation of PEI, whereas chloroquine did not affect PEI activity but increased that of quaternized counterparts [48]. On the other hand, PEI 1.8 kDa was completely ineffective in silencing GFP protein independently of the addition of chloroquine or bafilomycin A1, even at a N/P ratio of 30 (data not-shown). Although the buffering properties of low molecular weight PEI have been described [49], the poor cellular uptake of PEI 1.8 kDa complexes may make any later effects of these agents inside the endosomes undetectable. Therefore, PEI 25 kDa was selected as control polymer for the mechanistic studies and the corresponding complexes were prepared N/P of 6, reported to be the maximum amount of polymer that can be used to avoid toxicity [50-52]. As with the PEI 25kDa complexes, the silencing efficacy of DOPE-PEI complexes was not affected by the pre-treatment of cells with chloroquine. However, the silencing mediated by DPPE-PEI increased significantly (by 20%) after chloroquine treatment (fig. 2.10A). Similarly to PEI 25kDa, the inhibition of
the ATPase by bafilomycin A1 significantly decreased the GFP-downregulation mediated by DOPE-PEI complexes while DPPE-PEI efficacy was not affected (Fig. 2.10B).

**Fig. 2.10** Analysis of endosomal escape of DOPE-PEI and DPPE-PEI complexes (N/P 16). The silencing efficacy of complexes was measured after pre-incubation of cells with the endosomal acidification inhibitors (A) chloroquine and (B) bafilomycin A1 and compared with PEI 25 kDa (N/P 4). (one-way ANOVA, Turkey's test, * p < 0.05, n.s. no significant differences)
2.4. DISCUSSION

The development of novel materials and the optimization of existing ones for safe and efficient siRNA delivery are key to successful clinical application of gene silencing in cancer and other diseases. PEI-based delivery systems are attractive carriers because they can protect and efficiently deliver nucleotide-based molecules to target cells. Despite broad experience in the use of PEI, the balance between the efficacy and toxicity of this carrier is still sub-optimal. Recently, we found that phospholipid modification of low molecular weight PEI dramatically increased its gene down-regulation capacity while keeping cytotoxicity levels low [53]. In the present study, we aimed for a deeper understanding of the detailed structure and impact of DOPE-, DPPE- and PC-PEI conjugations on the complexes’ properties, intracellular delivery and gene down-regulation mediated by these PEI derivatives. Lipidation of PEI has usually been carried out in the context of plasmid DNA gene expression by cholesterol modification [54]. Regarding siRNA delivery, the modification of PEI with different fatty acids or alkane chains was reported to improve gene down-regulation efficiency that appeared to be dependent mostly on the level of substituted lipid and the ratio of polymer to siRNA. These features affect the siRNA binding affinity and other complex properties such as surface charge, which in turns affects uptake and intracellular trafficking [24, 55]. For example, Schroeder and collaborators have performed gel retardation studies demonstrating that the binding affinity of alkylated PEI compounds to siRNA decreased as the conjugation levels increased, and by reducing the binding affinity within the complex, the siRNA was readily released into the cytoplasm after cellular internalization [56]. Our results indicate that although the physicochemical properties and siRNA binding capacity of the conjugates were the same, their cellular interaction and silencing potency varied dramatically. DOPE, DPPE and PC conjugation did not change the size or the zeta potential of PEI complexes (fig. 2.6), N/P 16) but had a large impact on their transfection and ability to down regulate GFP expression (60%, 30%
and 5% decrease of GFP expression respectively, at a ratio of N/P 16). We attributed the specificity displayed by the phospholipid-PEI conjugates to a structure-specific interaction of the conjugates with the cell surface. In particular, the modification of PEI with DOPE and DPPE produced di-block amphiphiles able to self-assembly into micellar aggregates that completely changed PEI’s interaction with cell membranes. The interaction of phospholipid-PEI conjugates either in free form (fig. 2.5) or as complexes (fig. 2.9) with the cells was dose-dependent. The amount of siRNA formulated with DOPE-PEI or DPPE-PEI associated with cells increased with increasing N/P ratios. This was in sharp contrast with non-micellizable PC-PEI which was unable to interact with cells in free form or as a complex with siRNA. Interestingly, we observed that an excess of conjugate not only increase cellular association, as previously reported for other lipid-PEI derivatives [57], but rather promotes and accelerates siRNA internalization. As shown in (fig. 2.9C), more than 80% of the siRNA was already internalized during the first hour of incubation when complexes were prepared at N/P ratio of 25. Regarding the cell interaction of the conjugates in their free form, no toxic effect was observed unless high concentrations (≥ 125 µg/mL) were reached in contrast to certain toxicities observed for other derivatives at concentrations of 10-20µg/mL [55, 58]. The phospholipid-PEIs were 10 times less toxic than PEI 25 kDa (IC50= 123 and 100µg/mL vs IC50= 15µg/mL). Interestingly, their cytotoxicity became noticeable due to excessive cell interaction once the critical micellar concentration was exceeded (CMC= 97 µg/mL and 72 µg/mL, for DOPE-PEI and DPPE-PEI) supporting the idea of the structure-specific interaction of these conjugates with cells.

Although DPPE-PEI and DOPE-PEI carriers showed similar cell membrane interaction and siRNA uptake, DOPE-PEI displayed a more effective gene silencing. Studies to elucidate the mechanism of action of these PLPEI nanocarriers (fig. 2.10) show a positive lysomotropic chloroquine
dependency of DPPE-PEI but not DOPE-PEI and conversely a negative effect of Bafilomycin on DOPE-PEI, but not DPPE-PEI, pointing out mechanistic differences in their cytosolic trafficking. Chloroquine improved the silencing efficacy of DPPE-PEI, indicating the incomplete release of the internalized complexes from the endosomal compartment in the absence of this buffering agent (fig. 2.10A). On the other hand, bafilomycin inhibition of the active influx of water and ions essential for endosome acidification abolished the GFP down-regulation mediated by DOPE-PEI complexes and PEI 25 kDa, while DPPE-PEI efficacy was not affected (fig. 2.10B). The pH dependent-membrane activity of DOPE unit but not of DPPE is in agreement with previous studies with lipoplexes containing DOPE as a helper lipid for fusogenic functionality [59-61]. Zurhorn and co-workers showed that with the substitution of DOPE for DPPE, the transfection activity of the lipoplex decreased dramatically as a result of the inhibition of the intracellular delivery, confirming the fusogenic role of DOPE [62]. In addition, palmitic acid-substituted PEI (0.6-2kDa) efficacy did not assist for endosomal release and was affected by chloroquine treatment [58], pointing out the advantages of DOPE conjugation over other lipidic moieties in the optimization of lipid-PEI gene delivery systems.

The ability to condense siRNA, hydrodynamic size, zeta potential and enzymatic stability are often considered as important criteria to estimate the efficacy of lipid-PEI derivatives for delivery siRNA. This study highlights the importance of amphiphile structure and the lipid unit in promoting the interaction of with cell membranes, triggering the siRNA uptake and intracellular delivery. Besides higher transfection compared PEI 1.8 kDa and their safer toxicity profile compared to PEI 25 kDa, phospholipid-PEI can self-assemble into micellar structures and incorporate poorly soluble compounds in their core Combination of such improved features open new possibilities for their application as a potential co-delivery platform for siRNA/poorly soluble drugs combinations in a single carrier.
2.6 CONCLUSIONS

We have evaluated the feasibility and effectiveness of 3 structurally different phospholipid modification of PEI for siRNA delivery. The gene silencing efficacy of the conjugates was highly dependent on the nature of the lipid grafted and the structure of the conjugate. As compared to PAzPC or PC modified PEI, conjugation with PE phospholipids greatly improves the interaction of PEI 1.8 kDa with cell membranes and promotes siRNA uptake, especially when an excess of the polymer is present in the formulation. Besides their high cellular uptake, the endosomal escape mediated by DOPE-PEI conjugate may explain the superior silencing by DOPE-PEI complexes. In addition to their high transfection efficacy, phospholipid-PEI conjugates self-assembled into micellar structures that can incorporate poorly soluble compounds in their core. This opens new possibilities for their application as dual gene-drug nanocarriers.

3. HYPOTHESES#3 ‘DOPE-PEI-based MNPs can be used to deliver siRNA intracellularly, downregulate MDR1 gene expression and when used in combination with doxorubicin, they can overcome MDR in human breast cancer cells.’

SPECIFIC AIM#3 To develop and characterize PE-PEI-based, siRNA loaded MNPs, targeting the MDR1 gene (siMDR1) and evaluate the efficacy of the combination therapy of siMDR1 loaded MNPs and doxorubicin in overcoming MDR in human breast cancer cells.
3.1 BACKGROUND FOR SPECIFIC AIM#3

It was clear from the previous work that a DOPE-PEI was a better siRNA nanocarrier than PAzPC-PEI or DPPE-PEI. It was further decided to pursue all future work based on siRNA delivery carriers composed of DOPE-PEI. Also, it should be noted that all previous gene downregulation experiments were GFP based and at this point had no translational therapeutic benefit.

*Background for the choice of the therapeutic target.* Multiple or more commonly referred to as Multidrug resistance (MDR) is a major problem limiting the treatment of breast cancer. Several molecular mechanisms are involved in the development of a drug resistant phenotype [63]. One of the most studied resistance mechanisms is the reduction of intracellular drug concentration by transporter proteins that pump drug out of cells before they reach the site of action. Many of these transporters are members of the ATP-binding cassette (ABC) transmembrane protein superfamily, including P- glycoprotein (P-gp), multidrug resistance protein-1 (MRP-1) and breast cancer resistant protein. P- glycoprotein was the first to be described and is the best characterized to date. P-glycoprotein encoded by the MDR1 gene is a 170 kDa plasma membrane protein with 12 transmembrane domains and 2 ATP-binding domains. P-gp uses ATP energy to transport drugs and other xenobiotics from the intracellular to the extracellular compartment, although it may also interact with transmembrane substrates [64]. The localization of P-gp in normal tissues suggests that this transporter has the physiological function of detoxification and excretion of xenobiotics [63, 65]. In tumor tissue, intrinsic or induced overexpression of P-gp after exposure to chemotherapy agents has been extensively investigated as one primary reason for chemotherapy failure in different MDR cancer types [66-70]. In the case of breast cancer, induced expression of P-gp in cancer cells by exposure to anticancer drugs was detected in 52% of chemotherapy-treated patients [68, 71]. P-gp
expression has also been related to a significant increase in doxorubicin and taxol resistance of breast tumors regardless of prior treatment [69, 72].

Current siRNA therapy against P-gp. Apart from chemical P-gp inhibitors, the silencing of the MDR1 gene with small interfering siRNA (siRNA) can be a powerful tool to restore chemotherapy sensitivity in MDR cancer cells [73]. siRNA duplexes are short nucleotide molecules of about 21-25 base pairs that can trigger silencing of homologous gene expression by inducing degradation of the complementary mRNA [74]. Two advantages of the siRNA approach as compared to chemical inhibitors are its reduced toxicity in non-specific tissues and its high specificity. The main disadvantage of siRNA is its poor in vivo stability (rapid degradation in plasma and cellular cytoplasm, poor cellular uptake) that has limited its clinical application to date.

