Design, Synthesis, and in Vitro Gene Delivery Efficacies of Novel Cholesterol-Based Gemini Cationic Lipids and Their Serum Compatibility: A Structure−Activity Investigation

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Five cholesterol-based gemini cationic lipids, which differ in the length of the spacer [−$(CH_2)_n$−] chain between the head groups, have been synthesized. These lipids are useful as nonviral gene delivery agents, and all cholesterol-based gemini lipids (**2a**−**2e**) are better transfecting agents than their monomeric lipid counterpart **1**. Transfection efficiency of all the gemini lipids except lipid **2a** [−$(CH_2)_3$−] was maintained even when the serum was present during the transfection conditions as compared to the monomeric lipid **1**, with which a dramatic decrease in transfection efficiency was observed. With the increase in spacer chain length from propanediyl [−$(CH_2)_3$−] to pentanediyl [−$(CH_2)_5$−], transfection efficiency increased both in the absence and presence of serum. However, transfection efficiency decreased with further increase in the length from the pentanediyl [−$(CH_2)_5$−] to the dodecanediyl [−$(CH_2)_{12}$−] spacer. Among these gemini lipids **2c** showed the highest transfection activity, which was also greater than that of the commercially available formulation.

1. Introduction

Gene therapy, known as the fourth revolution in medicine, is the new hope for curing various genetic disorders by delivering the desired gene to the specific cells.[1] Viruses are the best means of delivering the genes,[2] but recent deaths in clinical trials have raised questions on the use of viruses as gene delivery vehicles.[3] Among nonviral vectors, cationic liposomes,[4] although less efficient, have a great potential for delivery of genetic therapeutic drugs, because of less toxicity, low immunogenicity, high DNA carrying capacity, their ease of large-scale production, and simplicity in preparation and administration.[5−10] Ever since its discovery,[11] cationic lipid-mediated gene delivery remains at the forefront of present day scientific research owing to its possible application in the field of gene therapy.[12]

Most of the cationic lipid formulations studied for gene delivery are based on either glycerol-[13] or cholesterol-based cytofectins.[14] For instance, 3β-[*N*,(*N*,*N*-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol),[a] a cationic cholesterol derivative, has been successfully used as a coaggregate with 1,2-dioleoyl-L-α-glycero-3-phosphatidyl ethanolamine (DOPE) to prepare liposomes that transfect mammalian cells efficiently.[15] Cationic lipid suspensions readily form complexes with the negatively charged DNA (gene) under ambient conditions. The molecular structure of cationic lipids is an important parameter that controls their DNA complexation and gene transfection activity. For instance, the functional group that links the polar head group and the hydrocarbon chains of such lipid molecules plays a crucial role in their utilization in gene transfer events. Thus, *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA), which contains an *ether* linkage between the head group and the long alkyl chains, shows greater *in vivo* transfection efficiency than the corresponding *ester* analogue *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTAP).[16,17] We have shown that the use of the *ether* linkage leads to a dramatic increase in transfection efficiencies of cholesterol-based cationic lipids as compared to the ester- or urethane-based cholesterol lipid analogues.[18,19] Hydrocarbon-chain-based lipids with varying oxyethylene linkages in cationic pseudoglyceryl lipids have also been studied for gene transfection activities. It has been observed that incorporation of oxyethylene units at linkages brought about significant enhancement in the *in vitro* gene delivery efficiencies of the cationic lipids in mammalian cells.[20,21] The presence of the ether linkage makes these compounds hydrolytically stable, and their aqueous suspensions were also found to have long shelf life.

Clearly, design and syntheses of new lipid systems with alternative structural types are crucial for the development of potent synthetic vectors for such applications. Gemini lipids are another class of lipids, where two lipid molecules are joined to each other *via* a spacer. We reported, for the first time, the synthesis and aggregation properties of various glycerol-backbone-based gemini lipids.[22,23] The effect of the spacer length on the aggregation properties of the gemini lipids was demonstrated in these investigations. There are, however, very few reports on the transfection properties of gemini lipids. Ahmad and co-workers have shown transfection properties of cardiolipin-based gemini lipids.[24,25] More recently, gemini surfactants based on lipophilic pyrilium salts have also been examined for gene delivery.[26] Recently, we have reported the synthesis of gemini cationic lipids based on an aromatic backbone between the hydrocarbon chains and headgroup.[27] However, there is no systematic study until now that examines the effect of the spacer on the gene transfection activities in a given series of gemini lipids. To our knowledge, there is no report on cholesterol-based gemini lipids as well.

Toward this end, we have designed five cholesterol-based gemini cationic lipids (**2a**−**2e**) having a polymethylene spacer between the cationic ammonium headgroups (Figure 1). These gemini lipids differ in the length of the spacer chain, varying from propanediyl to dodecanediyl. Though synthetically more

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[a] Abbreviations: DC-Chol, 3β-[*N*,(*N*,*N*-dimethylaminoethane)-carbamoyl] cholesterol; DOPE, 1,2-dioleoyl-L-α-glycero-3-phosphatidylethanolamine; DOTMA, *N*-[1-(2,3-dioleyloxy)]-*N*,*N*,*N*-trimethylammonium chloride; DOTAP, *N*-[1-(2,3-dioleyl)]-*N*,*N*,*N*-trimethylammonium chloride; MFI, mean fluorescence intensity; FACS, fluorescence-activated cell sorting.

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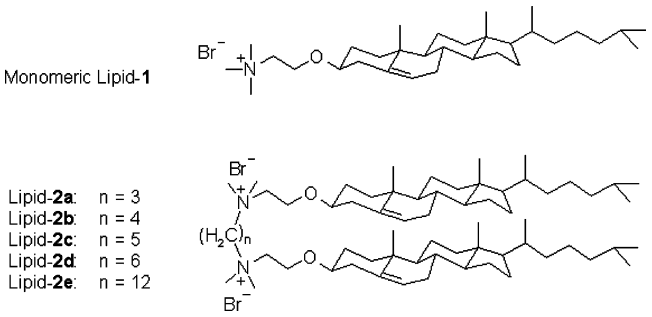


Figure 1. Molecular structures of cholesterol-based cationic monomeric lipid **1** and gemini lipids (**2a**−**2e**) synthesized and used for transfection studies.

demanding, an ether functionality was chosen between the cholesterol and headgroup mainly due to the enhanced biological activities of the cationic lipids having ether linkages compared to those of ester analogues, as mentioned earlier, and also due to the improved chemical stability of ether linkages.

Though unnatural, rigid cholesterol-based gemini lipids form an important class. These gemini lipids are expected to have different aggregation and transfection properties than those that are based on fatty acid chains. In this series of gemini lipids we have chosen a flexible hydrophobic polymethylene spacer such that the spacer between headgroups varies in length. In this paper, we describe the detailed synthetic procedures and optimized transfection activities of these biologically active compounds. To put our results in appropriate perspective, we compared the transfection activities of these new gemini cationic lipids (**2a**−**2e**) with those of the monomeric cholesterol-based lipid **1**. We have observed enhanced gene transfection activities for all these gemini lipids as compared to those of the monomeric one. Serum often inhibits the transfection efficiency of cationic liposomes. However, four of these gemini lipids except gemini lipid **2a** manifest significantly improved transfection activity even in the presence of serum.

