Effect of the Nature of the Spacer on Gene Transfer Efficacies of Novel Thiocholesterol Derived Gemini Lipids in Different Cell Lines: A Structure–Activity Investigation

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A structure–activity investigation was undertaken to see the effect of the nature of the spacer on the gene transfection efficacies of thiocholesterol-derived cationic gemini lipids possessing disulfide linkage between the cationic headgroup and the thiocholesterol moiety. Three gemini cationic lipids possessing hydrophobic flexible (-$(CH_2)_5$-; **1**), hydrophobic rigid (-C_6H_4-; **2**), and hydrophilic flexible (-CH_2-CH_2-O-CH_2-CH_2-; **3**) spacer segments were synthesized. In HeLa cells, lipid formulations **1** and **2** were found to be more effective as compared to lipid **3** formulation. In HT1080 cell line, the order of transfectability was **3** > **1** > **2**. Transfection studies in HeLa and HT1080 cell line also showed 40–50% transfection efficacy in the presence of 10% serum conditions. These formulations were also able to transfect gene across difficult cells like HaCaT. Cytotoxic studies showed the nontoxic nature of these lipid−DNA complexes at different N/P ratios used for transfection studies.

Introduction

Gene therapy for the treatment of various genetic disorders offers new hope for the coming years.[1] Developing clinically useful vectors for gene therapy depends upon the biosafety and gene transfer efficacies of the vectors used for delivering the therapeutic genes into the targeted body cells.[2,3] Two major categories of current transfection vectors are viral and nonviral. In spite of the high efficacies of the viral vectors, major disadvantages associated with the use of viral vectors include limited insert size, difficulty of large scale production, inflammatory and toxic reactions, strong immunogenic responses, and insertional mutagenesis.[4–7] Nonviral vectors include the use of surfactants, cationic lipids, polymers, peptides, and lipopolymers.[8–13]

From the last many years, search for cationic lipids of high efficiency is at the forefront for many scientists because of their easy production, high DNA carrying capacity, low toxicity, and minimum immunogenicity.[14–20] At molecular level, a cationic lipid consists of a hydrophobic part, a headgroup, and the linkage region between the hydrophobic and the headgroup. Various structure–activity investigations have been undertaken to elucidate the effect of the different parts of the cationic lipids on the transfection activities of their formulations.[20–28] Nakanishi et al. investigated the structure–activity relationship on gene transfection efficacies of cholesterol-based cationic lipids with different hydroxylated headgroups.[29,30] The structure–activity relationship was also examined on pyridinium-based cationic, dimeric, and oligomeric surfactants.[31] Various cholesterol-derivatized lipopolymers have also been studied for transfection activities by Kim and co-workers.[32,33]

Gemini surfactants comprise a dimeric form of surfactants that involve linking of two symmetrical surfactant units via a suitable spacer. Because of their unusual aggregation and biological properties, their use in gene delivery has also been investigated.[31,34–39] The unique physical properties of gemini surfactants motivated us to investigate for the first time the effect of the length of spacer on aggregation properties of gemini (symmetric dicationic) pseudoglyceryl and aromatic-derived gemini lipids.[40–45]

The functional group that links the polar headgroup and the hydrocarbon chains of lipid molecules plays a crucial role in their utilization in gene transfer events.[46,47] We have shown the effect of this linkage functionality on the gene transfection efficiencies of cholesterol-based lipids. It was observed that cholesterol-based cationic lipids bearing ether linkage between cholesterol backbone and headgroup possess greater transfection capability as compared to other linkages.[48,49] We have also studied the interactions of cholesterol and cholesterol based cationic lipids with phospholipids in bilayer membranes.[50–52] Recently we investigated the structure–activity relationship on cholesterol-based gemini lipids differing in the length and the nature of the spacer between headgroups, possessing ether linkage between the cationic headgroup and cholesterol moiety.[53,54] The high transfection activities, easy synthesis, and serum compatibility are the major advantages of these ether linkage-based cholesterol gemini cationic lipids, in spite of their nonbiodegradability due to the presence of nonscissile ether linkage.

Tang and Hughes pioneered the use of the disulfide bond as the linker functionality of cationic lipids.[55,56] The rationale behind this approach was to ensure collapsing of the lipid−DNA complex inside the cell cytoplasm after reduction of disulfide linker by the intracellular glutathione pool. Glutathione (GSH) is the most abundant low molecular weight thiol present in cells[57] and is involved in many important functions in the body, including the control of redox environment in cells.[58,59] GSH is found at high intracellular concentrations (~10 mM) in liver cells[60] and very low extracellular concentrations (<10 μM).[58,59] For example, blood plasma concentrations (2 μM)[61] are 1000-fold less than in erythrocytes (~2 mM).[62] This vast difference in potential "releasing agents" inside cells as compared to outside cells provides a potent mechanism for release of DNA from disulfide-based lipid−DNA complexes.[26,63–65]