3.2 MATERIALS

All materials were purchased from Sigma-Aldrich unless otherwise stated. Branched polyethylenimine with a molecular weight of 1.8 kDa was purchased from Polysciences, Inc (Warrington, PA). 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethyleneglycol)-2000] (PEG-PE), cholesterol, and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(glutaryl) (Glutaryl-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). All siRNA duplexes were purchased from Dharmacon (Lafayette, CO), namely, siRNA targeting Green Fluorescent Protein (GFP-siRNA): 5′-AUGAACUUCAGGGUCAGCUdTdT-3′ (sense), siRNA targeting MDR-1 (siMDR1): 5′-GGAAAGAAACCAACUGUCdTdT-3′ (sense) [75], and a non-targeting control siRNA, (siNegative): 5′-AGUACUGCUUACGAUACGGdTdT-3′(sense). FITC-labeled P-glycoprotein
antibody [UIC2] was purchased from Abcam (Cambridge, MA). Lipofectamine™ 2000 Reagent was purchased from Invitrogen (Carlsbad, CA). The CellTiter-Blue® Cell Viability Assay was purchased from Promega (Madison, WI). Cells from the C166 cell line (mouse yolk sac embryo) stably transfected with a plasmid reporter vector, pEGFP-N1, encoding for the enhanced green fluorescent protein, GFP, were obtained from the American Type Culture Collection (Manassas, VA). DMEM media was supplemented with 10% fetal bovine serum (FBS) and 0.2 mg/ml of Geneticin (G-418, Invitrogen, CA. Both MCF-7 cell lines were grown at 37°C under 5% CO2 in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% FBS and penicillin (100units/ml) and streptomycin (100 µg/ml). DMEM and penicillin/streptomycin stock solutions were purchased from Cellgro (Herndon, VA). Heat-inactivated FBS was purchased from Atlanta Biologicals (Lawrenceville, GA). Nuclease-free water was purchased from Qiagen (Germantown, MD).

3.3 METHODS

3.3.1 Synthesis of DOPE-PEI conjugates and preparation of DOPE-PEI/siRNA complexes

Previously described previously in the text (sections 2.3.1 and 2.3.6)

3.3.2 Preparation of micelle-like nanoparticles (MNPs)

The MNPs were assembled with DOPE-PEI: POPC: cholesterol: PEG-PE (4:3:3:0.3 mol/mol) and siRNA. First, 32 µg DOPE-PEI (21.7 µg as PEI) and 10 µg siRNA corresponding to an N/P ratio of 16 were diluted separately in BHG buffer and mixed to a final volume of 200 µL. A dry lipid film was prepared from the mixture of POPC, cholesterol and PEG-PE (6.8 µg, 3.5 µg, 2.5 µg; 3:3:0.3 mol/mol). The lipid film was hydrated with the preformed DOPE-PEI complexes and incubated for 1 h.
at room temperature with intermittent vortexing.

3.3.3 Particle size and zeta potential measurements by Dynamic light scattering (DLS)

The particle size and zeta potential of the formulations were measured using a Zeta Plus Particle Analyzer (Brookhaven Instruments Corp, Santa Barbara, CA). Scattered light was detected at 25°C at an angle of 90°. Samples (100 µL) of complexes and MNPs were diluted in 1.7 ml of nuclease-free water and measured immediately after preparation.

3.3.4 Green Fluorescent Protein (GFP) downregulation

In vitro GFP downregulation experiments were performed in stably transfected C166-GFP cells using GFP- targeted siRNA. A non-targeting control duplex (Negative-siRNA) was used as a non-specific control siRNA. Cells were seeded 24 h prior to transfection in 12-well plates at a density of 5 x 10^4 per well and complete medium was replaced with fresh serum-free medium. DOPE-PEI complexes at varying N/P ratios were added to cells to yield a final siRNA concentration of 100 nM. After 4h of incubation, the complexes were removed and fresh complete media was added. The cells were further incubated for 48 h. Thereafter, the cells were washed, trypsinized, and GFP downregulation analyzed by flow cytometry. In a second set of experiments, C166-GFP cells were treated, under the above conditions, with free siRNA or siRNA formulated in PEI 1.8 kDa complexes, DOPE-PEI complexes, or MNPs. All formulations were prepared at an N/P ratio of 16. Lipojectamine 2000 was used as a positive control according to the manufacture’s protocol.
3.3.5 Characterization of the MCF7/R and MCF7/S cells

Characterization of sensitive and resistant MCF-7 cells was performed with respect to (a) intracellular doxorubicin accumulation (b) cytotoxicity of increasing concentrations of doxorubicin towards MCF-7 R and S cell lines and (c) Expression of P-gp on MCF-7 R and S cell surface. The experimental protocols for these are briefly described below [53]:

(a) 5\(\mu\)g/ml Doxorubicin was added to MCF-7 R or S cells. After 1h of incubation, the drug was removed, the cells were washed with fresh media and observed under fluorescence microscopy.

(b) MCF7/R and MCF7/S cells were incubated with doxorubicin for 24 h after which cell viability assay using cell titer blue was performed. Relative viability is expressed as a percentage of non-treated cells.

(c) MCF7/R and MCF7/S cells were incubated with FITC-labeled antibody (anti P-gp). The fluorescence in the samples was analyzed by FACS.

3.3.6 P-glycoprotein downregulation

For P-glycoprotein silencing experiments, MCF-7 resistant cells (MCF7/ADR cells) were seeded in 12-well plates at a density of 5 x 10\(^4\) per well. After 24h, the cells were treated with DOPE-PEI complexes, MNPs or PEI 1.8 kDa complexes containing either siRNA targeting MDR1 (siMDR1) or a scramble siRNA (siNegative). All formulations were prepared at an N/P ratio of 16. Lipofectamine 2000 was used as a positive control. The final concentration of siRNA was 100 nM. After 4h of incubation, the treatments were removed and cells were re-incubated for 48 h. The cells were washed, detached by mechanical scarping and resuspended in BSA 0.5%. The cell suspensions were incubated with a FITC-labeled antibody against P-gp at 4ºC. After 40 min of incubation, the cells were washed
with BSA 0.5% and analyzed by flow cytometry.

3.3.7 Intracellular doxorubicin accumulation

MCF-7 resistant and sensitive cells were seeded in 12-well plates at a density of $5 \times 10^4$ - $8 \times 10^4$ per well, respectively. After 24h, the cells were treated with DOPE-PEI complexes or MNPs containing either siRNA targeting MDR1 (siMDR1) or a scramble siRNA (siNegative). All formulations were prepared at an N/P of 16. The final concentration of siRNA was 100 nM. After 4h of incubation, the treatments were removed and cells were re-incubated for 48 h. Cells were then incubated for 1h with doxorubicin (5µg/ml). After incubation, suspended cells were washed, trypsinized and fixed in a 4% formalin-phosphate-buffered solution. Accumulation of intracellular doxorubicin was analyzed by flow cytometry.

3.3.8 Cytotoxicity assays

To investigate the effect of time lag between siRNA and doxorubicin treatment on the drug toxicity, MCF-7 resistant cells were seeded in 96-well plates at 3,000 cells/well. After 24 h, the cells were treated with formulations prepared with siRNA targeting MDR1 (siMDR) and combined with doxorubicin (1µg/ml) after 0, 4, 8, 24 and 48 h. Control cells were treated only with doxorubicin. The cell viability was measured after 72 h of doxorubicin incubation with the CellTiter-Blue®. Briefly, 20 µL of CellTiter Blue was added to each well, and the plates were re-incubated for 2 h. The fluorescence was measured at excitation and emission wavelengths of 560 nm and 590 nm, respectively. Similarly, the effect of a scrambled siRNA (siNegative) sequence, in combination with doxorubicin, on the cytotoxicity profile of the MCF7/R and MCF7/S, was also investigated.
In another set of experiments, a 48 h time lag between siRNA and drug administration was selected and the effect of different drug incubation time was investigated in MFC-7 resistant cells and compared to that of sensitive cells. The cells were treated with siRNA (siMDR1 or siNegative) formulations as described above, and doxorubicin (1µg/ml) was added 48h post-siRNA treatment. Cell viability was measured after 24, 48, 72, 96 h of doxorubicin incubation with the CellTiter-Blue®. Similarly, the effect of co-administration of siRNA and doxorubicin was tested in MCF-7 resistant cells.

3.3.9 Statistical analysis

Results are presented as mean ± SD, and statistical significance of differences was evaluated by variance analysis, ANOVA; p-values smaller than 0.05 were considered to indicate a significant difference.

3.4 RESULTS

3.4.1 Nucleases protection

The DOPE modified PEI was synthesized as described earlier in this text (section 2.3.1). Also, the complex formation between siRNA and DOPE-PEI has been demonstrated previously in the text (section 2.3.6) and an N/P ratio of 16 was chosen for all the experiments listed here. siRNA is also protected from nucleases degradation at this N/P ratio for upto 20 minutes (fig. 2.6C). A gel retardation assay has been shown below which further proves that siRNA was protected for upto 2 hours in the DOPE-PEI complex (fig. 3.1).
DOPE-PEI/siRNA complexes protected the siRNA from degradation for up to 2 hours in the presence of RNAse III as compared to naked siRNA which degraded after 20 minutes.

3.4.2 Formulation and characterization of the Micelle like Nanoparticles (MNPs) encapsulating DOPE-PEI

MNPs incorporating DOPE-PEI/siRNA at N/P 16 were formulated with a view to improve biophysical characteristics. The hydrophobic interactions between the lipid part of DOPE-PEI and the free lipids led to the formation of MNPs as was suggested by the moderate increase in the size of DOPE-PEI/siRNA complexes from 127 ± 25 nm to 178 ± 34 nm in MNPs due to the incorporation of PEG and lipids, while the zeta potential decreased from 32 ± 5 mV to a near neutral surface charge of 3 ± 1 mV suggesting a PEG corona shielded the cationic charge contributed by the DOPE-PEI/siRNA complex.
3.4.3 Effect of the PEG/lipid layer on GFP downregulation

The GFP downregulation efficacy of the DOPE-PEI complexes has been shown earlier (fig. 2.8). However, the PEG chains could hinder the transfection ability of the complex alone and thus, affect GFP downregulation. Hence, it was relevant to evaluate to what extent this was true. The effect of the PEG/lipid layer on the silencing efficacy MNPs is shown in (fig. 3.2). The presence of PEG and lipids surrounding the complexes decreased the silencing efficacy of MNPs to a certain extent as compared to non-coated complexes. However, the ability of MNPs to deliver siGFP into cells and suppress GFP expression was high and comparable to that of Lipofectamine 2000 (positive control).

![Graph showing GFP expression](image)

**Fig. 3.2** Effect of PEG/lipid layer in MNPs silencing efficacy. C166-GFP cells (stably expressing GFP) were treated with the formulations prepared with GFP targeted siRNA (siGFP, black bars) or non-targeted siRNA (siNegative, white bars) at different N/P ratios. The siRNA concentration was 100
nM. After 4 h of incubation, complexes were removed and cells were incubated for 48 h. Cells were trypsinized and analyzed by flow cytometry. The down-regulation of GFP was measured by the decrease in the mean fluorescence of the treated cells compared with non-treated and expressed as % of non-treated control cells.

3.4.4 Characterization of the MCF7/R and MCF7/S cells

The MCF7/R and MCF7/S cells were characterized with respect to their ability to accumulate doxorubicin intracellularly, their respective cell viability in the presence of varying drug concentrations and surface P-gp expression. It was shown that the MCF7 wild type (MCF7/S cells) exhibited greater intracellular doxorubicin accumulation (fig. 3.3A) and increased cell death (fig. 3.3B) in the presence of the drug as compared to the doxorubicin resistant MCF7 cell line. For the P-gp downregulation study, the changes in P-gp surface protein expression were measured by flow cytometry (FACS) using a FITC-labeled antibody which recognizes an external conformational epitope of the P-gp protein. The overexpression of P-gp in control resistant cells was evident from a fluorescence increase in the presence of the antibody whereas this shift was not observed in sensitive cells that had low basal levels of P-gp (fig. 3.3C).
Fig. 3.3 Characterization of the MCF7 R and MCF7/S cell lines with respect to (A) intracellular doxorubicin accumulation (B) cell viability in the presence of doxorubicin and (C) surface P-gp expression. The shift in the green histogram in panel (C) to the right indicates overexpression of P-gp on the surface of MCF7/R cells as compared to MCF7/S cells. Data expressed as mean ± SD (n=3).

3.4.5 P-glycoprotein downregulation with DOPE-PEI formulations

As stated previously in the specific aims for this project, the final goal was to specifically downregulate MDR1 gene that encodes for P-glycoprotein (P-gp), which has been implicated widely for Multi-drug resistance (see background for this section). Down-regulation was evaluated in MCF-7/ADR cells using a siRNA targeting MDR1 gene (siMDR1). As mentioned earlier, the changes in P-gp surface protein expression were measured by flow cytometry (FACS) using a
FITC-labeled antibody which recognizes an external conformational epitope of the P-gp protein.

