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PAPER

Syntheses, Transfection Efficacy and Cell Toxicity Properties of Novel Cholesterol-based Gemini Lipids having Hydroxyethyl Head group†

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We have synthesized five new cholesterol based gemini cationic lipids possessing hydroxyethyl ($-CH_2CH_2OH$) function on each head group, which differ in the length of the polymethylene spacer chain. These gemini lipids are important for gene delivery processes as they possess pre-optimized molecular features, *e.g.*, cholesterol backbone, ether linkage and a variable spacer chain between both the headgroups of the gemini lipids. Cationic liposomes were prepared from each of these lipids individually and as a mixture of individual cationic gemini lipid and 1,2-dioleoyl phosphatidylethanolamine (DOPE). Each gemini lipid based formulation induced better transfection activity than that of their monomeric counterpart. One such gemini lipid with a $-(CH_2)_{12}-$ spacer, HG-12, showed dramatic increase in the mean fluorescence intensity due to the expression of green-fluorescence protein (GFP) in the presence of 10% FBS compared to the conditions where there was no serum. Other gemini lipids retained their gene transfection efficiency without any marked decrease in the presence of serum. The only exception was seen with the gemini with a $-(CH_2)_3-$ spacer, HG-3, which on gene transfection in the presence of 10% FBS lost ~70% of its transfection efficiency. Overall the gemini lipid with a $-(CH_2)_5-$ spacer, HG-5, showed the highest transfection activity at N/P (lipid/DNA) ratio of 0.5 and lipid : DOPE molar ratio of 2. Upon comparison of the relevant parameters, *e.g.*, %-transfected cells, the amount of DNA transfected to each cell and %-cell viability all together against Lipofectamine 2000, one of the best commercial transfecting agents, the optimized lipid formulation based on DOPE/HG-5 was found to be comparable. In terms of its ability to induce gene-transfer in the presence of serum and shelf-life DOPE/HG-5 liposome was found to be superior to its commercial counterpart. Confocal imaging analysis confirmed that in the presence of 10% serum using a Lipid : DOPE of 1 : 4 and N/P charge ratio of 0.75 with 1.2 μg DNA per well, HG-5 is better than Lipofectamine 2000.

Introduction

Cholesterol[1–6] and glycerol based[7–11] cationic lipids have been extensively used for gene delivery across a number of mammalian cells.[12] Some of these lipids with natural transfection enhancers *e.g.*, 1,2-dioleoyl-L-α-glycero-3-phosphatidylethanolamine (DOPE) express synergistically higher level of transfection in many cases, although a few lipids have shown efficient gene transfer activity without any helper lipids.[13] Molecular level structural variations in cationic lipids influence both biophysical characteristics of liposomes and their transfection efficiency. Reports describing variations in the headgroup structures,[4,5] hydrophobic moieties,[6] linker types,[13–16] linker lengths[17] and linker polarity[18] as well as balance between the head group and tail group have appeared in the literature.[3] A significant improvement in the transfection efficiency of cationic cholesterol based cytofectins was shown when the cationic center is linked to the steroid backbone *via* an ether type of linkage as opposed to their ester or urethane based counterparts.[13,14] High transfection efficacies of non-glycerol-based simple monocationic transfection lipids with hydroxyethyl ($-CH_2CH_2OH$) head groups were reported by Banerjee and coworkers.[19] The presence of hydroxyl function in the headgroup region of such lipids contributes favorably towards liposomal gene delivery.[20] These authors proposed a role of hydrogen-bonding interactions between the lipid headgroups and the cell surface of the biological membranes for the improvement in the transfection efficiency. Park *et al.* utilized OH group terminated dendrimers to avoid cytotoxicity and aggregation problems for polyamido amine based cytofectins.[21] Mahato *et al.*

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also showed that additional hydrogen bonding between cationic lipid and plasmid DNA enhances transfection activity.[22]

Gemini cationic lipids[23] represent another class of lipids which manifest significantly enhanced gene delivery capability.[24] However, there has been no attempt to explore the transfection activities mediated by the corresponding lipid systems having hydroxyethyl ($-CH_2CH_2OH$) moiety on the head group. Herein we present syntheses and transfection capabilities of the corresponding cholesterol based cationic gemini lipids.[25] Five new cholesterol based gemini lipids (HG-3, HG-4, HG-5, HG-6 and HG-12) have been synthesized (Fig. 1), where the two cationic cholesterol units are connected *via* an oligomethylene [$(-CH_2)_n-$] chain and each ammonium head groups also possess ($-CH_2CH_2OH$) moieties. In this work we demonstrate significant increases in transfection efficiency especially the amount of DNA transferred to each cell by some of these gemini lipids. Each gemini lipid formulation was found to be a better transfecting agent compared to its monomeric counterpart even in the presence of 10% FBS. In comparison to a commercially available transfection reagent Lipofectamine 2000, one of the gemini lipid, HG-5 showed better %-cell viability and higher mean fluorescence intensity (MFI) due to expressed green fluorescence protein (GFP) while the number of transfected cells mediated by either the commercial reagent or HG-5 was comparable. From confocal images it is clear that in the absence of serum, Lipofectamine 2000 is the best formulation. However, in the presence of 10% serum at optimized HG-5 : DOPE composition and gemini lipid/DNA ratio of 0.75, the gemini lipid performs better than Lipofectamine 2000.