2. Results and Discussion

2.1. Chemistry. Five cholesterol-based gemini lipids with a polymethylene spacer have been synthesized with different spacer lengths. These gemini lipids have been synthesized from the precursor cholest-5-en-3β-oxyethan-*N*,*N*-dimethylamine (**7**) by reacting it with corresponding α,ω-dibromoalkanes (Scheme 1). Compound **7** was synthesized from cholest-5-en-3β-oxyethane tosylate (**6**), which was synthesized by slightly modifying a reported procedure.[28] First, cholesterol (**3**) was tosylated using *p*-toluenesulfonyl chloride in pyridine−chloroform (v/v: 1/1) with a catalytic amount of DMAP for 6 h at 0 °C in 92% yield. Cholesterol tosylate (**4**) was then subjected to a reaction with ethylene glycol in dioxane under reflux for 4 h to afford cholest-5-en-3β-oxyethan-2-ol (**5**) in 85% yield. Compound **5** was then tosylated with *p*-toluenesulfonyl chloride in pyridine−chloroform (v/v: 1/1) for 6 h at 0 °C to get **6** in 90% yield. Reaction of the tosylate (**6**) with dimethylamine in dry MeOH in a screw-top pressure tube at 80 °C afforded the precursor **7** in quantitative yields. Gemini lipids with flexible hydrophobic spacers have been synthesized by reacting **7** with corresponding α,ω-dibromoalkanes in 50−60% yields. All the gemini lipids have been purified by repeated crystallizations from MeOH and EtOAc. Lipids **2a** and **2d** were found to be quite hygroscopic in nature. All the gemini lipids were characterized by ^{1}H−NMR, ^{13}C−NMR, ESI-MS, and elemental analysis as described in the Experimental Section. Lipid **1** was synthesized as reported previously.[28]

2.2. Aggregate Formation from Cationic Gemini Lipids. Upon hydration, all the gemini lipids except **2a** were found to get dispersed in water easily. Lipid **2a** could be also dispersed albeit with difficulty. All the lipid molecules mentioned here formed stable suspensions in water. The suspensions formed from the lipids were found to be translucent. Generally, TEM examination of air-dried, aqueous suspensions revealed the existence of closed aggregate structures for all the gemini lipids (not shown). The suspensions formed from **2a** showed some distinct aggregates. The aggregates formed from all the gemini lipids ranged from 30 to 90 nm in size. When compared, all gemini lipid aggregates were generally found to be smaller in size as compared to the aggregates of monomeric lipid **1**, which is found to be 100 ± 30 nm in size (not shown).

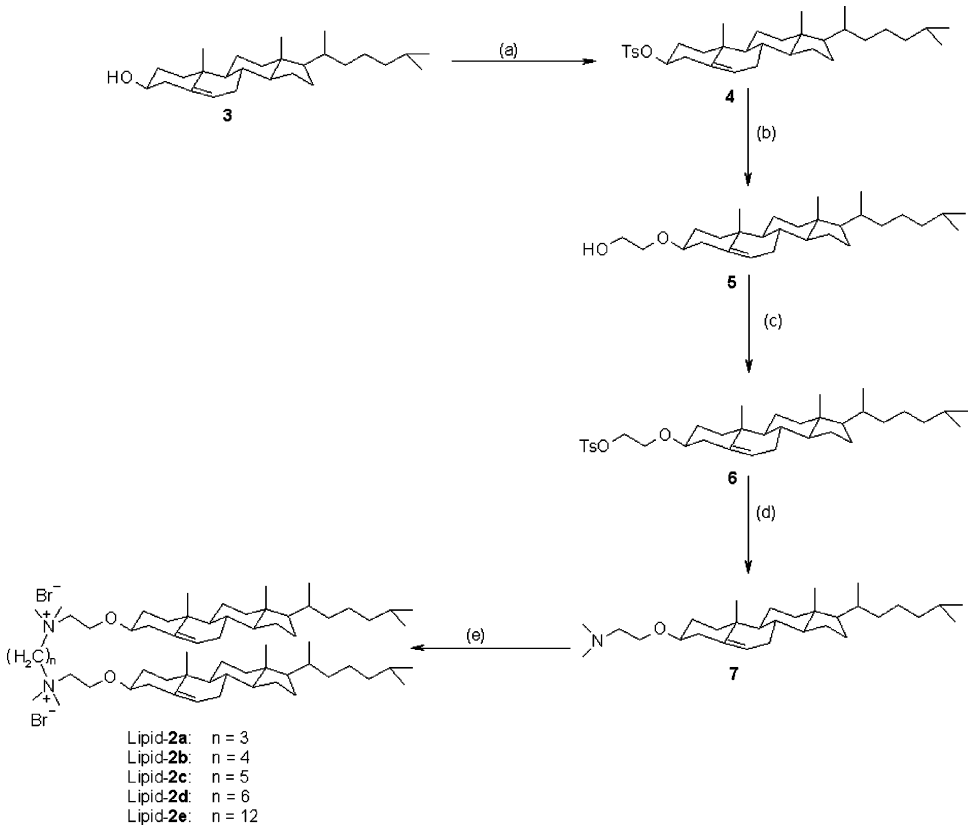
2.3. Mixed Liposome Formation with 1,2-Dioleoyl-L-α-glycero-3-phosphatidyl Ethanolamine (DOPE). Liposomes could be conveniently prepared from each of the gemini lipids with naturally occurring helper lipid (1,2-dioleoyl-L-α-glycero-3-phosphatidyl ethanolamine (DOPE)) by first subjecting the films of the lipid mixtures to hydration and repeated freeze−thaw cycles, followed by sonication at 60 °C for 15 min. All the gemini lipids formed optically transparent 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 3 months if stored at 4 °C.

2.4. Gel Electrophoresis. To characterize the electrostatic binding interactions between the plasmid DNA and the mixed cationic liposomes as a function of different N/P charge ratios (or lipid/DNA mol ratios), we performed conventional electrophoretic gel retardation assays (Figure 2). All gemini lipid suspensions were able to retard the plasmid DNA from the well at N/P ratio of 1.0. It should be noted that every gemini molecule possesses two hard charges, which indicates that the whole plasmid DNA gets retarded at lipid/DNA mol ratio of 0.5. Significant retardation of DNA was also observed at N/P ratio of 0.5 (or lipid/DNA mol ratio of 0.25) as well.

2.5. Transfection Biology. 2.5.1. Optimization of Lipid/DOPE Ratio. Naturally occurring lipids such as DOPE have been known to increase the efficiency of transfection in lipid-mediated gene transfer applications.[16] In order to find out the most effective formulations, transfections with identical lipid/DNA mol ratios (or N/P ratios), varying the mol ratio of the gemini lipids (**2a**−**2e**) in DOPE, were performed (Figure 3). To find out the optimized transfection efficiency, both the number of transfected cells and the mean fluorescence intensity have been 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.[29] These data were obtained from flow cytometric analysis.

All the gemini lipids are most effective at a lipid/DOPE mol ratio of 1:4, except lipid **2d**, which was found to be most effective at a lipid/DOPE mol ratio of 1:5. When the transfection efficiencies at their optimal lipid/DOPE ratio were compared, lipids **2c** and **2d** were found to be superior transfecting agents. Although the numbers of transfecting cells were high in the case of lipids **2a** and **2b**, the MFI was low. At this lipid/DOPE ratio, although the number of transfected cells were very high, they could transfect a considerably lesser amount of DNA.[29] Therefore, lipids **2a** and **2b** transfected more cells with low MFI, whereas lipids **2c** and **2d** were able to transfect a lesser number of cells but with high MFI. In contrast, the gemini lipid **2e** at

Scheme 1. Synthesis of Cholesterol-Based Gemini Lipids[a]



[a] Reaction conditions: (a) *p*-TsCl/Py/$CHCl_3$, 0 °C, 6 h; (b) ethylene glycol, 1,4-dioxane, 4 h, reflux; (c) *p*-TsCl/Py/$CHCl_3$, 0 °C, 6 h; (d) dimethylamine, MeOH, 80 °C, screw-top pressure tube; (e) MeOH−EtOAc, α,ω-dibromoalkanes, 80 °C, screw-top pressure tube, 48−72 h.

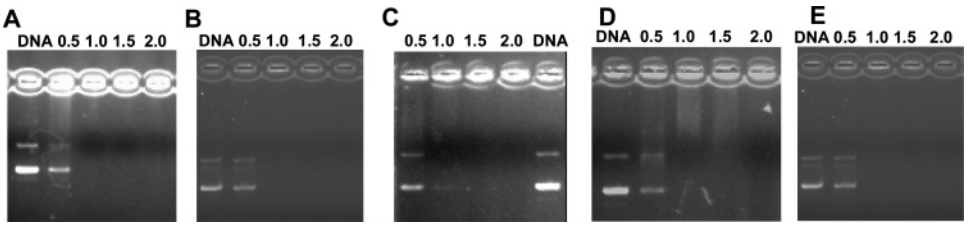


Figure 2. Electrophoretic gel patterns for lipoplex-associated DNA in gel retardation for gemini lipids: (A) **2a**, (B) **2b**, (C) **2c**, (D) **2d**, and (E) **2e**. The N/P ratios are indicated at the top of each lane.

different lipid/DOPE mol ratios was found to be less effective both in terms of the number of transfected cells and the MFI.

