Synopsis

The thesis entitled "**Novel Redox Responsive Cationic Lipids, Lipopolymers, Glycolipids and Phospholipid-Cationic Lipid Mixtures: Syntheses, Aggregation and Gene Transfection Properties**" elucidates the design, synthesis, aggregation and gene transfection properties of novel cholesterol based cationic lipids with ferrocene as the redox moiety, polyethylenimine based ferrocenylated lipopolymers and cholesterol based non-ionic glycolipids. The thesis also discusses the cationic phospholipid-cationic lipid mixtures as superior gene transfection agents. The work has been divided into six chapters.

Chapter 1. Introduction

Part A. Various Cholesterol based Systems for Applications as Biomaterials

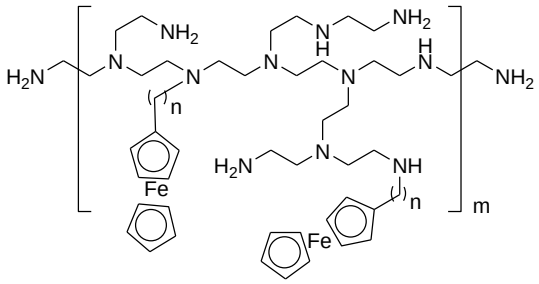
Liposomes composed of cationic lipids have become popular gene delivery vehicles. A great deal of research is being pursued to make efficient vectors by varying their molecular architecture. Cholesterol being ubiquitous component in most of the animal cell membranes is increasingly being used as a hydrophobic segment of synthetic cationic lipids. In this chapter we describe various cholesterol based cationic lipids and focus on the effect of modifying various structural segments like linker and the headgroup of the cationic lipids on gene transfection efficiency with a special emphasis on the importance of ether linkage between cholesteryl backbone and the polar headgroup. Interaction of cationic cholesteryl lipids with dipalmitylphosphatidycholine membranes is also discussed here. Apart from cholesterol being an attractive scaffold in

the drug/gene delivery vehicles, certain cholesteryl derivatives have also been shown to be attractive room temperature liquid-crystalline materials.

Part B. Diverse Applications of Ferrocene Derivatives

This chapter gives a brief overview of ferrocene chemistry followed by description of major applications of ferrocenyl derivatives in a variety of fields like catalysis, materials chemistry, electrochemical sensors, medicinal chemistry etc. We discuss the use of ferrocene as an electrochemical and redox active switch to achieve control over supramolecular aggregation. It also reviews ferrocene based amphiphiles including surfactants, lipids and polymers with an emphasis on the role of ferrocene over aggregate formation and their utilization in biological applications.

Chapter 2: Optimization of Redox Active Alkyl-Ferrocene Modified Polyethylenimines for Efficacious Gene Delivery in Serum



1a-c, n = 6, P8-C6-F1, P8-C6-F2, P8-C6-F3
2a-c, n = 11, P8-C11-F1 P8-C11-F2, P8-C11-F3

% ferrocene grafting, F1 = 15%, F2 = 25% and F3 = 50%

Figure 1. Structure of the alkyl-ferrocene modified 800 Da Branched Polyethylenimine.

In this chapter we present six new lipopolymers based on low molecular weight polyethylenimines (BPEI 800 Da) which are hydrophobically modified using ferrocene

terminated alkyl tails of variable lengths. The effects of degree of grafting, spacer length and redox state of ferrocene in the lipopolymer on the self assembly properties were investigated in detail by transmission electron microscopy (TEM), atomic force microscopy (AFM), dynamic light scattering (DLS) and zeta potential measurements. The assemblies displayed a redox induced increase in the size of the aggregates. The coliposomes comprising of the lipopolymer and a helper lipid 1,2-Dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE) showed excellent gene delivery capability in serum containing environment in two cancer cell lines (HeLa, U251 cells). Optimized formulations showed remarkably higher transfection activity than BPEI 25 KDa and even better than commercial Lipofectamine 2000 as evidenced from luciferase activity and EGFP expression analysis. Oxidation of ferrocene in lipopolymers led to reduced levels of gene transfection which was also followed by cellular internalization of fluorescently labeled *p*DNA using confocal microscopy. Cytotoxicity assay revealed no obvious toxicity for the lipopolyplexes in the range of optimized transfection levels. Overall, we have exploited the redox activity of ferrocene in PEI based polymeric gene carriers for trenchant control over gene transfection potential.