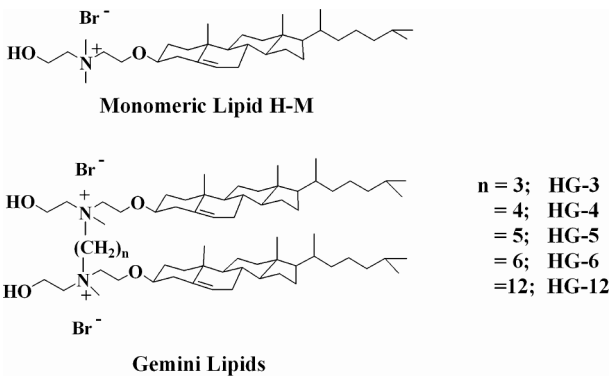
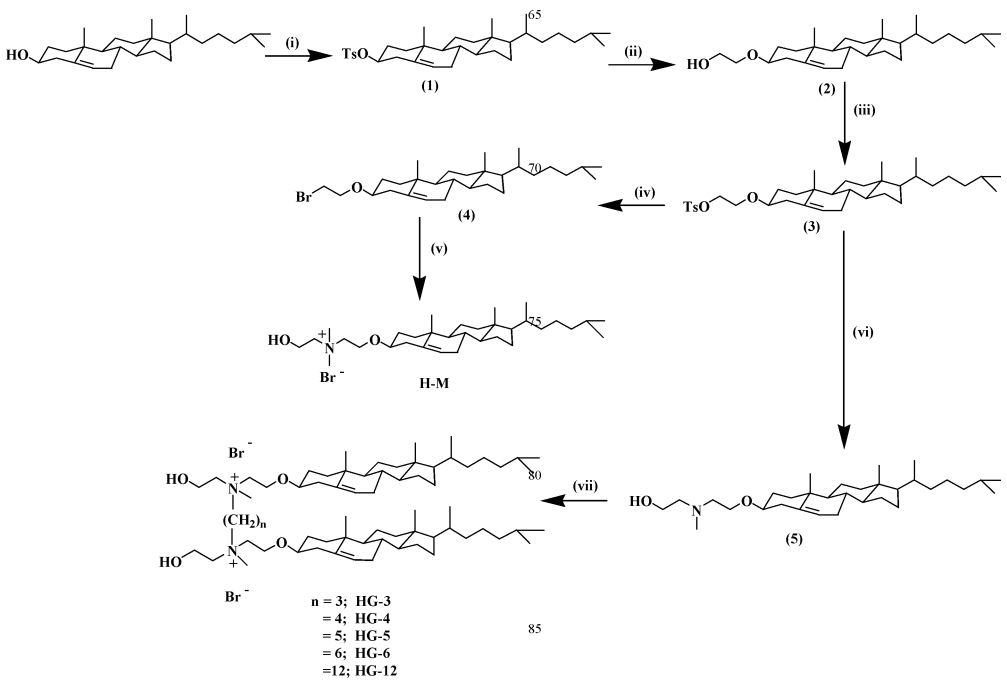


Fig. 1 Molecular structures of cholesterol based cationic monomeric and gemini lipids synthesized and used herein for gene transfection studies.

Results and Discussion

Chemistry. In this paper, we introduce a series of new cationic gemini cholesterol-based DNA transfection reagents. Five cholesterol based gemini lipids possessing hydroxyethylated headgroup and polymethylene spacer chain of variable lengths were synthesized (Scheme 1). The individual gemini lipids were obtained upon heating the key precursor cholest-5-en-3β-oxyethan-*N*-methyl-*N*-2-hydroxy-ethylamine (5) (0.2 mmol) with an appropriate α,ω-dibromoalkane (0.07 mmol) in dry MeOH-EtOAc (4 mL, v/v : 1/1) over a period of 48–72 h in a screw-top pressure tube, until TLC indicated complete disappearance of the starting dibromide. After that, the reaction mixture was cooled and the solvent was evaporated to furnish a crude solid. The residue was repeatedly washed with EtOAc to remove any residual cholest-5-en-3β-oxyethan-*N*-methyl-*N*-2-hydroxy-ethylamine (5). This was finally subjected to repetitive crystallizations from a mixture of MeOH and EtOAc to afford almost white solid in each case. The pure product yields ranged from ~40–50%. The purities of



Scheme 1 **Reaction conditions**. (i) *p*-TsCl, Py, $CHCl_3$, DMAP, 92.8%; (ii) Ethylene glycol, dioxane, reflux, 85%; (iii) *p*-TsCl, Py, $CHCl_3$, 90%; (iv) LiBr, DMF, N_2 atm., reflux, 95%; (v) *N*,*N*-dimethylethanolamine, EtOH, screw-top pressure tube, 81%; (vi) *N*-methylethanolamine, CH_3CN, reflux, 97%, (vii) $Br–(CH_2)_n–Br$, EtOH, screw-top pressure tube, 70 °C, 40–50%.