2.5.2. Optimization of N/P Ratio. After optimizing the lipid/DOPE ratio for every lipid, all the gemini lipids were tested by taking the identical amount of DNA (0.8 μg) and varying the amount of lipid using the respective optimized lipid/DOPE mol ratio (Figure 4). Lipid **2a** was able to transfect to a maximum extent of 40% of the cells with MFI of ∼125 at N/P ratio of 0.25, whereas lipid **2b** could transfect approximately 50% of the cells with a nearly identical MFI at the same N/P ratio. Transfection efficiency was found to decrease at higher N/P ratios, especially in terms of MFI. Gemini lipid **2c** was able to transfect only 40% of cells at N/P of 0.5 or 0.75, but the MFI observed was found to be very high as compared to that showed by either lipids **2a** or **2b**. The MFI observed with lipid **2c** was ∼150, which was approximately 2 times greater than that affected by gemini lipids **2a** and **2b** at N/P ratio of 0.5. Gemini lipid **2d** showed a maximum transfection efficiency of 40−60% with an MFI of ∼120 at N/P ratio of 0.5. At N/P ratio of 0.25, the maximum MFI of ∼145 was observed, but the number of transfected cells observed was less as compared to that at N/P ratio of 0.5. But with the dodecamethylene-spacer-based gemini lipid (**2e**), the transfection efficiency decreased drastically. Nearly 50% of all the cells get transfected with a low MFI of ∼60, when gemini lipid **2e** was used at N/P ratio of 0.5 with EGFP-c3. The number of transfected cells at the N/P ratio of 1.0 was high, but the MFI was found to be very low. All the gemini lipids were found to be better transfecting agents than the monomeric counterpart **1**. The number of transfecting cells was 60% in the case of monomeric lipid **1**, but the MFI observed was very less. All the gemini lipids followed a bell-shaped graph, when transfection efficiencies were plotted against N/P ratio. When all the gemini lipids were compared at N/P ratio of 0.5 (Figure S2, Supporting Information), lipid **2c** was found to be the best transfecting agent in terms of MFI, although the number of transfected cells was found be less. The transfecting efficiency increased with the increase in the spacer chain length from lipid **2a** to lipid **2c** and then decreased while going from lipid **2c** to **2d** or **2e**. Lipid **2c** was found to have the maximum MFI, which was in fact comparable to that of formulation **E**, one of the best currently commercially available transfecting agents.

2.5.3. Effect of Serum. One of the major drawbacks of the known cationic lipids for their *in vivo* use is the inhibition of

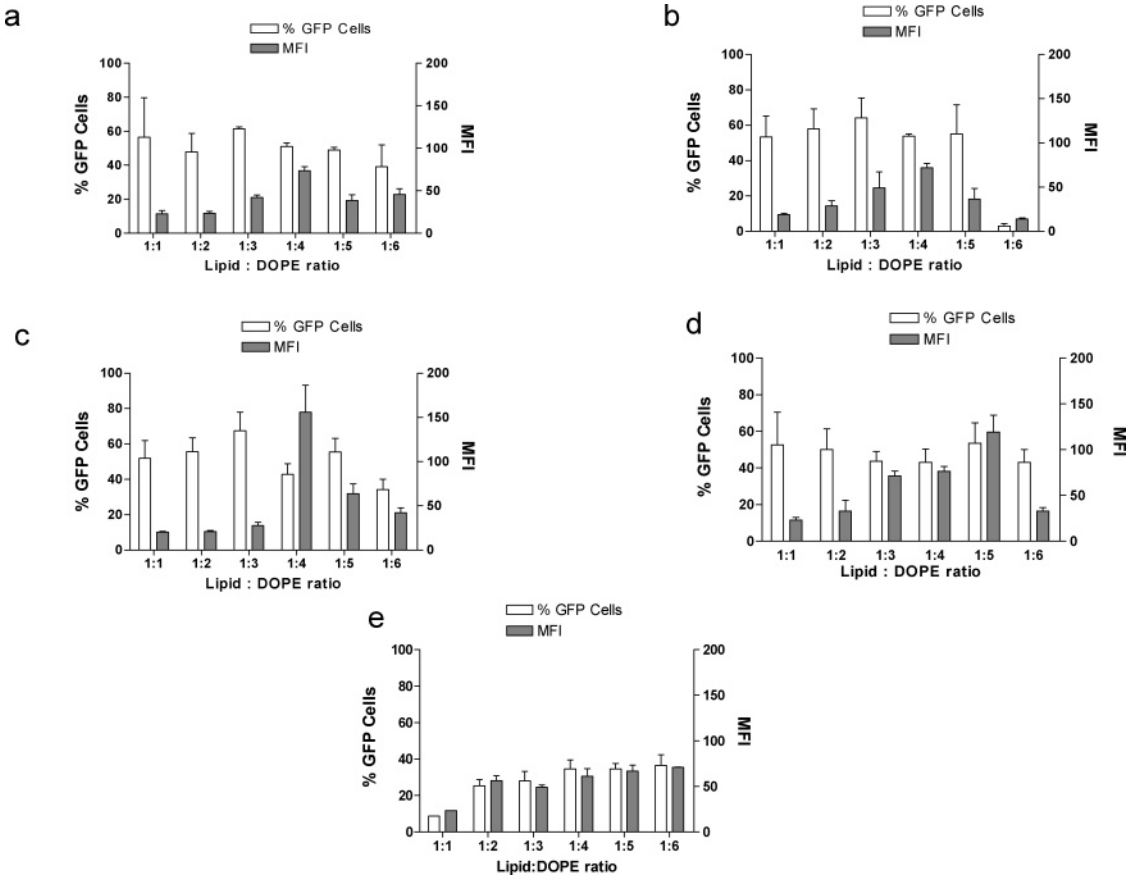


Figure 3. Transfection efficiencies of EGFP-c3 DNA of cholesterol-based gemini lipids with various compositions of DOPE: (a) **2a**, (b) **2b**, (c) **2c**, (d) **2d**, and (e) **2e**. The concentration of DNA = 0.8 μg/well, and lipid was used at N/P ratio of 0.5. Data are expressed as the number of transfected cells and MFI as obtained from flow cytometry analysis.

the transfection efficiency of cationic liposomes in the presence of serum. Very few cationic lipids are known which demonstrate transfection activity in the presence of serum,[10,21,30] although their efficiency is quite low. To our knowledge there is no gemini lipid known in the literature that shows transfection activity in the presence of serum. For instance, monomeric lipid **1**, which is found to be a good transfecting agent as a coliposome with DOPE, loses much of its efficiency as a transfection reagent in the presence of serum. Maintenance of the transfection efficiency in the absence of serum even for *in vitro* experiments is also cumbersome often due to the toxic nature of the synthetic cationic lipid formulations.

Therefore, in order to investigate the effect of serum on the gene transfection efficiencies of cholesterol-based gemini lipids, we performed transfection experiments in the presence of 10% serum with the optimized lipid/DOPE formulation at different N/P ratios using plasmid EGFP-c3 and analyzed the data by flow cytometry (Figure 4). The transfection efficiency of monomeric lipid **1** decreased drastically, whereas in the case of the commercially available reagent, formulation **E**, there was ~70% decrease in the MFI of the transfected cells in the presence of serum. In the case of gemini lipid **2a**, there was no change in the number of transfected cells, but the MFI decreased in the presence of serum. This shows that lipid **2a** is able to transfect the same number of cells in the presence of serum, but the amount of DNA delivered gets reduced in the presence of serum, whereas in the case of lipid **2b** the reduction in transfection efficiency was not so appreciable as compared to that of lipid **2a**. Serum hardly inhibits the transfection efficiency of lipid **2c** both in terms of the percentage of transfected cells and the MFI. As a matter of fact there was little enhancement in the transfection efficiency in the presence of serum in case of lipid **2c**, both in terms of percentage of transfected cells and MFI. At N/P ratio of 0.25, gemini lipid **2c** showed an ~1.5-fold increase in MFI, and the transfection efficiency was found to better than that of formulation **E** (Figure 4). Lipid **2c** is able to transfect nearly 50% of the cells with MFI of ~175 in the presence of serum at N/P ratio of 0.5−0.75, which is greater than formulation **E**. In the case of the gemini lipid **2d**, the presence of serum did not inhibit the transfection efficiency at all. In fact, there was some increase in the number of transfected cells and the MFI in the presence of serum in this instance. To our pleasant surprise, there was a significant increase in the transfection efficiency of gemini lipid **2e** in the presence of serum as compared to the one carried out in the absence of serum. Lipid **2e** was able to transfect only to the extent of 35−40% of the cells with MFI of ~60 in the absence of serum, but in the presence of serum, the transfection efficiency increased to 60−70% with MFI of ~110 at N/P ratio of 0.5. Similarly, in the presence of serum, all gemini lipids followed a bell-shaped curve, possessing maximum transfection at N/P ratio of 0.5−0.75.

