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Novel Methods for Delivering and Promoting the Endosomal Escape of Nucleic Acid Based Drugs: Chiral Polyamines and Hydrophobic Nanoparticle-Containing Liposomes

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NOVEL METHODS FOR DELIVERING AND PROMOTING THE ENDOSOMAL ESCAPE OF NUCLEIC ACID BASED DRUGS: CHIRAL POLYAMINES AND HYDROPHOBIC NANOPARTICLE-CONTAINING LIPOSOMES

BY

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ABSTRACT

Nucleic acid based drugs such as plasmid DNA (pDNA), small interfering RNA (siRNA), short hairpin RNA (shRNA), micro RNA (miRNA), and both antisense and antigene oligonucleotides, are potentially potent and specific compounds for therapeutic applications. Many major life threatening ailments might be treated using these molecules, and many polynucleotide products are currently in advanced clinical development. However, their successful therapeutic application is hindered due to limited delivery to their site of action in either the cytosol or the nucleus of a cell. Some of the barriers in the path of successful delivery of these biomolecules to their site of action have been addressed. However, endosomal entrapment followed by maturation to lysosomes and degradation of these compounds inside the cell is one remaining major hurdle. This dissertation describes two novel siRNA delivery techniques which present distinct advantages in their respective areas of application while, at the same time, constitute promising platforms for developing therapeutic biologicals. Chapter 2 focuses on liposomal delivery vehicles containing hydrophobic nanoparticles in their bilayers, which encapsulate the nucleic acid based drugs and promote endosomal escape by nanoparticle induced fusion with the endosomal membranes. Specifically we use metal nanoparticles in specialized liposomes for the efficient delivery of small interfering RNA (siRNA). Manuscript 2 focuses on novel chiral cationic polymers – polyethyleneimines (PEIs) – that form complexes with the negatively charged nucleic-acid based drugs and promote endosomal escape via a proton sponge effect. Specifically we use of chiral cationic polyamines for two intriguing applications: fabrication of chiral covalently-

linked microcapsules, and enantiospecific delivery of siRNA to Huh 7 cells. We found that two of the designed polymers improved transfection efficiency relative to commercially available transfection reagents with lower cell toxicity. In total this dissertation presents work that demonstrates novel and efficient delivery strategies that promote endosomal escape and enhance the intracellular activity of nucleic acid based drugs.

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I would also like to Thank Kim Andrews for her positive support and guidance through out the entire time. My friends Hope, Sreeram Raju, Divya, Srinivas, Shalu, Vishal jiju, Daniela, Rosemary Gatti, Alizandra, Elizabeth, Lexi, Steffani and Mayurika, Thank you all. I am proud and thankful for my team at CCE, Providence, Tamara Bolotow, Cessie and Dr. Edward Bozzi who gave me financial stability and positive guidance all the time.

I wouldn't have stood strong in my pursuit if I didn't have the love and support of my family, specially, my sisters Sushma and Payal. I love my dear nephews Laksh and Agastya and my new niece Myra and hope that one day they will be able to read this piece of work.

I was emotional focused in my journey because I have the passionate, unconditional love of my fur babies, Eva-Jones and Dollops. I am very grateful for their “paw-love”.

This thesis is dedicated to my loving parents, without whom this day in my life would have not been possible. I love you both immensely. Thank you Ma and Papa.

PREFACE

This dissertation has been written in a manuscript format. Chapter 1 includes brief background material on the current state of the art regarding delivery methods for nucleic acid based drugs. Chapter 2 is in manuscript format of the journal *American Chemical Society* and is pending submission to the journal. Chapter 3 is in manuscript format of the journal *Bioorganic & Medicinal Chemistry Letters* and has been published. Chapter 4 focuses on Future Work for manuscript 1. Chapter 5 includes brief summary and conclusions tying the two manuscripts into a single whole. The appendices include work conducted by the author but unrelated to the dissertation theme. The Bibliography lists all the sources used or consulted in writing the entire dissertation in the style of American Chemical Society format in alphabetical order by the last name of the first author.

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INTRODUCTION

Pharmaceutical research, in both academia and in industry, is increasingly focused on the development of biotechnology-derived and genetically-engineered nucleic acid based drugs such as plasmid DNA (pDNA), small interfering RNA (siRNA), short hairpin RNA (shRNA), micro RNA (miRNA), and antisense- and antigene-oligonucleotides as potential therapeutics. These molecules can be highly target-specific and potent, and they may be used to treat various life-threatening ailments{Alvarez-Salas, 2008 #67}. Allied Market Research™ has predicted that the global siRNA therapeutics market alone is expected to reach $1.2 billion by the year 2020. While promising as therapeutic agents, the delivery of these molecules is one of the most challenging aspects of their development. Nucleic acid based drugs are very hydrophilic, are of high molecular weight, are often chemically and enzymatically unstable, and are highly charged molecules{Oliveira, 2006 #65}. These molecules face many hurdles before they reach their target site of action, such as rapid renal clearance, serum degradation, opsonization, reticuloendothelial system (RES) uptake and metabolism, insufficient tissue and cell internalization, endosomal degradation, and immunosensitization.{Wang, 2010 #69} Hence, in order to use these drugs therapeutically, it is necessary to develop vehicles and methods for their efficient delivery to their site of action.

Among various potential delivery approaches, the use of self-assembling lipids in order to develop vesicular delivery vehicles has proven to be one of the successful and feasible approaches.[5] In particular, small unilamellar vesicles (SUVs) such as liposomes

are popular carriers for nucleic acid based drugs because of their favorable characteristics such as biocompatibility, biodegradability, spontaneous self-assembly, the ease of large-scale production, and suitability for clinical application.{Musacchio, 2011 #71} Some of the barriers in the path of efficient delivery of nucleic acid based drugs to their site of action have been addressed using liposomal delivery systems{Schroeder, 2010 #70}. For instance, the stability and plasma half life of liposomes may be enhanced by adding neutral or charged lipids, cholesterol, and polyethylene glycol (PEG) to the formulation. The lipids stabilize the liposomal bilayer by reducing repulsion between similar charges, and by inducing steric hindrance to the liposome surfaces. Similarly, cholesterol embeds in the hydrophobic domains of the bilayer and enhances structural rigidity and facilitates cellular uptake by improving endosomal internalization. PEG chains extend out of the lipid bilayer and provide a shield of steric hindrance on the positive charge that prevents interaction with opsonins and subsequent RES uptake. However, PEG chains also interfere with cellular uptake and endosomal escape. Hence, transient PEG coating strategies have been utilized, wherein exchangeable or reducible PEG linkages such as PEG-ceramide, disulfide-PEG, and orthoester-PEG lipids are used{Romberg, 2008 #72}. Moreover, the attachment of targeting ligands at the distal end of PEG moieties has assisted in cell specific uptake. However, despite many efforts and advances in these delivery vehicles, endosomal degradation of their cargo remains one of the pivotal challenges{Varkouhi, 2011 #73}. Hence, there is a pressing need to develop novel techniques to promote the endosomal escape of biologics before they are degraded in endosomes. In this investigation we evaluated novel methods to promote the endosomal escape of liposome-encapsulated polynucleotides to address this issue.

Another option for cellular deliveries, specifically of negatively charged polynucleotides and nucleic acids based drugs are cationic polymers{Tamura, 2010 #81}. Among these cationic polymers, polyethylenimines (PEIs) are most popular due to their relatively higher transfection efficiency. Under physiological conditions, PEIs are protonated and can form ionic complexes with nucleic acids, which are then taken up by the cells via caveolae- or clathrin-mediated pathways, resulting in efficient transfection{Gunther, 2011 #82}. The transfection efficiency of PEIs is also attributable to their facilitated endosomal escape due to a proton sponge effect{Behr, 1997 #84;Boussif, 1995 #83;Sonawane, 2003 #85}. The proton sponge effect takes place due to the acidification of endosomes after internalization that leads to protonation of the amine groups of the PEIs, which causes an influx of additional protons and chloride ions. Consequently, water molecules enter the endosome to equilibrate the resulting osmotic imbalance, resulting in rupture of endosomal membranes due to inflation{Tseng, 2009 #86}. Although there are many reported examples of PEIs used for siRNA and DNA delivery, many of these delivery vehicles suffer from high cytotoxicity. The physicochemical properties of the PEIs play an important role in determining their relative efficiency and toxicity. For example, increased molecular weight of polyethylene imines (PEIs) increases their cytotoxicity, whereas very low molecular weight PEIs depict poor transfection efficiency{Fischer, 1999 #88;Godbey, 1999 #87}. Thus, the development of PEI delivery agents, which exhibit both delivery efficacy and minimal toxicity, remains a challenge. In this investigation we evaluated novel chiral PEIs as efficient and relatively less toxic delivery vehicles.

CHAPTER 1

MANUSCRIPT 1

Preparing to submit it to American Chemical Society

Discovery of an Innovative Pathway for Endosomal Escape of Nucleic Acid-Based Drugs: Nanoparticle Induced Fusogenicity in Liposomal Bilayers.

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ABSTRACT

In this work we describe novel compositions and methods of use of formulations designed for the efficient delivery of nucleic acid-based drugs to their site of action. We discovered that the incorporation of hydrophobic metal nanoparticles into the membranes of vesicular delivery vehicles, such as liposomes, enhance the endosomal membrane fusogenicity and endosomal escape of these delivery vehicles. This observed enhancement in fusogenicity is attributed to nanoparticle-mediated promotion of inverted hexagonal phase formation in lipid bilayers. As a result, the vesicular delivery vehicles more readily fuse with the endosomal bilayer, thereby leading to enhanced endosomal escape of the vesicular delivery vehicle, thus facilitating the delivery of biologically active molecules to their sites of action. We successfully demonstrated inverted hexagonal phase formation due to the presence of hydrophobic nanoparticles of gold or silver in the bilayers using ^{31}P-NMR spectroscopy. The promotion of inverted hexagonal phase formation was greater for higher concentrations and larger sized nanoparticles were incorporated into the liposomes. The enhancement in transfection efficiency due to the presence of nanoparticles was demonstrated for pDNA and siRNA for eGFP expression using fluorescence microscopy and FACS analysis, whereas that for pDNA of mFXRa1 was shown using Western blot.

1. Introduction

Pharmaceutical research, in both academia and in industry, is increasingly focused on the development of biotechnology-derived and genetically-engineered nucleic acid

based drugs such as plasmid DNA (pDNA, small interfering RNA (siRNA), short hairpin RNA (shRNA), micro RNA (miRNA), antisense, and antigene oligonucleotides as potential therapeutics. These molecules are highly specific and potent and can be used to treat various life-threatening ailments.[1] Allied Market Research™ has predicted that the global siRNA therapeutics market alone is expected to reach $1.2 billion by the year 2020. While promising as therapeutic agents, the delivery of these molecules is one of the most important and challenging aspects of their development, and is the focus of extensive industrial and academic research efforts.[2] Nucleic acid-based drugs are very hydrophilic, are of high molecular weight, are often chemically- and enzymatically unstable, and are highly charged molecules.[3] If administered naked, these molecules face many hurdles before they reach their target site of action, such as rapid renal clearance, serum degradation, opsonization, RES uptake and metabolism, insufficient tissue and cell internalization, and endosomal degradation, as well as immunosensitization.[4] Hence, in order to use these drugs therapeutically, it is necessary to develop vehicles for their efficient delivery to their site of action. Among various potential formulation methods, the use of self-assembling lipids and polymers in order to develop vesicular delivery vehicles such as lipsomes and polymersomes has proven to be one of the successful and feasible approaches. Despite many efforts and advances in these delivery vehicles, endosomal degradation of their cargo remains one of the pivotal challenges. Hence, there is a pressing need to develop novel techniques to promote endosomal escape of biologics before they are degraded in the endosome. The present investigation describes a novel method designed to address this need.