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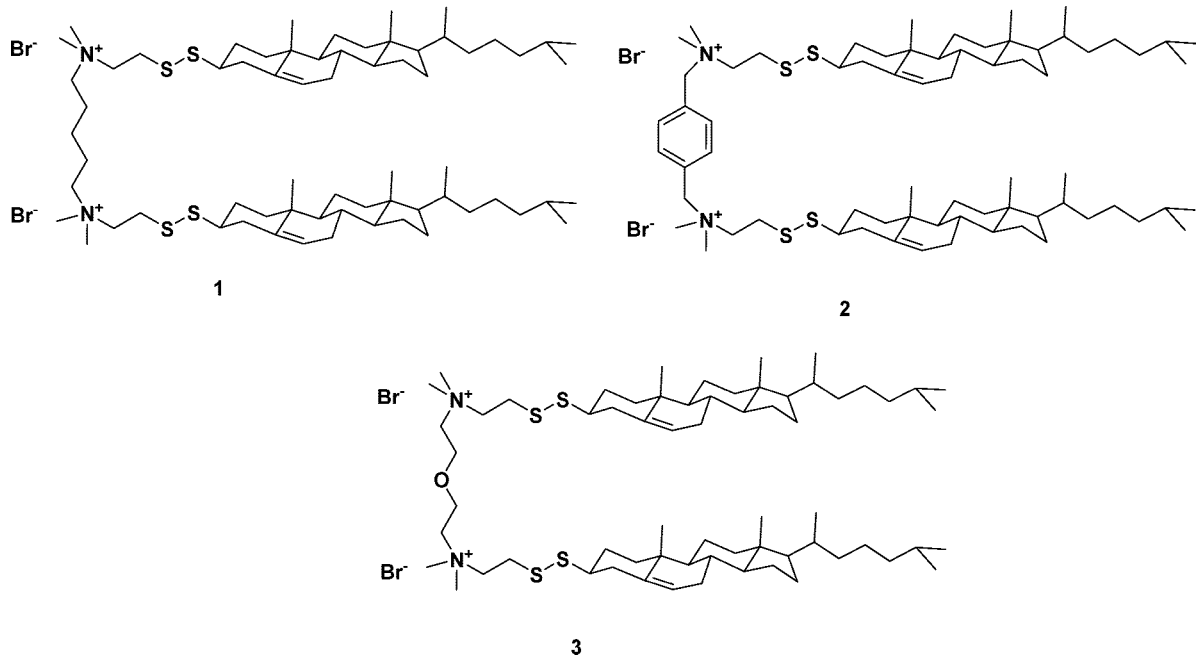


Figure 1. Molecular structures of thiocholesterol-derived cationic gemini lipids possessing -$(CH_2)_5$- (**1**), -C_6H_4- (**2**), and -CH_2-CH_2-O-CH_2-CH_2- (**3**) spacer, synthesized, and studied.

In this present work, we present for the first time the synthesis and transfection properties of three thiocholesterol-derived gemini cationic lipids possessing disulfide linkages and differing in the nature of the spacer between cationic headgroups (Figure 1). We varied the spacer in these lipids from hydrophobic flexible -$(CH_2)_5$- to hydrophobic rigid (-C_6H_4-) to hydrophilic flexible (-CH_2-CH_2-O-CH_2-CH_2-) spacer to examine the role of the spacer on gene transfection activities in different cell lines. Here we incorporated disulfide linkage between the cationic headgroup and the thiocholesterol backbone, making the gemini lipids biodegradable as well. Transfections have been performed in four different cell lines, HeLa, HT1080, PC3AR, and HaCat, each of which is different from each other in their origin and characteristics. HeLa is a cervical cancer cell line, whereas HT1080 is a human fibrosarcoma cell line. PC3AR is prostate cancer line and HaCaT represents human keratinocyte cell line. Cytotoxic studies confirmed the nontoxic nature of these lipid−DNA complexes at different N/P ratios used for transfection studies. Electron microscopy studies showed the vesicle-like nature of the lipid−DOPE[a] aggregates and elongated aggregates of lipoplexes.

Results and Discussion

Chemistry. Earlier work from this laboratory demonstrated that the nature of the linkage between the headgroup and the cholesterol backbone plays an important role in modulating the gene delivery efficacies.[48,49] Ether linkage between the cationic headgroup and the cholesterol units in such lipids were found to be the best for transfecting agents. Later on, cholesterol-based gemini lipids based on ether linkage, differing in the length of the spacer between headgroups, were shown to be better transfecting agents.[53,54] In this work, we describe the effect of introducing a disulfide linkage between the cationic headgroup and the thiocholesterol backbone. Disulfide linkage makes these gemini lipids highly biocompatible and reducible. This is designed to make the release of DNA from its lipoplexes easier.

For the synthesis of the precursor *N*,*N*-dimethylaminoethyl 3-cholesteryl disulfide (**6**) for gemini lipids, first thiocholesterol (**4**) was reacted with 2-pyridyl disulfide in dry chloroform under nitrogen atmosphere to get 2-pyridinyl 3-cholesteryl disulfide (**5**) in 80% yields (Scheme 1).[66] Reaction of the **5** with *N*,*N*-dimethylaminoethylthiol hydrochloride in the presence of triethyl amine yielded the precursor **6** in 80% yields.[66] Gemini lipids were synthesized by reaction of **6** with corresponding dibromides in sealed tube for 48–96 h. All the final gemini lipids were purified by repeated crystallizations from MeOH−EtOAc mixture until TLC ensured their purity and were characterized by ^{1}H NMR, mass spectra, and elemental analysis.

Aggregate Formation from Cationic Gemini Lipids. Upon hydration, all the gemini lipids were found to get dispersed in water easily. Each lipid molecule mentioned here formed stable suspension in water and was found to be translucent. TEM examination of air-dried, aqueous suspensions revealed the existence of vesicular aggregate structures for all the gemini lipids (not shown).