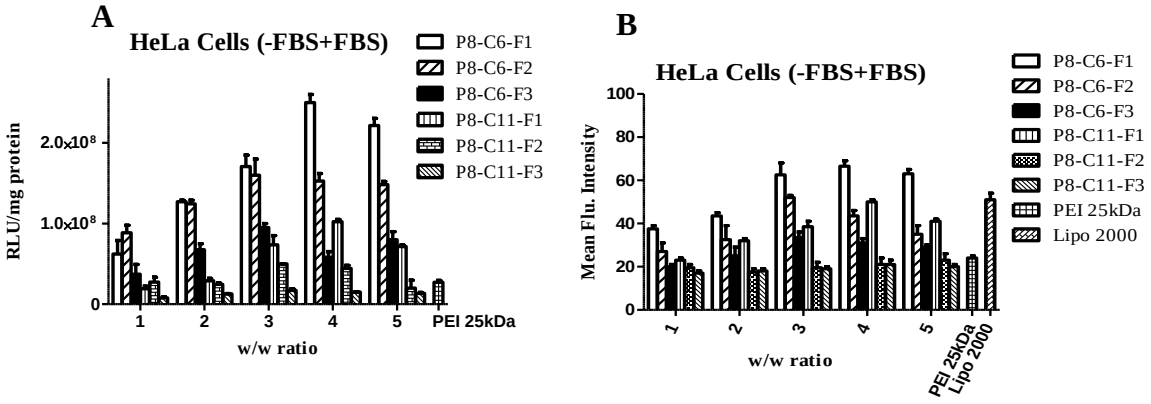
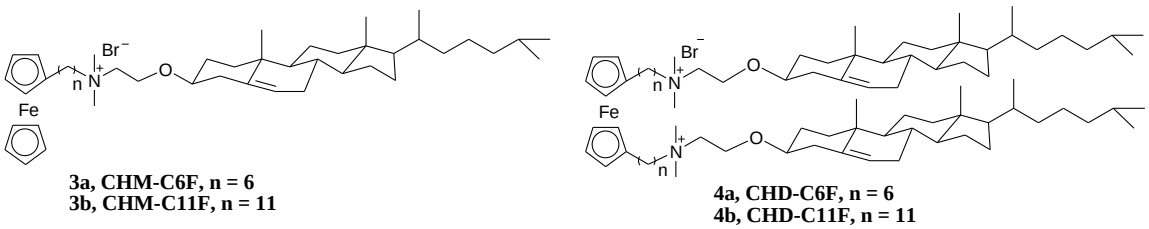


Figure 2. Maximum transfection efficacies of optimized redox lipopolymer/DOPE formulations by (A) Luciferase Assay and (B) Flow cytometry (GFP expression).

Chapter 3. Membranes derived from Redox-active Cholesterol based Cationic Lipids and their Interactions with DNA and Phospholipid Membranes



3a, CHM-C6F, n = 6
3b, CHM-C11F, n = 11

4a, CHD-C6F, n = 6
4b, CHD-C11F, n = 11

Figure 3. Molecular structures of the electroactive cholesterol based monomeric and gemini lipids.

This chapter describes the synthesis and aggregation properties of two series of redox-active ferrocene containing monomeric and gemini cationic lipids with cholesterol as a hydrophobic domain. These cationic lipids are modified at their headgroup region using ferrocene terminated alkyl chains of differing length. All the four cationic lipids formed stable suspensions in water. Aggregation behavior of these cationic lipids in aqueous suspensions in their unoxidized and oxidized state was studied using TEM, DLS, zeta potential measurements and XRD studies. Cationic lipids with ferrocene in natural, reduced state were found form bigger sized vesicles which upon oxidation became smaller aggregates with increased zeta potential. XRD results indicate the existence of nice lamellar arrangements of the lipid bilayers. Thermotropic phase transition behavior of DPPC membranes incorporated with cationic ferrocene lipids was also studied using differential scanning calorimetry. Finally, we assayed *p*DNA (plasmid DNA) binding ability of all the four cationic lipids using ethidium bromide intercalation assay where all the cationic lipid formulations showed excellent DNA binding capability. In the experiments involving SDS-induced release of DNA, we observed that redox-active