Liposomal delivery systems are popular carriers for nucleic acid-based drugs because of their favorable characteristics such as biocompatibility, biodegradability, spontaneous self-assembly, the ease of large-scale production, and suitability for clinical application[5]. Some of the barriers in the path of efficient delivery of nucleic acid based drugs to their site of action have been addressed using liposomal delivery systems[6]. For instance, the stability and plasma half life of liposomes may be enhanced by adding neutral lipids, cholesterol, and polyethylene glycol (PEG) to the lipid formulation. The neutral lipids stabilize the liposomal bilayer by reducing repulsion between similar charges. Similarly, cholesterol embeds in the hydrophobic domains of the bilayer and enhances structural rigidity as well as facilitates cellular uptake by improving endosomal internalization. PEG chains extend out of the lipid bilayer and provide a shield of steric hindrance on the surface of the liposome which reduces interactions with opsonins and subsequent RES uptake. However, PEG chains also interfere with cellular uptake and endosomal escape. Hence, transient PEG coating strategies have been utilized, wherein exchangeable or reducible PEG linkages such as PEG-ceramide, disulfide-PEG, and orthoester-PEG lipids are used[7]. Moreover, the attachment of targeting ligands at the distal end of PEG moieties has improved cell-specific uptake.

Endosomal degradation is still one of the major barriers to the efficient delivery of nucleic acid-based drugs[8]. Various approaches, such as fusion in the endosomal membrane, a proton sponge effect, pore formation in the endosomal membrane, and photochemical disruption of the endosomal membrane have been assessed in order to address this issue[9]. From among these strategies, the fusion of the liposomal and

endosomal membranes and the subsequent release of the liposomal cargo into the cytosol has been perhaps the most promising one[10]. This membrane fusion occurs via inverted hexagonal (H_{II}) phase formation between liposomal and endosomal bilayers. The H_{II} phase formation can be enhanced by increasing negative interfacial curvature of the liposomal bilayer using lipids with appropriate critical packing parameters[11]. For instance, by using lipids with higher unsaturation in their chains generates a kink that assists in H_{II} phase formation by increasing negative interfacial curvature[12-14]. A packing frustration is generated in the hydrophobic domains of the lipids while the H_{II} phase is being formed due to creation of voids around the hydrophilic channels of the H_{II} phase[15]. In this investigation we hypothesized that the presence of free flowing hydrophobic nanoparticles (NPs) in the liposomal bilayers would increase their negative interfacial curvature as well as satisfy the packing frustration during H_{II} phase formation by filling up the voids in the hydrophobic domains. Hence, various hydrophobic metal NPs of different sizes were incorporated into the liposomal bilayers at different concentrations, and the enhancement in fusogenicity and subsequent increase in transfection efficiency of these NPs-containing liposomal delivery vehicles was demonstrated in this work.

Materials

The eGFP expressing plasmid DNA (pDNA) was obtained from Origene, eGFP specific silencing siRNA from Qiagen and mouse FXRa1 protein expressing plasmid construct from Dr. Mathew Stonner (Biomedical and Pharmaceutical Sciences, University of Rhode Island). The non-ionic lipid DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine), the cationic lipid DOTMA (1,2-di-O-octadecenyl-3-trimethylammonium propane

chloride salt), and the anionic lipid (1,2-dipalmitoyl-sn-glycero-3-phospho-L-serine sodium salt) were obtained from Avanti Polar Lipids, Inc. (Alabama, USA). Oleic acid-coated hydrophobic SPIO maghemite NPs (5 nm, 24 mg mL^{-1}, or 187.9 mM Fe2O3) dispersed in chloroform were purchased from Ocean Nanotech (Springdale, AR, USA). Dodecanethiol-coated hydrophobic gold (2 nm) and silver (4 nm) NPs dispersed in hexane were obtained from Nanocomposix (San Diego, CA, USA). The transfection reagent GenJet-II and the cell culture medium, Gibco's OPTIMEM-1 (Reduced FBS), was obtained from ThermoFisher Scientific. Huh-7 cells were purchased from ATCC and primary antibody for mouse FXR (H-130) (SC-13063) and mouse GAPDH from Santacruz Inc. Negative control siRNA (20 nmol) was purchased from Qiagen.

2. Methods

2.1. Preparation of liposomes

Liposomes were prepared by thin film hydration method.[16] Briefly, a chloroform solution of cationic lipid DOTMA and non-ionic lipid DSPC at an equal molar ratio with/without hydrophobic NPs at various ratios of lipid molecules : NPs was prepared in a glass vial. A thin uniform film was then prepared by rapidly evaporating the organic solvent under vacuum for 2 hours in order to remove the trace solvent. This film was then hydrated using 0.5 ml phosphate buffer saline (PBS) with/without nucleic acid (pDNA or siRNA) by vortexing for 30 seconds. The resulting dispersion was then sonicated using a bath sonicator for 30 minutes. The total lipid concentration in the liposomes was 2 mM. The total pDNA concentration was 40 ng/µl and that of siRNA was 400 nM, respectively, in the liposomes containing corresponding nucleic acids.

2.2. Preparation of multi lamellar vesicles (MLVs)

MLVs were prepared by a thin film hydration method in order to study potential fusogenicity enhancement. Briefly, a chloroform solution of cationic lipid DOTMA and anionic lipid DPPS at an equal molar ratio with/without hydrophobic NPs at various ratios of lipid molecules : NPs was prepared in a sterile glass vial. A thin uniform film was then prepared by rapidly evaporating the chloroform under vacuum for 2 hours in order to remove the excess solvent,. This film was then hydrated using 2 ml (for AgNPs-containing MLVs) or 4 ml (for AuNPs-containing MLVs) of citrate buffer (10 mM in 10% D2O, pH 4.0) by vortexing for 30 seconds. The total lipid concentration was 15 mM. The resulting dispersion was then sonicated using a bath sonicator for 30 minutes. The morphology of the liposomes was observed using cryo-TEM (JEM-2100F, Jeol USA Inc., MA, USA) and the phase transition was detected using ^{31}P-NMR (Varian 500 MHz, Agilent Technologies, CA, USA) analysis.

2.3. Dynamic light scattering (DLS)

A Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) was used to perform DLS experiments in order to measure the size of liposomes. A suspension of each liposome preparation (0.5 ml) was deposed into disposable polystyrene cuvettes (Sarstedt AG & Co., Newton, NC, USA) having a 1 cm path length. The temperature was set to 25 °C for the analysis and samples were allowed to equilibrate for 60 seconds before the measurement. The particle size was then determined at a manual setting of 15

counts with 10 seconds per count and a measurement angle of 173° Backscatter (NIBS Default).

2.4. Cryogenic transmission electron microscopy (Cryo-TEM)

Cryo-TEM was performed in order to determine the morphology of the liposomes. The samples were prepared at 37 °C using a Vitrobot (FEI Company), a PC-controlled robot for sample vitrification. Quantifoil grids were used with 2-μm carbon holes on 200 square mesh copper grids (Electron Microscopy Sciences, Hatfield, PA). After immersing the grid into the sample, it was then removed, blotted to reduce film thickness, and vitrified in liquid ethane. Imaging was performed in a cooled microscopy stage (Model 915, Gatan Inc., Pleasanton, CA) at 200 kV using a JEOL JEM-2100F TEM (Peabody, MA).

2.5. Phosphorus-31 nuclear magnetic resonance spectroscopy

The ^{31}P-NMR spectra were acquired on an Agilent NMRS 500 NMR spectrometer operating at 202.3 MHz using a 5-mm OneNMR probe. NMR data were collected for 60 K scans with a 35.7-kHz sweep width using 131 K data points. Acquisition time was 1.3 s with a relaxation delay of 0.5 s. The data were processed with Mnova program V8.1 Mesterlab research SL. A line broadening of 50 Hz was applied to all spectra. All spectra were indirectly referenced to H_3PO_4 set to 0 ppm. Data were acquired without spinning.

2.6. Cell transfection

Huh-7 cells at $2x10^5$ cells/well concentration were seeded in 12 well plates and transfected in the presence of 1 ml/well OPTIMEM-1 using 50 µl/well liposomes for eGFP expressing pDNA experiments. For mFXRa1 protein expressing pDNA experiments, Huh-7 cells were seeded at $4x10^5$ cells/well concentration in 6 well plates and transfected in the presence of 2 ml/well OPTIMEM-1 using 100 µl/well liposomes. For eGFP specific silencing siRNA experiments, 12 well plate conditions as described above were used and Huh-7 cells were first transfected with eGFP expressing pDNA at 2 µg/well concentration using GenJet. The medium was then replaced after 12 hours in order to remove left-over GenJet reagent and the cells were transfected using 50 µl/well liposomes comprising eGFP specific silencing siRNA.

2.7. Fluorescence microscopy

Cells transfected using eGFP expressing pDNA or eGFP silencing siRNA were observed 48 hours after transfection under a fluorescence microscope (Eclipse TE2000-E, Nikon Instruments Inc., NY, USA) at a 10x magnification set up in order to detect the eGFP fluorescence in the cells. Images were taken in order to observe the relative eGFP fluorescence in the cells.

2.8. FACS analysis

FACS analysis was performed in order to count eGFP-expressing cells using a flow cytometer (BD FACSVerse™, BD Biosciences, CA, USA). The well plates were removed from the incubator 48 hours after transfection and the medium was discarded.

The cells were then washed twice with 1 ml of PBS equilibrated at 37 °C. The cells were then trypsinized using 1 ml of trypsin equilibrated at 37 °C. DMEM (1 ml) containing 10 % FBS, 1 % NEA, and 1 % PS equilibrated at 37 °C was added into the wells. The plate was then shaken and the contents (total 2 ml) were transferred to 15 ml tubes. The tubes were then centrifuged for 5 minutes at 1000 rpm and 5 °C, supernatant was discarded, and 2 ml of PBS stored at 5 °C was added to these tubes. This step was repeated one more time and then 2 ml of room temperature PBS was added to the tubes. The cells were then suspended by pipetting up and down and then analyzed using the flow cytometer with laser set up for counting eGFP expressing cells.

2.9. Western blot

The Western blotting technique was used in order to determine the protein levels of mFXRa1 protein from the Huh7 whole cell lysates. Cells were ground 48 hours after transfection in 1x sucrose-Tris buffer using a mechanical homogenizer. The whole cell lysate was then centrifuged at 15000g for 15 minutes to obtain the clear lysate. The protein concentration was determined using the Micro-BCA method from Thermo-Fischer's Pierce Protein protocol. Thirty µg of protein was loaded onto an SDS PAGE gel followed by a semi-wet transfer of the separated proteins from the gel to a PVDF membrane. The membrane containing proteins were blocked in 5% milk/TBST buffer for 3 hours followed by treatment with the primary antibody of mFXRa1 (1:1000) in 5% milk/TBST overnight at 4 °C. The membrane was then washed and incubated with the secondary antibody (1:4000) in 5% milk for 1 hour at room temperature on a lab shaker. The membranes were washed three times using 1x Tris-sucrose buffer containing (10%)

Triton X-100 (TBST) and were then imaged using the chemi-luminescent substrate from Thermo-Fischer Scientific. Protein expression was quantified using a Typhoon 9000-FLA imager and normalized against GAPDH, as an internal housekeeping gene.