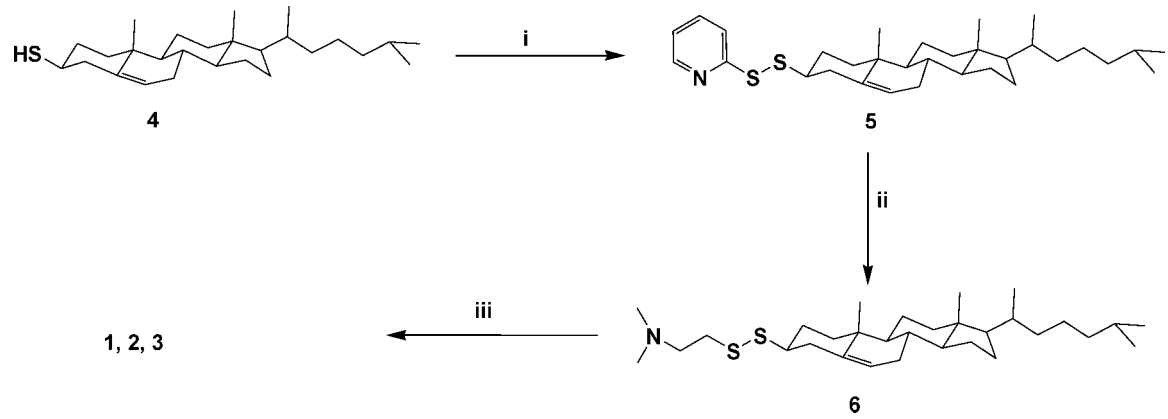
Mixed Liposome Formation with 1,2-Dioleoyl-L-α-glycero-3-phosphatidyl Ethanolamine (DOPE). Liposomes could be conveniently prepared from each gemini lipid with naturally occurring helper lipid DOPE by first subjecting the films of lipid mixtures to hydration, repeated freeze–thaw cycles, followed by sonication at 70 °C for 15 min. All the gemini lipids formed optically clear suspensions. Vesicles were prepared under sterile conditions and were resonicated for 5 min at room temperature before transfection experiments. The vesicular suspensions were sufficiently stable and no precipitation was observed within three months if stored at 4 °C.

Transfection Biology

Optimization of Lipid−DOPE Ratio. Naturally occurring lipid such as DOPE has been known to increase the efficiency of transfection in lipid mediated gene transfer applications.[46] To find out the most effective formulations, transfections with identical lipid/DNA mol ratio (or N/P ratio), varying the mol ratio of gemini lipids (**1**, **2**, **3**) in DOPE were performed. To

[a] Abbreviations: DOPE, 1,2-dioleoyl-L-α-glycero-3-phosphatidylethanolamine; MFI, mean fluorescence intensity; FACS, fluorescence-activated cell sorting.

Scheme 1[a]



[a] Reagents and conditions: (i) $CHCl_3$, 2-pyridyl disulfide, 48 h; (ii) $CHCl_3$, triethyl amine, *N*,*N*-dimethylaminoethylthiol hydrochloride, 24 h; (iii) MeOH−EtOAc, dibromopentane, dibromoethoxy ethane, or dibromo *para*-xylene, 48–96 h.

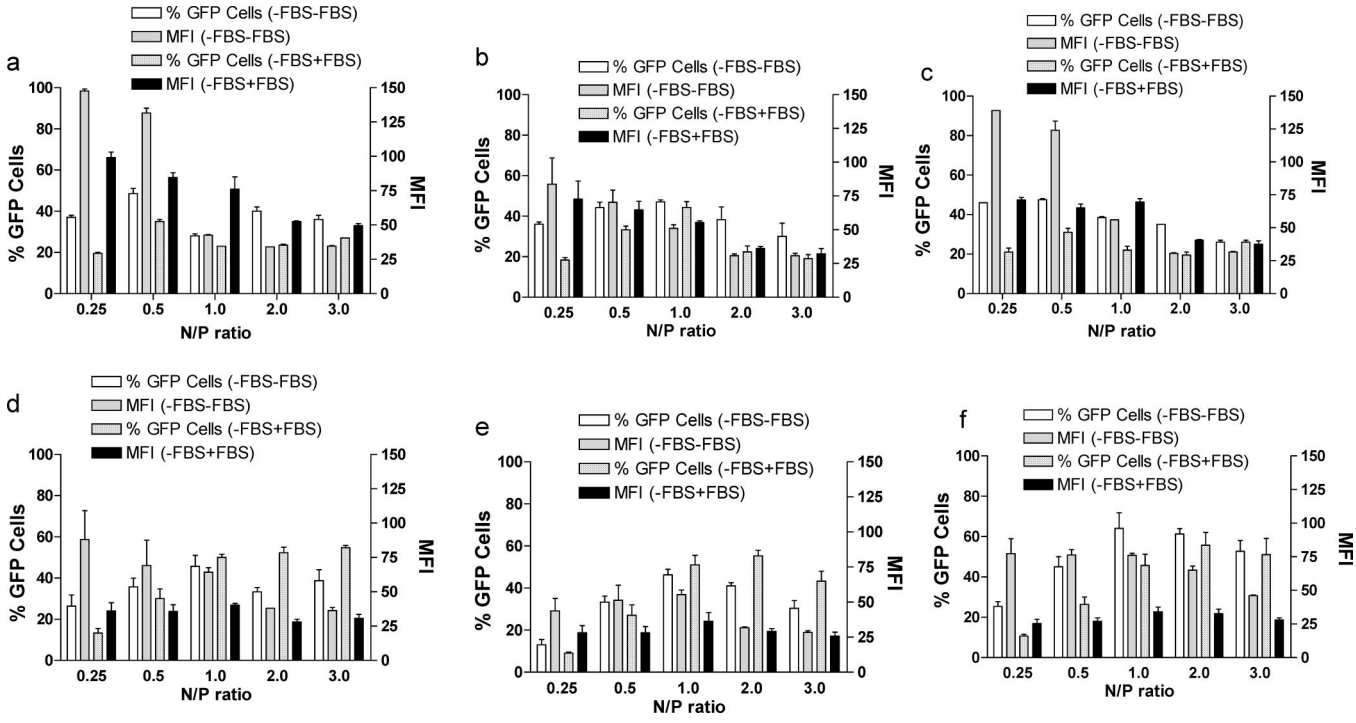


Figure 2. Transfection efficiencies of gemini lipids using optimized lipid−DOPE formulations in HeLa and HT1080 cells at various N/P ratios in the absence and presence of serum. HeLa cells: (a) Lipid **1**; (b) Lipid **2**; (c) Lipid **3**. HT1080 cells: (d) Lipid **1**; (e) Lipid **2**; (f) Lipid **3**.