monomeric lipids (**3a-b**) were found to be more efficient in facilitating the release of DNA from the liposome-DNA complex in the presence of negatively charged SDS micelles than their gemini counterparts (**4a-b**).

Chapter 4. Redox-responsive Gene Delivery by Ferrocene containing Cationic Cholesteryl Lipids in Serum

This chapter describes the transfection efficacy of redox-active monomeric and gemini cationic lipids with cholesterol backbone. The transfection efficiency of all the lipids could be tuned by changing the oxidation state of the ferrocene moiety. Gene transfection capability was assayed in terms of EGFP expression using pEGFP-C3 plasmid DNA in three cancer cell lines of different origin, namely Caco-2, HEK293T and HeLa in the presence of serum.

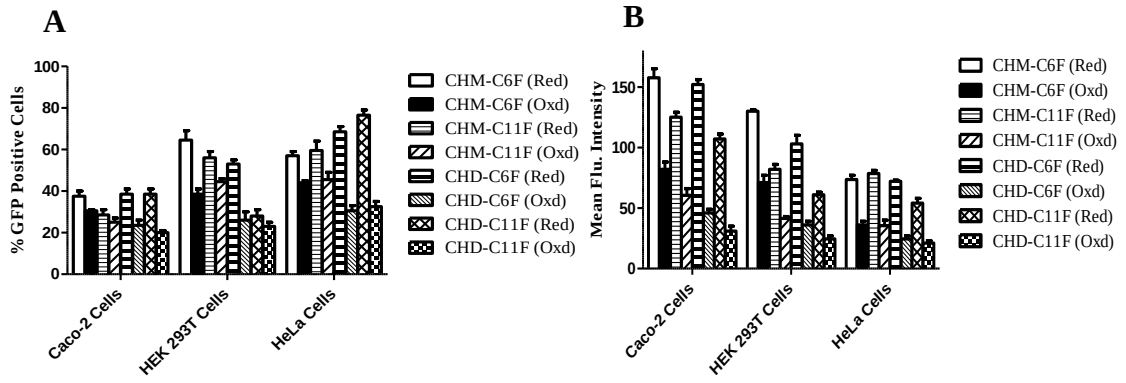


Figure 4. Effect of oxidation state of ferrocene on maximum transfection efficacies of monomeric and gemini lipids in three different cell lines (Caco-2, HEK 293T and HeLa).

Cationic liposomal formulations with ferrocene in its reduced state were observed to be potent transfectants reaching the EGFP expression levels even better than

commercial lipofectamine 2000 in the presence of serum as evidenced by flow cytometry. EGFP expression was further substantiated using fluorescence microscopy studies. All liposomal formulations containing oxidized ferrocene displayed diminished levels of gene expression and interestingly, these results were consistent for each formulation in all the three cell lines. Assessment of EGFP expression mediated by both reduced and oxidized ferrocene containing formulations was also undertaken following cellular internalization of labelled *p*DNA using confocal microscopy and flow cytometry. Lipoplexes derived from different liposomal formulations with reduced and oxidized ferrocene were characterised using TEM, AFM, zeta potential and DLS measurements. Overall, we demonstrate here controlled gene transfection levels using redox driven, transfection efficient cationic monomeric and gemini lipids.