All the gemini lipids were found to be better transfecting agents than the monomeric one even in the presence of serum. Even the transfection efficiency of gemini lipid **2c** was found to be greater than one of the best commercially available reagents, formulation **E**, which is also known to show transfection in the presence of serum. Gemini lipid **2e** showed a 2-fold increase in the transfection efficiency in the presence of serum both in terms of the number of transfected cells and the MFI as compared to that in the absence of serum. Flow cytometric scans of transfection experiments using lipid the

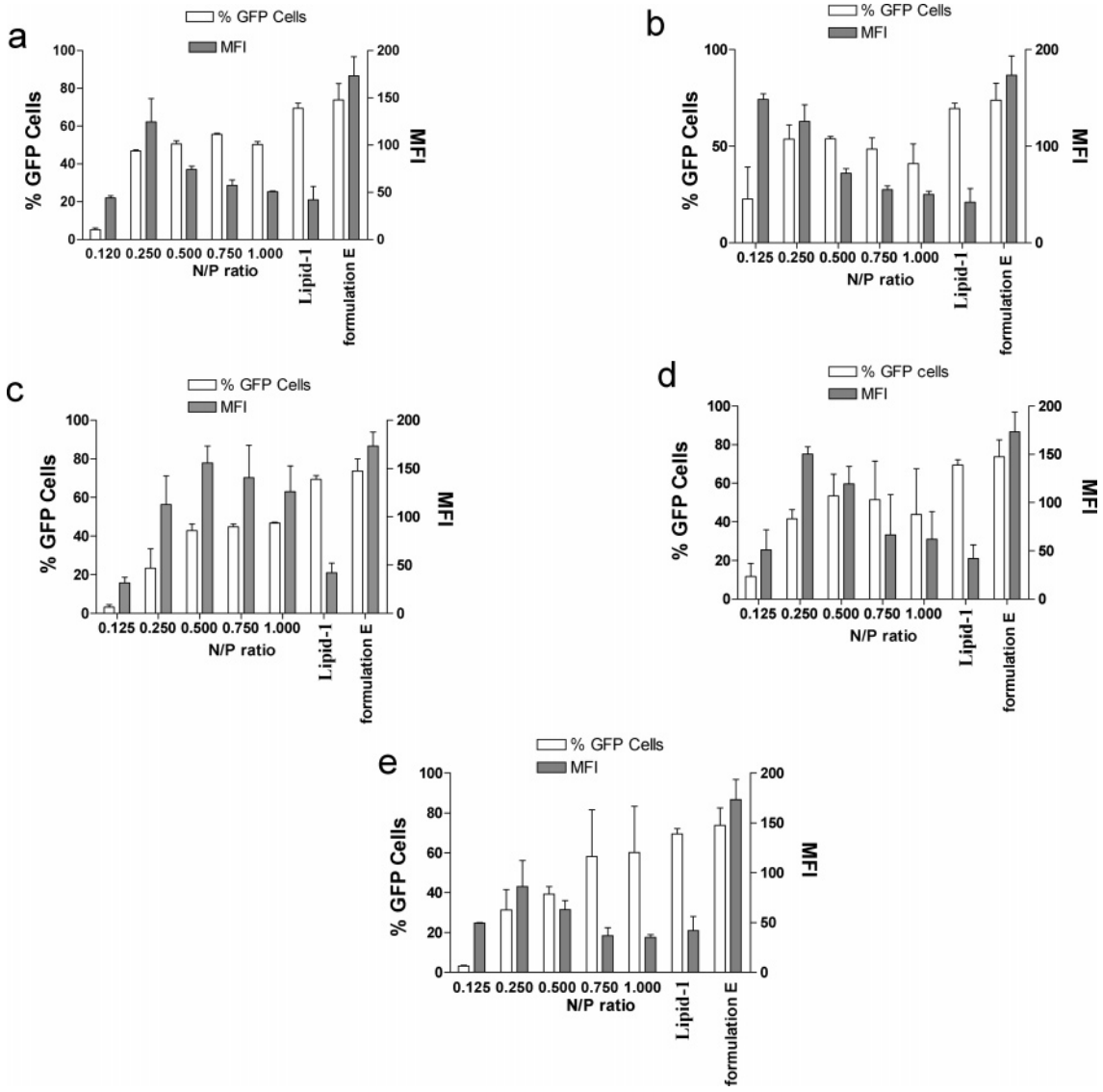


Figure 4. Transfection efficiencies of gemini lipids using optimized lipid/DOPE formulations at various N/P ratios in the absence and the presence of serum: (a) **2a**, (b) **2b**, (c) **2c**, (d) **2d**, and (e) **2e**. The concentration of DNA = 0.8 μg/well. Data are expressed as the number of transfected cells and MFI as obtained from flow cytometry analysis.

2e/DOPE (1:4 mol ratio) formulation at N/P ratio of 0.5 in the absence (−FBS−FBS) and presence (−FBS+FBS) of serum are shown in Figure 5. Overall, there was a nearly 2-fold increase in the number of transfected cells (M2 and M3 region), whereas there was a 3-fold increase in number of highly transfected cells (see M3 region) in the presence of serum. This suggests that there are some serum components that must be facilitating the efficient transfection activity with the liposome prepared from this gemini lipid. This was found to happen only in the case of the gemini lipid **2e**, suggesting that there are some structural features at the aggregation level in this lipid that support the enhancement of gene transfection activities in the presence of serum.

2.5.4. Effect of the Variation of the Amount of DNA. To see whether variation in the amount of the DNA affects the transfection efficiency of gemini lipids, we have performed transfection experiments with all the gemini lipids at N/P ratios of 0.25 and 0.5, varying the amount of the DNA from 0.4 to 2.0 μg/well. In the case of lipid **2a**, there was no enhancement in the transfection efficiency at high DNA amounts, although there was a little improvement when 0.4 μg of DNA was used (not shown). Similarly in case of lipid **2b**, at both N/P ratios, transfection efficiencies decreased at higher DNA amounts, but at N/P ratio of 0.25, using 0.4 μg of DNA there was little improvement in the gene transfection efficiency. There was an increase in the transfection efficiency with an increase in the amount of DNA at both N/P ratios in the case of lipid **2c**, the maximum being observed using 1.2−1.6 μg of DNA. At a N/P ratio of 0.5, using 1.6 μg of DNA nearly 70% of the cells get transfected with MFI of ∼160, which is comparable with formulation **E**, both in terms of percentage of transfected cells and MFI (Figure 6). As already shown, MFI comparable to that of formulation **E** was observed using 0.8 μg of DNA at a N/P ratio of 0.5, whereas the number of transfected cells was only 40%. Now using 1.6 μg of DNA at N/P ratio of 0.5 the number of transfected cells increased to 70%, which is comparable to that of formulation **E** without any change in MFI. In the case of lipid **2d** there was no enhancement in gene transfection efficiency, except the little improvement in the MFI at N/P ratio of 0.5 using 0.4 μg of DNA (not shown). We have shown that lipid **2e** shows better transfection efficiency in the presence of serum; therefore, transfection studies with varying DNA amounts were performed in the presence of serum for the lipid **2e**/DOPE (1:4) formulation. With the increase in the amount of DNA,

	M 2	M 3
Lipid **2e** (-FBS-FBS)	22% (GFP Cells)	11 % (GFP Cells)
Lipid **2e** (-FBS+FBS)	31% (GFP Cells)	28 % (GFP Cells)