find out the optimized transfection efficiency, both the number of transfected cells and the mean fluorescence intensity were considered. The mean fluorescence intensities (MFI) defined for GFP positive cells reveal that the level of GFP expression with a higher MFI value correlates positively with a high GFP expression. These data obtained from flow cytometric analysis indicated that the optimization lipid−DOPE ratios for **1** and **3** is 1:5, whereas for **2**, the optimized ratio is 1:4 (not shown).

Optimization of Lipid/DNA Charge Ratio. Transfection experiments at different N/P ratios were performed in HeLa and HT 1080 cell lines using optimized lipid−DOPE ratios, as shown in Figure 2. Formulations based on **1** and **3** were found to be more effective as compared to **2** in HeLa cells. Both **1** and **3** transfected nearly 40–50% cells with MFI of ∼125 at N/P ratio of 0.25–0.5 (Figure 2a,c). At high charge ratios, the transfection efficiency decreased especially in terms of MFI. These results clearly showed that both **1** and **3** formulations were highly effective at low N/P ratios where we observed the movement of the plasmid DNA from the well in gel electrophoresis experiments as described later. This indicates that complete gel retardation of the DNA and charge neutralization of the DNA are not necessary for efficient transfection and use of such low concentration of lipids would hardly be toxic to cells. In the case of HT1080 cells, optimization N/P ratio for lipid formulations was found to be 1.0 where formulation of **1** showed ∼40% transfection efficiency with MFI of ∼70 (Figure 2d). Formulation **2** was found to be the least efficient in HT1080 cell line, transfecting 40% cells with MFI of ∼50 (Figure 2e), whereas **3** was most efficient, transfecting ∼60% cells with MFI of ∼75 at N/P ratio of 1.0 (Figure 2f). Therefore, the transfection efficacy in HT1080 cell line decreases in the order **3** > **1** > **2**.

Effect of the Serum. One of the serious shortcoming of the cationic lipid-mediated gene delivery, especially involving in vivo trials, is that the transfection is inefficient, particularly in the presence of serum proteins. Serum proteins that are negatively charged are known to interact with cationic lipids, thereby competing with DNA for cationic lipids, leading to inhibition of transfection. To have better potential, especially

Table 1. Transfection Efficacies of Different Lipid Formulations in PC3AR and HaCat Cells

	PC3AR cells		HaCat cells	
lipid formulation	% GFP cells	MFI	% GFP cells	MFI
Formulation A	12	54	11	32
Formulation B	54	239	28	57
lipid **1**	54	260	22	37
lipid **2**	61	418	21	28
lipid **3**	37	105	5	18

in gene therapy, it is important to have reagents that are capable of delivering and expressing an external gene inside a cell in the presence of serum. There are only a few reports of cationic lipids that induce transfection activity, even in the presence of serum.[28,53,54,67,68] Therefore, we planned to study the efficiencies of these thiocholesterol-based gemini lipids in the presence of serum in HeLa and HT1080 cells (Figure 2).

In the case of formulation of **1**, there was a 2-fold decrease in the MFI in HeLa cells in the presence of 10% serum conditions, transfecting nearly 35% of cells with MFI of ∼75 (Figure 2a). Formulation of **2** was able to transfect a maximum of 40% cells with a MFI of ∼50 (Figure 2b), whereas only 30% transfected cells were observed with a MFI ∼60 in the case of formulation **3** by FACS analysis (Figure 2c). In HT1080 cells, although these lipid formulations were able to transfect a high number of cells, the MFI intensity observed was low (Figure 2d−f). Nearly 50–60% of the cells were found to be transfected with MFI of ∼25. The decrease in the MFI is expected, as in the presence of serum, and negatively charged serum proteins start competing with negatively charged DNA for binding to gemini cationic lipids, which probably leads to the dissociation of the lipoplexes. This resulted in the delivery of a lower number of the plasmid DNA in the cells, leading to low MFI.

Transfection Studies in PC3AR and HaCaT Cell Lines. To further explore the efficacy of these formulations, we chose two other cell lines, PC3AR and HaCaT, and compared the efficacies of these formulations with commercially available reagents "Formulation A" and "Formulation B". Table 1 shows the transfection efficacies of different lipid formulations in PC3AR and HaCaT cells. Table 1 clearly showed that the gemini lipid formulations were much superior to Formulation A in PC3AR cells. Comparing with Formulation B, **3** was not found to be superior, whereas formulation **1** showed comparable transfection efficacy as compared to Formulation B. On the other hand, lipid **2** formulation showed nearly two time higher MFI with comparable number of GFP positive cells. As HaCaT cell lines are very difficult to transfect, we observed 11% and 28% GFP cells with Formulation A and Formulation B respectively. In case of lipid **1** and **2** formulations, ∼20% cells showed GFP expression, whereas only 5% efficacy was observed in case of lipid **3** formulation.

Cytotoxicity Studies. MTT assay based cytotoxic studies have been performed in HeLa cell lines at different N/P ratios at which transfection experiments were performed. The cell viability results (not shown) clearly demonstrate that all the lipid formulations are virtually nontoxic in the entire range of lipid/DNA charge ratios used in the present study.