3. **Results and Discussion**

3.1. Schematic, size, and morphology of liposomes

Figure 1 depicts the chemical structures of the materials used and the schematic of liposomes manufactured in this work. As shown in Figure 1, the hydrophobic NPs made of gold (Au) and silver (Ag) are coated with hydrophobic chains of dodecanethiol (Figures 1a and 1b, respectively). The chemical structures of the cationic lipid DOTMA, the non-ionic lipid DSPC, and the anionic lipid DPPS are shown in Figures 1c, 1d, and 1e, respectively. Figure 1e shows the schematic of model liposomes examined in this study, comprising active agents or therapeutic agents inside the aqueous core and hydrophobic NPs in the bilayer. Since a typical liposomal bilayer width is 5 nm, hydrophobic NPs of up to 5 nm diameter can be incorporated into this bilayer, which may lead to bulging in the liposomal bilayer. Moreover, the presence of hydrophobic NPs in the bilayer may increase the negative interfacial curvature of the bilayer and enhance its fusogenicity by satisfying packing frustration during H_{II} phase formation, subsequently increasing the transfection efficiency of the liposomes. However, it is important to determine the impact of incorporating the NPs into the bilayer on the size and morphological characteristics of the liposomes. The size of liposomes manufactured in this work was measured using DLS and their morphological characteristics were observed using cryo-TEM. A representative DLS plot and cryo-TEM image of AuNPs-

containing liposomes are shown in Figure 2. The size and shape of the liposomes was not affected due to the incorporation of hydrophobic AuNPs into the bilayer. The size of the liposomes with or without hydrophobic AuNPs was 203-225 nm and the polydispersity index was 0.277-0.299. Similar results were obtained for liposomes comprising AgNPs or MNPs with and without loading pDNA or siRNA.

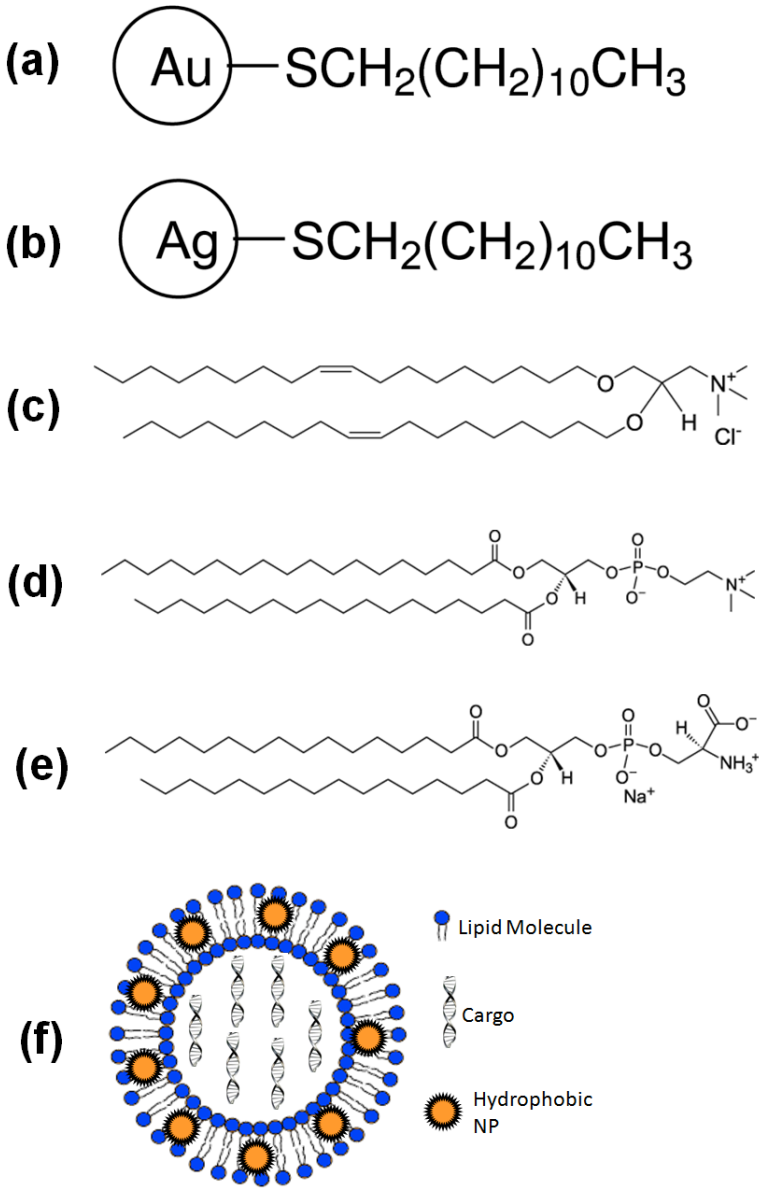

Figure 1.(a) AuNPs and (b) AgNPs coated with dodecanethiol. The chemical structures of (c) DOTMA, (d) DSPC, and (e) DPPS. The schematic of a model liposome comprising active agents or therapeutic agents ("cargo") inside the aqueous core, with hydrophobic NPs incorporated into the bilayer (f).

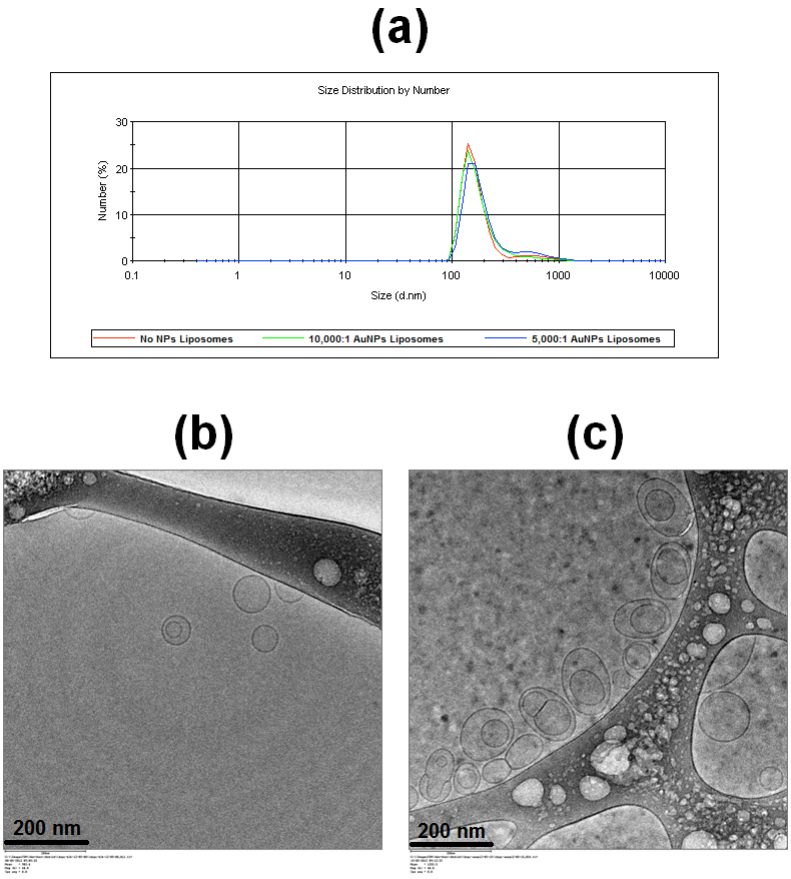

Figure 2. (a) the size distribution of liposomes with and without AuNPs. Cryo-TEM images of liposomes without (b) and with (c) AuNPs. The size and shape of the liposomes was not affected due to the incorporation of hydrophobic AuNPs into the bilayer.

3.2. Enhancement in bilayer fusogenicity due to presence of NPs

The release of liposome content into the cytosol is problematic due to the entrapment of liposomes and their contents within the endosomal compartments after endocytosis. One of the major pathways for endosomal escape of the liposomal cargo depends on the fusion between liposomal and endosomal bilayers. This fusion occurs via H_{II} phase formation (Figure 3a). As described herein, we hypothesized that after endocytosis of hydrophobic NPs-containing liposomes, the presence of hydrophobic NPs in the liposomal bilayer will promote fusion, thereby enhancing the release of liposomal cargo into the cytosol. This mechanism of action has been illustrated in Figures 3b and 3c. Briefly, free flowing hydrophobic NPs present in the liposomal bilayer would be expected to occupy the voids generated during H_{II} phase formation between liposomal and endosomal bilayers that would relax the packing frustration and ease the fusion process.

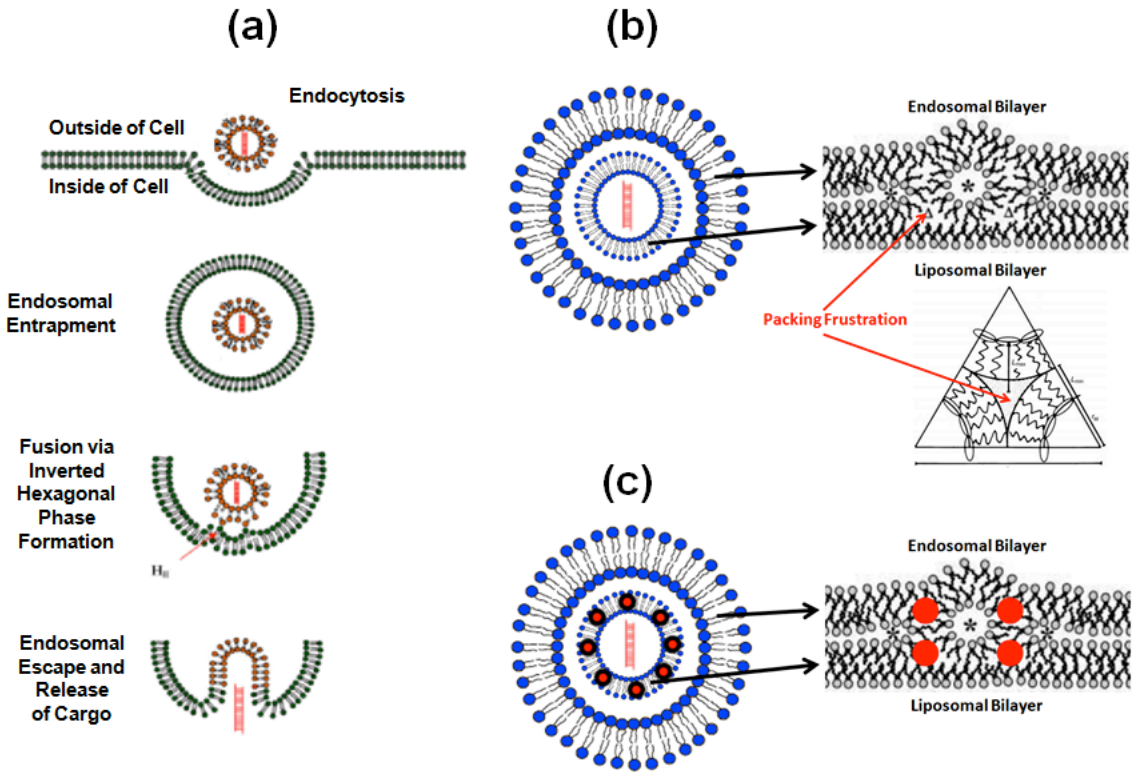

Figure 3. The liposomes taken inside the cell by endocytosis are entrapped in the endosomal membranes, followed by endosomal escape. The liposomes then release their cargo into the cytosol by fusing with the endosomes (a). This fusion between liposomal and endosomal membranes occurs via inverted hexagonal (H_{II}) phase formation that generates packing frustration in both the bilayers (b). The presence of hydrophobic nanoparticles relaxes this packing frustration and promotes fusion via H_{II} phase formation (c).

The enhancement in fusogenicity was assessed by measuring the H_{II} phase transition temperature and by ^{31}P-NMR analysis. The morphology of MLVs produced for this measurement has been depicted in Figure 4a. As shown in Figure 4b, these MLVs depicted a characteristic ^{31}P-NMR profile with a high field peak and a low field shoulder

pattern, which transformed into a low field peak and a high field shoulder upon transition to the H_{II} phase. As illustrated in Figures 4b and 4c, the phase transition temperature was reduced a from 50 °C to 40 °C upon incorporating 2 nm AuNPs at 10,000:1 lipid molecules : NPs ratio into the bilayers. Although the phase transition temperature was not further reduced, the intensity of the low field peak was higher for a 5,000:1 lipid molecules : NPs ratio at 40 °C for this system (Figure 4d). Moreover, the phase transition temperature was further reduced to 35 °C upon the incorporation of 4 nm AgNPs at a 10,000:1 lipid molecules : NPs ratio into the bilayers (Figures 4e). These data suggest that the phase transition from bilayer to H_{II} was induced not only by increasing the concentration of the NPs but also by an increase in their size.