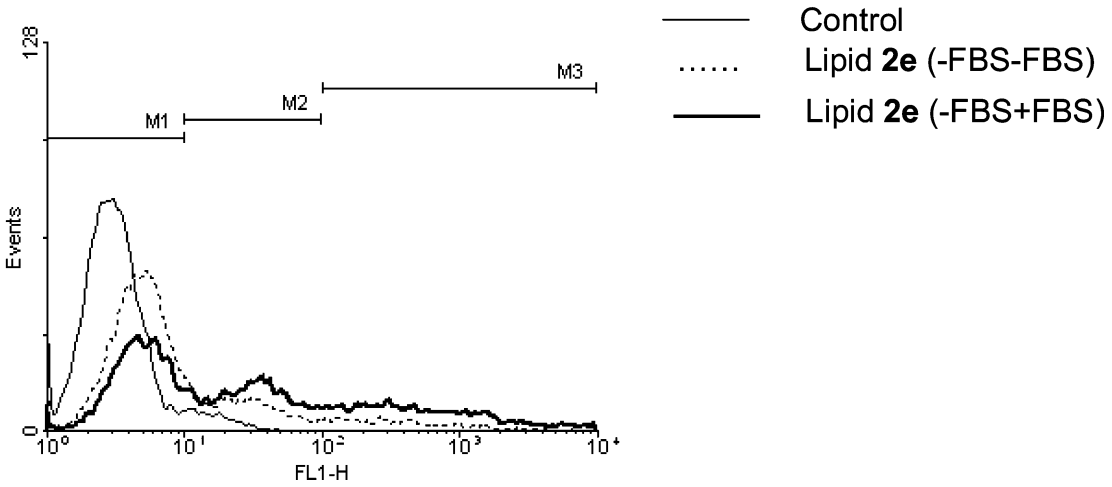


Figure 5. Flow cytometric scans of transfection experiments using the lipid **2e**/DOPE (1:4 mol ratio) formulation, when transfection experiments were performed in the absence (−FBS−FBS) and presence (−FBS+FBS) of serum. Untransfected cells, used as the control, are also shown. M1 marks the population of cells having fluorescence under control, M2 marks the population of cells with medium fluorescence intensity, and M3 marks transfected cells with high fluorescence intensity.

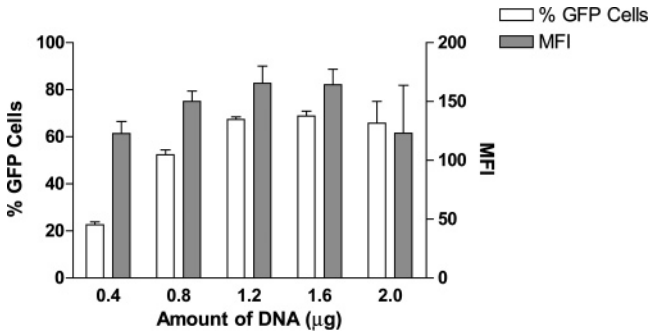
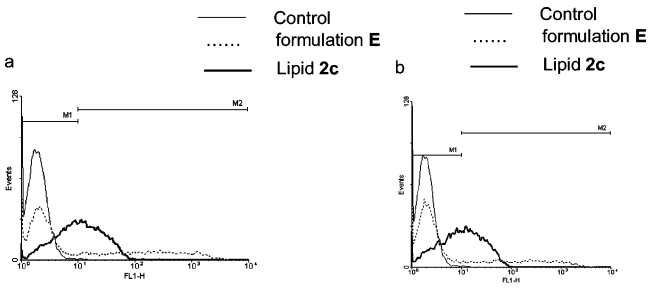


Figure 6. Effect of variation of the amount of DNA on the gene transfection efficiency of the gemini lipid **2c**/DOPE (1:4 mol ratio) formulation at N/P ratio of 0.5.



	30 % FBS	50 % FBS
Lipid **2c**	42 ± 12	40 ± 10
Formulation **E**	33 ± 7	25 ± 5

Figure 7. Flow cytometric scans of gene transfection performed using the lipid **2c**/DOPE (1:4 mol ratio) formulation at N/P ratio of 2.0 and formulation **E** in high percentages of serum: (a) 30% serum; (b) 50% serum.

there is little increase in the number of transfected cells without any significant change in MFI. Lipid **2e** is able to transfect nearly 65% of the cells with an MFI of ∼100 (not shown). Therefore, DNA variation experiments suggest that among the gemini lipids, lipid **2c** shows the maximum transfection comparable to that of formulation **E**, whereas lipid **2e** shows a very good transfection efficiency of 65% with an MFI of 100 in the presence of serum.

2.5.5. GFP Assay at High Serum Concentrations. To see the effect of the high concentrations of serum on gene transfection efficiencies, we have performed the gene transfection efficiencies using the best lipid **2c**/DOPE (1:4) formulation. Flow cytometric scans of transfection experiments using the gemini lipid **2c**/DOPE (1:4) formulation and the formulation **E** in the presence of 30% and 50% serum concentrations are shown in Figure 7. FACS profiles indicate that the lipid **2c**/DOPE formulation is able to transfect nearly 40% of the cells at a high percentage of serum as well at N/P ratio of 2.0, whereas formulation **E** is able to transfect only 25% of the cells at 50% serum concentration. In terms of fluorescence intensity, formulation **E** is able to transfect a lesser number of cells with high MFI, whereas the lipid **2c**/DOPE (1:4) formulation transfects more cells with a low MFI. Here it should be noted that in formulation-**E**-based transfection, an EC buffer and enhancer are also used, whereas gemini lipid formulations are easy to prepare. The low MFI intensity observed in the case of the lipid **2c**/DOPE (1:4) formulation is expected as, at such high serum concentrations, negatively charged serum proteins start to compete with DNA for lipid complexation, therefore reducing the number of copies of the plasmid delivered inside the cell.

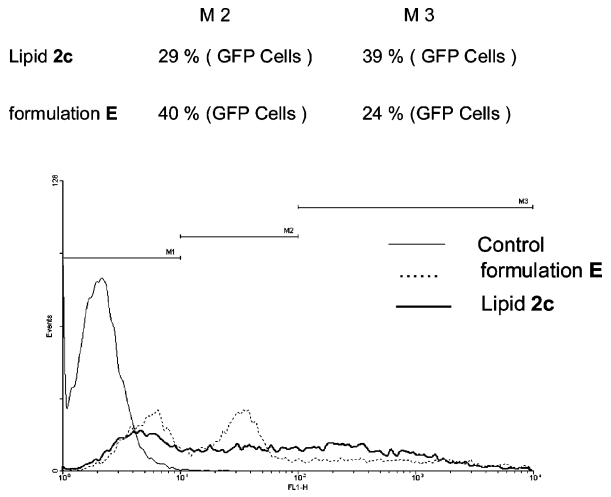


Figure 8. Flow cytometric scans of transfection experiments using the lipid **2c**/DOPE (1:4 mol ratio) formulation and formulation **E**. Untransfected cells, used as the control, are also shown. M1 marks the population of cells having fluorescence under control, M2 marks the population of cells with medium fluorescence intensity, and M3 marks transfected cells with high fluorescence intensity.

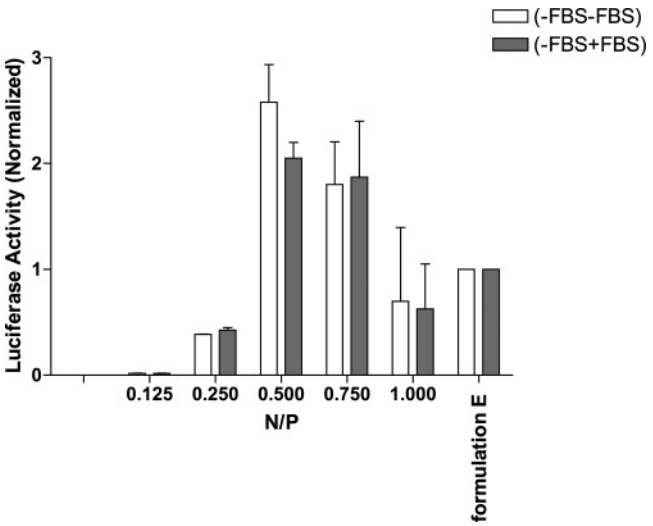


Figure 9. Transfection efficiency of the gemini lipid **2c**/DOPE (1:4 mol ratio) formulation at different N/P ratios as evidenced by luciferase expression in the absence and presence of serum. Shown are the data normalized with respect to formulation **E**.

2.6. Comparison of Gemini Lipid 2c and Formulation E. **2.6.1. GFP Expression.** Flow cytometric scans of transfection experiments using the gemini lipid **2c**/DOPE (1:4) formulation and formulation **E** are shown as an overlay in Figure 8. These profiles have been divided into three regions: M1 presents the number of cells under control (untransfected), M2 presents the transfected cells with medium fluorescence intensity, whereas M3 presents highly fluorescent transfected cells. It has been observed that the number of transfected cells using formulation **E** and the gemini lipid **2c** is almost the same, but the number of highly fluorescent cells (see region M3) is almost 2 times greater in the case of the lipid **2c** formulation as compared to formulation **E**, whereas the number of low fluorescent cells (see region M2) is high in the case of formulation **E** as compared to that of the gemini lipid **2c**. This proves that formulation **E** is able to transfect a comparable number of cells as gemini lipid **2c**, but gemini lipid **2c** transfects more cells with high fluorescent intensity.