Characterization of Lipid−DNA complexes

Gel Electrophoresis. We performed conventional electrophoretic gel retardation assays to characterize the electrostatic binding interactions between the plasmid DNA and the mixed (lipid−DOPE) cationic liposomes as a function of different N/P charge ratios (or lipid/DNA mole ratios), as shown in Figure 3. Among the three gemini lipids, **1** was found to be most effective in DNA retardation. No movement of the plasmid DNA was observed at N/P ratio of 1.0 in the case of **1** lipid-based formulation. It should be noted that every gemini molecule possesses two permanent charges, which indicates that the whole plasmid DNA gets retarded at lipid/DNA mol ratio of 0.5. In case of **2** and **3** lipid-based formulations, we observed that the movement of the small amount of the DNA occurred from the well at N/P ratio of ∼1.0.

To demonstrate the degradation of the gemini cholesteryl lipids possessing disulfide linkage, mixed lipid vesicles (0.2 mM) containing optimized DOPE concentration were first incubated with 10 mM glutathione (GSH). Then the gel electrophoresis using GSH-treated vesicles and GSH-untreated vesicles (as control) were performed to assess the ability of the resulting systems toward lipoplex formation. GSH treated vesicles were found unable to bind with 0.2 μg of the plasmid DNA at N/P ratio of 3.0 as compared to untreated vesicles where gel retardation was observed as shown in Figure 4.

Electron Microscopy and DLS Studies. We characterized the optimized lipid−DOPE based liposomes and lipoplexes using optimized formulations by transmission electron microscopy. Lipid **1** based formulations formed mostly the vesicular aggregates of 70–90 nm in size, although some larger aggregates of 100–140 nm were also seen, as shown in Figure 5. Aggregates from **2** and **3** were found to be in the range of 50–80 nm in size. DLS studies showed the liposome aggregates of 100–130 nm in size, as shown in Table 2. For TEM studies, samples are usually prepared after wicking of the excess solvent, followed by air drying. Therefore, during this process, there can be shrinkage of the aggregates because of the removal of the excess solvents, which leads to the formation of smaller size aggregates. Morphology of lipid/plasmid complexes was also visualized under transmission electron microscope after negative staining using uranyl acetate, as shown in Figure 6. All these formulations showed a tendency to form "elongated" structures after DNA complexation. It appears the lipid vesicles are organized on the templates of the plasmid DNA surface. DLS studies showed that the lipoplexes from lipid **1** based formulations are smaller in size as compared to lipid **2** and **3** based lipoplexes, as shown in Table 2.

Conclusions

Gene delivery by cationic liposomes offers certain advantages over the viral vectors. Structure–activity investigations are the need of gene therapeutic research to find out the best formulations for gene transfer for different cell lines, where tailor-made lipids have a potential for safe and efficient gene delivery. Nature remains as the ultimate source of inspiration for chemists to synthesize suitable lipids for efficient biological activities. Taking that inspiration from naturally occurring cardiolipins, we initiated a program to find out the structure–activity investigations on different types of synthetic gemini lipids. Three thiocholesterol-based gemini lipids differing in the nature of the spacer between the cationic headgroups, possessing disulfide linkages between the headgroup and the thiocholesterol moiety have been synthesized. Their liposomal formulations with DOPE were studied for transfection activities in four different cell lines. Transfection efficacy of these formulations was found to be dependent upon the cell line, as **1** and **3** formulations were found to be effective in HeLa cell lines, whereas **3** was found to be more effective than **1** in HT1080 cell lines. In PC3AR cells, **2** was found to be the best, which was also able to transfect very

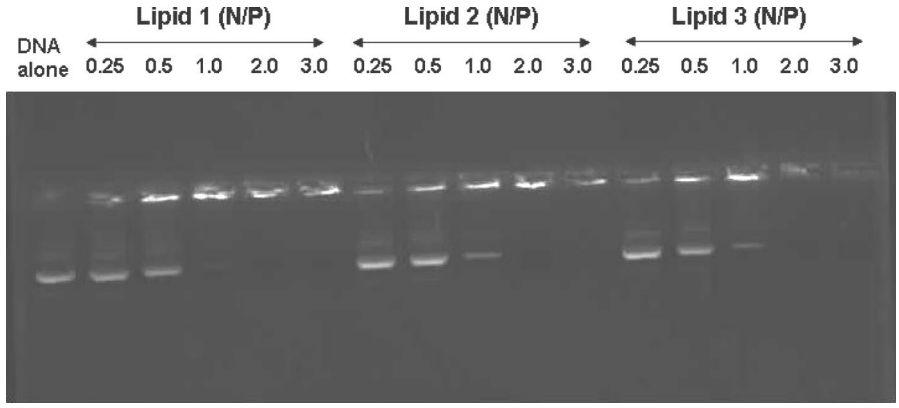


Figure 3. Electrophoretic gel patterns for lipoplex-associated DNA in gel retardation for gemini thiocholesterol lipids. The N/P ratios are indicated at the top of each lane; 0.2 μg of the DNA was complexed with liposomes at various N/P ratios for 30 min; and lipoplexes were run electrophoretically on 1% agarose gel.