3.3. Hydrophobic NPs improved transfection efficiency

The expression of eGFP was significantly increased by using liposomes containing AuNPs as compared to those without NPs. As depicted in Figure 5a, no fluorescence was detected in cells transfected using blank liposomes, whereas the positive control cells transfected using the commercial reagent Genjet exhibited a substantial number of cells with green fluorescence. The number of cells showing green fluorescence was higher for AuNPs-containing liposomes as compared to those using liposomes without NPs. In order to quantify this effect, the cells showing green fluorescence were measured using FACS analysis that confirmed the enhancement in transfection efficiency due to the presence of AuNPs. As shown in Figure 5b, the number of cells showing eGFP fluorescence were 1.28 times higher when transfected using AuNPs-containing liposomes as compared to those transfected using liposomes without

AuNPs. Student-t test was conducted in order to determine the statistical significance with a p-value of 0.02.

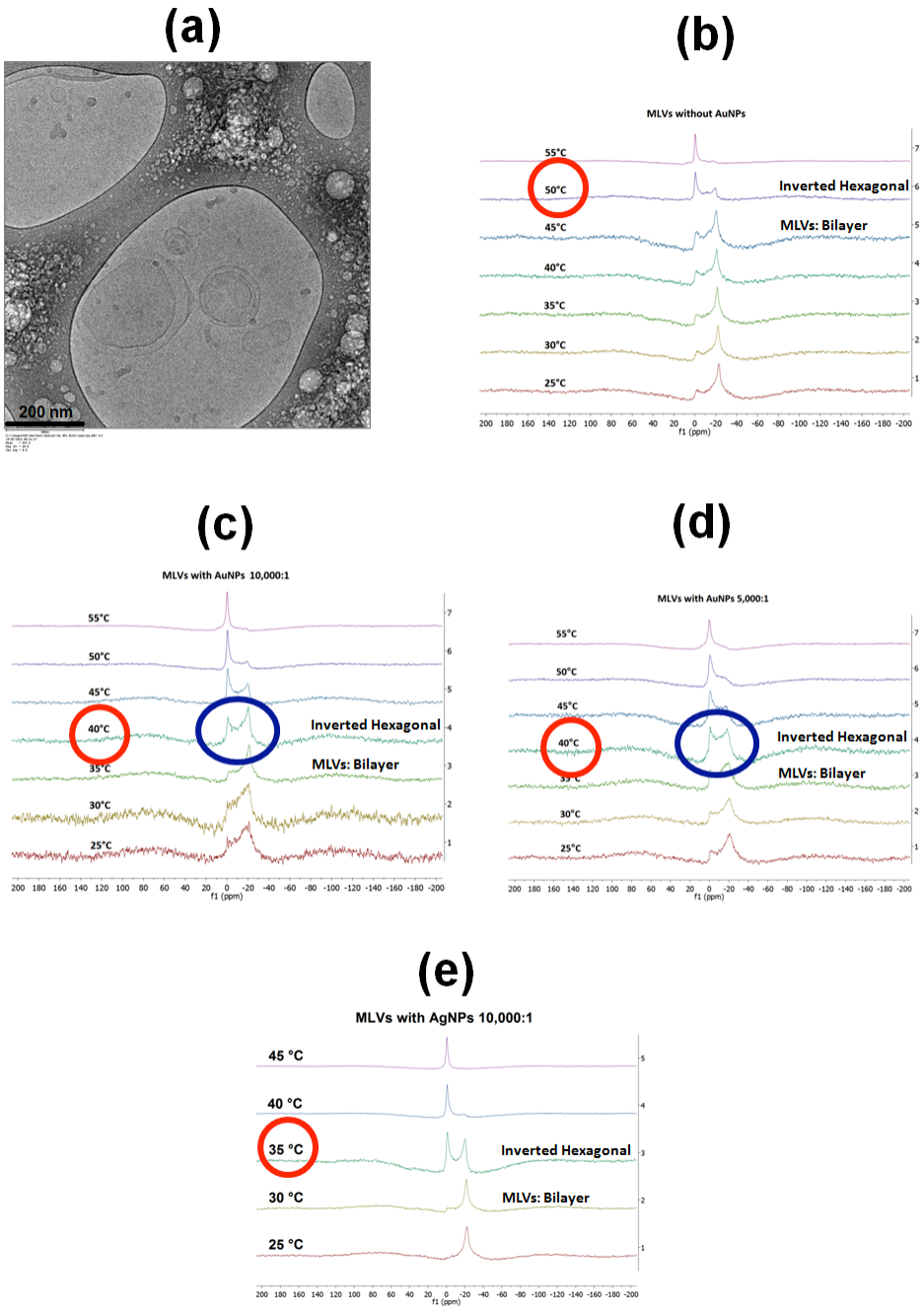

Figure 4. (a) Cryo-TEM image of MLVs simulating endosomal entrapment condition. The H_{II} phase transition for MLVs (b) without NPs, (c) with 10,000:1 lipid molecules : AuNPs, (d) 5,000:1 lipid molecules : AuNPs, and (e) 10,000:1 lipid molecules : AgNPs

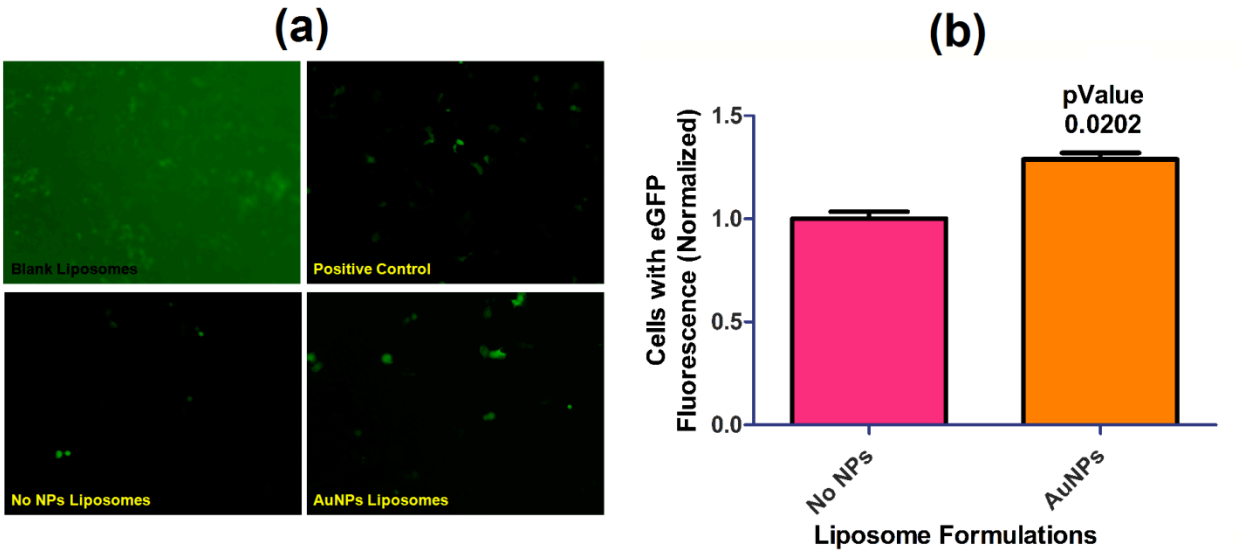

Figure 5. (a) fluorescence microscopic images and (b) bar chart summarizing the number of cells with eGFP fluorescence counted using FACS for cells transfected with pDNA using liposomes with and without AuNPs.

The enhancement in transfection efficiency of eGFP expressing pDNA due to the presence of NPs in the liposomal bilayer was dependent on the size of the NPs and correlated well with the results of ^{31}P-NMR spectroscopy. As was revealed by ^{31}P-NMR spectroscopy, membrane fusogenicity was increased by increasing the size of NPs. It might therefore be inferred that liposomes containing relatively larger NPs in their bilayer would likely exhibit higher transfection efficiency. The dot plots obtained by FACS analysis, shown in Figure 6a, illustrate the number of cells without eGFP fluorescence (depicted as blue dots) as compared to those with eGFP fluorescence (depicted as pink dots). The cells transfected using blank liposomes do not show any pink dots, whereas the positive control cells transfected using commercial reagent GenJet depicted a significant number of pink dots. Similarly, liposomes without any NPs showed very few pink dots as

compared to those for AgNPs-containing liposomes in their corresponding dot plots. After counting these eGFP expressing cells, it was determined that the transfection efficiency of eGFP expressing pDNA was enhanced 8-fold by incorporating 4 nm AgNPs into the pDNA-containing liposomes (Figure 6b) as compared to a 1.28-fold increase by incorporating 2 nm AuNPs (Figure 5b). Thus, liposomes containing the larger AgNPs that displayed higher fusogenicity also exhibited higher transfection efficiency as compared to liposomes containing smaller AuNPs.

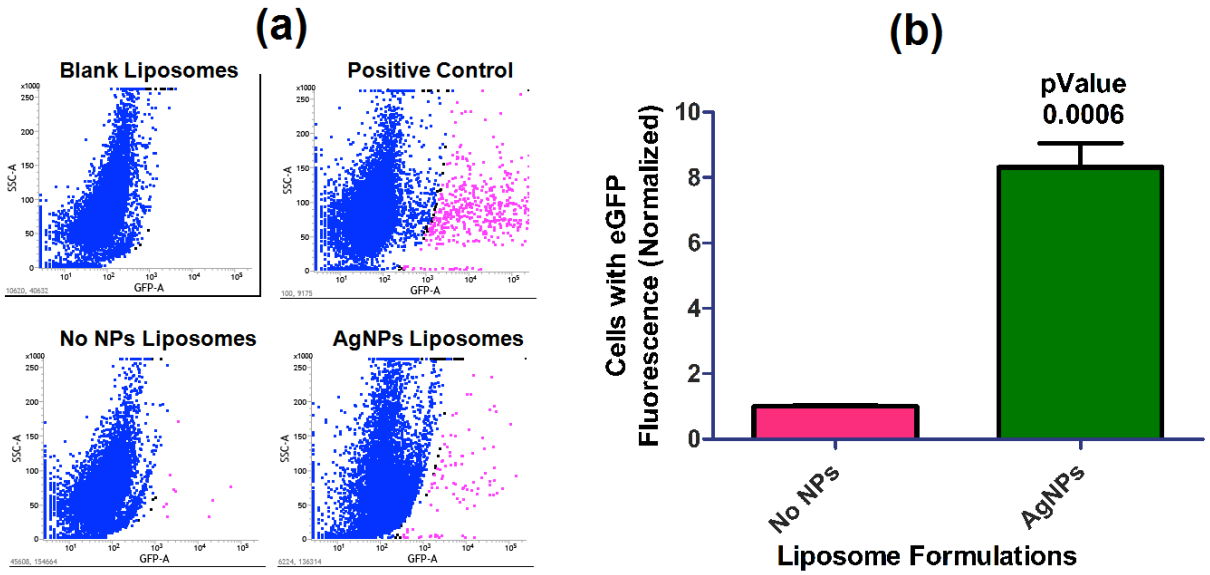

Figure. 6 (a) Dot plots generated using FACS and (b) bar chart representing the number of cells displaying eGFP fluorescence, counted using FACS for cells transfected with pDNA using liposomes with and without AgNPs.