DOPE-PEI complexes and MNPs effectively delivered siMDR1 into in MCF-7 resistant cells and down-regulated P-gp expression (figs. 3.4d and 3.4e, respectively). siMDR1 formulations (green histograms) showed less fluorescence than control cells (filled histograms) suggesting that less P-gp was expressed and presented on the cell surface. Thus, less fluorescent antibody was attached. By contrast, those cells treated with siNegative formulation (orange histograms) showed fluorescence similar to control cells. The same results were obtained with Lipofectamine 2000 used as a positive control for siRNA transfection (fig. 3.4f). No changes in P-gp expression were observed when the cells were treated with free siMDR1 or PEI complexes (figs. 3.4b and 3.4c, respectively).
Fig. 3.4 Decrease in the P-gp expression in resistant MCF-7 cells mediated by DOPE-PEI complexes and MNPs. MCF-7 resistant cells were treated with different formulations prepared either with siRNA targeting MDR-1 (siMDR) or scramble siRNA (siNegative). After 4h, the treatments were removed and fresh medium was added. The cells were re-incubated for 48 hours. The cells were detached by mechanical scrapping, resuspended in BSA 0.5%, incubated with FITC labeled antibody against P-gp and analyzed by Flow cytometry. Filled histograms correspond to non-treated cells (black, in the absence of antibody and blue in the presence of antibody), green histograms correspond to formulations prepared with siRNA targeting MDR-1 and orange histograms correspond to formulations prepared with scramble siRNA. A shift to the left in flow cytometry histograms is
indicative of P-gp downregulation.

3.4.6 Intracellular accumulation of doxorubicin

In the previous experiment, it was shown that P-gp was successfully downregulated. The direct effect of this is expected to be increase in intracellular doxorubicin which was measured in MCF-7 cells. This would reinstate the fact whether or not the P-gp silencing achieved by DOPE-PEI formulations could effectively inhibit the drug efflux activity of the transporter. Doxorubicin fluorescence increased ~2-fold in resistant cells treated with DOPE-PEI or MNPs (fig. 3.5A).

Formulations loading non-targeted siRNA (siNegative) did not change the amount of drug inside the cells. This latter finding confirms the specificity of the silencing and discards the probable notion of drug accumulation due to polymer permeabilization of cell membrane. As expected, the amount of drug in sensitive cells was considerably high compared to resistant cells (notice the different scale bar in figs. 3.5A and 3.5B). In sensitive cells, the intracellular doxorubicin was not affected by the siRNA, siMDR1 or siNegative, employed (fig. 3.5B). At these experimental conditions, the decrease of P-gp in the sensitive strain may not play a significant role in the accumulation of the drug.
Fig. 3.5 Intracellular doxorubicin levels in (A) resistant and (B) sensitive MCF-7 cells mediated by DOPE-PEI complexes and MNPs. MCF-7 resistant and sensitive cells were treated with different formulations prepared either with siRNA targeting MDR-1 (siMDR) or scramble siRNA (siNegative). Control cells were treated only with medium. After 4 hours, media was changed. Cells were reincubated for 48 h. Cells were then treated for 1h with doxorubicin (5µg/ml). Then washed, trypsinized and analyzed by flow cytometry. The accumulation of doxorubicin within the cells was measured by the increase in the mean fluorescence. Data are expressed as the mean ± SD (n=3). (* p < 0.001 vs siMDR-1 formulations).
3.4.7 Cytotoxicity studies

P-gp silencing and doxorubicin administration were combined to see if there was any improvement in the final drug cytotoxicity against resistant MCF-7 cells. siRNA and doxorubicin were sequentially administered with a time lag of 48 h in order to maximize P-gp down-regulation. Cell viability in resistant cells was measured after different drug incubation times and compared to that in sensitive cells (fig. 3.6). The pre-treatment of resistant cells with either DOPE-PEI complexes or MNPs loaded siMDR-1 improved doxorubicin cytotoxicity. Significant differences (p<0.001) compared to drug-only treated cells were observed at all incubation time points (fig. 3.6A). After 1 day of drug treatment, 95% of control resistant cells were viable. However, cells treated with siMDR-1 formulations had cell viability values of 45 % and 65%, respectively, for complexes and MNPs. After 2-3 additional days of drug treatment, the viability of siMDR-1 treated cells dropped to 16% compared to 40% for drug-only control cells.

For sensitive cells, the toxicity profiles of the treatments were similar and did not improve with longer incubation times (fig. 3.6B). A significant improvement of drug efficacy was detected only for DOPE complexes after 24 h of doxorubicin treatment. The inherent sensitivity of the MCF-7 wild strain to doxorubicin may explain these results. A comparison of doxorubicin toxicity towards resistant and sensitive cells indicated that the combination of P-gp silencing and doxorubicin restored the sensitivity to doxorubicin in resistant cells to sensitive cell values (figs. 3.6A and 3.6B). It is important to note that scrambled siRNA formulations, used as a negative control for P-gp silencing specificity, did not improve doxorubicin cytotoxicity (fig. 3.7).
**Fig. 3.6** Doxorubicin cytotoxicity in (A) resistant and (B) sensitive MCF-7 cells after treatment with DOPE-PEI complexes and MNPs. MCF-7 resistant and sensitive cells were treated with formulations prepared with siRNA targeting MDR-1 (siMDR). Control cells were treated only with medium. After 4 hours, the media was changed. Cells were reincubated for 48 hours. Cells were treated with doxorubicin (1µg/ml) for 24, 48, 72 and 96 h and cell viability was measured. Data are expressed as the mean ± SD (n=3). (p < 0.001 for DOPE-PEI treatments vs only-drug treated cells, for all time points in resistant cells).
Fig. 3.7 P-gp silencing specificity: Resistant (A) and sensitive MCF-7 cells (B) were treated with formulations prepared with siRNA targeting MDR-1 (siMDR-1) or scramble siRNA (siNegative). Control cells were treated with medium. After 4 hours, the media was changed. Cells were reincubated for 48 hours and treated either with media (- Doxorubicin) or with doxorubicin (+ Doxorubicin), (1µg/ml). Cell viability was measured. Data are expressed as the mean ± SD (n=3).
In the second set of experiments, the effect of different time lags between siRNA and drug administration on doxorubicin toxicity was studied. As shown in (fig. 3.8), the effectiveness of doxorubicin was significantly improved by P-gp silencing mediated by DOPE-PEI complexes regardless of the time lag, including simultaneous administration of siRNA and doxorubicin (time lag=0 h) (p<0.001 vs doxorubicin only).

**Fig. 3.8** Doxorubicin cytotoxicity in resistant MCF-7 cells after treatment with DOPE-PEI complexes at different time lag (h). MCF-7 resistant and sensitive cells were treated with formulations prepared with siRNA targeting MDR-1 (siMDR). Control cells were treated only with medium. After different time points post-siMDR-1, cells were treated with doxorubicin (1µg/ml) for 72 h, and viability was measured. Data are expressed as the mean ± SD (n=3). (p < 0.001 for DOPE-PEI treatments vs dox-only treated cells, for all time points in resistant cells).
3.5 DISCUSSION

Recent studies have shown that silencing of MDR-1 gene using siRNA can improve the effectiveness of anticancer drugs on MDR tumors [76-78]. In the case of breast cancer, the availability of \textit{in vitro} models and clinical data make this strategy even more attractive [79, 80]. However, two main problems need to be resolved. The first is related to the inherent instability of siRNA that requires its association with viral or synthetic carriers. The development of an ‘ideal delivery system’ highly efficient, non-toxic and non-immunogenic is still a major challenge. The second problem lies with the optimization of the combination of P-gp downregulation and the anticancer drug administration for maximal therapeutic synergy. Some authors have shown that the sequential administration of siRNA/drug permits sufficient time to achieve substantial downregulation of P-gp and consequently maximum cell killing when the drug is administered to the cells [78, 81] By contrast, other authors suggested that the combination of drug and siRNA in a single carrier could promote the synergy of the treatments by temporally co-localizing them in the tumor cells [79, 82, 83]. In any case, it is desirable that a versatile carrier fit both administration schedules.

With this background in mind and based on findings from our previous work, we synthesized and evaluated a DOPE-PEI conjugate for siRNA delivery and toxicity. The conjugates were tested for P-gp downregulation and combined sequentially or in co-therapy with doxorubicin to overcome MDR in breast cancer cells. As previously shown, the conjugation of DOPE did not diminish siRNA condensation capacity of PEI. Small complexes (<200 nm) were spontaneously formed when siRNA was mixed with either modified or non-modified PEI. More importantly, the silencing efficacy of PEI was dramatically improved by DOPE conjugation and via previous mechanistic studies we have shown that DOPE-PEI complexes improved transfection efficacy by both greater cellular uptake and
endosomal escape.

These complexes were then formulated in MNPs for improved biocompatibility. The lipid moiety of DOPE-PEI can hydrophobically interact with PEG-PE and free lipids to MNPs. We previously reported that MNPs had reduced cytotoxicity and improved in vivo stability as compared to plain PEI complexes [30, 84]. Here, the benefits of MNPs were also demonstrated for the DOPE-PEI complex. The positive surface of DOPE-PEI complexes (32±5 mV) decreased in MNPs to an almost neutral and more biocompatible one (3±1 mV). The presence of PEG surrounding DOPE-PEI complexes did not significantly influence the silencing efficacy of MNPs but considerably decreased their cytotoxicity compared to Lipofectamine 2000 or non-coated complexes especially in sensitive MCF-7 cells. MNPs could provide a useful platform for future in vivo targeted siRNA applications.

DOPE-PEI formulations were tested for P-gp downregulation capability. The presence of P-glycoprotein on the surface of resistant cells decreased after treatment of the cells with DOPE-PEI or MNPs loading siMDR-1. This P-gp downregulation was translated into an effective inhibition of the drug efflux activity. The amount of doxorubicin inside MDR-1 treated cells doubled as compared to that of control cells. It is important to note that the reduction of P-gp expression and augmentation of the drug accumulation do not necessarily correlate with a complete reversion of MDR phenotype. In our case, P-gp downregulation mediated by DOPE- PEI and MNPs restored doxorubicin sensitivity in resistant cells (figs. 3.6A and 3.6B). Pre-treatment of resistant cells with siMDR-1 formulations before administration of doxorubicin led to a 2-fold drop in cell viability, similar to values in sensitive cells. So far, most of the studies focused on combined use of P-gp siRNA and anticancer drugs (doxorubicin, paclitaxel) reported successful results using a time lag of 24 to 48 h between
treatments. Yadav and collaborators studied the effect of separation duration (from 1 to 48 h) between siRNA and paclitaxel formulated in biodegradable nanoparticles. Significant enhancement in cytotoxicity was observed only in resistant ovarian cancer cells when paclitaxel was administered after 24h of P-gp downregulation. Similarly, separated preparations of cationic liposomes of siRNA and doxorubicin showed a therapeutic improvement in mice but resulted in severe aggregation and doxorubicin leakage when co-encapsulated. In this sense, one of the advantages of DOPE-PEI is that the effectiveness of doxorubicin was improved regardless of the time lag between siRNA formulation and drug and included simultaneous administration of the treatments (fig. 3.8).

3.6 CONCLUSIONS

We have demonstrated the feasibility and effectiveness of DOPE-PEI formulations to deliver siRNA and modulate the expression of P-glycoprotein. The down-regulation of P-gp translated into an effective inhibition of the doxorubicin efflux activity, enhancement of intracellular doxorubicin accumulation and restoration of drug sensitivity in resistant human breast cancer cells.
4. HYPOTHESIS #4 ‘DOPE-PEI based nanocarriers will deliver siRNA to tumors in vivo.’

SPECIFIC AIM#4 To evaluate the biodistribution of DOPE-PEI/siRNA-based nanocarriers and the role of PEGylation in the organ distribution profile and blood circulation time.