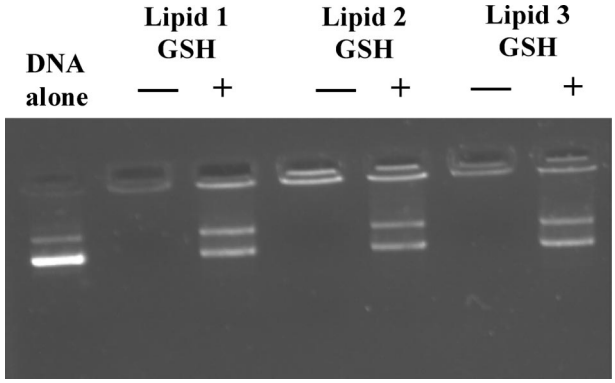


Figure 4. Electrophoretic gel patterns for lipoplex-associated DNA in gel retardation for gemini thiocholesterol lipids using 10 mM glutathione (GSH) treated (+) and untreated (−) vesicles at N/P ratio of 3.0. A total of 0.2 μg of the DNA was complexed with 10 mM glutathione (GSH) treated (+) and untreated (−) vesicles at N/P ratio of 3.0 for 30 min, and lipoplexes were run electrophoretically on 1% agarose gel.

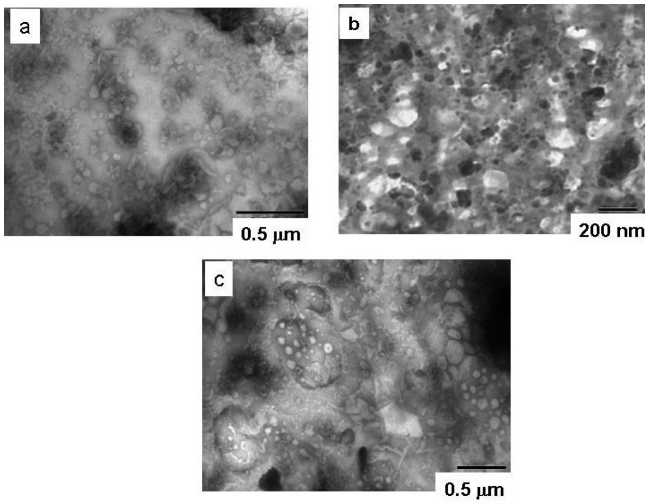


Figure 5. Transmission electron micrographs for the liposomes prepared from optimized gemini lipid:DOPE coliposomes (a): **1**; (b): **2**; (c) **3**.

difficult transfectable HaCaT cells as well. All the formulations were found to be practically nontoxic. Electron microscopy showed their tendency to form "elongated" structures after DNA complexation. Whatever may be the actual mechanism[69–71] for transfection mediated by this class of gemini lipid formulations,

Table 2. Hydrodynamic Diameters of the Optimized Lipid−DOPE Coliposomes and Lipoplexes

	hydrodynamic diameter (nm)	
	liposomes	lipoplexes
lipid **1**	104	56
lipid **2**	120	144
lipid **3**	128	138

the interesting results obtained with these novel disulfide possessing gemini lipids should be of interest to researchers working in the field of gene therapy using nonviral vectors.

Experimental Section

Materials and Methods. All reagents, solvents, and chemicals used in this study were of the highest purity available. The solvents were dried prior to use. Column chromatography was performed using 60–120 mesh silica gel. NMR spectra were recorded using Jeol JNM λ−300 (300 MHz for ^{1}H) spectrometer. The chemical shifts (δ) are reported in ppm downfield from the internal standard, TMS, for ^{1}H NMR. Mass spectra were recorded on a Kratos PCKompact SEQ V1.2.2 MALDI-TOF spectrometer or on a MicroMass ESI-TOF spectrometer or Shimadzu table-top GC-MS or ESI-MS (HP1100LC-MSD). Infrared (IR) spectra were recorded on a Jasco FT-IR 410 spectrometer using KBr pellets or neat. Gemini lipids were synthesized as described below and were characterized fully by their ^{1}H NMR, mass spectra, and elemental analysis.

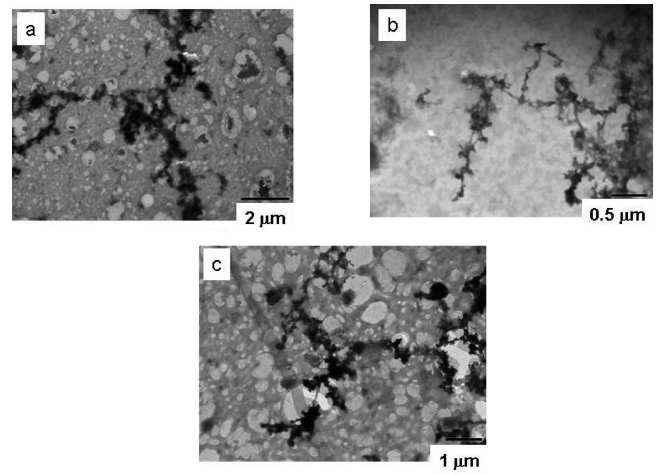


Figure 6. Transmission electron micrographs for the lipoplexes prepared from optimized gemini lipid−DOPE liposome/DNA complexes at the optimized N/P ratio. (a) **1**; (b) **2**; (c) **3**.

Synthesis. 2-Pyridinyl 3-cholesteryl Disulfide (5).[66] Thiocholesterol (**4**; 0.8 g, 2 mmol) and 2,2-pyridyl disulfide (0.48 g, 2.2 mmol) were dissolved in 5 mL of dry chloroform. The reaction mixture was stirred at room temperature for 48 h under nitrogen. Solvent was then removed by rotary evaporation under reduced pressure. The residue was washed with 20 mL of methanol and filtered. The crude product was purified by silica gel column chromatography [hexane to hexane/ethyl acetate (20/1)]. $R_f = 0.8$, [hexane/ethyl acetate (2/1)]. A white solid (0.8 g, 80% yield) was obtained. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.66 (s, 3H); 0.85–2.01 (m, 38H); 2.36 (d, 2H, $J = 8.0$ Hz); 2.72–2.82 (1H, m); 5.33 (1H, d); 7.03–7.07 (1H, m); 7.59–7.64 (1H, m); 7.77 (1H, d, $J = 9.0$); 8.44 (1H, d, $J = 6.0$). ESI-MS: m/z 512.5 ($M + H^+$). HRMS for $C_{32}H_{50}NS_2$: calcd 512.3384, found 512.3368.