2.6.2. Luciferase Assay. To quantify the transgenic expression for the gemini lipid **2c**, we used luciferase gene expression assay in HeLa cells. Transfection efficiencies using the lipid **2c**/DOPE (1:4) formulation at different N/P ratios as obtained from luciferase assay, normalized with respect to formulation **E**, are shown in Figure 9. Upon transfection with the luciferase gene, pGL3-control, it was observed that the total amount of the luciferase (protein) formed, or the average transgene expression, was more than 2 times higher than the formulation **E**. Serum does not inhibit the luciferase activity of the lipid **2c**/DOPE formulation.

2.7. Characterization of Lipid−DNA Complexes by TEM. The morphology of the lipid/plasmid complexes was visualized under electron microscope after negative staining using uranyl acetate. The complexes formed with different gemini lipids at their optimized transfection ratios with plasmid DNA revealed some distinct morphological changes depending on the length of the spacer in between the cationic ammonium headgroups of the gemini lipid molecules (Figure 10). The lipoplexes formed from gemini lipid **2a**/DNA had the tendency to form elongated organizations like a network of rods. Gemini lipid **2b**- and **2c**-based lipoplexes showed aggregated structures, but these lipoplex aggregates were larger in size ranging from 100 to 200 nm. In the case of lipid **2d**-based lipoplexes, globular aggregates of 200−400 nm size were observed along with the presence of some uncomplexed, naked DNA. Lipid **2e**/DNA lipoplexes showed the existence of irregular morphologies. As the lipid **2e**/DOPE formulations were the least efficient transfecting agents in the absence of serum and better transfecting agents in the presence of serum, it is possible that these different morphologies could as well be responsible for their high transfection efficiency in the presence of serum.

2.8. Cytotoxicity Assay. MTT-based cell viability assays were performed in HeLa cells across the entire range of lipid/DNA charge ratios (N/P) used in the actual transfection experiments. The percentage cell viabilities of all the gemini lipids except lipid **2e** were found to be very high at all the concentrations used for the transfection experiments (Figure 11). The most potent transfecting gemini lipid **2c** was found to be nontoxic at all concentrations studied.

2.9. Conclusions. For the first time, synthesis of five cholesterol-based gemini cationic lipids with varying spacer chain lengths between the cationic ammonium headgroups has been achieved. These lipids form stable suspensions in water easily. Electron microscopy revealed the presence of closed membranous aggregates in these lipid aggregates. These gemini lipids in the presence of helper lipid, DOPE, showed a significant enhancement in the gene transfection activities as compared to their monomeric lipid counterpart. With the increase in length of the spacer from trimethylene to pentamethylene, the transfection efficiency was found to increase, whereas further increase in the spacer length to dodecamethylene led to a decrease in the transfection activity. All the gemini lipids were found to be more effective than monomeric lipid **1**. Gemini lipid **2c** was found to be the most effective lipid, which showed transfection activity greater than that of formulation **E**, one of best-known commercially available transfecting reagents. Lipid **2c** was nearly 2 times more effective than formulation **E**. All the gemini-lipid-based DOPE formulations did not possess any toxicity at the concentrations at which transfections were performed.

Serum is known to be a major impediment to the applications of cationic lipids for their use *in vivo*. So there is always a challenge to design such cationic lipids, which can be useful for transfection studies in the presence of serum. All the gemini

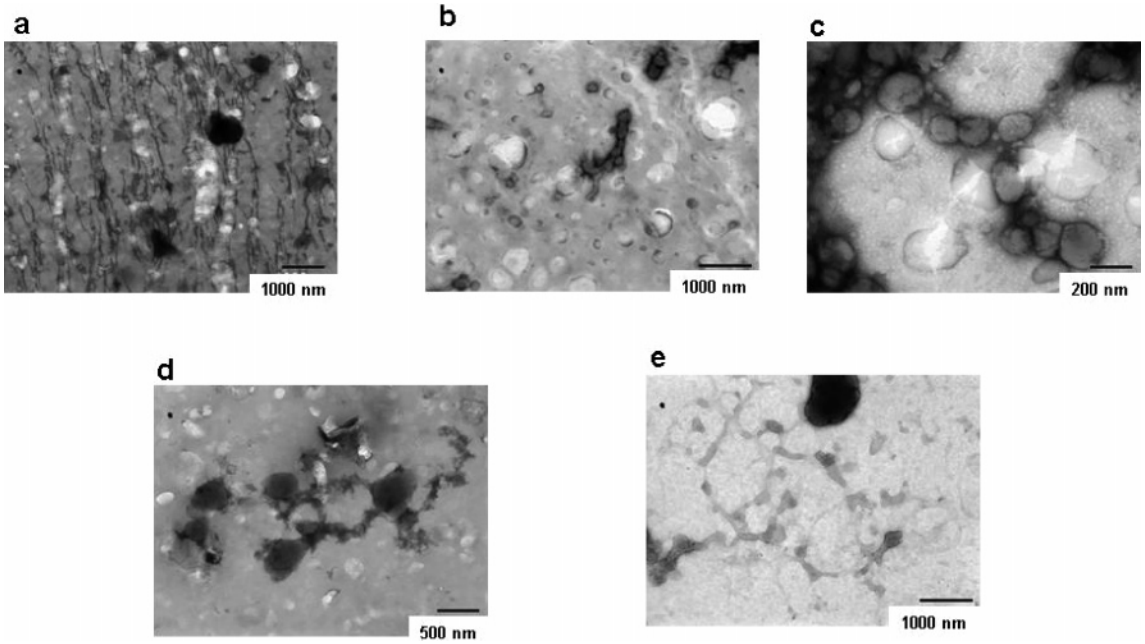


Figure 10. Transmission electron micrographs for the lipoplexes prepared from the most effective gemini lipid/DOPE liposome/DNA complexes at the optimized N/P ratio: (a) **2a**, (b) **2b**, (c) **2c**, (d) **2d**, and (e) **2e**.

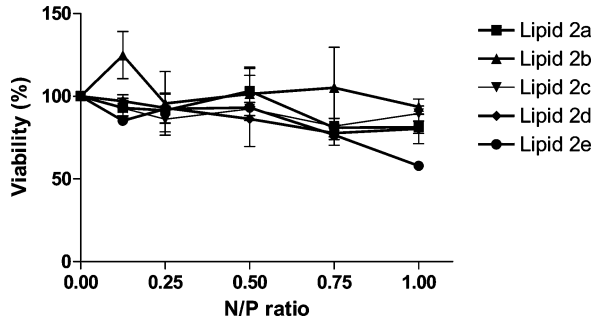


Figure 11. MTT assay-based cellular cytotoxicity of gemini lipids (**2a**−**2e**) against HeLa cells. The percent viability values shown are the average of triplicate experiments performed on the same day.

lipids except lipid **2a** showed enhanced transfection activity in the presence of serum as well, whereas transfection by monomeric lipid **1** got diminished dramatically in the presence of serum. Lipid **2e** showed an unusual feature of enhanced gene transfection in the presence of serum as compared to that in the absence of serum. To see the generality of the transfection by gemini lipids, transfection experiments performed in the U373 cell line, using the best gemini lipid **2c**, show 60−70% transfection efficiency with an MFI of 100 (Figure S2, Supporting information). Transmission electron micrographs of the lipoplexes showed distinct morphological changes depending upon the length of the spacer between the cationic headgroups.

We have been able to reduce their cytotoxicity and enhance the transfection efficiency significantly. The simplicity of the use of molecular building blocks like cholesterol and their high chemostability and shelf life make these formulations particularly attractive.

Although the DNA delivery to eukaryotic cells involves several steps[31] and the mechanisms of many of these steps are not very clear at the molecular level, it would be important to note some correlation between the molecular structures of these new gemini lipids, the membrane level properties, and the transfection properties. Whatever may be the actual mechanism for transfection mediated by this class of gemini lipid formulations, the interesting and meaningful results obtained with these novel cholesterol-based gemini lipids should be of interest to researchers working in the field of gene therapy using nonviral vectors.

3. Experimental Section

3.1. 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 a Jeol JNM λ-300 (300 MHz for ^{1}H and 75 Hz for ^{13}C) spectrometer. The chemical shifts (δ) are reported in ppm downfield from the internal standard, TMS, for ^{1}H−NMR and ^{13}C−NMR. Mass spectra were recorded on a Kratos PCKompact SEQ V1.2.2 MALDI-TOF spectrometer, a MicroMass ESI-TOF spectrometer, or a 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, ^{13}C−NMR, mass spectra, and elemental analysis.