The enhancement in transfection efficiency for pDNA was further confirmed by Western blotting. As shown in Figure 7a, pDNA transfected Huh-7 cells using liposomal formulations containing AuNPs in their bilayers depicted higher protein expression of

mFXRa1 as compared to those transfected using liposomes without NPs. The mFXRa1 protein expression on the Western blot was normalized using the housekeeping gene GAPDH. When the protein expression was quantified it was noted that the transfection efficiency of AuNPs-containing liposomes was twice that of liposomes without NPs (Figure 7b). Thus, it was confirmed not only by using two different genes (eGFP and mFXRa1) but also by using two different bio-analytical techniques (FACS and Western blot) that the presence of NPs in the liposomal bilayers enhanced the transfection efficiency of the corresponding pDNA.

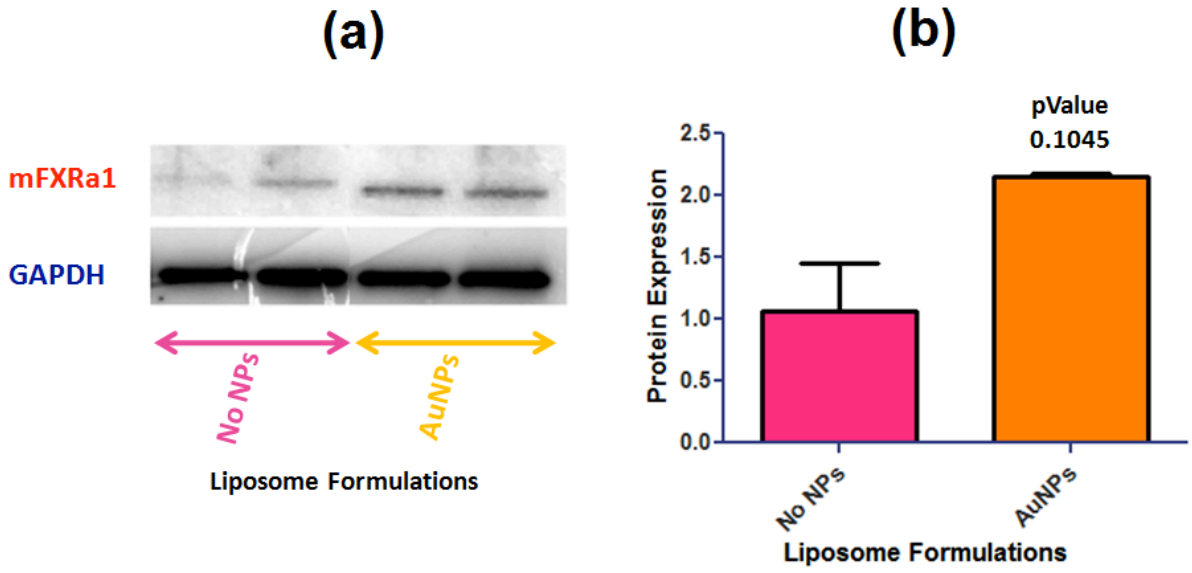

Figure 7. (a) Western blot image and (b) bar plot of the quantification of mFXRa1 protein for cells transfected with mFXRa1 expressing pDNA using liposomes with or without AuNPs.

Another nucleic acid-based, bioactive compound, siRNA, was also investigated in order to confirm the enhancement in transfection efficiency due to NPs-induced fusogenicity of the liposomal bilayers. The eGFP specific silencing siRNA can inhibit the

expression of eGFP protein. As shown in Figure 8, the number of cells showing eGFP fluorescence as measured by FACS analysis was lower for NPs-containing liposomes as compared to liposomes without NPs. These results also correlated well with the increase in fusogenicity and eGFP expression noted in pDNA experiments. For example, AuNPs with a 2 nm diameter exhibited lower fusogenicity (Figures 4c and 4e) and lower transfection efficiency for pDNA (Figures 6a and 6b) as well as siRNA (Figure 8) as compared to that for larger sized AgNPs with a 4 nm diameter. Although 5 nm MNPs were larger than AgNPs of 4 nm diameter, AgNPs containing liposomes exhibited higher transfection efficiency than liposomes containing MNPs of 5 nm diameter. This discrepancy could be explained due to difference in the hydrophobic coating material of the NPs. The AuNPs and AgNPs used in this work were coated with dodecanethiol chains, whereas the MNPs were coated with oleic acid chains. The dodecanethiol chain is shorter, with only 12 carbon atoms, as compared to oleic acid chains, with 18 carbon atoms. Further, the dodecanethiol chain is completely saturated whereas the oleic acid chain has one double bond that generates a kink in the chain. Thus, it might be inferred that the longer chain with a kink may restrict the motion of MNPs in liposomal membranes, and reduce their capacity to relax packing frustration, which might lead to lower fusogenicity as compared to AgNPs. However, the nature of this observation could not be assessed using ^{31}P-NMR spectroscopy due to the magnetic nature of the MNPs, which is incompatible with this analytical technique.

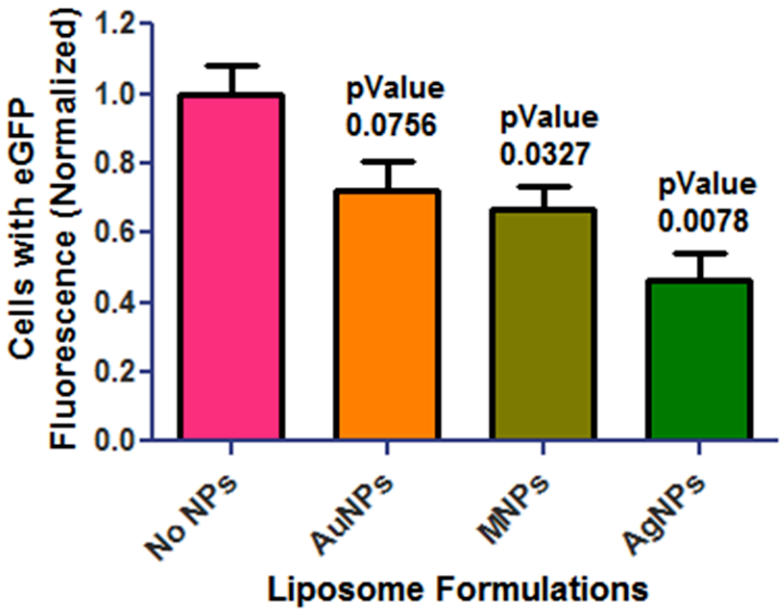

Figure 8. Bar chart for number of cells with eGFP fluorescence counted using FACS for cells transfected with pDNA using GenJet followed by siRNA using liposomes with and without NPs.

5. Conclusions

Upon entering a cell, liposomes containing nucleic acid-based bioactive molecules tend to be taken up by endocytosis, and their therapeutic action depends upon escape from these endosomes into the cytosol. As demonstrated in this work, NPs incorporation into liposomal membranes induced fusogenicity in the liposomal bilayers and led to higher transfection efficiency and biological activity for two major nucleic acid based drugs - pDNA and siRNA. These phenomena might be attributed to the use of hydrophobic NPs in liposomal membranes as a novel method of endosomal escape. This approach could further benefit currently popular strategies such as magnetically guided delivery of nucleic acid-containing liposomes to their target tissues and enhance the

therapeutic effect due to NPs induced higher efficiency. Further, techniques such as radio-frequency heating or laser excitation might further enhance the fusogenicity of these metal NPs-containing liposomes due to the generation of heat and also due to the rupture of endosomal membranes resulting from laser-induced vibration of membrane-encapsulated metal NPs.

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CHAPTER 2

MANUSCRIPT 2

Novel Chiral Cationic Polyamines as Effective and Less Toxic Delivery Vehicles for siRNA.

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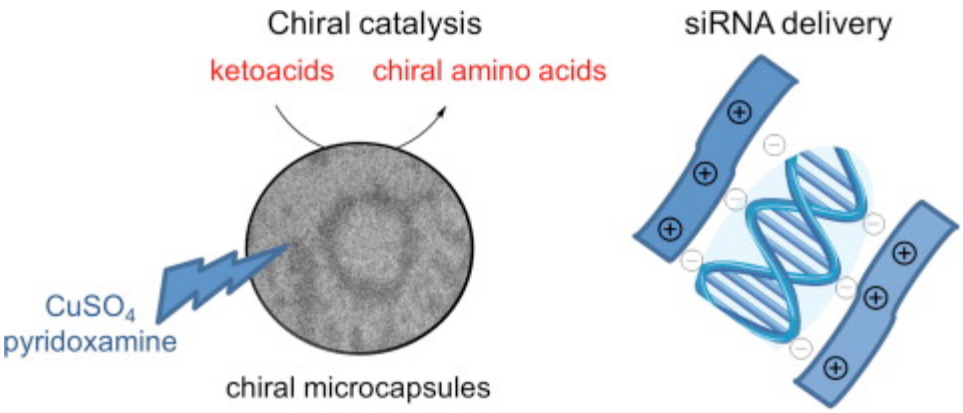
Abstract

Reported herein is the use of chiral cationic polyamines for two intriguing applications: fabrication of chiral covalently-linked microcapsules, and enantiospecific delivery of siRNA to Huh 7 cells. The microcapsules are easily fabricated from homochiral polymers, and the resulting architectures can be used for supramolecular chiral catalysis and many other potential applications. Enantiospecific delivery of siRNA to Huh 7 cells is seen by one 'enantiomer' of the polymers delivering siRNA with significantly improved transfection efficiency and reduced toxicity compared to the 'enantiomeric' polymer and commercially available transfection reagents. Taken together, the use of these easily accessible polyamine structures for diverse applications is highlighted in this Letter herein and can lead to numerous future research efforts.

Graphical abstract

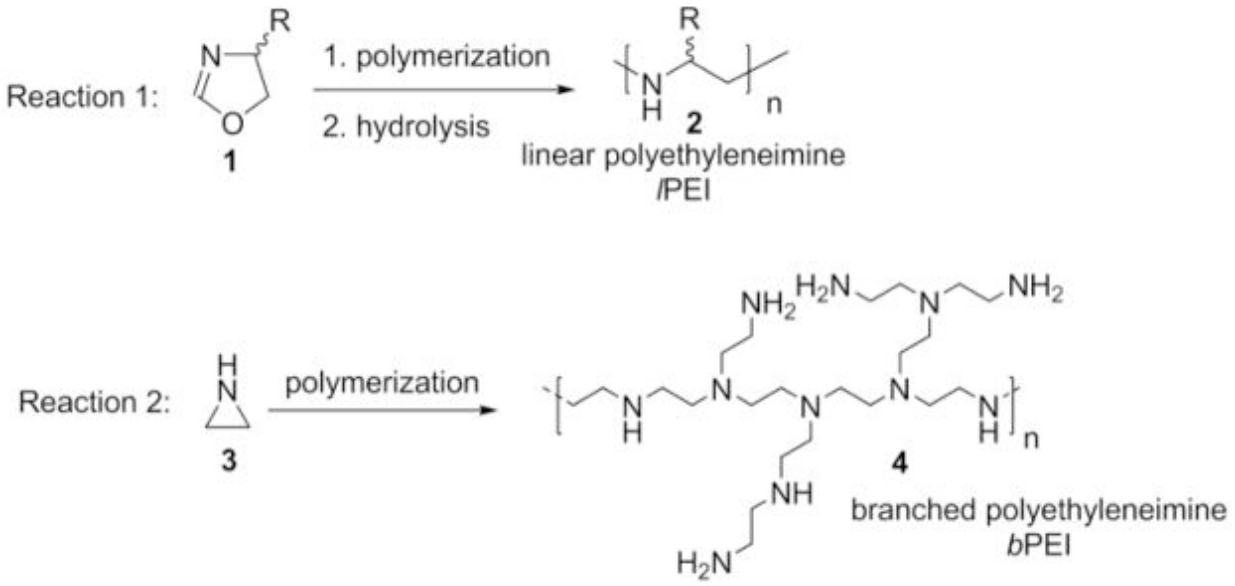


Keywords

Polyamine; Chirality; siRNA; Transfection.