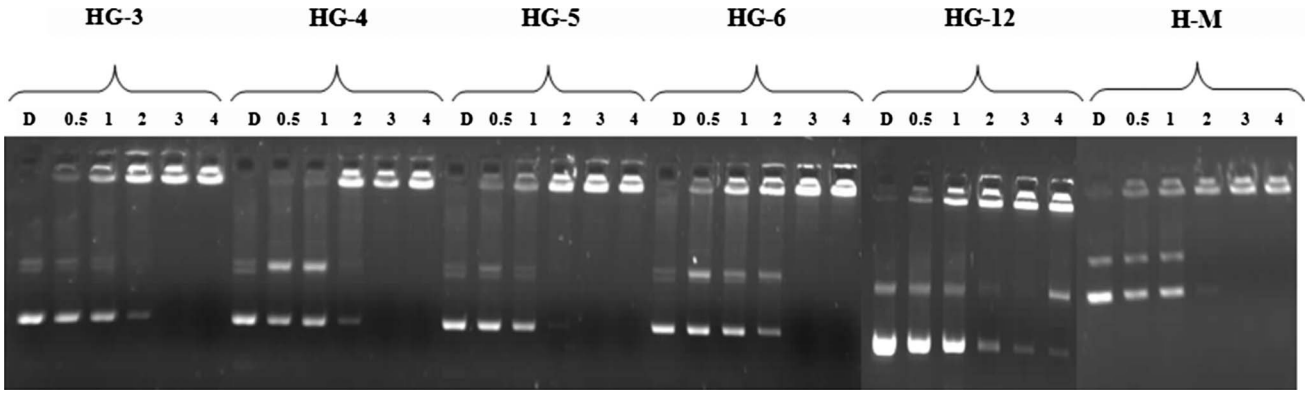


Fig. 2 Electrophoretic gel patterns for lipoplex-associated DNA in gel retardation for gemini lipids of various spacers. The N/P ratios are indicated at the top of each lane. 0.2 μg of plasmid DNA was complexed with liposome at various N/P ratios for 30 min, and lipoplexes were run electrophoretically on 1% agarose gel.

these lipids were ascertained from TLC; the R_f ranged from 0.2 to 0.3 in 10 : 1 $CHCl_3$/MeOH and each new gemini lipids was fully characterized by ^{1}H-NMR, ^{13}C-NMR, mass spectrometry, and CHN analysis, *cf.* experimental section.

Aggregate formation from cationic gemini lipids. Each gemini lipid as well as their monomeric counterpart, when dispersed in water formed stable suspensions. Briefly each lipid film was subjected to hydration followed by freeze-thaw cycles, vortexing and bath sonication to form lipid suspension. While all other gemini lipids as well as the monomer were easily dispersible in aqueous medium, it took longer to form aqueous suspension from the gemini lipid HG-3. Each lipid suspension remained as a stable suspension with no increase in optical scatter as long as it was kept under sterile conditions at 4 °C. Each lipid suspension was characterized by DLS methods (not shown) and their hydrodynamic diameters (average) ranged from ~50 to ~225 nm.

Transfection biology

All cationic gemini lipid based formulations including their monomeric counterpart were found to readily form lipoplexes with plasmid DNA (see below). However, transfection abilities of the neat lipid suspensions were rather modest when compared with the commercially available transfecting agent, Lipofectamine 2000 (not shown). Naturally occurring lipids such as DOPE were found to increase the efficiency of transfection significantly.[14] It may however, be noted that the positive control Lipofectamine 2000 reagent also contains DOPE in its formulation.

Mixed liposomes of cationic gemini lipids with 1,2-dioleoyl-*L*-α-*glycero*-3-phosphatidylethanolamine (DOPE). Mixed liposomes were prepared from different molar ratios of DOPE: gemini lipid, in a similar way described for the liposome preparation of the gemini lipids alone. Each lipid or mixed lipid suspensions were prepared under sterile conditions, stored at 4 °C and were re-sonicated at (35 KHz) for 5 min at room temperature before transfection experiments. Precipitation was observed with some of the formulations having high molar ratio of DOPE upon storage. Accordingly only freshly prepared mixed liposomes were used for transfection experiments.

Gel electrophoresis. Electrophoretic gel retardation assay was performed to probe the electrostatic binding of plasmid DNA with various cationic lipids, which retards the movement of DNA towards anode. Visible bands appeared due to intercalation of ethidium bromide in DNA. As a function of N/P charge ratio, the DNA binding affinity of each lipid was measured on the basis of band intensity outside the well on 1% agarose gel (Fig. 2). All lipid suspensions were able to bind 100% DNA (0.2 μg) at N/P charge ratio of ~3 irrespective of the spacer length. As each gemini lipid has two positive charges on each molecule, complete binding lipid/DNA molar ratio was found to be ~1.5 which was slightly higher than the binding ratio of the monomer. Thus the insertion of spacer between two $HO–CH_2–CH_2–N^+$-head groups as well as the spacer length influenced DNA binding affinity to some extent.

Gene transfection

Optimization of lipid : DOPE ratio. To achieve maximal gene transfection out of the gemini lipids developed in this work, we included in mixed liposomes various percentages of DOPE, a naturally occurring lipid, which is known to increase the efficiency of other lipid mediated gene transfer.[25] In order to find out the most efficient combination, transfection experiments were performed keeping constant lipid/DNA mol ratio (N/P ratio) with varying mol ratio of DOPE in individual gemini lipids (HG-3, HG-4, HG-5, HG-6 and HG-12) (Supporting Information, Figure S2†). Flow cytometry analysis shows the most efficient molar combination of lipid and DOPE by taking both, %GFP (Green Fluorescence Protein positive) cells and MFI (Mean fluorescence Intensity) of GFP positive cells into consideration. The MFI defined for GFP positive cells indicate that the levels of GFP expression with a higher MFI value correlate positively with a high GFP expression.[26] All the DOPE : lipid combinations were optimized separately both in the absence of serum (–FBS–FBS) and the presence of serum (–FBS+FBS) conditions.