4.1 BACKGROUND FOR SPECIFIC AIM#4

It has been shown previously that the DOPE-PEI based nanocarriers successfully brought about downregulation of P-gp in vitro. As the next logical step we wanted to evaluate if the siRNA delivery system could downregulate the MDR1 gene transcriptionally and translationally, in vivo. The in vivo studies have been divided into two sections viz. biodistribution and therapeutic efficacy studies. The biodistribution study is described in this section.

4.2 MATERIALS

All materials were purchased from Sigma-Aldrich unless specified otherwise. Branched polyethylenimine with a molecular weight of 1.8 kDa was purchased from Polysciences, Inc (Warrington, PA). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethyleneglycol)-2000] (PEG-PE) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(glutaryl) (Glutaryl-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). Nuclease-free water was purchased from Qiagen (Germantown, MD). Fluorescein labeled siRNA (FL-siRNA) was obtained from Dharmaco Technologies (Lafayette, CO).
4.2.1 Cell culture

4T1 cells were obtained from the American Type Culture Collection (Manassas, VA). DMEM media supplemented with 10% fetal bovine serum (FBS), penicillin (100units/ml) and streptomycin (100µg/ml) was used for the culturing of the cells and they were grown at 37°C under 5% CO2. Penicillin/streptomycin stock solutions were purchased from Cellgro (Herndon, VA). Heat-inactivated FBS was purchased from Atlanta Biologicals (Lawrenceville, GA).

4.2.2 Animals

Female Balb/c mice (6-8 weeks) were purchased from Charles River Laboratories (Wilmington, MA).

4.3 METHODS

4.3.1 Preparation of formulations

The DOPE-PEI conjugate, DOPE-PEI/FL-siRNA complexes and their respective MNPs were prepared as described in the text before (see sections 2.3.1, 2.3.6 and 3.3.2). Another PEGylated formulation viz. DOPE-PEI/FL-siRNA + PEG-PE (1:10 w/w) was prepared by adding a DOPE-PEI/siRNA complex solution prepared in BHG at N/P 16, to a previously freeze dried lipid film of PEG-PE (PEI-PE:PEG-PE was 1:10 w/w) and allowed to stand at room temperature with intermittent shaking, for 30 minutes.

4.3.2 Size and zeta potential measurements for DOPE-PEI/PEG-PE (1:10w/w)

Briefly, 100µl of the formulation was diluted suitably in nuclease free BHG buffer at pH 7.4 and size and zeta potential measurements were made using a Malvern Zetasizer Nano instrument.
4.3.3 Animal treatments

Female balb/c mice were s.c injected into the right flank with 0.2 million 4T1 cells resuspended in sterile PBS. Once the tumors were ca. 100 mm$^3$, the animals were randomly assigned to 4 treatment groups viz. naked siRNA, PEI/siRNA, DOPE-PEI/siRNA and MNPs of DOPE-PEI complexes (DOPE-PEI: POPC: Chol: PEG-PE 4:3:3:0.3 mol/mol). The fluorescein labeled-siRNA (FL-siRNA) was used for preparation of the complexes and the siRNA dose was 1.2mg/kg. All the formulations were prepared in nuclease-free, filtered HEPES buffered glucose (BHG), pH 7.4 at N/P 16. In vivo imaging was performed at four time points (1h, 4h, 8 h, 24h). For every time point n=3. The Kodak Image Station- in vivo imager (Dr. Amiji’s lab) was used for in vivo imaging. Ex vivo imaging was also performed for all the time points, for the same mice after sacrificing them and harvesting their organs. For ex vivo imaging, the Xenogen IVIS Lumina (Dr. Heather Clark’s lab) was used.

In another set of experiments, mice were treated as exactly as the first set except for female balb/c mice bearing 4T1 tumors ca. 100 mm$^3$, were randomly assigned to 4 treatment groups and were injected with the formulations listed below, via the tail vein route: naked FL-siRNA, PEI/FL-siRNA, PEI-PE/FL-siRNA and PEI-PE/PEG-PE (1:10 w/w) which replaced the MNPs in the first set. 4 hours post injection, the mice were sacrificed, the organs were harvested and blood was collected via cardiac puncture. The organs were homogenized in cold BHG, centrifuged to obtain a clear supernatant and the serum was collected from the derived blood. The organ homogenates and serum were transferred to a 96 well plate and the fluorescence from the samples was read in a fluorescence plate reader at $\lambda_{\text{ex}}$ 485/20, $\lambda_{\text{em}}$ 528/20. Fluorescence values were converted into percent injected dose/gm tissue (%ID/g tissue) or (%ID/ml of serum) from standard curves plotted separately for every tissue type, obtained by spiking varying amounts of FL-siRNA in the organ supernatants.
or serum. The percentage of recovery was approximately 80% for all the organs except for the tumor (56%).

4.3.4 Statistical analysis

The data was analyzed using GraphPad Prism 5.04. Two way ANOVA and One way ANOVA followed by Bonferroni’s multiple comparisons test analysis were used where appropriate. A p-value less than 0.05 was considered to be statistically significant denoted by *

4.4 RESULTS

4.4.1 Size and zeta potential measurements for DOPE-PEI/PEG-PE (1:10 w/w)

The size of the DOPE-PEI/PEG-PE (1:10w/w) formulation was ca. 150 nm±25nm, PDI 0.310 and the average zeta potential was found to be about 6.7mV±3mV. The formulation was stable in isotonic conditions, 150mM NaCl, for upto 24 hours i.e. no appreciable changes in size or zeta were recorded. The formation of the PEG-PE coating around the DOPE-PEI/siRNA core can be proposed from the fact that the average size increased from about 130nm for DOPE-PEI/siRNA complexes to about 150nm for the PEGylated formulation and the zeta potential drastically changed from an average of about 40mV for DOPE-PEI/siRNA complexes to about 7mv after the addition of PEG-PE. It should be noted that the reported zeta potential for PEG_{2000}-PE micelles is about -33mV [85].

4.4.2 In vivo and ex vivo imaging

The first set of in vivo imaging experiments was unsuccessful because no fluorescence signal was obtained from the mice organs or the tumor despite shaving the area around the tumor. This was
probably due to the weak green fluorescent signal of the FL-siRNA further impeded by the soft tissue of the animal. So, as one possible solution, **ex vivo** imaging of the animals was performed using the IVIS Lumina instrument. The same animals that were used for **in vivo** imaging were euthanized, the organs harvested and analyzed for fluorescence. This venture also proved to be almost futile with all the animals at every time point, recording high auto fluorescence. Disappointingly, the MNPs didn’t seem to work for the purposes of **in vivo** siRNA delivery and the formulation was discontinued for the next series of experiments which were carried out at the 4 h time point. Since, the MNPs did not seem to work we switched to DOPE-PEI/PEG-PE (1:10 w/w), the rationale being to include PEG for **in vivo** stability and since another group in our lab was investigating a similar micellar system for pH-sensitive gene delivery [43]. We did have moderate success with the animals treated with DOPE-PEI/PEG-PE (1:10 w/w) formulation vs non treated mice in the tumor. Increased fluorescence signal of FL-siRNA was detected in the tumor (4h time point) but not in the rest of the organs/treatments.
**Fig. 4.1** *Ex vivo* tumor fluorescence obtained from DOPE-PEI/siRNA + PEG-PE (1:10 w/w) micellar treatment after 4 hours as compared to tumor obtained from naked siRNA treatment group

4.4.2 *Tissue homogenization*

Tissue homogenization studies were performed since the *ex vivo* imaging failed to produce desired results. When the above experiment was repeated with the same four groups used in the second set of experiments viz. naked siRNA, PEI/siRNA, DOPE-PEI/siRNA and DOPE-PEI/PEG-PE (1:10 w/w) micelles complexed with siRNA; 4h-post injection, the fluorescence of naked siRNA in serum was non-detectable (**fig. 4.2**). The DOPE-PEI/PEG-PE (1:10w/w) carrier exhibited significantly higher injected dose of siRNA in the serum than the rest of the formulations presumably due to prolonged blood circulation time. This formulation also delivered FL-siRNA to the tumor, significantly better than naked siRNA (**fig. 4.3**). This could be attributed to improved circulation in blood and better accumulation in the tumor via the EPR effect. A high amount of naked siRNA accumulated in the kidney, PEI seemed to have a preferential affinity towards the heart tissue, the DOPE-PEI group delivered FL-siRNA to the lung tissue in a significantly high amount and it was noticed that PEGylation decreased non-specific binding in most cases.
Fig. 4.2 Distribution of fluorescein-labeled siRNA (FAM-siRNA, naked or complexed in a formulation) in serum 4 hours post injection. One way ANOVA followed by Bonferroni’s multiple comparisons test was performed for data analysis. * indicates significantly higher levels than naked siRNA group, # indicates significantly higher levels than PEI/siRNA group and ” indicates significantly higher levels than DOPE-PEI group
Fig. 4.3 Biodistribution of fluorescein-labeled siRNA (FL-siRNA, either naked or complexed in formulations) 4 hours post injection. Two way ANOVA was performed for data analysis. * indicates significantly higher levels than naked siRNA group, # indicates significantly higher levels than PEI/siRNA group and ″ indicates significantly lower levels than DOPE-PEI group.

4.5 DISCUSSION

4T1 cells were chosen to induce tumors since it is a well established animal model closely mimicking human metastatic breast cancer (Pulaski et al. 2000) and it is fairly simple to induce these tumors quickly in female Balb/c mice. It should be noted that the %ID/g of organ or %ID/ml of serum was calculated by transforming the fluorescence observed with the help of standard curves plotted for each tissue type and taking into account organ recovery values calculated separately.

In serum, it has been found that pharmacokinetics of siRNA clearance are determined by the modifying moiety as is observed in our case with DOPE-PEI complexed FL-siRNA; it is seen to
circulate for a longer time in serum as compared to naked siRNA and PEI complexed siRNA. It has been suggested that the lipid modification of siRNA increases interaction with albumin and thus, increases circulation time by decreasing glomerular filtration [86]. Addition of the PEG layer further increased the serum concentration. This could be explained on basis of the reasons stated above (RNase degradation protection and prevention of RES uptake).

As seen in (fig. 4.3), there was a statistically significant accumulation of FL-siRNA 4 hours post DOPE-PEI/PEG treatment in the tumor as compared to the naked form. It has been shown that PEGylation prevents RNase degradation [18]. This feature combined with the decreased RES uptake that PEG imparts, probably helped the formulation to circulate in vivo for a longer time and resulted in greater accumulation in the tumor possibly via the EPR effect (since no targeting moiety is present).

A high liver uptake of FL-siRNA was seen, irrespective of the formulation. Leaf Huang et. al in 2006 found that their PEGylated nanocarrier of ca. 150nm was found to accumulate in higher amount in the tumor than the liver, 4 hours post injection. This was attributed to the inability of the carrier to penetrate liver sinusoids fenestrae (pore size ca. 100nm) but could extravaste through leaky tumor vasculature (pore size ca. 400nm). We, however, didn’t obtain this pattern. It has also been observed that the most stable polyplexes experienced fast deposition in the liver whereas PEGylated polyplexes exhibited lesser uptake in spleen and liver [87]. Our data is consistent with this observation (fig. 4.3).

In the lungs, it was observed that the PEI/siRNA delivered FL-siRNA fluorescence was for all practical purposes same as naked FL-siRNA (fig. 4.3). This could be explained on the basis of the micro environment that the formulation is exposed to, before it comes into contact with the airway cell membrane i.e. the penetration of the mucus layer, the surfactant layer and the pereciliary fluid layer.
covering the airway cells [88]. It is well known that mucus contains a high percentage of mucins which are heavily glycosylated glycoproteins and form negatively charged mucin polymers [89]. It is possible that the positively charged PEI polymer interacts preferentially with the mucin, releasing the siRNA which eventually gets degraded and eliminated. This would best answer why we didn’t see an increase in fluorescence with PEI delivered FL-siRNA as compared to naked siRNA. For reasons unknown, the lungs of the mice treated with DOPE modified PEI complexed with FL-siRNA exhibited a high siRNA uptake. This suggested that the attachment of DOPE to the PEI backbone, decreased PEI interaction with the airway mucus and thus, increased its siRNA payload delivering capability significantly. Further investigation so as to why this phenomenon occurs, is needed.