Polyethyleneimines (PEIs) are a well-studied class of polymers.1,2 These polymers are synthesized commercially via the ring opening of aziridine (Scheme 1, Reaction 2),3–5 although this process leads to highly branched polymers6 with significant polydispersity indexes. The controlled synthesis of linear PEIs occurs via the cationic ring opening of oxazolines,7–9 followed by hydrolysis of the resulting formamides (Scheme 1, Reaction 1). Using chiral oxazolines as substrates for the polymerization reaction provides straightforward access to homochiral PEIs,10–12 with chiral centers at every polymer repeat unit.

Scheme 1



General synthetic methods for linear and branched polyethyleneimine (PEI)

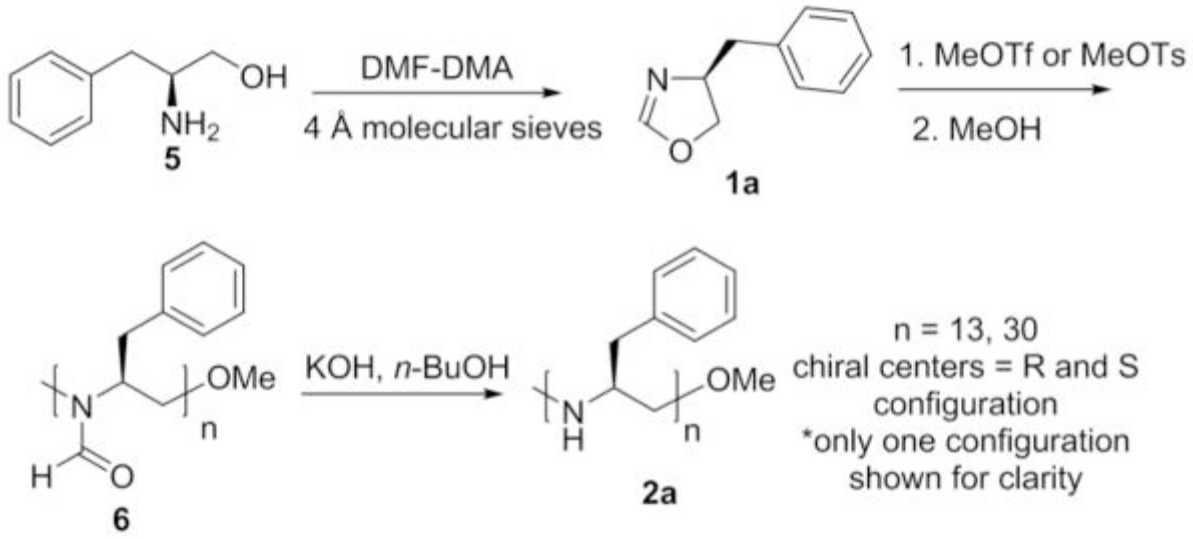
The significant interest in PEIs is driven largely by various applications of PEIs in fields including chiral catalysis,13–16 drug delivery,17,18 and oligonucleotide complexation and delivery.19,20 PEIs have also been covalently linked to form PEI-derived microcapsules,21 which have been used for site-isolated catalysis.22,23 In one

example, the Lewis basic PEI catalyzed a reaction in the same reaction vessel as a Lewis acidic nickel catalyst, which was used to catalyze the second reaction.24,25

Use of the same PEI scaffold for multiple applications has rarely been reported, although such multi-purpose polymers would have significant operational advantages. Reported herein is the use of a single PEI scaffold for two purposes: the fabrication of covalently-linked chiral microcapsules, and the efficient delivery of siRNA to Huh7 cells.26

The chiral PEIs were synthesized via the cationic polymerization of 4-benzyl-2-oxazoline (1a) (both R and S configurations), followed by the hydrolysis of the initially formed polyformamide (Scheme 2). The resulting polymers were characterized by 1H NMR spectroscopy, and the results were in agreement with literature-reported spectra.11 Using this methodology, polymers with 13 and 30 repeat units were formed, with both R and S configured side chains.

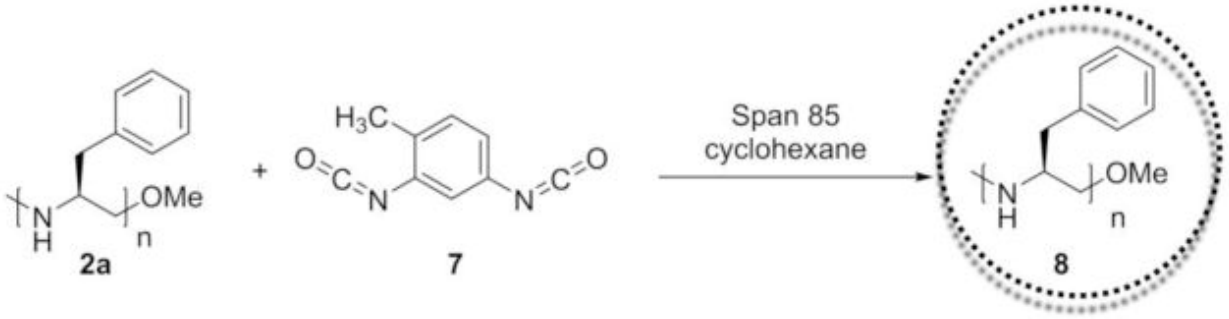
Scheme 2



Scheme 2 Synthesis of chiral polymers 2a

Once synthesized, the homochiral PEIs were cross-linked to form homochiral microcapsules following the procedure developed by McQuade and co-workers.22 Briefly, polymers 2a were dissolved in methanol, and added to a solution of 2% Span 85, followed by the addition of 2,4-tolylene diisocyanate (TDI, compound 7) (Equation 1), which cross linked the microcapsules to form a polyurea coating.25 The resulting polyurethane-type structures have been shown to be stable in a variety of aqueous media.27,28After thorough solvent evaporation, chiral microcapsules were obtained.

Equation 1



Equation 1 Synthesis of chiral covalently-linked microcapsules

The resulting microcapsules were imaged using transmission electron microscopy (TEM), and some images are shown in Figure 1. The diameters of the particles ranged from 57 nm–250 nm, with an average diameter of 141 nm (± 35 nm; 62 particles measured). These new supramolecular architectures contain narrow size distributions and

uniform structures, in good agreement with literature-reported results for achiral microcapsule analogues.21

Figure 1

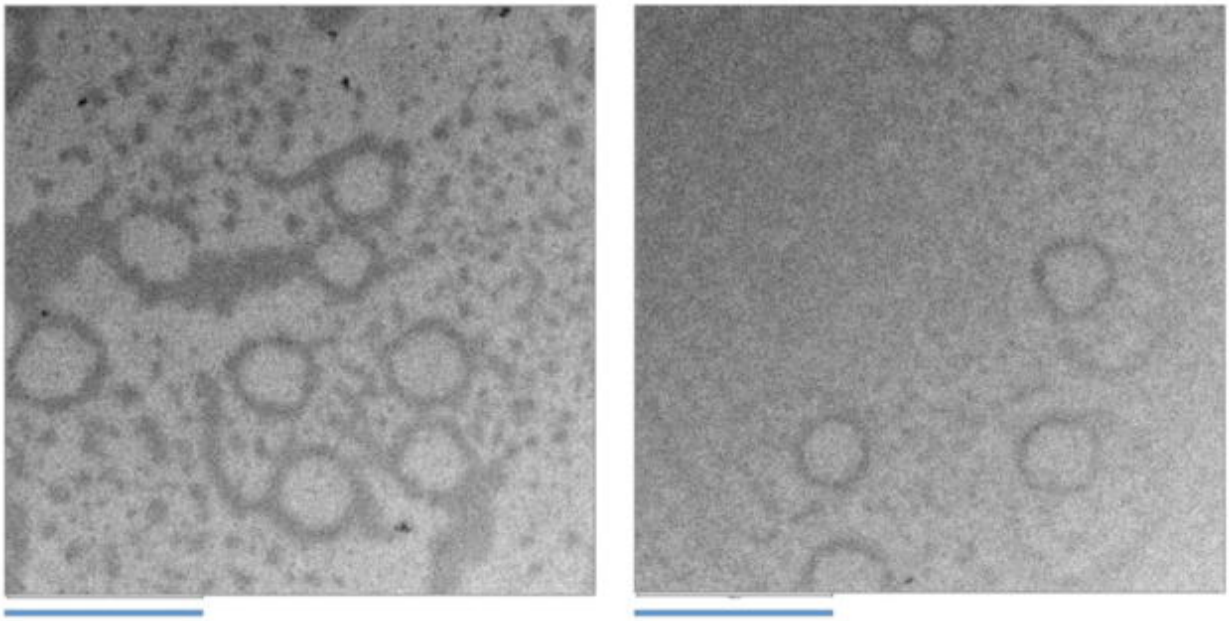
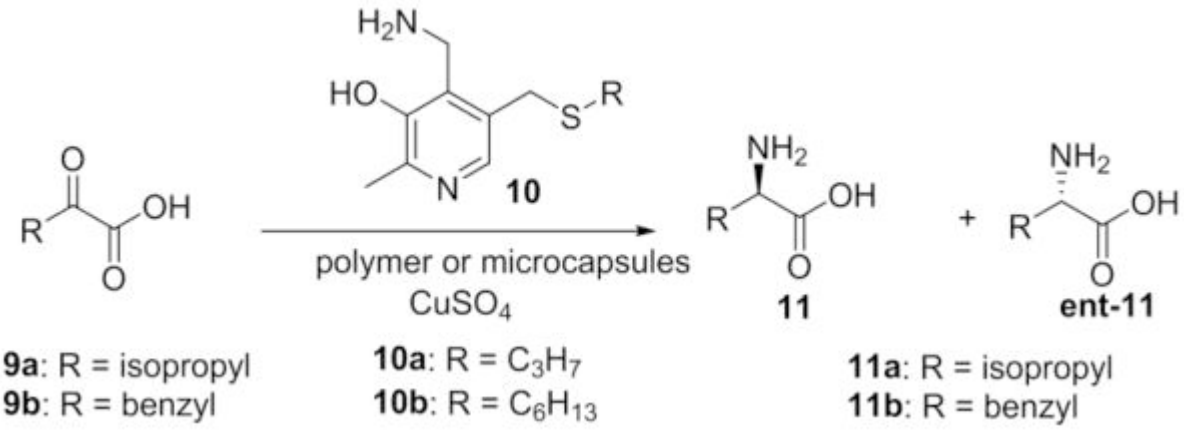


Figure 1 TEM images of chiral microcapsules 8(Blue line represents a 500 nm scale)

The newly formed microcapsules contain a variety of features that make them particularly amenable to supramolecular chiral catalysis, including: (a) multiple chiral centers, covalently confined in a small space; (b) multiple amino groups that can be protonated or deprotonated over a wide pH range;29 and (c) a hydrophobic core resulting from the hydrophobic benzyl side chains.30

To investigate the effect of capsule formation on the resulting supramolecular chiral environment, the newly synthesized chiral microcapsules were used as catalysts for the transamination reaction of ketoacids to amino acids (Equation 2). Obtaining good enantioselectivities in such transamination reactions has been an ongoing research problem.10 Preliminary results indicate that the microcapsule-catalyzed reactions proceeded with significantly higher enantioselectivities compared to the polymer-catalyzed reactions (up to 20% enantiomeric excess (ee) obtained for the synthesis of L-valine, under conditions where the polymer itself yielded 4% ee). Efforts to optimize the reaction conditions are in progress.

Equation 2



Equation 2 Enantioselective transamination of ketoacids 9 to amino acids 11

Interestingly, the chiral PEIs also functioned as efficient siRNA delivery agents. Although there are many reported examples of PEIs used for siRNA and DNA delivery,31–33 many of these delivery vehicles suffer from high cytotoxicity.34 The

development of gene delivery agents that are both effective and less toxic remains a highly relevant research objective.