Chapter 5: Synthesis of 'Click Chemistry' Mediated Glycolipids: Their Aggregation Properties and Interaction with DPPC Membranes

This chapter describes the synthesis and aggregation properties of cholesterol based glycolipids along with their interaction with a model phosphatidylcholine membranes. Three series of non-ionic glycolipids with hydrophobic cholesterol backbone and various monosaccharide and disaccharide sugars as the hydrophilic polar domain have been synthesized. These were conjugated to the cholesteryl backbone via oligooxyethylene spacers of different lengths (n = 1, 3 and 4) using Cu (I) catalyzed Huisgen [3+2] cycloaddition, which is popularly known as 'Click Chemistry'. All the synthetic glycolipids (5a-d, 6a-d and 7a-d) formed vesicular aggregates in aqueous medium as confirmed by TEM and DLS. XRD studies with the cast films of lipids

revealed that the bilayer width increased with increase in the length of oligoethylene spacer unit that has been incorporated between the hydrophobic and hydrophilic domains. Also, within the same series containing a particular oligoethylene unit, bilayer widths were found to be more for the lipids containing disaccharides as their headgroup than monosaccharides.

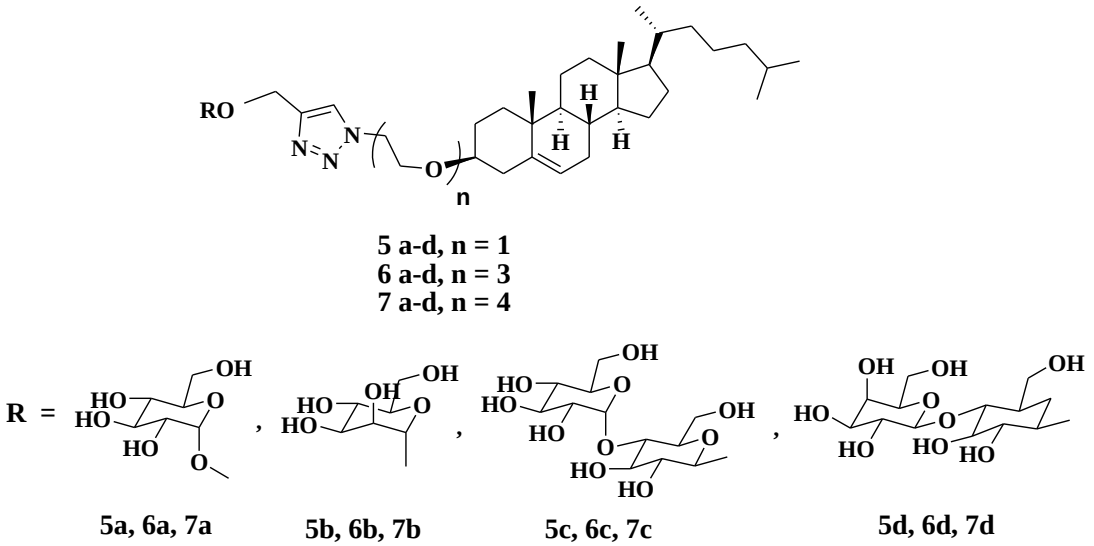


Figure 5. Molecular structures of various cholesterol-based glycolipids.

Calorimetry studies of the coaggregates containing naturally occurring 1, 2-dipalmitoylphosphatidylcholine (DPPC) and various mol-% of each of the glycolipids revealed that more than 30 mol-% of glycolipids are required to completely abolish the phase transition of DPPC membranes. These results were further supported by fluorescence anisotropy measurements of the co-aggregates using 1, 6-diphenylhexatriene (DPH) as a probe. Fluorescence anisotropy of the neat vesicles revealed that **9a** and **9c** were more rigid than DPPC vesicles in the solid-like gel phase, while the glycolipids with longer oxyethylene spacers (n = 3 and 4) were less rigid than the DPPC vesicles.

Chapter 6. Hydrophobic Moiety Decides the Synergistic Increase in Transfection Efficiency in Cationic Phospholipid/Cationic Lipid mixtures

This chapter describes the effect of inclusion of cationic lipid/cationic gemini lipids into the membranes of a cationic phospholipid on the gene delivery efficiency across HeLa and HEK293T cell lines. Although all the three cationic lipids have the same quaternary ammonium moiety as their headgroup, they differ from each other in terms of their hydrophobic moiety and in the number of cationic headgroups. Chol-N is a cholesterol based monocationic lipid, while $2C_{14}$-N and $2C_{14}$N-5-N$2C_{14}$N are monomeric and gemini cationic lipids respectively with pseudoglycerol backbone consisting of tetradecyl (n-$C_{14}H_{29}$) chains. Each of the three cationic lipids under the current investigation, namely, Chol-N, $2C_{14}$-N and $2C_{14}$N-5-N$2C_{14}$N were added in different ratios to EtDMoPC and the resultant mixed membranes were studied for the biophysical characterization and gene delivery efficacies.