High levels of FL-siRNA were also observed in the kidney; this an expected phenomenon since naked siRNA is rapidly excreted by the kidneys and this supersedes nuclease degradation [90]. Another reason for higher renal uptake is explained on basis of interaction between the negatively charged basal membrane of the Bowman’s capsule [87] and our excessively positively charged complexes (N/P 16). It is interesting to note that PEGylation decreases this to a certain extent as compared to PEI/siRNA; though this is not statistically significant (fig. 4.3).

Another interesting thing to note is that in all organs some fluorescence is seen in the case of treatment with naked siRNA. This seems unusual but this has been seen with radioactively labeled siRNA [87]. The siRNA fragments (caused due to nuclease degradation) with the radiolabel, linger in the organ system for a long time for reasons unknown and it is a possibility that this is the same case with the FL-siRNA.
4.6 CONCLUSIONS

DOPE modified PEI, especially the PEGylated carrier, seems to be a promising nanocarrier for in vivo siRNA delivery. Not only did it significantly increase serum siRNA levels after 4 hours as compared to non-phospholipid modified and non-PEG containing PEI nanocarriers; it also successfully improved siRNA delivering capability to the tumor due to a combination of factors viz. reduction in non-specific tissue interactions, siRNA protection from RNAse and the EPR effect. These results, therefore, suggest a prospective application of the DOPE-PEI/PEG-PE micelles for in vivo siRNA delivery.

HYPOTHESIS#5 ‘DOPE-PEI based nanocarriers containing siRNA targeted against the MDR1 gene in combination with doxorubicin will exhibit enhanced anti-tumor efficacy by downregulating MDR1 and thus, overcome MDR in a s.c. MCF7/ADR tumor xenograft model.’

SPECIFIC AIM#5 To evaluate the therapeutic efficacy and MDR1 gene downregulation capacity of the DOPE-PEI/siRNA and doxorubicin combination therapy.

5.1 BACKGROUND FOR SPECIFIC AIM#5

Resistance to chemotherapy has been a serious challenge in effective breast cancer treatment. The development of multidrug resistance (MDR), mediated by the overexpression of adenosine triphosphate (ATP) binding cassette (ABC-transporters) is believed to play an important role in poor efficacy of cancer chemotherapy [91, 92]. P-glycoprotein, a 170-kDa plasma membrane glycoprotein, was the first ABC transporter that consistently identified to be overexpressed in breast cancer cell lines displaying
MDR. P-glycoprotein which uses ATP to actively pump cytotoxic drugs out of the cells, is responsible for the efflux of chemotherapeutic drugs such as vinblastine, doxorubicin, and paclitaxel which lead to decrease in accumulation of effective drug concentration in tumor cells and failure of chemotherapy [93]. P-gp, thus, as discussed before in this text, is an attractive target to circumvent MDR as it is over-expressed from 0-30% in newly diagnosed to over 70% of relapsed form of breast cancer [69].

Numerous studies have aimed to overcome MDR phenomenon by different strategies. One of these approaches which have been studied extensively is focus on the use MDR modulators. However, results are not promising and their shortcoming attributed to their non-specific action on P-gp and/or off target distribution which leads to intolerable side effects and toxicity. Efforts still continue to discover not toxic MDR inhibitors which lack pharmacokinetic interactions with anticancer drugs [94-100]. The difficulties encountered with MDR inhibitors have led alternative approaches to overcome MDR. Novel approaches are based on selective regulation of the oncogene expression [101, 102]. Apart from antisense oligonucleotides [103] and ribozymes [104], more recently, RNA interference has emerged as a powerful technique for the selective post-transcriptional gene silencing through catalytic degradation of mRNAs triggered by short double-stranded RNAs [73, 101, 102]. The major bottleneck in the development of therapeutic RNAi is efficient delivery of siRNA into the cell of interest following systemic administration [105]. With the development of nanotechnology, nano-formulations have been widely used to circumvent MDR [106-109]. One of the most investigated nanocarriers for in vitro and in vivo delivery of DNA system is polyethylenimine (PEI); which function as transfection reagents based on their ability to compact genetic materials into nano-scale complexes. This feature offers protection of DNA them from harsh enzymatic degradation and change intracellular trafficking to increase the drug concentration in MDR cancer cells (uptake through endocytosis and escape from lysosome by proton
sponge effect). But on the other hand, representation of higher transfection correlates with increased non-specific toxicity due to excessive interactions with cell membranes by cationic charges associated with PEI. These biocompatibility issues have been addressed by different methods such as coupling hydrophobic or hydrophilic moieties [110-112] degradable acrylates [113] or incorporation of PEI complexes into liposomal formulations [114].

Recent studies in our lab have shown successful transfection effect of DNA and siRNA with phosphatidylcholine (PC)-modified PEI [84, 115]. Also we have reported similar transfection efficacy to that of PEI with generated micelle-like nanoparticles (MNPs) but with less toxicity and better biocompatibility properties. MNPs form based on the combination of a covalent conjugate between phospholipid and polyethylenimine (PLPEI) further associated with PEG and lipids [115]. Further studies, as discussed previously in this text, demonstrated the feasibility of using siRNA to inhibit P-gp expression with modified phospholipid (dioleoylphosphatidylethanolamine) and PEI (DOPE-PEI) in MCF-7 human breast cancer and [53].

The first leg of our in vivo study demonstrated the tissue distribution of the PEGylated and non-PEGylated DOPE-PEI/siRNA nanocarriers and showed that these nanocarriers could efficiently deliver siRNA to the tumor and improve blood distribution profile in vivo. The next phase of the in vivo study was the evaluation of the therapeutic efficacy of the combination therapy of siRNA nano-formulations with doxorubicin in a s.c. MCF7/ADR tumor xenograft model in nude female Balb/c mice.
5.2 MATERIALS

All materials were purchased from Sigma-Aldrich unless otherwise stated. Branched polyethyleneimine [16] with a molecular weight of 1.8 kDa was purchased from Polysciences, Inc (Warrington, PA). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethyleneglycol)-2000] (PEG-PE) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(glutaryl) (Glutaryl-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). All siRNA duplexes were purchased from Dharmacon (Lafayette, CO), namely, siRNA targeting MDR-1 (siMDR1): 5’- GGAAAAGAAAACACUGUCdTdT-3’ (sense) [75] and a non-targeting control siRNA, (siNegative): 5’-AGUACUGCUUACGAUACGGdTdT-3’ (sense). FITC-labeled P-glycoprotein antibody [UIC2] was purchased from Abcam (Cambridge, MA). Negative control FITC-IgG1 antibody for flow cytometry was procured from Santacruz Biotech Inc. (Dallas, TX). The CellTiter-Blue® Cell Viability Assay was purchased from Promega (Madison, WI). DMEM media was supplemented with 10% fetal bovine serum (FBS) and 0.2 mg/ml of Geneticin (G-418, Invitrogen, CA). Both MCF-7 sensitive and MCF7/ADR cell lines were grown at 37°C under 5% CO2 in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% FBS and penicillin (100units/ml) and streptomycin (100µg/ml). DMEM and penicillin/streptomycin stock solutions were purchased from Cellgro (Herndon, VA). Heat-inactivated FBS was purchased from Atlanta Biologicals (Lawrenceville, GA). Nuclease-free water was purchased from Qiagen (Germantown, MD). The RNAeasy kit for mRNA isolation was obtained from Qiagen. The First Strand cDNA synthesis kit and the SYBR green kit for qRT-PCR were obtained from Roche, USA. Primers for the dsMDR1 gene (5’-ATATCAGCAGCCCACATCAT-3’) and (5’-GAAGCACTGGGGATGTCGGGT-3’) and for the housekeeping gene GAPDH (5’-
GCCAAAAGGGTCATCATCTC-3’) and (5’-GTAGAGGCAGGGATGATGTTC-3’) were obtained from Invitrogen, CA. The Aspartate aminotransferase (AST)/Alanine aminotransferase (ALT) assay kit was purchased from the biomedical research service center at SUNY Buffalo (Buffalo, NY).

5.2.1 Animals

Nude female Balb/c mice (6-8 weeks) were procured from Charles River Laboratory (CRL) in Wilmington, MA.

5.3 METHODS

5.3.1 Characterization of MCF7 and MCF7/ADR cells

The MCF7/ADR cells were cultured for 4 weeks in complete DMEM media containing 5µM doxorubicin hydrochloride (dox). The IC₅₀ values of the cells towards doxorubicin were then calculated using the cell titer blue viability assay and employing the pseudo-Hill plot. Briefly, 10,000 MCF7 and MCF7/ADR cells were seeded in 96 well plates with complete DMEM media and after 24 hours cells were treated with varying doxorubicin concentrations in serial dilutions. After 24 hours, the cell titer blue viability assay was performed as per manufacturer protocol. In addition, the surface expression of P-gp in both the cell lines was evaluated using flow cytometry by employing the FITC-labeled anti-P-gp (UIC2) antibody (as described in section 3.3.5)
5.3.2 Establishment of orthotopic and subcutaneous (s.c.) MCF7/ADR tumor xenografts in female nude mice

Currently, there is a lot of conflicting literature with reference to the development of the MCF7/ADR tumor xenograft model in nude mice with respect to dependence of the tumor proliferation in the mice on the presence/in the absence of the female hormone β-estradiol \([81, 116-120]\). To first evaluate orthotopic and s.c. tumorigenesis for MCF7/ADR cells (that were previously cultured in the presence of 5 \(\mu M\) dox) in female nude mice, two groups of \(n=3\) nude female mice (6-8 weeks) were injected with these cells into the mammary fat pad and one group (\(n=3\)) were injected with these cells s.c. over the right flank. Note- MCF7/ADR cells were cultured in the absence of the dox, a week before animal injections to avoid deleterious effects of the presence of the drug in cells during inoculation in mice.

The 3 groups were injected as stated below:

**Group#1** – 5 million MCF7/ADR cells were resuspended in Matrigel® to yield a total volume of 100\(\mu\)l and injected into the mammary fat pad.

**Group#2** – Silastic tubing was cut into 1cm x 1cm lengths and one end was plugged with a silicone rubber sealant. Estradiol was mixed with cholesterol at a weight ratio of 1:100 and ground in a mortar and pestle. About 2.3 mg of the mixture was added into each tube, followed by sealing as before. The filled implants were wiped clean, sterilized with UV radiation overnight and stored sterile before use. These were then implanted surgically at the back of the head of the three female nude mice. A couple of days were allowed for the mice to recover and 5 million MCF7/ADR cells resuspended in Matrigel was injected into the mammary fat pad of these mice as performed previously for group#1.

**Group#3** – 5 million MCF7/ADR cells were resuspended in Matrigel to yield a total volume 100\(\mu\)l and this cell suspension was injected s.c. near the right flank region of the female nude mice.
Five weeks post injection, qRT-PCR was performed to detect the MDR1 levels in MCF7/ADR orthotopic tumors and were compared with MDR1 levels in tumors obtained from the s.c. MCF7/ADR tumors. Tumors in female nude mice from MCF7 sensitive cells grown in the presence of β-estradiol were also used for MDR1 gene expression evaluation (provided by Dr. Tao Wang at CPBN). The qRT-PCR sample preparation and analysis process has been briefly described below:

Tumors were isolated after sacrificing the animals. Isolated tumors were homogenized and processed for RNA isolation using RNAeasy from Qiagen ®. Isolated RNA was treated with DNAse to eliminate any possible contaminating genomic DNA. RNA was quantified and quality was evaluated using a ND-1000 NanoDrop spectrophotometer. Primer sequences for both the MDR1 (gene of interest) and GAPDH (housekeeping gene) were adopted from (Wu, Cancer Res. 2003). The primers were however, evaluated using the Invitrogen OligoPerfect™ for GC content and annealing temperature. Primers were also evaluated for primer-dimer formation. GAPDH was used as an appropriate internal loading control for qRT-PCR. cDNA synthesis and subsequent PCR amplification was performed using the First strand cDNA synthesis TM from Roche ®. Finally, qRT-PCR was performed on the tumor samples for each treatment using the LightCycler® 480 qRT-PCR machine from Roche™ in our department. The assay was run in 96-well optical reaction plates. Data was normalized to GAPDH and relative quantification method was used for data analysis.