***N*,*N*-Dimethylaminoethyl 3-Cholesteryl Disulfide (6).**[66] To a solution of **5** (1.0 g, 2 mmol) in dry $CHCl_3$ (20 mL), triethylamine (5 mL) and *N*,*N*-dimethylaminoethylthiol hydrochloride (1.4 g, 10 mmol) were added. The reaction mixture was stirred at room temperature for 24 h. After the completion of the reaction, the solution was washed with 2% aqueous KOH (50 mL) and 50 mL of brine. The oil layer was dried over anhydrous Na_2SO_4, filtered, and evaporated. The crude product was purified by silica gel column chromatography [hexane/ethyl acetate (3/1) to (2/1)]. $R_f = 0.2$, [hexane/ethyl acetate (2/1)]. A colorless viscous liquid (0.8 g, 80% yield) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.67 (s, 3H); 0.84–1.64 (m, 33H); 1.76–2.02 (m, 5H); 2.26 (s, 6H); 2.34 (s, 1H); 2.57–2.62 (t, 2H, $J = 6.0$ Hz); 2.78–2.82 (t, 2H, $J = 6.0$ Hz); 5.36 (s, 1H). ESI-MS: m/z 506.2 ($M + H^+$).

General Synthesis of Gemini Lipids. A solution of *N*,*N*-dimethylaminoethyl 3-cholesteryl disulfide (**6**; 0.2 mmol) and an appropriate dibromo compound (0.07 mmol) in dry MeOH-EtOAc (4 mL, v/v:1/1) was refluxed over a period of 48–96 h in screw-top pressure tube, after which TLC indicated complete disappearance of the starting dibromide. Then reaction mixture was cooled and the solvent was evaporated to furnish a crude solid. It was repeatedly washed with ethyl acetate to remove any of unreacted *N*,*N*-dimethylaminoethyl 3-cholesteryl disulfide (**6**), and the residue was finally subjected to repeated crystallizations from a mixture of MeOH and ethyl acetate. This afforded white solid in each case. The purities of these lipids were ascertained from TLC (silica gel), and the R_f ranged from 0.2 to 0.3 in 10:1 $CHCl_3$/MeOH. The product yields ranged from 50 to 60%. All the new gemini lipids were fully characterized by ^{1}H NMR, mass spectrometry, and C, H, N analysis. Pertinent spectroscopic and analytical data are given below.

Lipid 1. ^{1}H NMR ($CDCl_3$) δ 0.67 (6H, s); 0.84–2.32 (86H, m); 2.72 (2H, m); 3.12 (4H, s); 3.39 (12H, s), 3.76 (8H, m), 5.38 (2H, d). ESI-MS: 1161.7 ($M^{+2} + Br^-$), 606.5, 592.5, 540.5 ($M^{+2}/2$). Anal. ($C_{67}H_{120}N_2S_4Br_2$) C, H, N.

Lipid 2. ^{1}H NMR ($CDCl_3$) δ 0.67 (s, 6H); 0.84–2.32 (m, 80H); 2.33 (br. m, 4H); 2.73 (br s, 2H); 3.30 (br s, 16H); 3.79 (br s, 4H); 5.38 (s, 2H); 7.80 (s, 4H). ESI-MS: 1195.6 ($M^{+2} + Br^-$), 557.5 ($M^{+2}/2$). Anal. ($C_{70}H_{118}N_2S_4Br_2$) C, H, N.

Lipid 3. ^{1}H NMR ($CDCl_3$) δ 0.65 (6H, s); 0.83–2.30 (80H, m); 2.73 (4H, m); 3.05 (2H, m); 3.46 (12H, s); 4.36 (12H, m); 5.36 (2H, d). ESI-MS: 1163.7 ($M^{+2} + Br^-$), 541.5 ($M^{+2}/2$). Anal. ($C_{66}H_{118}N_2OS_4Br_2$) C, H, N.

Liposome Preparation. Individual lipid or its mixture with DOPE in the desired mol ratio was dissolved in chloroform in autoclaved Wheaton glass vials. Thin films were made by evaporation of the organic solvent under a steady stream of dry nitrogen. Last traces of organic solvent were removed by keeping these films under vacuum overnight. Freshly autoclaved water (Milli-Q) was added to individual film such that the final concentration of the cationic lipid was 0.5 mM. The mixtures were kept for hydration at 4 °C for 10–12 h and were repeatedly freeze–thawed (ice-cold water to 70 °C) with intermittent vortexing to ensure hydration. Sonication of these suspensions for 15 min in a sonicator bath at 70 °C afforded closed, cationic liposomes, as evidenced from transmission electron microscopy. Liposomes were prepared and kept under sterile conditions. Formulations were stable and, if stored frozen, possessed long shelf life.

Transmission Electron Microscopy. Freshly prepared aqueous suspensions of each cationic lipid (0.5 mM) or their lipoplexes were examined under transmission electron microscopy by negative staining using 1% uranyl acetate. A 10 μL sample of the suspension was loaded on to Formvar-coated, 400 mesh copper grids and allowed to remain for 1 min. Excess fluid was wicked off the grids by touching their edges to filter paper, and 10 μL of 1% uranyl acetate was applied on the same grid, after which the excess stain was similarly wicked off. The grid was air-dried for 30 min, and the specimens were observed under TEM (JEOL 200-CX) operating at an acceleration voltage of 120 keV. Micrographs were recorded at a magnification of 5000–20000×.