Each gemini lipid at certain lipid : DOPE mol ratio, showed most efficient transfection activity involving maximum number of cells with higher amount of DNA to each cell on average. HG-3 and HG-4 showed the highest transfection efficiency at lipid : DOPE ratio ~1 : 1 while gemini lipids, HG-5 and HG-12 showed their highest efficacy at lipid : DOPE ratio of ~1 : 2

in absence (–FBS–FBS) and presence (–FBS+FBS) of serum. However, HG-6 showed the highest activity at 1 : 2 lipid : DOPE ratio in absence of serum and 1 : 4 ratio in the presence of serum respectively. Comparison of flow cytometry (FACS) analysis of each optimized formulation shows HG-5 and HG-12 formulations to be more efficient than others. Although HG-3 and HG-4 showed MFI value at the optimized lipid : DOPE ratio, they showed a lower number of transfected cells compared to the optimized formulations based on HG-5 and HG-12. A lipid : DOPE ratio (1 : 1) was found to be good for HG-3 and HG-4 in terms of greater DNA transportation to each cell on average. But for HG-5 and HG-12 at optimized lipid : DOPE ratio of 1 : 2, less DNA was transferred to more cells. HG-6 showed moderate efficacy of DNA transfer both in terms of the number of cells and the amount of DNA to each cell.

Lipid-to-DNA charge ratio. A second level of optimization was performed to determine the best transfecting composition for the delivery of both a relatively higher amount of DNA and to larger number of cells. Each experiment was performed in duplicate using 0.8 μg DNA per well with variation of lipid concentration in terms of optimized lipid : DOPE mol ratio (Fig. 3). Each gemini lipid was invariably a better transfecting agent than the monomeric counterpart in terms of both number of transfected cells and MFI. Although the number of transfecting cells was ~65% in case of the monomeric lipid "H-M", the MFI observed was only ~20.

In absence of serum (–FBS–FBS), the lipid HG-3 was able to transfect to a maximum of 60% of the cells with MFI of only 20 at N/P ratio of 1, whereas lipid HG-4 could transfect approximately 75% of the cells with nearly ~30 MFI at the same N/P ratio. Transfection efficiency was found to be saturated at these N/P ratios in terms of both% GFP cells and MFI. Although lipid HG-5 was able to transfect 75% of cells at N/P of 0.75 or 1, the MFI of ~40 was found to be less as compared to the MFI of ~80 at N/P of 0.25, which showed only ~70% of GFP positive cells. Thus HG-5 presented an optimized condition of the number of transfected cells and the amount of DNA transported to each cell at N/P of 0.25. HG-6 showed a maximum transfection efficiency of ~70% with a MFI of ~40 at N/P ratio of 0.75. Although at N/P ratio of 0.50, a maximum MFI of ~65 was observed the number of transfected cells was less as compared to that using N/P ratio of 0.75. On the other hand, HG-12 showed maximum transfection efficiency of ~75% with MFI ~25 at N/P charge ratio of 1. Here, MFI showed no change with variation of N/P ratio but %-transfection efficiency decreased at a lower ratio. If we compare the transfection efficiency of each gemini lipid at N/P charge ratio of 0.25, transfection efficiency as well as MFI both follows the same trend of initial increase from HG-3 to HG-5 and then decrease till HG-12 as the spacer length increases. HG-5 showed maximum transfection efficiency among all five gemini lipids in the series.

Luciferase assay. To quantify the transgenic expression for the gemini lipid HG-5, we used luciferase gene expression assay in HeLa cells (Fig. 4). Transfection efficacies of lipid HG-5 : DOPE (1 : 2) formulation at different N/P ratios were obtained from luciferase assay. Experiments were performed in absence of serum (–FBS–FBS). 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 very high at N/P charge ratio of 0.25 compared to other N/P ratios. Further we performed experiment with all lipid formulations other than HG-5 at their optimized DOPE molar and N/P charge ratio, which were obtained by FACS analysis of eGFP-C3 based transfection expression. We found that here also HG-5, with lipid : DOPE (1 : 2) at N/P charge ratio of 0.25 was the best among all, while HG-6 with lipid : DOPE (1 : 2) at N/P charge ratio of 0.5 was far better than others except HG-5. These results agreed with the results obtained with pEGFP-C3 based transfection experiments.

Effect of blood serum. During lipofection experiments, lipoplexes were incubated with cells for 6 h in appropriate cultural conditions without serum (–FBS–FBS) or with serum (–FBS+FBS). Blood serum, which consists of anionic proteins, competes with DNA for complexation with cationic lipids. Thereby serum promotes dissociation of lipoplexes, which in turn decreases the number of DNA molecules delivered for transfection and finally transfection efficiency of formulation. Maintenance of the transfection efficiency in the absence of serum even for *in vitro* experiments is also cumbersome due to the toxic nature of the synthetic cationic lipid formulations. There are only a few examples of cationic lipids, which show transfection activity even in the presence of serum.[8,27–29] Thus, in addition to strong DNA binding property and easy release of DNA from such complexes,[30,34] a good transfecting agent should have adequate stability of lipoplexes in blood serum.

To evaluate the transfection efficiency in the presence of 10% serum, we performed transfection similarly to the way it was performed in the absence of serum (–FBS–FBS) except that lipoplex was incubated with cells in the presence of 10% FBS containing Dulbecco's modified eagles medium (DMEM). This was also optimized toward appropriate molar ratio of DOPE for transfection efficiency, which gave identical optimal DOPE/lipid ratio for all the gemini lipids except that of HG-6, which showed an optimization of lipid : DOPE at 1 : 4 (rather than 1 : 2 in absence of serum) (Supporting Information, Figure S3†). Each of the DOPE formulations for every gemini lipid was used to determine N/P charge ratio using plasmid pEGFP-C3 and the resulting data were analyzed by flow cytometry (Fig. 5). The transfection efficiency of monomeric lipid 'H-M' decreased drastically in the presence of serum. Also there was a ~60% decrease in MFI of the transfected cells in the presence of serum for Lipofectamine 2000 (Fig. 6). However, in the case of HG-3 and HG-4, there were no changes in MFI, although there was a decrease in the number of transfected cells in the presence of serum. The transfection efficiency of HG-5 decreased both in terms of the percentage of transfected cells and the MFI at N/P charge ratio of 0.125, 0.25, 0.75 and 1. But at 0.5 N/P ratio we noticed no significant decrease in the number of transfected cells and considerable increase in MFI. HG-5 showed maximum transfection efficiency at N/P 0.5 rather than 0.25 in the absence of serum (Fig. 5).