5.3.3 Preparation and characterization of the formulations

The DOPE-PEI was synthesized and DOPE-PEI/siRNA complex was prepared as described before (section 2.3.1 and section 2.3.6. respectively). The DOPE-PEI/PEG-PE (1:10w/w)/siRNA complex was prepared as described before (section 4.3.1). Both the formulations were evaluated for size and zeta potential using the Malvern zetasizer instrument. Briefly, 100µl complexes at the N/P ratio of 16
(corresponding to 40µg siRNA), were diluted to 1ml with nuclease free water and size and zeta potential were measured.

Transmission electron microscopy (TEM) images were also obtained to assess the size and morphology of the formulations. For this, 10 µL of the sample was placed on the Formvar-coated copper grids (Electron Microscopy Science, Hatfield, PA) and negatively stained with 50 µL of 1% (w/v) uranyl acetate for 2-5 minutes. A Whatman filter paper was used to drain excess liquid, the grids were allowed to air-dry for a minute. Images were acquired using the JEOL 100X transmission electron microscope (Peabody, MA). Atleast 30 measurements were made for each formulation to determine mean particle size.

5.3.4 Therapeutic efficacy experiment

Thirty female nude mice were injected s.c. near the right flank with five million MCF7/ADR cells/mouse resuspended in Matrigel for a total volume of 100µl. Once the tumor volume reached ca. 60mm³ the mice were randomly assigned to different treatment groups. The DOPE-PEI and DOPE-PEI/siRNA were prepared as described previously in the text (sections 2.3.1 and 2.3.6). The treatment groups and the dosing regimen and have been described below:

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepes buffered glucose (BHG)</td>
<td>5</td>
</tr>
<tr>
<td>siMDR1 + doxorubicin.HCl</td>
<td>5</td>
</tr>
<tr>
<td>DOPE-PEI/siMDR1 (N/P 16) + doxorubicin.HCl</td>
<td>5</td>
</tr>
<tr>
<td>DOPE-PEI/siNegative (N/P 16) + doxorubicin.HCl</td>
<td>5</td>
</tr>
<tr>
<td>(DOPE-PEI/siMDR1)/PEG-PE (1:10 w/w) + doxorubicin.HCl</td>
<td>5</td>
</tr>
</tbody>
</table>
Tumor volume was measured as \( V = \frac{1}{2} \times (\text{smaller diameter})^2 \times \text{larger diameter} \). The Relative tumor volume (RTV) was calculated, defined as \( \frac{V_n}{V_o} \), where \( V_n \) was the volume in mm\(^3\) on day ‘n’ and \( V_o \) at the start of treatment. Mean RTV (RTV\(_m\)) and standard deviation were calculated for each group. All injections were administered via the tail vein method. In addition, the group DOPE-PEI/siRNA + doxorubicin (co-administration) was included to evaluate the effect of the combination therapy of the siRNA and the dox, when administered concurrently.

**Note-** For the co-administration group, DOPE-PEI/siRNA was injected and immediately followed by another separate injection of free doxorubicin HCl. One such dosing regimen per week.

The dose for siMDR1 i.e. siRNA against MDR1 was 1.2 mg/kg (optimized from previous biodistribution experiment) and dose for dox was 2 mg/kg [121, 122]. The dosing regimen was one naked siRNA or formulation containing siRNA injection followed by one doxorubicin HCl injection after 48 hours; one such regimen per week for five weeks.

Animals were monitored for body weight and tumor volume twice a week. Animals were sacrificed a week after the last doxorubicin injection (5th dose). The blood was collected via cardiac puncture, serum was obtained by centrifugation of the freshly collected blood samples at 2,000xg for 30 minutes at 4 °C and stored at -80°C for further analysis. The tumors were excised and flash frozen in liquid nitrogen for further analysis.

**5.3.5 Evaluation of repeated dose toxicity in mice**

The percent change in body weight was calculated at the end of the study and reported as a preliminary indication of general toxicity. The serum aspartate amino transferase (AST) and alanine amino transferase (ALT) levels were also measured and reported as a more reliable indication of repeated dose toxicity. AST and ALT values were measured using the manufacturer’s standard kinetic assay protocol.
5.3.6 qRT-PCR for evaluation of MDR1 levels in excised tumors

Tumors were excised post-mortem and qRT-PCR for evaluation of transcriptional MDR1 levels was performed as described before (section 5.3.2).

5.3.7 Flow cytometry to evaluate P-gp downregulation

Tumors were minced using two surgical blades with vortexing in a 50ml tube containing 1ml cold PBS. Cell suspension was collected (500mcl x 2 aliquots) and aliquot 1 was incubated with FITC-labeled anti P-gp antibody and aliquot 2 was incubated with non-specific IgG1-FITC antibody (same antibody concentrations for each sample) for 1 hour on ice. Samples were suitably diluted with sheath liquid, flow cytometry was performed and mean fluorescence intensity (MFI) was corrected for fluorescence due to non-specific binding.

5.3.8 Statistics

Students t-test, Two way ANOVA and One way ANOVA followed by Bonferroni multiple comparisons test in GraphPad Prism 5.0 were employed for statistical data analysis, where appropriate. For in vivo experiments data plotted as mean ± SD (n=5).

5.4 RESULTS

The MCF7/ADR cells were cultured in the presence of 5µM dox for four weeks with a view to increase the resistance of the cells towards doxorubicin. To confirm this, a simple experiment to check the IC₅₀ values of the wild strain vs the resistant was performed, using the cell titer viability assay. The pseudo-Hill plot was constructed to determine the IC₅₀ values. At the end of the four weeks of dox treatment, the
MCF7/ADR cells exhibited an approx. 500-fold higher IC₅₀ as compared to the MCF7/S strain as shown in the table below.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MCF7 ADR</th>
<th>MCF7 Sensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IC₅₀ values</strong></td>
<td>4254.3 µM</td>
<td>8.2 µM</td>
</tr>
</tbody>
</table>

**Table 5.1** IC₅₀ values for MCF7/ADR and MCF7/S cells towards doxorubicin. HCl

Further, the surface P-gp expression for both cell lines was evaluated using flow cytometry by employing FITC-labeled P-gp antibody. It was seen that the surface expression of P-gp was significantly higher in MCF7/ADR cells when compared to MCF7/S cells which exhibited lower levels of P-gp. The increase in dox resistance of the MCF7/ADR cells correlated well with increased surface P-gp expression observed.

**Fig. 5.2** P-gp levels in MCF7/S vs MCF7/ADR using flow cytometry. Data plotted as mean ± SD (n=3).

Data analyzed by student’s t- test in GraphPad Prism 5.0. ** denotes p-value < < 0.05
After the MCF7/ADR cells were successfully characterized for dox resistance and surface P-gp expression, nude female Balb/c nude mice were injected orthotopically and s.c with these cells to check for tumorigenesis. The tumors were allowed to develop for five weeks after which the mice from each group were sacrificed and their tumors excised. qRT-PCR was performed and the MDR1 transcriptional levels in the tumors were obtained. It was seen that not only were the tumors in the two orthotopic tumors very similar in volume (mean volume approx. 40 mm$^3$) but the MDR1 expression was also similar. This suggested that in contrast to MCF7/S tumors, the growth of MCF7/ADR orthotopic tumors is estrogen-independent since implantation of the estradiol pellet didn’t affect tumor volume or MDR1 gene expression, 5 weeks post tumor inoculation. Note- The MCF7/S tumor was obtained from Dr. Tao Wang in our lab. Briefly, this tumor was developed by injecting 2 million MCF7/S cells s.c. over the right flank of female nude mice surgically implanted with estradiol pellets over the back of the neck of the mouse. This tumor was also evaluated for MDR1 levels as a control.

The qRT-PCR data clearly showed that the MDR1 levels in the MCF7/ADR orthotopic tumors (about 40mm$^3$ after five weeks) in the presence/absence of the estradiol was very similar i.e. no statistical significance. On the other hand the s.c. tumors (about 100mm$^3$) had a lower MDR1 level albeit significantly higher than the MDR1 levels in the MCF7/S tumor. The s.c. model was chosen for the final therapeutic efficacy experiment since larger tumors were obtained in a shorter period of time and the tumors retained high MDR1 levels.
**Fig. 5.3** qRT-PCR to evaluate MDR1 gene expression in orthotopic and s.c. tumors (5 weeks post inoculation). Data plotted as mean ± SD (n=2) in all groups except MCF/S where only one tumor was analyzed. H- β-estradiol pellet. *, # and ^ denote treatment groups statistically very significant from orthotopic MCF7/sensitive (MCF7/S) tumor. “ denotes groups are statistically significant from s.c. MCF7/ADR tumor.

The batch of synthesized formulations to be employed for the therapeutic efficacy experiment were characterized with respect to size and zeta potential as described (Table 5.4).
<table>
<thead>
<tr>
<th>Formulations</th>
<th>Size (nm)</th>
<th>Zeta (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOPE-PEI/siRNA</td>
<td>156.4 ± 68.67</td>
<td>48.4 ± 8.4</td>
</tr>
<tr>
<td></td>
<td>PDI 0.227</td>
<td></td>
</tr>
<tr>
<td>(DOPE-PEI/PEG-PE)/siRNA</td>
<td>146.9 ± 55.75</td>
<td>13.4 ± 6.49</td>
</tr>
<tr>
<td></td>
<td>PDI 0.222</td>
<td></td>
</tr>
</tbody>
</table>

**Table 5.4** Size and zeta potential for the *in vivo* formulations

Additionally, TEM images have been shown for the DOPE-PEI/siRNA and (DOPE-PEI/PEG-PE)/siRNA complexes. It should be noted that the dye used for negative staining in the sample preparation (uranyl acetate) is a hydrophilic dye and stains hydrophilic moieties. The DOPE-PEI/siRNA micelles appeared like ovoid particles, their mean particle size being 110.41nm ± 20.41nm. These particles sometimes appeared in concentric ovoid structures. Most of the particles bear structures encapsulated within them perhaps, suggesting siRNA encapsulation inside the PLPEI nanocarrier, though this could not be conclusively proven using this technique. The (DOPE-PEI/PEG-PE)/siRNA micelles also appeared ovoid and seemed to encapsulate a payload like the DOPE-PEI/siRNA micelles. The mean particle size for the (DOPE-PEI/PEG-PE)/siRNA complex was 95.81nm ± 38.70nm.
(B) (DOPE-PEI/PEG-PE)/siRNA

Fig. 5.5 Transmission electron microscope images of DOPE-PEI/siRNA (A) and (DOPE-PEI/PEG-PE)/siRNA (B). For both formulations morphology appeared ovoid encapsulating a payload, possibly siRNA (inconclusive). Mean particle size for DOPE-PEI/siRNA was ca. 120nm while for (DOPE-PEI/PEG-PE)/siRNA, mean particle size was ca. 115nm. Note- Images in the inset are higher magnifications of respective samples shown for better clarity. For each sample n=30 measurements.
The therapeutic efficacy experiment was started after the tumor volume reached 60mm$^3$ and mice were randomized into the six treatment groups described before. The dosing regimen was carried on for 5 weeks and five days after the final dose, the mice were sacrificed. The therapeutic efficacy experiment data is shown in the (fig. 5.6). It was found that the mean relative tumor volumes (RTVm) were significantly smaller in all treatment groups as compared to all of the control groups. It is important to note that the same DOPE-PEI nanocarrier complexed with scrambled siRNA did not have any therapeutic advantage and hence, proves the relevance of the MDR1 siRNA sequence specificity for therapeutic efficacy. Free therapeutic siRNA in combination with doxorubicin also did not confer any therapeutic benefit proving that the nanocarrier is needed to deliver the siRNA into the tumor to see beneficial effect.