The following 4 polymers were investigated as potential siRNA delivery agents: R-2a-13; S-2a-13; R-6-13; and S-6-13, where the R/S designation refers to the chirality of the side chain and the number 13 refers to the number of repeat units in the polymers. The efficacy of these polymers in transfecting an Alexa488-labeled control siRNA sequence35 to Huh7 cells36 was measured by determining the intracellular fluorescence 24 hours post-transfection. The results obtained using the chiral polyamines were compared to results obtained using commercially available transfection reagents: Genjet siRNA Transfection Reagent (SignaGen Laboratories); HiPerFect Transfection Reagent (Qiagen Laboratories);37 and Lipofectamine 2000 (Invitrogen Technologies).

Figure 2 shows a graph of the intracellular fluorescence of Huh7 cells following their incubation with Alexa-labeled siRNA with various delivery reagents. The intracellular fluorescence obtained with compounds S-6-13 and S-2a-13 is substantially higher than the fluorescence observed with positive controls Lipofectamine and Genjet, indicating the polymers' ability to transfect siRNA efficiently (Table 1). More interestingly, compounds R-2a-13 and R-6-13, which are identical except for the three-dimensional configuration of the benzyl group, transfect siRNA with approximately the same efficiency as Lipofectamine and Genjet, and substantially lower than the "enantiomeric" polymers.

Figure 2

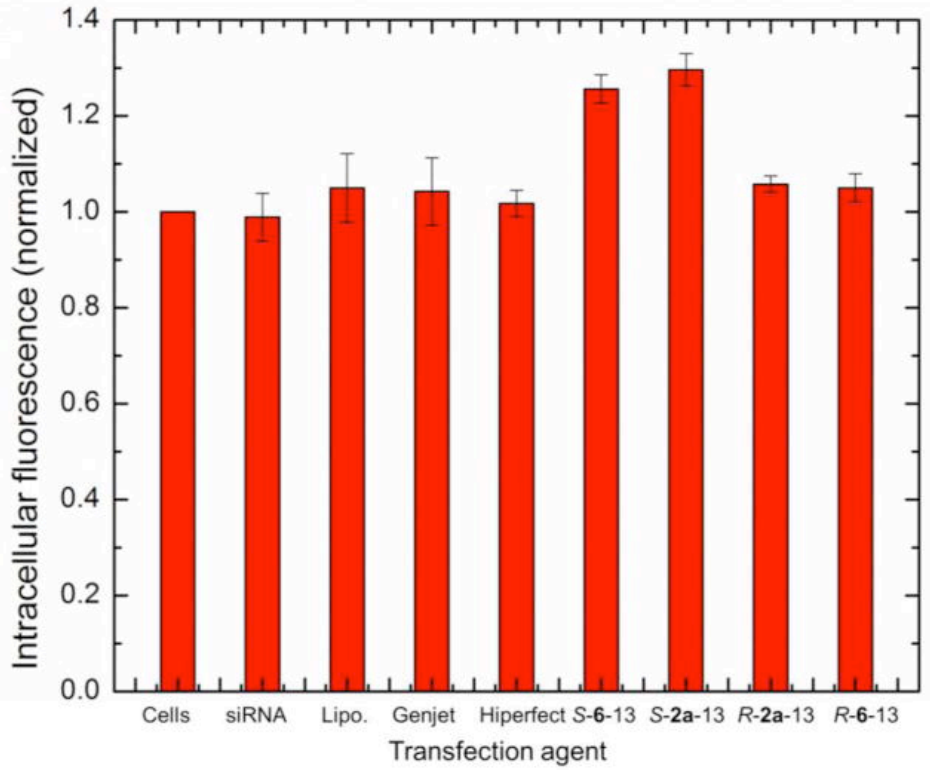


Chart of the intracellular fluorescence of Huh7 cells after transfection with siRNA (all PEIs were used at a 1000 nM final concentration)

Table 1

Transfection agent	**Intracellular fluorescence (normalized to 1.00 for cells alone)**
S-**6**-13	1.26
S-**2a**-13	1.30
Lipofectamine	1.05
Genjet	1.04
R-**2a**-13	1.06
R-**6**-13	1.05

Table 1 Transfection efficiencies of chiral PEIs and commercial transfection agents

The chirality of the side chains of the PEIs thus has a direct and measurable effect on the ability of PEIs to transfect siRNA efficiently: S chiral centers (compounds S-6-13 and S-2a-13) transfect siRNA more efficiently than the R analogues. Such a result may seem intuitive: that the interaction of two chiral macromolecules (chiral PEI and chiral siRNA) depends on the three-dimensional configuration of both molecules. This intuition is borne out by the results of this study, which is the first direct proof that the chirality of a polyamine directly impacts its transfection efficiency. Similar effects of the chirality on transfection efficiency were recently observed for the lipid delivery agent 1,2-dioleoyl-3-

trimethylammonium-propane (DOTAP).39 In that report, the R enantiomer performed better than either the S enantiomer or the racemic DOTAP mixture.

The toxicity of the newly synthesized PEIs was tested using an MTT assay.40 After 24 hours of incubation, the absorbance of the cells was quantified and the cell viability was calculated. Using 1000 nM of S-2a-13 reduced the cell viability to 88%, and 1000 nM of S-6-13 reduced it to 82%. By comparison, Lipofectamine reduced cell viability to 89%, and compounds R-2a-13 and R-6-13 reduced viability to 78% and 71%. Thus, the toxicity of the chiral PEIs, like the transfection efficiency, depends on the three-dimensional configuration of the benzylic side chains.

The differences in transfection efficiency and toxicity mean that the S- and R-configured PEIs likely have fundamentally different three-dimensional architectures. The relationship between the chirality of individual stereocenters and the overall polymer configuration has been studied for related polymers using circular dichroism spectroscopy.41–43 These differences in chirality affect the polymers' solubility44 and their interactions with DNA,45 and as shown here, their transfection efficiencies.

In summary, chiral polymers 6 and 2a were synthesized via straightforward, well-precedented procedures. These polymers were used for two novel applications: the fabrication of chiral, covalently-linked microcapsules, and the transfection of siRNA to Huh7 cells. The chiral microcapsules can be used for a number of potential applications in supramolecular chiral catalysis and in supramolecular enantiomer separations. The

chirality-dependent siRNA transfection also provides an intriguing platform for further investigation. In particular, polymer S-2a-13 demonstrated good transfection efficiency and limited toxicity, and will be used for further biochemical investigations. The results of these and other experiments will be reported in due course.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material

Acknowledgments

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CHAPTER 3

FUTURE WORK

In the current dissertation work we demonstrated that the fusogenicity of liposomal delivery vehicles could be enhanced by incorporating hydrophobic NPs in their bilayers. The enhancement in fusogenicity of the liposomal bilayers was depicted by promotion of H_{II} phase formation using both gold and silver NPs coated with dodecanethiol chains. The transfection efficiency of eGFP expressing pDNA, mFXRα1 expressing pDNA, and eGFP specific siRNA in Huh-7 cells was significantly enhanced due to incorporation of gold, silver, and magnetic NPs in the liposomal bilayers. Thus, this enhancement could be attributed to the NP-induced fusogenicity in the bilayer. The fusogenicity was slightly increased by increasing the concentration of NPs in the bilayer and was significantly increased by incorporating larger sized NPs. The NP size induced increase in fusogenicity could be correlated with enhanced transfection efficiency of eGFP expressing pDNA as well as eGFP specific siRNAs for the corresponding liposomal formulations.

We hypothesize that the fusogenicity can be further enhanced by using radiation and the future work will be focused on this aspect. MNPs exhibit Brownian and Neel relaxations upon exposure to the alternating radiofrequency radiations that lead to rotation of the entire particle and change in the direction of its magnetization, respectively {Nedelcu, 2008 #10}. These relaxations cause movement of the MNPs in the

surrounding medium as well as increase in their temperature {Fortin, 2008 #11}. Thus, the MNPs can fill up the voids generated during H_{II} phase formation thereby satisfying the packing frustration. Moreover, the enhanced temperature and vibration of the MNPs can induce rapid pore formation in the endosomal membrane during H_{II} phase formation. Similarly, upon exposure to the near infra-red and UV radiation, AuNPs demonstrate increase in temperature {An, 2013 #12}. In the proposed future work the hydrophobic NPs such as MNPs and AuNPs will be incorporated into the bilayer of liposomes containing nucleic acid based drugs. These liposomes will be used to transfect the cells. The cells will then be exposed to the radiofrequency and near infra red/UV radiation for MNPs and AuNPs containing liposomes, respectively. The enhancement in transfection efficiency due to the radiation will be determined by running the same experiment without exposure to the radiation. The cells will be exposed to the radiation post transfection at different starting time points and the optimum time and duration of exposure will be determined. Further, in-vivo experiments will be conducted in mice by dosing them with these specialized liposomes containing nucleic acid based drugs via tail vein injection. The animals will then be exposed to the radiation and the enhancement in protein production with or without radiation exposure will be compared. Fluorescent labeled lipids and nucleic acid based drugs will be used in order to determine the location of the liposomes and exposure to the radiation will be initiated when the fluorescence is detected in the endosomes as tiny dots in the cells. Thus, homogeneous spreading of the fluorescence in the cells post radiation exposure may indicate endosomal escape.

Mammalian hepatocytes express asialoglycoprotein receptor (ASGP-R) exclusively and in high numbers {Ashwell, 1982 #13}. The human ASGP-R is a transmembrane protein with two homologous subunits (H1 and H2), which recognizes and binds desialylated glycoproteins with terminal galactose or N-acetylgalactosamine residues {Ashwell, 1982 #13}. The uptake of receptor-ligand complex by the hepatocytes occurs via receptor-mediated endocytosis after the binding process. The ASGP-R is then recycled back to the surface, whereas the ligand is degraded into the lysosomes by the enzymes {Geffen, 1992 #14}. The ASGP-R has been used for liver specific delivery for a long time due to its abundant and exclusive expression on the hepatocytes and highly efficient uptake via endocytosis {Wu, 2002 #15}. The major ligands for ASGP-R are galactose, N-acetylgalactosamine and glucose and the key factors affecting ligand-receptor binding include isomeric forms of sugar, galactose density and branching, spatial geometry and galactose linkages {D'Souza, 2015 #16}. Akinc et al. developed liver targeting approach using an exogenous ligand containing a multivalent N-acetylgalactosamine (GalNAc)-cluster, which binds with high affinity to the ASGP-R {Akinc, 2010 #18}. In this work the GalNAc moiety was conjugated to the distal end of a 2,000 MW polyethylene glycol (PEG) utilizing a distearyl (C18) lipid (GalNAc–PEG–DSG) providing a stable hydrophobic anchor for the targeted PEG–lipid to the iLNP. Such PEG-lipids with targeting ligand to the distal end of the PEG will be used in our specialized liposomes containing hydrophobic NPs in order to target the liver.

In the third part of the future work, liver targeting approach using our specialized liposomes will be combined with controlled radiation exposure. Typically process of

ASGP ligand-receptor binding occurs within 8.7 minutes followed by cell internalization in next 2.3 minutes {Schwartz, 1982 #19}. The dissociation of ASGP-R and its return to the cell membrane then takes place in next 4.2 minutes {Schwartz, 1982 #19}. Hence, we hypothesize that the hepatocyte targeting liposomes containing NPs will be taken up by the cells in approximately 11 minutes post transfection and if the cells are exposed to the radiation it may lead to endosomal escape and highly efficient transfection of nucleic acid based drugs. Therefore, in-vitro work in intact hepatocytes will be conducted in order to test the proof of concept using specialized liposomes containing targeting moiety and NPs followed by in-vivo work using fluorescently labeled lipids/nucleic acid based drugs.