Lipid HG-5 was able to transfect nearly 55% of the cells with a MFI of ~65 in the presence of serum at N/P ratio of 0.5. In the case of HG-6, the presence of serum inhibited transfection efficiency in terms of the number of transfected cells although there were little increases in MFI in most cases. Surprisingly this lipid showed no significant change in either %transfected cells as well as MFI at optimized N/P charge ratio which had been shifted from 0.5 to

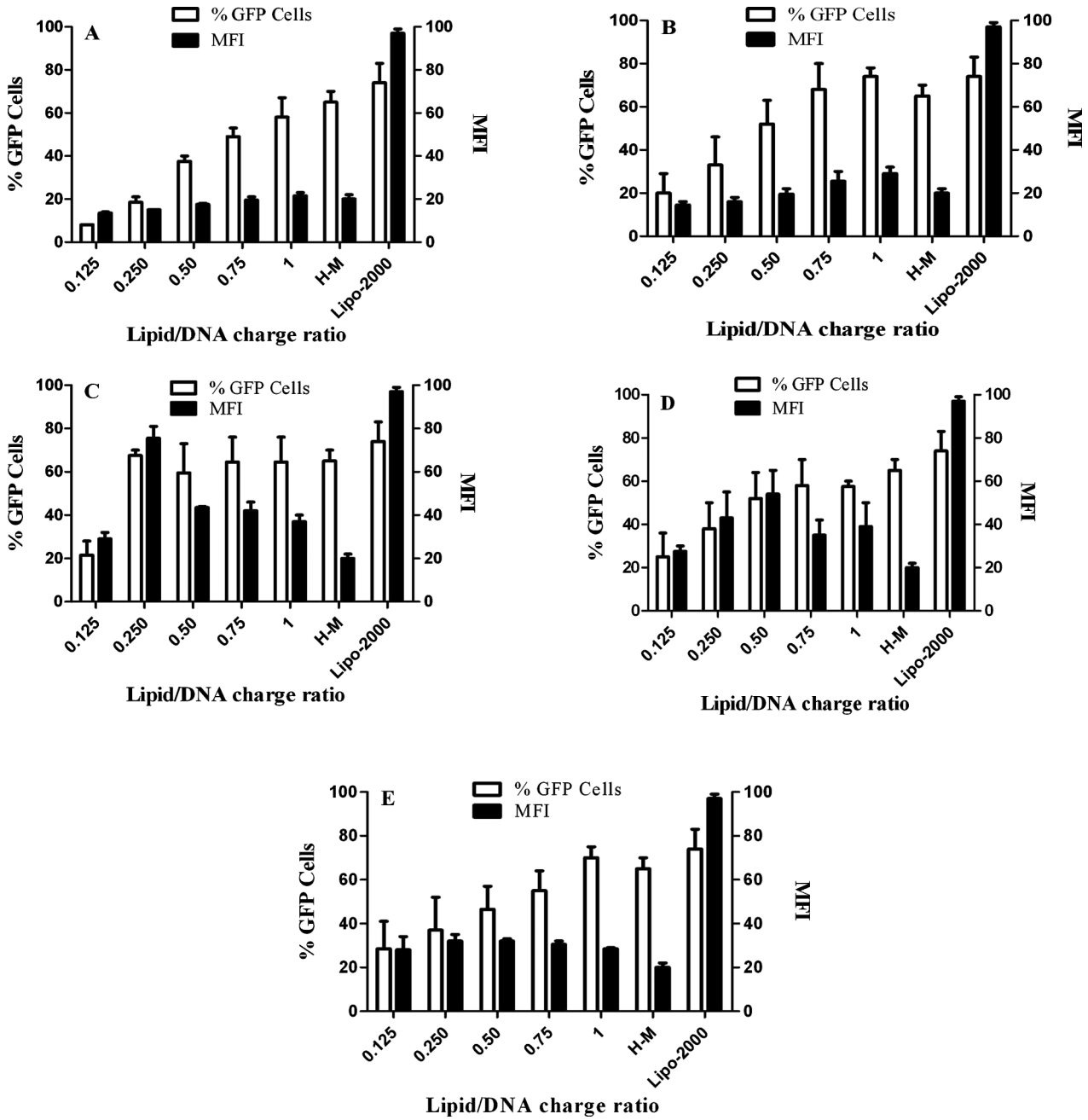


Fig. 3 Transfection efficacies of the optimized gemini lipid : DOPE formulations at various N/P ratios in absence of serum in comparison to lipid monomer and commercial transfecting agent Lipofectamine 2000. (A) HG-3; (B) HG-4; (C) HG-5; (D) HG-6 and (E) HG-12. Concentrations of DNA = 0.8 mg/well. Data are expressed as the number of transfected cells and MFI as obtained from the flow cytometry analysis.

0.75 in the presence of serum. There was a significant increase in the transfection efficiency of gemini lipid HG-12 in presence of serum as compared to the one carried out in the absence of serum in terms of the MFI although the number of transfected cells was not affected at N/P ratio of 0.5 but at other N/P charge ratios, the number of transfected cells was affected with small decreases in the number of transfected cells with considerable increases in MFI.