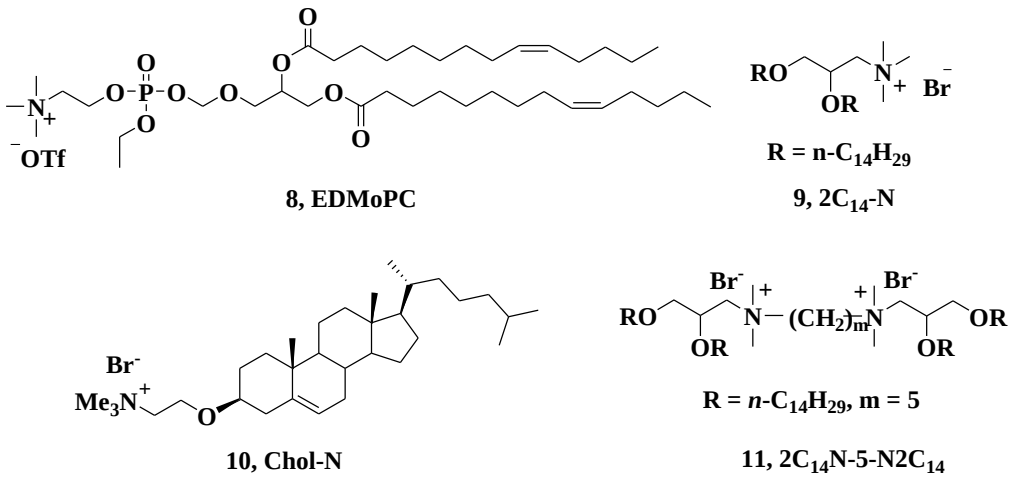


Figure 6. Molecular structures of cationic lipids used in this study.

All the formulations were characterized using dynamic light scattering and zeta potential measurements to obtain their hydrodynamic diameters and surface charge properties respectively. Their DNA binding ability was also studied by measuring changes in zeta potential and gel electrophoresis of the lipoplexes formed by the coliposomal formulations and *p*DNA at different Lipid/DNA weight ratios. The gene delivery efficacies of various formulations were studied in terms of EGFP expression using pEGFP-C3 plasmid DNA in two different cell lines, namely HeLa and HEK293T. In the absence of serum we found that the formulation (EtDMoPC+2C_{14}N-5-N2C_{14}N) showed better transfection efficiency than the individual lipids. However, in the case of others, i.e., (EtDMoPC+Chol-N) and (EtDMoPC+2C_{14}-N) formulations, there was a slight decrease in transfection efficiency compared to the individual lipids. In the presence of serum, the formulations (EtDMoPC+2C_{14}-N) and (EtDMoPC+2C_{14}N-5-N2C_{14}N) showed significantly higher transfection efficacies compared to their individual lipids. Fusion assay using labelled cationic lipid formulations and unlabelled anionic liposomes revealed that lipoplexes prepared from EtDMoPC+ 2C_{14}-N and EtDMoPC+ 2C_{14}N-5-N2C_{14} exhibited much higher fusogenicity as compared to the lipoplexes prepared using EtDMoPC+Chol-N as well as the individual lipids. Thus, the liposome formulations which showed better transfection activity fused more readily with the anionic liposomes than did the formulations with poorer activity. Overall, we found that the hydrophobic domain of the cationic lipid/cationic gemini lipid that is added to cationic phospholipid has an important role on the transfection efficiency of the mixed formulations. Additionally the cytotoxicity studies revealed that each of these formulations was not significantly toxic making them viable for applications *in vivo*.