3.2. Synthesis. 3.2.1. Cholest-5-en-3*β*-tosylate (4). To an ice-cooled solution of cholesterol (**3**) (5.0 g, 0.013 mol) in dry pyridine (5 mL) and dry chloroform (5 mL), tosyl chloride (3.7 g, 0.02 mol) was added. A catalytic amount of DMAP was also added. The reaction mixture was then allowed to stir at 0 °C for 6 h. To the reaction mixture, chloroform (35 mL) was added, and the reaction mixture was washed with 1 N HCl (2 × 50 mL), water (50 mL), and brine (50 mL); the organic layer was separated and dried over anhydrous Na_2SO_4. Chloroform from this solution was evaporated to leave a residue. From the residue cholest-5-en-3β-tosylate (**4**) was recrystallized using chloroform and methanol. Yield: white solid, 6.49 g, 0.012 mmol, 92.8%. Mp: 133 °C. Lit mp: 132−133 °C.[32] IR (neat) (cm^{-1}): 2949, 2867, 1598, 1495, 1467, 1366, 1188, and 1174. ^{1}H−NMR ($CDCl_3$, 300 MHz): δ: 0.65 (s, 3H), 0.84−2.29 (m, 41H), 2.44 (s, 3H), 4.32 (m, 1H), 5.30 (d, 1H, J = 4.5 Hz), 7.31−7.34 (d, 2H, J = 8.1 Hz), 7.78−7.81 (d, 2H, J = 8.1 Hz).

3.2.2. Cholest-5-en-3*β*-oxyethane (5). Chlolest-5-en-3β-tosylate (**4**) (3.5 g, 6.5 mmol) was taken in anhydrous dioxane. To this dry ethylene glycol (10 g, 0.16 mol) was added, and the mixture was refluxed under nitrogen for 4 h. The solution was cooled, and the solvent was removed under vacuum. A white residue was obtained which was dissolved in chloroform (50 mL) and washed with water. The organic layer was separated, washed with $NaHCO_3$ (50 mL),

water (50 mL), and brine (50 mL), and dried over anhydrous Na_2SO_4. Finally, this solvent was removed in vacuo and the product, cholest-5-en-3β-oxyethane (**5**), was purified by column chromatography over silica gel using a mixture of petroleum ether and ethyl acetate. Yield: white solid, 2.3 g, 5.3 mmol, 85%. Mp: 99−100 °C. Lit mp: 97−98 °C.[32] IR (neat) (cm^{-1}): 3438, 2936, 2868, 1466, and 1376. ^{1}H−NMR ($CDCl_3$, 300 MHz): δ: 0.67 (s, 3H), 0.85−2.35 (m, 41H), 3.20 (m, 1H), 3.57−3.60 (t, 2H), 3.72 (t, 2H), 5.34 (d, 1H). ESI-MS: 453 ($M + Na^+$).

3.2.3. Cholest-5-en-3β-oxyethane tosylate (6). To an ice-cooled solution of cholest-5-en-3β-oxyethan-2-ol (**5**) (2.2 g, 5.1 mmol) in dry pyridine (5 mL) and dry chloroform (5 mL), tosyl chloride (1.5 g, 7.86 mmol) was added. The reaction mixture was allowed to stir at 0 °C for 6 h. To the reaction mixture chloroform (40 mL) was added, and then the reaction mixture was washed with 1 N HCl (2 × 50 mL), water (50 mL), and brine (50 mL). Finally, the organic layer was separated and dried over anhydrous Na_2SO_4. The solvent was removed using a rotary evaporator, and the product, cholest-5-en-3β-oxyethane tosylate (**6**), was purified by column chromatography over silica gel using a mixture of petroleum ether and ethyl acetate. Yield: 2.68 g, 7.04 mmol, 90.0%. IR (neat) (cm^{-1}): 2935, 2886, 1598, 1465, 1360, 1189, and 1177. ^{1}H−NMR ($CDCl_3$, 300 MHz): δ: 0.67(s, 3H), 0.85−2.25 (m, 41H), 2.44 (s, 3H), 3.06−3.13 (m, 1H), 3.63−3.66 (t, 2H), 4.13−4.16 (t, 2H), 5.31 (d, 1H), 7.32−7.35 (d, 2H), 7.79−7.82 (d, 2H). ESI-MS: 607 ($M + Na^+$).[33]

3.2.4. Cholest-5-en-3β-oxyethan-*N*,*N*-dimethylamine (7). Dimethylamine was dissolved in dry ethanol in a screw-top pressure tube at 0 °C. The cholest-5-en-3β-oxyethane tosylate (**6**) (250 mg, 0.5 mmol) dissolved in dry MeOH (2 mL) was carefully added to the dimethylamine solution at 0 °C. The reaction mixture inside the pressure tube was screw capped and heated at 80 °C for 24 h. The remaining dimethylamine was evaporated, and the solvent was removed by rotary evaporation to afford a yellowish mass. The crude product was dissolved in $CHCl_3$ (25 mL) and washed with saturated $NaHCO_3$ (25 mL), water (25 mL), and then brine solution (25 mL). The final product was a yellowish solid (206 mg, 0.5 mmol, 99%). Mp: 145 °C. $R_f = 0.3$ ($CHCl_3$). FT-IR (neat) (cm^{-1}): 2936, 2866, 1465, 1377, 1274, 1195, 1110, and 1078. ^{1}H−NMR (300 MHz, $CDCl_3$): δ: 0.67 (s, 3H), 0.85−2.20 (m, 41H), 2.26 (s, 6H), 2.38 (m, 1H), 2.47−2.51 (t, $J = 6.0$ Hz, 2H), 3.11−3.18 (m, 1H), 3.54−3.59 (t, $J = 6.0$ Hz, 2H), 5.34 (d, $J = 4.5$ Hz, 1H). ^{13}C−NMR ($CDCl_3$, 75 MHz): δ: 11.66, 18.54, 19.18, 20.87, 22.40, 22.65, 23.69, 24.10, 27.80, 28.05, 28.13, 31.68, 31.73, 35.61, 36.01, 36.65, 37.06, 38.87, 39.33, 39.59, 42.09, 45.63, 49.97, 55.99, 56.55, 59.05, 65.72, 79.15, 122.27, 140.66. ESI-MS: 458 ($M^+ + 1$).

3.2.5. General Method for the Synthesis of Gemini Lipids (2a−2e). A solution of cholest-5-en-3β-oxyethan-*N*,*N*-dimethylamine (**7**) (0.2 mmol) and an appropriate α,ω-dibromoalkane (0.07 mmol) in dry MeOH−EtOAc (4 mL, v/v: 1/1) was refluxed over a period of 48−72 h in a screw-top pressure tube, until TLC indicated complete disappearance of the dibromide. After that, the reaction mixture was cooled and the solvent was evaporated to furnish a crude solid. This was repeatedly washed with ethyl acetate to remove any of the cholest-5-en-3β-oxyethan-*N*,*N*-dimethylamine (**7**), and the residue was finally subjected to repeated crystallizations from a mixture of MeOH and ethyl acetate. This afforded a white solid in each case. The product yields ranged from 50% to 60%. The purities of these lipids were ascertained from TLC; the R_f ranged from 0.2 to 0.3 in 10:1 $CHCl_3$/MeOH. All the new gemini lipids were fully characterized by ^{1}H−NMR, ^{13}C−NMR, mass spectrometry, and C, H, N analysis. Pertinent spectroscopic and analytical data are given below.

3.2.6. Lipid 2a. ^{1}H−NMR (300 MHz, $CDCl_3 + CD_3OD$): δ: 0.68 (s, 6H), 0.85−2.34 (m, 84H), 3.32 (br s, 14H), 3.74 (br s, 8H) 3.92 (br s, 4H), 5.37 (d, $J = 4.5$ Hz, 2H). ^{13}C−NMR ($CDCl_3 + CD_3OD$, 75 MHz): δ: 11.74, 18.60, 19.23, 21.01, 22.45, 22.68, 23.74, 24.18, 27.91, 28.03, 28.11, 31.75, 31.80, 35.69, 36.09, 36.68, 36.85, 38.64, 39.41, 39.64, 39.41, 39.64, 42.21, 49.99, 56.06, 56.62, 61.54, 62.10, 64.56, 79.90, 122.40, 139.66. ESI-MS: 478.5 (M^{+2}/2), 1035.4 ($M^{+2} + Br^-$). Anal. ($C_{65}H_{118}N_2O_2Br_2 \cdot 4.5H_2O$) C, H, N.