All gemini lipids were found to be significantly better transfecting agents than their monomeric species even in the presence of serum. The transfection efficiency of gemini lipid HG-5 in the presence of serum was also found to be at least comparable with one of the potent, commercially available reagents, Lipofectamine 2000, which is also known to show transfection in the presence of serum. Gemini lipid HG-6 retains its transfection efficiency in presence of serum in terms of the %-transfection activity as well as MFI while HG-12 showed a two-fold increase in the transfection efficiency in presence of serum in terms of MFI than that in absence of serum. This suggests that there are some serum components that must be facilitating the transfection activity

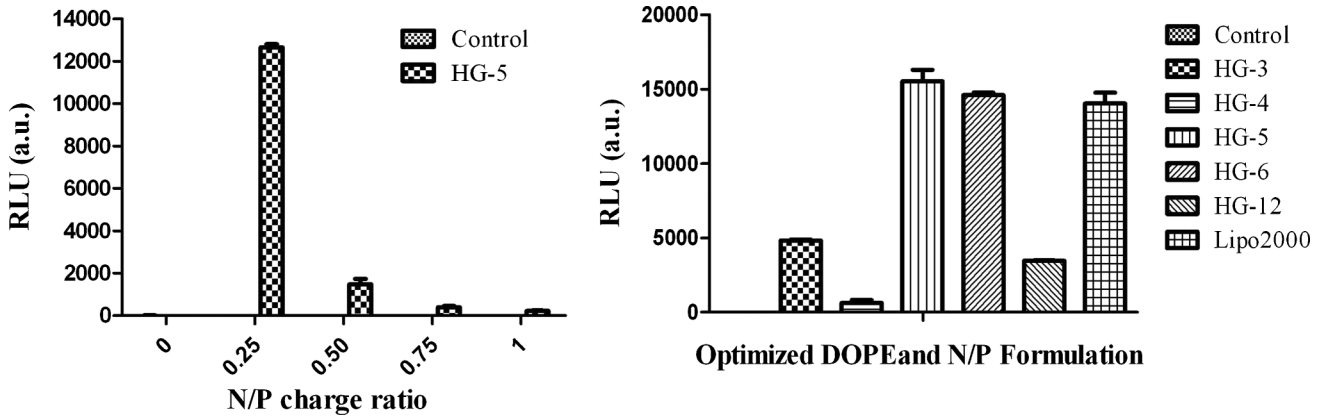


Fig. 4 Transfection efficacies of optimized gemini lipid: DOPE formulations at various N/P ratios in absence of serum. (A) Comparative luciferase activity of HG-5; (B) comparative luciferase activity of all the. gemini lipids at optimized lipid: DOPE molar ratio. Concentrations of DNA = 0.8 μg/well. Data are expressed as relative light unit (a.u.) obtained from luciferase activity assay for 20 μL of cell lysate.

with the liposomes prepared from this class of gemini cholesterol lipid.

To probe the effect of higher serum concentrations on the transfection, we performed experiment in high FBS condition (–FBS+FBS). Lipoplex suspensions with 10, 20, 30, 40 and 50% FBS were added to 70% confluent HeLa cells and allowed them to grow for 6 h. Then the medium was removed and cells were grown in a medium having 10% serum for 42–48 h and examined using FACS. Among all the gemini lipids, HG-5 was most efficient. HG-5 formulation, optimized with DOPE at a molar ratio of 1 : 2 (lipid : DOPE) and lipid : DNA charge ratio at 0.5 (N/P charge ratio) was used for this experiment. FACS profiles (Fig. 7) indicate that such a formulation was able to transfect nearly 45% of the cells with a good MFI of 70 in presence of 30% serum. At 50% serum condition only 15% cells were transfected with low MFI of ~35. In terms of fluorescence intensity, formulation HG-5 was able to transfect less number of cells with moderate MFI even at 50% of serum condition. Low MFI intensity observed in the case of lipid HG-5 : DOPE (1 : 2) formulation is expected as at such high serum concentrations, negatively charged serum proteins start to compete strongly with DNA for lipid complexation, reducing the number of plasmids available for cellular delivery.

Effect of the amount of DNA on gene transfection efficiency. To determine the DNA loading capacity of individual lipid formulations with optimized DOPE molar ratio and N/P charge ratio, we performed transfection with all the gemini lipids, varying the amount of DNA from 0.4–2.0 μg/well. We used 2 : 1 molar ratio of DOPE : Lipid for HG-5. An increase in transfection efficiency was observed with an increase in the amount of the DNA from 0.4 μg to 1.2 μg at optimized DOPE/lipid as well as N/P ratios with the most efficiently transfecting lipid, HG-5. At N/P ratio of 0.25, using 1.2 μg of DNA nearly 75% of the cells were transfected with a MFI of ~120, which was higher than the MFI obtained using Lipofectamine 2000 (Fig. 8).

Cytotoxicity assay. Toxicity of each co-liposomal formulations (cationic lipid with optimized DOPE molar concentration obtained in transfection experiments) as well as each liposomal formulation (cationic lipid alone at different concentrations), was determined using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay following literature procedure.[31,32] MTT-based cell viability assays were performed on HeLa cells using 0.2 μg DNA per well in 96 well plates. Experiments were performed in triplicates. Percentage cell viabilities of all the lipids except lipid HG-12 were found to be very high at all the concentrations and over the entire range of lipid : DNA charge ratios (N/P) used for the transfection experiments (Figure S4C†). Each liposomal formulation showed very low cytotoxicity even at very high lipid concentrations, compared to concentrations used for transfection experiments (Figure S4A and S4B†). The most potent transfecting gemini lipid HG-5 was found to be particularly non-toxic at optimized Lipid : DOPE molar and N/P charge ratio.