Plasmid DNA. pEGFP-c3 (Clontech, U.S.A.), which encodes for an enhanced green fluorescence protein (GFP) under a CMV promoter, was amplified in *Escherichia coli* (DH5α) and purified using Qiagen Midi Prep Plasmid Purification protocol (Qiagen, Germany). Purity of the plasmid was checked by electrophoresis on 1.0% agarose gel. Concentration of the DNA was estimated spectroscopically by measuring the absorption at 260 nm and confirmed by gel electrophoresis. The plasmid preparations showing a value of $OD_{260}/OD_{280} > 1.8$ were used.

Cell Culture. Cells (HeLa, HT1080, PC3AR) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma), supplemented with 10% fetal bovine serum (FBS) in T25 culture flasks (Nunc, Denmark), and were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were regularly passaged by trypsinization with 0.1% trypsin (EDTA 0.02%, dextrose 0.05%, and trypsin 0.1%) in PBS (pH 7.2). HaCat cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma), supplemented with 10% heat inactivated fetal bovine serum (FBS).

Cytotoxicity. Toxicity of each cationic lipid formulation toward HeLa cells in the presence of 10% FBS was determined using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide reduction assay following literature procedures.[72,73] Nearly 15000 cells/well were plated in 96-well plate. After 24 h, optimized lipid/DOPE formulations were complexed with 0.2 μg of the DNA at various N/P ratios for 30 min. DNA−lipid complexes were added to the cells in the absence of serum. After 6 h of incubation, lipoplexes were removed and 200 μL of media with 10% FBS was added. After 42 h, 20 μL of MTT solution was added and the cells were incubated further for 4 h. Blue formazan crystals were seen at well when checked under microscope. Media was removed and 200 μL of DMSO was added per well. The absorbance was measured using microtiter plate reader.

Transfection Procedure. All transfection experiments were carried out in HeLa Cells in antibiotic-free media unless specified otherwise. In a typical experiment, 24-well plates were seeded with 45000 cells/well in antibiotic-free media 24 h before transfection such that they were at least ∼70% confluent at the time of transfection. For transfection, lipid formulation and DNA were serially diluted separately in DMEM containing no serum to have the required working stocks. DNA was used at a concentration of 0.8 μg/well unless specified otherwise. The lipid and DNA were complexed in a volume of 200 μL upon incubation of the desired amount of lipid formulation and plasmid DNA together at room temperature for about 30 min. The lipid concentrations were varied so as to obtain the required lipid/DNA (N/P) charge ratios. Charge ratios here present the ratio of charge on cationic lipid (in mol) to nucleotide base molarity and were calculated by considering the average nucleotide mass of 330. After 30 min of complexation, 200 μL of media were added to the complexes (final DNA concentration = 12.12 μM). Old medium was removed from the wells, cells were washed with DMEM, and lipid−DNA complexes in 200 μL of media were added to the cells. The plates were then incubated for 6 h at 37 °C in a humidified atmosphere containing 5% CO_2. At the end of the incubation period, medium was removed and cells were washed with DMEM, 500 μL of DMEM containing 10% FBS was added per well. Plates were further incubated for a period of 42 h before checking for the reporter gene expression. GFP expression was examined by fluorescence microscopy and was quantified by flow cytometry analysis. Transfections with Lipofectin

and Lipofectamine 2000 have been performed according to given protocols. In the manuscript, Lipofectin is designated as "Formulation A" and Lipofectamine 2000 as "Formulation B".

For transfections in the presence of serum, lipid, and DNA were separately diluted in serum-free media, as already mentioned, and the complexation was done in serum-free media (200 μL) for 30 min. The complex was then diluted to 400 μL with DMEM containing 20% FBS so as to achieve a final serum concentration of 10%. The cells were then incubated with this complex for 6 h. At the end of the incubation period, medium was removed, and cells were washed with DMEM, 500 μL of DMEM containing 10% FBS was added per well.

Gel Electrophoresis. To examine the complexation of DNA with cationic lipid suspensions at different lipid−DNA ratios, we prepared lipid−DNA complexes at different lipid−DNA charge ratios in an identical manner as was done with transfection experiments. After 30 min of incubation, these complexes were electrophoretically run on a 1.0% agarose gel. The uncomplexed DNA moved out of well but the DNA that was complexed with lipid remained inside the well.[20]

Flow Cytometry. The reporter gene expression was examined by fluorescence microscopy at regular intervals and was quantified 48 h post-transfection by flow cytometry. Percentage of transfected cells were obtained by determining the statistics of cells fluorescing above the control level, wherein nontransfected cells were used as the control. Approximately 10000 cells were analyzed to achieve the statistical data. For flow cytometry analysis, ∼48 h post-transfection, old medium was removed from the wells; cells were washed with PBS and trypsinized by adding 100 μL of 0.1% trypsin. To each well, 200 μL of PBS containing 20% FBS was added. Duplicate cultures were pooled and analyzed by flow cytometry immediately using Becton and Dickinson flow cytometer equipped with a fixed laser source at 488 nm.

FACS Analysis. FACS data were analyzed by public domain WinMDI software to eliminate data from cell debris (particles smaller than cells), dead cells, and clumps of two or more cells. Subcellular debris and clumps can be distinguished from single cells by size (estimated by the intensity of low angle forward scatter). Dead cells have lower forward-scatter and higher side-scatter than living cells. The FACS scans have been configured to display the fluorescence signals only from those particles with a specified set of scatter properties, namely, living single cells. This is called a scatter-gated fluorescence analysis. The data from dead cells were eliminated by gating out brightly fluorescent cells.

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Supporting Information Available: Elemental analysis and ESI-MS spectra of all gemini lipids. This material is available free of charge via the Internet at http://pubs.acs.org.

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