**Note:** siMDR1- siRNA targeting MDR1, siscr- scrambled siRNA, DOPE-PEI : PEG-PE (1:10w/w) and Co-administration – DOPE-PEI/siMDR1 + doxorubicin.HCl administered together
**Fig. 5.6** Relative tumor volume (RTV) for different treatments. Mean ± SD plotted (n=5). Data analyzed using two way ANOVA in GraphPad prism 5.0. p < 0.05 considered statistically significant. ^, * and # denote the respective groups are statistically very significant with respect to BHG, free siMDR1 and DOPE-PEI/siscr.

The volume and weights of all the excised tumors for all groups was recorded. The ratio of post-mortem mean tumor volume (TVm) in all treatment groups to post-mortem TVm in control and ratio of post-mortem mean tumor weight (TWm) in treatment groups to post-mortem TWm in control were plotted. The data seen here is consistent with tumor volume data where all treatment groups exhibit significantly lower post-mortem tumor volumes and tumor weights.
Fig. 5.7 Ratio of mean post-mortem (A) Tumor volumes (B) Tumor weights. Mean ± SD plotted. n=5 per treatment group. Data analyzed using one way ANOVA subjected to Bonferoni’s multiple comparison test in GraphPad Prism 5.0. p-value < 0.05 was considered to be statistically significant. #, ^ and * denote the respective groups are statistically significant with respect to BHG, free siMDR1 and DOPE-PEI/siscr

As a measurement of general toxicity that could be a resultant of the prolonged dosing regimen of the phospholipid-modified polymeric formulations, mice body weights were monitored throughout the study. At the end of the study, percent change in mean mice body weights from day 0 of the treatment was calculated and shown in the table below. Serum aspartate and alanine amino transferases viz. AST and ALT were also analyzed as a more reliable measure of repeated dosing toxicity. It was seen that there was no significant loss in body weight in any of the treatment groups. Also, there was no significant/alarming spike in serum AST and ALT levels for any of the treatment groups.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent change in body weight (from day 0)</th>
<th>AST (IU/L)</th>
<th>ALT (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHG buffer/control</td>
<td>100.03 ± 0.42</td>
<td>11.21 ± 0.14</td>
<td>14.97 ± 0.13</td>
</tr>
<tr>
<td>Free siMDR1</td>
<td>100 ± 0.12</td>
<td>14.13 ± 0.34</td>
<td>16.82 ± 0.13</td>
</tr>
<tr>
<td>DOPE-PEI/siMDR1</td>
<td>100.07 ± 1</td>
<td>13.24 ± 0.18</td>
<td>19.36 ± 0.29</td>
</tr>
<tr>
<td>DOPE-PEI/siscr</td>
<td>100.02 ± 0.28</td>
<td>12.91 ± 0.06</td>
<td>16.21 ± 0.08</td>
</tr>
<tr>
<td>DOPE-PEI/PEG-PE</td>
<td>100.05 ± 0.6</td>
<td>20.27 ± 0.52</td>
<td>21.24 ± 0.31</td>
</tr>
<tr>
<td>Co-administration</td>
<td>100.06 ± 0.73</td>
<td>11 ± 0.11</td>
<td>16.55 ± 0.14</td>
</tr>
</tbody>
</table>
Table 5.8 Evaluation of repeated dosing toxicity in mice by measurement of changes in body weight and serum AST, ALT levels

qRT-PCR was performed on the excised tumors for evaluation of the transcriptional mRNA levels of the MDR1 gene. It was seen that in all the treatment groups, MDR1 was significantly downregulated (fig. 5.8). Interestingly, DOPE-PEI/PEG-PE and co-administration groups showed a significantly higher gene downregulation than the DOPE-PEI group itself. However, this did not translate to proportional tumor volume reduction difference between the respective treatment groups.

Fig. 5.8 qRT-PCR data for tumors from all groups. Data plotted as mean ± SD. (n=5; performed in duplicates). One way ANOVA- Bonferroni’s multiple comparison test in GraphPad prism 5.0. p-value < 0.05 was considered to be statistically significant. #, ^ and * - Respective treatment groups are statistically
significant than free siMDR1 and DOPE-PEI. ” - Treatment groups statistically significant than DOPE-PEI/siMDR1.

Flow cytometry was also performed to check the P-gp protein level expression on the surface of the tumor cells. The FACS data seemed to correlate well with the qRT-PCR data. The P-gp protein expression was downregulated in all the treatment groups and as is with the qRT-PCR data, a similar pattern is seen where the P-gp expression is lower in the PEGylated nanocarrier and the co-administration group as compared to the DOPE-PEI group; though the P-gp levels in all treatments were significantly lower than the control groups (fig. 5.9).

![Flow cytometry data of tumors for evaluation of surface P-gp expression. Mean ± SD plotted (n=5). One way ANOVA-Bonferroni multiple comparison test on GraphPad prism 5.0. p-value < 0.05 was considered to be statistically significant. ^, * and # denote the respective groups are statistically very significant.](image-url)
significant with respect to BHG, free siMDR1 and DOPE-PEI/siscr. ” – Treatment groups statistically significant from DOPE-siMDR1

The PCR and the flow cytometry data combined show that the treatments were able to transcriptionally and translationally downregulate MDR1 and thus, P-gp effectively in solid tumors. This clearly explains the reduced tumor volumes in the treatment groups over the controls.

5.5 DISCUSSION

The second leg of the in vivo study was the therapeutic efficacy study. The aim was to prove that the PLPEI-based nanocarriers could deliver therapeutic siRNA into solid tumors and inhibit tumor growth. The MCF7/ADR cells were grown in the presence of high concentration of dox to make the cells more drug resistant. These cells were then injected orthotopically and s.c to develop tumors which were then characterized with respect to MDR1 gene expression. The final experiment was carried out in an s.c tumor xenograft model.

The formulations were characterized with respect to their size and zeta potential before the dosing regimen. Additionally, TEM was performed as a supplementary tool for characterization of the nanocarriers. The dosing regimen for all treatments (except the co-administration group) was a naked siRNA or siRNA containing formulation injection followed by doxorubicin.HCl (dox) after 48 hours. This regimen was decided based on our previous in vitro findings [53] and available literature which proves sequential dosing regimen of siRNA and doxorubicin shows significant MDR1 downregulation [81, 123]. All the treatments exhibited decreased tumor volume with respect to controls. Also, since no statistical significance was seen within the treatment groups and since they were all non-toxic, it could be
concluded, within the frame of the therapeutic efficacy experiment, that PEGylation didn’t impart any therapeutic advantage to the DOPE-PEI nanocarrier. Also, the timing of the dox administration didn’t seem to have an impact on regimen efficacy as both groups DOPE-PEI/siRNA with a 48 hour lag dox administration and the dox co-administration group demonstrated similar efficacy. Interestingly, this is in agreement to our in vitro findings (fig. 3.6).

qRT-PCR and flow cytometry data exhibited similar pattern where all treatment groups downregulated MDR1 and P-gp significantly as compared to controls. The PCR and flow cytometry data, in addition to the free siMDR1 control group, more importantly, clear recent concerns that continuous si/shRNA dosing can show tumor inhibition not due to mRNA downregulation but due to non-specific activation of the innate immune system [123].

It was seen that the DOPE-PEI/PEG-PE and co-administration groups exhibited a higher transcriptional and translational MDR1 gene downregulation. Several complex factors could be suggested, having a bearing on this observation viz. the time of excision of tumor after the last dose, mRNA turnover, nature of the tumor xenograft etc. In relation to the current experiment, the slow growth of the s.c MCF7/ADR xenograft tumors could play a major role so as to why tumor volume differences between different treatment groups were not seen even as PCR and FACS data pointed out MDR1 levels were lower in the PEGylated DOPE-PEI and co-administration group. It is conceivable that these tumors would need a longer time to manifest tumor volume differences due to the low MDR1 and P-gp levels. Probably, if the tumors would have been monitored for a longer time, differences in volume could have been seen. In the case of the co-administration group, there is literature which shows that the co-administration of a cytotoxic drug (in this case dox) and the MDR1 modulator, (in this case the siRNA), exhibits synergistic effects in MDR1 downregulation [79, 82, 95, 124, 125], albeit in all these cases the drug and the siRNA were in the same formulation and were found to be in spatial proximity; which in our
case cannot be proved without further investigation. However, it has been shown that free doxorubicin injected I.V. has a plasma half-life of about 16 ± 3 hours in nude, tumor bearing mice [126] and previous biodistribution studies prove that our DOPE-PEI nanocarrier can deliver siRNA to the tumor in four hours (about 5% injected dose of siRNA found in tumor after 4 hours) and plasma levels showed about 10% of injected dose after four hours (figs. 4.2 and 4.3). So it is safe to say that in the co-administration group, the nanocarrier and dox can temporally co-exist in vivo.

It has also been shown that in low doses, dox behaves more like an anti-angiogenic agent, expanding interstitial space, vessel diameter and blood perfused area than a tumor cytotoxic drug [127]. In the co-administration group, it is possible that the carrier penetration was improved by this phenomenon (similar to tumor priming), although without microvessel density and drug distribution studies, this cannot be said to have occurred here. The similar qRT-PCR and FACS data however, hint that the siRNA nanocarrier could have been benefited by the anti-angiogenic effect of dox and so we observed improved gene and protein downregulation with the co-administration group. With regards to the PEGylated DOPE-PEI group, PEGylation of the DOPE-PEI nanocarrier has been shown to improve the blood circulation time (fig. 4.3) of the carrier and was probably therefore, more effective in delivering siRNA to the tumor due to EPR and thus, exhibiting improved gene downregulation.

The PCR and the FACS data, thus, in contrast to efficacy data, suggest towards some benefit to PEGylation of the PLPEI nanocarrier and the time of the drug administration. In a nutshell, the DOPE-modified PEI nanocarrier was successfully able to deliver siRNA to tumors, downregulate MDR1 and thus, the dox could better inhibit tumor growth due to decreased P-gp expression on the tumor cells allowing the drug to exert its action more efficiently.
5.6 CONCLUSIONS

We were successfully able to develop PLPEI-based nanocarriers that could self-assemble and electrostatically interact and complex siRNA. In addition to standard DLS measurements, the nanocarriers were characterized using TEM. It was seen that the combination treatment of PLPEI/siRNA nanocarriers and doxorubicin exhibited significantly lower tumor volumes as compared to the appropriate control groups. Further, qRT-PCR and FACS data clearly showed that the decrease in tumor volumes in the treatment groups was because of significant transcriptional and translational downregulation of MDR1. Thus, it could be concluded that our nanocarriers could effectively deliver siRNA to tumors, were able to successfully downregulate the MDR1 gene and thus, reverse the phenomenon of MDR in MCF7/ADR tumor xenografts.

6. HYPOTHESIS#6 ‘Transferrin-modified micelles based on PEGylated DOPE-PEI modified with PEG can be loaded with siRNA for intracellular delivery and gene silencing in vitro.’

SPECIFIC AIM#6 To formulate DOPE-PEI based transferrin (Tf)-conjugated micelles for prospective utilization as targeted siRNA delivery system in vitro.

6.1 BACKGROUND FOR SPECIFIC AIM#6

The nanocarriers we have used until this point have exploited the passive EPR effect as the strategy for tumor accumulation. Here we go a step further in a bid to make our carrier targeted so as to improve tumor accumulation for possible future in vivo application. Transferrin (Tf) was chosen as the targeting moiety for the reasons stated below and preliminary data obtained until this point has been shown.
Rationale for selection of Tf as the targeting moiety. The major drawbacks of non-viral vectors are the lack of cell specificity and their limited ability to transfect cells. Coupling of cell-binding ligands such as transferrin (Tf) to polycations can be a relevant strategy to overcome these barriers [128, 129]. Transferrin receptors are expressed on the surface of cerebral endothelial cells, hepatocytes and highly proliferating cells such as erythroblasts and tumor cells [130]. The inclusion of transferrin into polyplexes may thus, enhance their specificity towards target sites (e.g. tumor, brain, liver) and at the same time shield their positively charged surface due to its anionic nature, leading to an inhibition of non-specific interactions after systemic application. In this project, we have developed a novel multifunctional carrier consisting of DOPE-modified polyethylenimine micelles grafted with PEG and Tf–PEG for delivery of siRNA as a payload. We have already demonstrated that DOPE-PEI achieves high levels of gene silencing and self-assemble into micellar structures. Here, preliminary in vitro transfection studies carried out with Tf micelles has been presented. Our future goal is to try to increase the bioavailability and efficacy of the formulation at the tumor site. DOPE-PEI micelles have been modified with PEG keeping in mind the in vivo application; to improve the in vivo stability and biocompatibility along with Tf to increase tumor specificity.