3.2.7. Lipid 2b. ^{1}H−NMR (300 MHz, $CDCl_3$): δ: 0.67 (s, 6H), 0.85−2.35 (m, 86H), 3.32 (m, 2H), 3.34 (br s, 12H), 3.74 (br s, 4H) 3.93 (br s, 4H), 4.02 (br s, 4H), 5.36 (d, $J = 4.5$ Hz, 2H). ^{13}C−NMR ($CDCl_3$, 75 MHz): δ: 11.86, 18.70, 19.36, 19.94, 21.019 22.53, 22.80, 23.85, 24.28, 28.00, 28.14, 28.21, 31.83, 31.89, 35.78, 36.17, 36.75, 36.90, 38.75, 39.49, 39.72, 42.29, 50.04, 56.16, 56.67, 61.09, 61.80, 65.29, 79.99, 122.43, 139.77. ESI-MS: 485.9 (M^{+2}/2). Anal. ($C_{66}H_{120}N_2O_2Br_2$) C, H, N.

3.2.8. Lipid 2c. ^{1}H−NMR (300 MHz, $CDCl_3$): δ: 0.67 (s, 6H), 0.85−2.35 (m, 88H), 3.20 (m, 2H), 3.39 (br s, 12H), 3.81 (br. s, 4H) 3.90 (br s, 8H), 5.36 (d, $J = 4.5$ Hz, 2H). ^{13}C−NMR ($CDCl_3$, 75 MHz): δ: 11.83, 18.69, 19.34, 21.04, 21.81 22.52, 22.77, 23.82, 24.30, 27.98, 28.14, 28.21, 31.81, 31.88, 35.76, 36.12, 36.75, 36.88, 38.74, 39.48, 39.68, 42.27, 50.02, 56.13, 56.67, 61.87, 63.69, 65.45, 79.91, 122.43, 139.76. ESI-MS: 492.4 (M^{+2}/2), 1064.7, 1066.9 ($M^{+2} + Br^-$). Anal. ($C_{67}H_{122}N_2O_2Br_2$) C, H, N.

3.2.9. Lipid 2d. ^{1}H−NMR (300 MHz, $CDCl_3$): δ: 0.67 (s, 6H), 0.85−2.31 (m, 90H), 3.20 (m, 2H), 3.40 (br s, 12H), 3.81−3.92 (br m, 12H), 5.36 (d, $J = 4.5$ Hz, 2H). ^{13}C−NMR ($CDCl_3$, 75 MHz): δ: 11.79, 18.64, 19.31, 20.99, 21.76, 22.49, 22.73, 23.80, 24.23, 24.67, 27.91, 28.08, 31.76, 31.85, 35.73, 36.12, 36.70, 36.85, 38.69, 39.43, 39.66, 42.23, 49.97, 56.09, 56.62, 61.90, 63.51, 65.70, 79.78, 122.27, 139.82. ESI-MS: 499.2 (M^{+2}/2). Anal. ($C_{68}H_{124}N_2O_2Br_2 \cdot 2.5H_2O$) C, H, N.

3.2.10. Lipid 2e. ^{1}H−NMR (300 MHz, $CDCl_3$): δ: 0.67 (s, 6H), 0.85−2.30 (m, 102H), 3.20 (m, 2H), 3.39 (br s, 12H), 3.68 (br m, 4H), 3.89−3.92 (br m, 8H), 5.34 (d, $J = 4.5$ Hz, 2H). ^{13}C−NMR ($CDCl_3$, 75 MHz): δ: 11.83, 18.67, 19.31, 21.01 22.52, 22.72, 22.77, 23.80, 24.25, 25.94, 27.96, 28.09, 28.18, 28.59, 31.81, 31.86, 35.74, 36.14, 36.73, 36.85, 38.74, 39.46, 39.68, 42.26, 50.01, 51.83, 56.11, 56.65, 61.93, 63.41, 66.23, 79.93, 122.45, 139.74. ESI-MS: 485.9 (M^{+2}/2). Anal. ($C_{74}H_{136}N_2O_2Br_2$) C, H, N.

3.3. 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. The last traces of organic solvent were removed by keeping these films under vacuum overnight. Freshly autoclaved water (Milli-Q) was added to the 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 60 °C) with intermittent vortexing to ensure hydration. Sonication of these suspensions for 15 min in a sonicator bath at 60 °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.

3.4. Transmission Electron Microscopy. Freshly prepared aqueous suspensions of each cationic lipid (0.5 mM) or lipoplex were examined under transmission electron microscopy by negative staining using 1% uranyl acetate. A 10 μL sample of the suspension was loaded on 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 for 1 min, 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 5000−20 000X.

3.5. 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). The purity of the plasmid was checked by electrophoresis on 1.0% agarose gel. The concentration of DNA was estimated spectroscopically by measuring the absorption at 260 nm and confirmed by gel electrophoresis. Plasmid preparations showing a value of $OD_{260}/OD_{280} > 1.8$ were used.

3.6. Cell Culture. Cells (HeLa, U373) 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).

3.7. Cytotoxicity. The 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.[34,35]

3.8. 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 45 000 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 by incubating the desired amount of lipid formulation and 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 the 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 was added to the complexes (final DNA concentration = 12.12 μM). Old medium was removed from the wells, the cells were washed with DMEM, and the lipid−DNA complexes in 200 μL 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, the medium was removed and the 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. Control transfections were performed in each case by using commercially available transfection reagent "Effectene" using standard conditions specified by the manufacturer. Here, "Effectene" is represented by formulation **E**. All the experiments were done in duplicate, and the results presented are the average of at least two such independent experiments done on two different days. For comparison, transfections using lipid **1** were performed using the optimized lipid/DOPE ratio[19] of 1:1 at a N/P ratio of 1.0, using 0.8 μg of plasmid DNA.

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, the medium was removed and the cells were washed with DMEM; 500 μL of DMEM containing 10% FBS was added per well. For transfections at 30% and 50% of serum concentrations, complexes were diluted with DMEM containing 60% FBS or with neat FBS, respectively.

3.9. 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 the transfection experiments. After 30 min of incubation, these complexes were electrophoretically run on a 1.0% agarose gel. The uncomplexed DNA moved out of the well, but the DNA that was complexed with lipid remained inside the well.[36]

3.10. Flow Cytometry. The reporter gene expression was examined by fluorescence microscopy at regular intervals and was quantified 48 h post-transfection by flow cytometry. The percentage of transfected cells was obtained by determining the statistics of the cells fluorescing above the control level wherein nontransfected cells were used as the control. Approximately 10 000 cells were analyzed to achieve the statistical data, which have been presented as the average of at least two independent measurements. For flow cytometry analysis, ∼48 h post-transfection, the old medium was removed from the wells; the 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 a Becton and Dickinson flow cytometer equipped with a fixed laser source at 488 nm.

3.11. Luciferase Assay. The efficiency of transfection was determined by checking the activity of luciferase protein expressed using a single-luciferase assay kit provided by Promega (U.S.A.) following manufacturer's protocol. For a typical assay in a 24-well plate, ∼48 h post-transfection, the old medium was removed from the wells and the cells were washed twice with 200 μL of PBS. To each well 100 μL of cell lysis buffer was then added, and the cells were lysed for 30 min in a horizontal rocker at room temperature (RT). The cell lysate was transferred completely to Eppendorf tubes and centrifuged (4000 rpm, RT) for 2 min; the supernatant was transferred to Eppendorf tubes and stored in ice. For the assay, 10 μL of this supernatant and 10 μL of luciferase assay substrate (Promega) were used. The lysate and the substrate were both thawed to RT before performing the assay. The substrate was added to the lysate, and the luciferase activity was measured in a luminometer (Turner designs, 20/20, Promega, U.S.A.) in standard single-luminescence mode. The measurement was performed for 10 s. A delay of 2 s was given before each measurement. The protein concentration in the cell lysate supernatant was estimated in each case using Bradford's method with bovine serum albumin as a standard.[37] Comparison of the transfection efficiencies of the individual lipids was made based on data for luciferase expressed as either relative light units (RLU) or relative light units (RLU)/μg of protein.

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Supporting Information Available: Elemental analysis values, structure−activity graph, and flow cytometric scans of transfection experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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