Comparison against Lipofectamine 2000. A comparative study of the effective formulation of gemini lipid against its monomeric counterpart and Lipofectamine 2000 was performed. The transfection experiments as well as MTT assays were conducted both in the absence as well as the presence of serum (Fig. 9A and 9B). Monomeric lipid showed ~70% transfected cells with a MFI of ~20 and 80% cell viability in absence of serum. However, in presence of serum these showed merely ~20% transfection and low MFI with slightly better %-cell viability in both cases of lipid alone and lipid/DNA complex of identical cationic lipid concentration.

In contrast each gemini lipid showed much better transfection efficiency and better%-cell viability except HG-12, which was found to be slightly more toxic at optimized N/P charge ratio, compared to its monomer. Lipofectamine 2000 was found to be a better transfecting agent compared to gemini lipid HG-5/DOPE formulation, but at the same time it showed very high levels of toxicity both as such and in the form of lipoplexes and showed only ~20% less cell viability in either case.

We compared optimized HG-5/DOPE formulation, with Lipofectamine 2000, in 10% serum containing DMEM (Fig. 10). HG-5 at Lipid : DOPE molar ratio of 1 : 2 and N/P ratio of 0.5 using 1.2 μg DNA/well in 10% serum condition afforded ~75% transfection with MFI of ~120 while Lipofectamine 2000 showed ~70% transfection with a MFI of only 50, using manufacturer's protocol. Thus HG-5 showed better serum compatibility than the Lipofectamine 2000 toward transfection experiments.

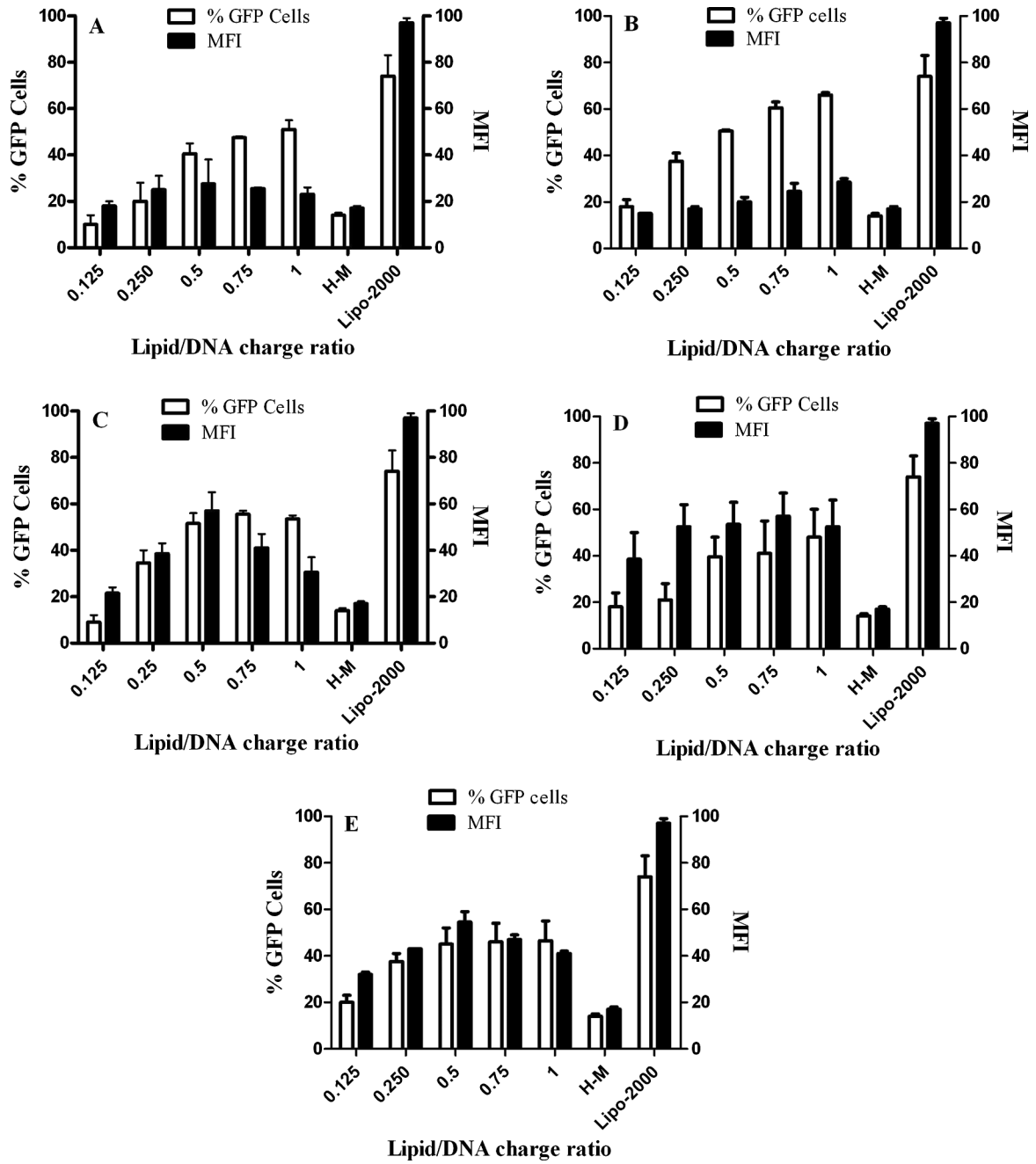


Fig. 5 Transfection efficiencies of optimized gemini lipid : DOPE formulations at various N/P ratios in presence of serum in comparison to monomeric lipid and Lipofectamine 2000. (A) HG-3; (B) HG-4; (C) HG-5; (D) HG-6 and (E) HG-12. Concentrations of DNA = 0.8 μg/well. Data are expressed as the number of transfected cells and MFI as obtained from the flow cytometry analysis.