6.2 MATERIALS

All materials were purchased from Sigma-Aldrich unless otherwise stated. 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (PEG-PE) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(glutaryl) (Glutaryl-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). siRNA targeting Green Fluorescent Protein (GFP-siRNA): 5’-AUGAACUUCAGGGUCAGCUdTdT-3’ (sense), scrambled control siRNA, (siNegative): 5’-
AGUACUGCUUACGAGAUACGGdTdT-3’ (sense) and 6-carboxyfluorescein (FAM or FL) - labeled siRNA (siGLO® siRNA) were purchased from Dharmaco (Lafayette, CO).

6.2.1 Cell culture

HeLa cells (cervical cancer cells) and NIH3T3 (fibroblasts) were obtained from the American Type Culture Collection (Manassas, VA) and was cultured using DMEM media supplemented with 10% fetal bovine serum (FBS) and penicillin (100 units/ml) and streptomycin (100 µg/ml), grown at 37°C under 5% CO₂ Dulbecco’s. DMEM was purchased from Cellgro (Herndon, VA). Heat-inactivated FBS was purchased from Atlanta Biologicals (Lawrenceville, GA). Stably GFP expressing HeLa cells were obtained from Cell Biolabs, Inc. (San Diego, CA) and grown at 37°C under 5% CO₂ using DMEM supplemented with 10% FBS and 10µg/ml Blasticidin S HCl (Invitrogen, CA). Nuclease-free water was purchased from Qiagen (Germantown, MD).

6.3 METHODS

6.3.1 Effect of PEG on colloidal stability of micellar nanocarriers

Micellar nanocarriers with varying % PEG were incubated with 150 mM NaCl for up to 24h at RT following which their size and zeta potential was measured using a Brookhaven zeta plus particle analyzer.

6.3.2 Effect of PEG on cellular uptake and gene silencing

HeLa cells were treated with DOPE-PEI micellar formulations with varying PEG amounts prepared with FL-siRNA for 4h. Naked siRNA and DOPE-PEI complexes (no PEG content) were used as
controls. HeLa–GFP expressing cells were treated with DOPE-PEI based formulations prepared with siRNA targeting GFP (siGFP) or scramble (siNegative) at N/P 16 for 4 h. Non-treated cells were used as control. Cellular uptake and gene silencing was studied by flow cytometric analysis.

6.3.3 Synthesis of the DOPE-PEI Tf micelles

Step#1 Synthesis of PEG-PEI-DOPE. Into the solution of DOPE-PEI (20 mg) in chloroform and 25 μL of triethylamine, poly(ethylene glycol)-succinimide carboxymethyl (mPEG-SCM) (molecular weight 2000, 15.0 mg, 7.51 μM) was added. The reaction mixture was stirred for 4 h at room temperature, the reaction mixture evaporated to dryness and dialyzed using cellulose ester membrane (MWCO. 3500) against water.

Step#2 Synthesis of Mal-PEG-PEI-DOPE. The reaction condition was same as the synthesis of PEG-PEI-DOPE except instead of using mPEG-SCM, bifunctional Mal-PEG-2000-NHS was used.

Step#3 Activation of transferrin (Tf). Into the solution of transferrin (4.8 mg) in PBS, pH 8.0 was added Traut's reagent (Pierce, Rockford, IL) according to manufacturer's protocol. The reaction was continued for 1 h at room temperature. The thiolated Tf was purified using Zeba™ spin desalting column (Pierce) following manufacturer's protocol.

Step#4 Preparation of micelles. The chloroform solution of PEI-DOPE, PEG-PEI-DOPE and Mal-PEG-PEI-DOPE in the molar ratio of 75:22.5:2.5 was mixed and evaporated to make a lipid film. The lipid film was hydrated with PBS.

Step#5 Preparation of Tf-micelles. The micelles solution prepared in step 4 were incubated with thiolated Tf solution overnight at 4 °C. The solution was dialyzed using cellulose ester
membrane (MWCO, 100,000) to get rid of excess Tf. The dialysate was further diluted to prepare the micelle solution as 1 mg/mL of total PEI.

6.3.4 Size and zeta potential of Tf micelles complexed with siRNA

The Tf micellar/siRNA complexes were characterized by Dynamic light scattering (DLS). The particle size and zeta potential of the formulations was measured by using a Zeta Plus Particle Analyzer (Brookhaven Instruments Corp, Santa Barbara, CA). Scattered light was detected at 25°C at an angle of 90°. Samples (50 µL) were diluted in 1.7 ml of nuclease-free water and measured immediately after preparation.

6.3.5 Gel retardation assay

For gel retardation studies, DOPE-PEI complexes containing 25% PEG were complexed with siRNA and siRNA with varying N/P ratios and were electrophoresed through a 0.8% agarose gel, using the E-Gel electrophoresis system (Invitrogen Life Technologies) and evaluated under UV-light.

6.3.6 Cell uptake studies

HeLa and NIH3T3 fibroblast cells were treated with the targeted and non-targeted formulations containing 25% PEG prepared with FAM-siRNA at N/P 16 and uptake was studied after 1h and 4h using flow cytometry. HeLa cells were also treated with non-targeted micelles and Tf micelles complexed with FAM-siRNA at N/P 16 after pre-treatment with free transferrin and cellular uptake was evaluated using FACS analysis.
6.3.7 GFP downregulation studies

HeLa–GFP cells were treated with targeted and non-targeted formulations prepared with siRNA targeting GFP (siGFP) or scrambled (siNegative) for 1 or 4 h and flow cytometry was performed.

6.4 RESULTS

6.4.1 Effect of the varying amount of PEG on size and zeta measurements

The first step towards formulating this carrier was to evaluate the optimal amount of PEG that would strike the right balance needed for stability and cellular uptake and silencing efficacy. As a first step, it was shown that increasing amounts of PEG (in the form of PEG-PEI-DOPE) added to DOPE-PEI micellar nanocarriers, shield their positive charge and improves their colloidal stability (see fig. 6.1)

![Graph showing size and zeta potential of PEG formulations]

**Fig. 6.1 (A)** Size and colloidal stability of different mol% PEG formulations as indicated by the change in size after incubation with 150 mMNaCl for up to 24h at RT. **(B)** Zeta potential as a function of PEG mol%
6.4.2 Effect of varying PEG amount on cellular uptake

Also, when HeLa cells were treated with DOPE-PEI based micellar nanocarriers, it was seen that increasing amounts of PEG prevented their cellular uptake (see fig. 6.2).

![Graph showing siRNA uptake mediated by different mol% PEG in DOPE-PEI nanocarriers.](image)

**Fig. 6.2** siRNA uptake mediated by different mol% PEG in DOPE-PEI nanocarriers. HeLa cells were treated with formulations prepared with fluorescein-siRNA for 4h. Naked siRNA and DOPE-PEI complexes (no PEG content) were used as controls.

6.4.3 Effect of varying PEG amount on GFP downregulation

A similar trend as the above study was noticed when the HeLa-GFP cells were treated with siGFP containing formulation with varying amounts of PEG. As the mol% PEG increased, the GFP downregulating potential of the nanocarriers decreased (see fig. 6.3)
**Fig. 6.3** Green Fluorescent Protein (GFP) silencing by different mol% PEG in DOPE-PEI nanocarriers. HeLa –GFP expressing cells were treated with formulations prepared with siRNA targeting GFP (siGFP) or scramble (siNegative) for 4 h. Non-treated cells were used as control.

### 6.4.4 Synthesis of transferrin-modified micelles

From the above experiments, the Tf micelles were synthesized stepwise as described in the methods section 5.3.3 as PEI-DOPE, PEG-PEI-DOPE and Mal-PEG-PEI-DOPE in the molar ratio of 75:22.5:2.5 (see fig. 6.4). The Tf micelles complexed with siRNA at N/P 16 were prepared as shown in the schematic below. Their size was found to be 182nm±3.6nm, PDI 0.210 and the zeta potential was found to be 14.96±1.32 mV.
Fig. 6.4 Scheme for formation of transferrin micelles complexed with siRNA (DOPE-PEI: PEG 2000–PE:TF- PEG 2000–PE 75:22.5:2.5) N/P 16

6.4.5 Gel retardation assay

Once the amount of PEG was optimized, this formulation of Tf micelles was evaluated for its siRNA complexing capability. The Tf micelles demonstrated successful siRNA complexation. The siRNA was completely absorbed in the PEI corona of the micelles by electrostatic interactions at N/P ratios ≥ 3 (fig. 6.5)
Fig. 6.5 Binding of 25%PEG/DOPE-PEI to siRNA at different N/P ratios demonstrated by the gel retardation assay. 1: free siRNA, 2: N/P= 1, 3: N/P= 3, 4: N/P =6

6.4.6 Cell uptake studies with Tf micelles

It was seen that modification of DOPE-PEI: PEG-DOPE-PEI, 75:25 mol ratio (mixed micelles) with Transferrin (TF) overcame the low cellular association of 25% PEG-micelles towards HeLa cells (human cervix carcinoma, highly expressing TF receptor) (fig. 6.6A). This improvement was not observed when a non-cancer fibroblast cell line was used (fig. 6.6B). The pre-incubation of HeLa cells with free Tf, also called a competition assay, significantly decreased siRNA uptake suggesting that Tf-targeted-micelles mediated ligand dependent uptake.
Fig. 6.6 siRNA uptake mediated by Transferrin targeted and non-targeted formulations towards (A) HeLa and (B) NIH3T3 fibroblast cells. (C) Competition study performed in HeLa cells. The siRNA uptake mediated by TF-micelles was compared in the absence (-TF) or in the presence (+TF) of free transferrin.

6.4.7 GFP downregulation studies

The presence of 25% PEG in the DOPE-PEI micelles decreased the silencing efficiency of DOPE-PEI conjugate (fig. 6.7A) but the inclusion of Tf in such micelles completely restored and prolonged their capacity to down-regulate GFP in cancer cells (fig. 6.7B)

![Graph A](image)

![Graph B](image)

Fig. 6.7 (A) Green Fluorescent Protein (GFP) silencing by targeted and non-targeted micelles. HeLa–GFP expressing cells were treated with formulations prepared with siRNA targeting GFP (siGFP) or scramble (siNegative) for 1 or 4 h. (B) Kinetics of GFP-silencing mediated by targeted and non-targeted formulations
6.5 DISCUSSION

This DOPE-PEI core-based PEGylated transferrin-labeled system developed in this section has been designed for a potential future tumor targeted system for *in vivo* application. Another potential use of this system, in the future, could be for simultaneous delivery of siRNA and drug since the simultaneous delivery of drug and siRNA can promote the synergy of the treatments by temporally co-localizing them in the tumor cells. The preliminary results obtained until this point have been presented here. As shown in the data above, we were able to successfully develop and characterize the DOPE-PEI core based PEGylated Tf-labeled micelles. The PEG amount was optimized for future *in vivo* application. This carrier exhibited siRNA condensing capacity, transferrin ligand mediated cellular uptake and intracellular siRNA delivery which translated into effective and sustained gene downregulation in a stably GFP expressing cancer cell line.

6.6 CONCLUSIONS

Transferrin-targeted, phospholipid-modified nanocarriers based on previously proven, non-toxic lipiddated PEI seems to be a promising nanocarriers from the studies presented. It could also become a useful carrier for future siRNA/drug delivery suitable for intravenous injection. This carrier was able to to load siRNA and facilitate the uptake by means of targeted interaction with cancer cells. Thus, it possesses the potential to increase the bioavailability and efficacy of siRNA/anticancer drug combinations in the future.
BIBLIOGRAPHY