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SUMMARY AND CONCLUSIONS

Despite of the significant potential of nucleic acid based drugs as therapeutic agents for several life threatening ailments, their successful application is majorly limited due to insufficient delivery of these drugs to their site of action. One of the major barriers in the path of their delivery is endosomal degradation, which occurs followed by cellular uptake. Liposomal vehicles such as SNALPs (stable nucleic acid lipid particles) developed by Tekmira is one of the leading strategies for therapeutic application due to its feasibility of large scale and cGMP manufacture, storage stability for up to 2 years, lower in-vivo toxicity, and higher potency and encapsulation efficiency. On the other hand, poly-cationic polymers are majorly used in intact cells for transfection in order to evaluate initial effectiveness of nucleic acid based drugs during discovery stages as well as to understand the etiology of diseases. These poly-cationic polymers are highly efficient in cellular uptake via interaction with negatively charged cell membrane and endosomal escape via proton sponge effect mechanism, however, exhibit higher toxicity. In this work we demonstrated that the efficiency of SNALP type vehicles was significantly enhanced using hydrophobic NPs, which could be attributed to the ability of these vehicles in efficiently delivering the cargo to the site of action via improved endosomal escape. Whereas, our novel chiral polyamines exhibited higher transfeciton efficiency as well as lower toxicity. Thus, our novel liposomal- and chiral polyamine based vehicles could be beneficial in improving the efficiency of nucleic acid based drugs for therapeutic application and discovery purposes, respectively.

In manuscript 1 we demonstrated that the fusogenicity of liposomal delivery vehicles could be enhanced by incorporating hydrophobic NPs in their bilayers. The enhancement in fusogenicity of the liposomal bilayers could be depicted by promotion of H_{II} phase formation using both gold and silver NPs coated with dodecanethiol chains. The transfection efficiency of eGFP expressing pDNA, mFXRα1 expressing pDNA, and eGFP specific siRNA in Huh-7 cells was significantly enhanced due to incorporation of gold, silver, and magnetic NPs in the liposomal bilayers. Thus, this enhancement could be attributed to the NP-induced fusogenicity in the bilayer. The fusogenicity was slightly increased by increasing the concentration of NPs in the bilayer and was significantly increased due to using larger sized NPs. The NP size induced increase in fusogenicity could be correlated with enhanced transfection efficiency of eGFP expressing pDNA as well as eGFP specific siRNAs for the corresponding liposomal formulations. This approach could further benefit currently popular strategies such as magnetically guided delivery of nucleic acid-containing liposomes to their target tissues and enhance the therapeutic effect due to NPs induced higher transfection efficiency. Further, techniques such as radio-frequency heating or laser excitation might further enhance the fusogenicity of these metal NPs-containing liposomes due to the generation of heat and also due to the rupture of endosomal membranes resulting from laser-induced vibration of membrane-encapsulated metal NPs.

In manuscript 2 we showed that Chiral Polyamines presented another efficient technique for the delivery of siRNA with less toxicity post cell transfection. chiral

polymers **6** and **2a** were synthesized via straightforward, were used for two novel applications: the fabrication of chiral, covalently-linked microcapsules, and the transfection of siRNA to Huh7 cells. The chirality-dependent siRNA transfection also provides an intriguing platform for further investigation. In particular, polymer *S*-**2a**-13 demonstrated good transfection efficiency and limited toxicity, and will be used for further biochemical investigations. The results of these and other experiments will be reported in due course.

APPENDICES

The first part of the Appendix focusses on investigating role of Nuclear receptors Farnesoid X Receptors (FXR) and Estrogen Receptors in transcriptional regulation of Bile Salt Export pump (BSEP). The human Bile Salt Export pump (BSEP) is a key player in maintaining the overall Bile acid homeostasis of the body and is regulated by Farnesoid X Receptors (FXR) in an isoform-dependent manner. Disruption of BSEP leads to development of severe pathological conditions such as Intrahepatic Cholestasis of Pregnancy (ICP) and Hepatocellular Carcinoma (HCC) showing increased serum bile acid levels in the body. The goals of the proposed research focuses on investigating the underlying mechanisms involved in the etiology of Hepatocellular Carcinoma by establishing the connection between FXR, the key Bile acid regulator and Estrogen Receptors and its variants in the Intrahepatic Cholestasis of Pregnancy (ICP).

The final part of the appendix presents a published manuscript that focusses on evaluation of biological molecules (chalcones, flavones and chromenes) in development, as potent farnesoid x receptor (FXR) antagonists.

APPENDIX 1

Manuscript 1: Estrogen and Estrogen Receptor-α-Mediated Trans repression of Bile Salt Export Pump.

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Estrogen and Estrogen Receptor-α-Mediated Transrepression of Bile Salt Export Pump

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Abstract

Among diseases unique to pregnancy, intrahepatic cholestasis of pregnancy is the most prevalent disorder with elevated serum bile acid levels. We have previously shown that estrogen 17β-estradiol (E2) transrepresses bile salt export pump (BSEP) through an interaction between estrogen receptor (ER)-α and farnesoid X receptor (FXR) and transrepression of BSEP by E2/ERα is an etiological contributing factor to intrahepatic cholestasis of pregnancy. Currently the mechanistic insights into such transrepression are not fully understood. In this study, the dynamics of coregulator recruitment to BSEP promoter after FXR activation and E2 treatment were established with quantitative chromatin immunoprecipitation assays. Coactivator peroxisome proliferator-activated receptor-γ coactivator-1 was predominantly recruited to the BSEP promoter upon FXR activation, and its recruitment was decreased by E2 treatment. Meanwhile, recruitment of nuclear receptor corepressor was markedly increased upon E2 treatment. Functional evaluation of ERα and ERβ chimeras revealed that domains AC of ERα are the determinants for ERα-specific transrepression on BSEP. Further studies with various truncated ERα proteins identified the domains in ERα responsible for ligand-dependent and ligand-independent transrepression. Truncated ERα-AD exhibited potent ligand-independent transrepressive activity, whereas ERα-CF was fully capable of transrepressing BSEP ligand dependently in vitro in Huh 7 cells and in vivo in mice. Both ERα-AD and ERα-CF proteins were associated with FXR in the coimmunoprecipitation

assays. In conclusion, E2 repressed BSEP expression through diminishing peroxisome proliferator-activated receptor-γ coactivator-1 recruitment with a concurrent increase in nuclear receptor corepressor recruitment to the BSEP promoter. Domains AD and CF in ERα mediated ligand-independent and ligand-dependent transrepression on BSEP, respectively, through interacting with FXR.

Among diseases unique to pregnancy, intrahepatic cholestasis of pregnancy (ICP) is the most prevalent disorder (1,–3) in pregnant women. ICP predominantly occurs in the late stages of pregnancy and spontaneously recovers after birth (3). Although ICP is a relatively mild disorder for the mother, it poses significant risks of complications to the fetus, including preterm delivery, respiratory stress, and prenatal mortality (1,–4). One of the characteristic clinical manifestations of ICP is markedly elevated levels of serum bile acids, indicating the disruption of bile acid homeostasis in ICP patients (5,–7).

Bile acid homeostasis is achieved through a tightly regulated enterohepatic circulation of bile acids. Canalicular secretion of bile acids through bile salt export pump (BSEP) is the rate-limiting step in such circulation (8, 9). Modulation of BSEP expression or function by inherited or acquired factors has a profound impact on the biliary and intrahepatic bile acid levels. Indeed, the impairment of BSEP expression or function has been directly linked to such diseases as progressive familial intrahepatic cholestasis type 2 (10, 11), benign recurrent intrahepatic cholestasis (12, 13), and ICP (14,–16).

Under physiological conditions, BSEP expression is coordinately regulated by distinct but related transactivation pathways (17,–21), notably the bile acids/farnesoid X receptor (FXR) signaling pathway (17, 18). Activation of FXR by bile acids strongly induces

BSEP expression in vitro and in vivo (17, 18). Such feed-forward regulation of BSEP by bile acid/FXR is considered a major mechanism for preventing excessive accumulation of toxic bile acids in hepatocytes.

We previously reported that BSEP expression was significantly repressed in the late stages of pregnancy in mice and inversely correlated with serum estrogen 17β-estradiol (E2) levels (22). Further studies showed that E2 repressed BSEP expression in vitro and in vivo through estrogen receptor (ER)-α, and such repression was resulted from a cross talk between the E2/ERα and bile acids/FXR signaling pathway. It is thus concluded that E2-mediated transrepression of BSEP represents an etiological contributing factor to ICP. However, the underlying mechanisms of such transrepression are not fully understood.

In this study, we demonstrated that E2 repressed BSEP expression through decreasing recruitment of coactivator peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1) with a concurrent increase in recruitment of nuclear receptor corepressor (NCoR) to the BSEP promoter. Further studies revealed that domains AD and CF in ERα mediated ligand-independent and ligand-dependent transrepression on BSEP, respectively, through interacting with FXR.

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APPENDIX 2

Manuscript 2: Transcriptional Dynamics of Bile Salt Export Pump during Pregnancy: Mechanisms and Implications in Intrahepatic Cholestasis of Pregnancy.

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Transcriptional Dynamics of Bile Salt Export Pump during Pregnancy: Mechanisms and Implications in Intrahepatic Cholestasis of Pregnancy

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Abstract

Bile salt export pump (BSEP) is responsible for biliary secretion of bile acids, a rate limiting step in the enterohepatic circulation of bile acids and transactivated by nuclear receptor farnesoid x receptor (FXR). Intrahepatic cholestasis of pregnancy (ICP) is the most prevalent disorder among diseases unique to pregnancy and primarily occurs in the third trimester of pregnancy with a hallmark of elevated serum bile acids. Currently, the transcriptional regulation of BSEP during pregnancy and its underlying mechanisms and involvement in ICP are not fully understood. In this study, the dynamics of BSEP transcription *in vivo* in the same group of pregnant mice before, during and after gestation were established with *in vivo* imaging system (IVIS). BSEP transcription was markedly repressed in the later stages of pregnancy and immediately recovered after parturition, resembling the clinical course of ICP in human. The transcriptional dynamics of BSEP was inversely correlated with serum 17β-estradiol (E2) levels before, during and after gestation. Further studies showed that E2 repressed BSEP expression in human primary hepatocytes, Huh 7 cells and *in vivo* in mice. Such transrepression of BSEP by E2 *in vitro* and *in vivo* required estrogen receptor α (ERα). Mechanistic studies with chromatin immunoprecipitation (ChIP), protein co-immunoprecipitation (Co-IP) and bimolecular

fluorescence complementation (BiFC) assays demonstrated that ERα directly interacted with FXR in living cells and *in vivo* in mice. In conclusion, BSEP expression was repressed by E2 in the late stages of pregnancy through a non-classical E2/ERα transrepressive pathway, directly interacting with FXR. E2-mediated repression of BSEP expression represents an etiological contributing factor to ICP and therapies targeting the ERα/FXR interaction may be developed for prevention and treatment of ICP.

Keywords: BSEP, Bile acids, 17β-estradiol, FXR, ERα

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APPENDIX 3

Manuscript 3: Synthesis and biological evaluations of chalcones, flavones and chromenes as farnesoid x receptor (FXR) antagonists

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Synthesis and biological evaluations of chalcones, flavones and chromenes as farnesoid x receptor (FXR) antagonists

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Highlights

- New series of chalcone and chromene are first reported as potent FXR antagonists.
- A chromene compound (11c) significantly reduce the plasma and hepatic triglyceride level and plasma ALT level in KKay diabetic mice.
- Pharmacological role of FXR antagonist and its potential in the disease treatment is revealed.

Abstract

Farnesoid X receptor (FXR), a nuclear receptor mainly distributed in liver and intestine, has been regarded as a potential target for the treatment of various metabolic diseases, cancer and infectious diseases related to liver. Starting from two previously identified chalcone-based FXR antagonists, we tried to increase the activity through the design and synthesis of a library containing chalcones, flavones and chromenes, based on substitution manipulation and conformation (ring closure) restriction strategy. Many

chalcones and four chromenes were identified as microM potent FXR antagonists, among which chromene 11c significantly decreased the plasma and hepatic triglyceride level in KKay mice.

Graphical abstract