Confocal studies. Cells were examined under confocal microscope (Zeiss LSM-510 Meta Apochromat) at objective of Plan Apochromat 63x/1.4 Oil DIC and analyzed by Zeiss LSM image browser. For confocal studies, we selected HG-5 at lipid : DOPE (1 : 4) and N/P (0.5) for (–FBS–FBS) (Fig. 11B) using 0.8 μg DNA and (Fig. 11C) using 1.2 μg DNA. We used 1 : 4 lipid : DOPE 1 : 4 and N/P charge ratio of 0.75 and the experiment was done in presence of serum (–FBS+FBS) using 1.2 μg DNA per well (Fig. 11D). Lipofectamine 2000, was used as a positive control by manufacturer's protocol both in the absence (–FBS–FBS) (Lipo2000) (Fig. 11F) and in the presence (–FBS+FBS) (Lipo2000*) (Fig. 11E) of 10% serum. HeLa cells, without transfection were taken as negative control (Fig. 11A). From confocal images, it is clear that in the absence of serum, Lipofectamine 2000 is the most effective formulation but in the presence of 10% serum, HG-5 is better than Lipofectamine 2000. Results in support of such conclusions are obtained from FACS analysis.

Conclusions

In conclusion, for the first time, syntheses of five cholesterol based gemini cationic lipids with varying spacer chain length

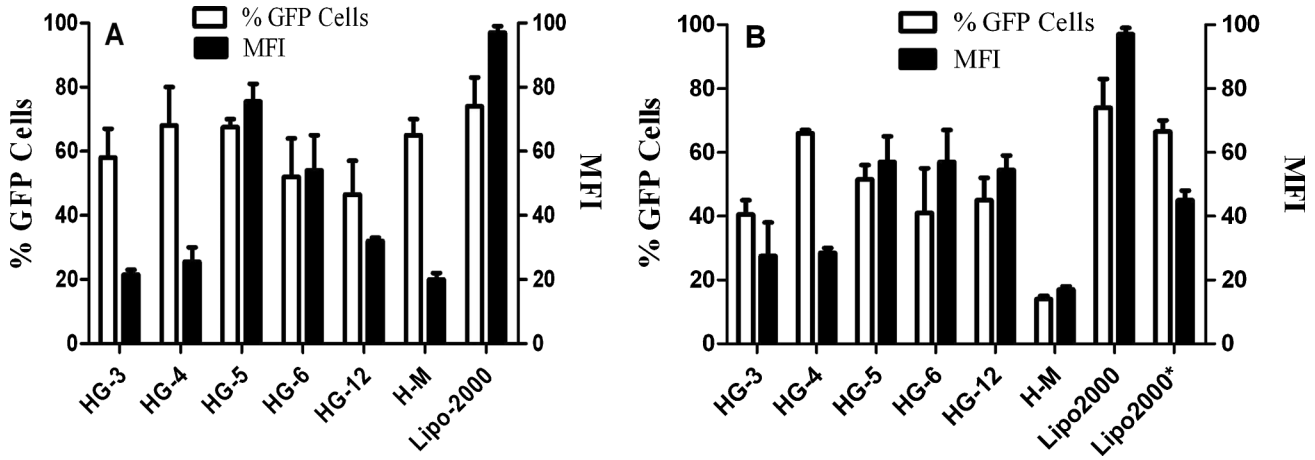


Fig. 6 Transfection efficiencies of optimized gemini lipid : DOPE formulations at optimized N/P ratios in the absence and presence of serum in comparison to monomeric lipid based formulation and Lipofectamine 2000. Comparative transfection efficiencies (A) without serum and (B) in presence of 10% serum. Concentrations of DNA = 0.8 μg/well. Data are expressed as the number of transfected cells and MFI as obtained from the flow cytometry analysis. Lipo2000* shows transfection activity of Lipofectamine 2000, when experiment was done in 10% FBS using our optimized conditions.

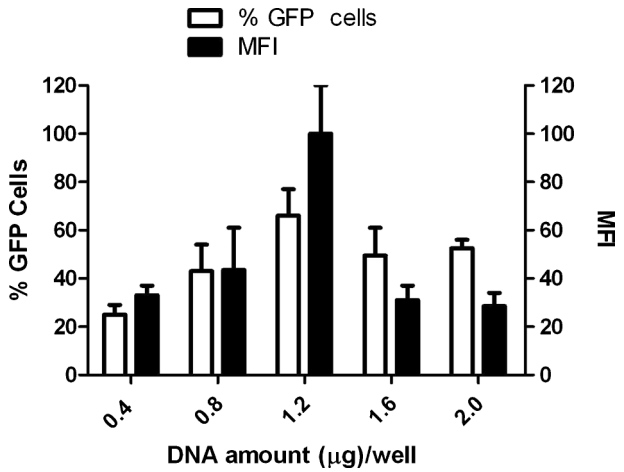


Fig. 7 Effects of variation of percentages of serum on the gene transfection efficiency of HG-5L/DOPE (1 : 2) formulation at N/P ratio of 0.5.

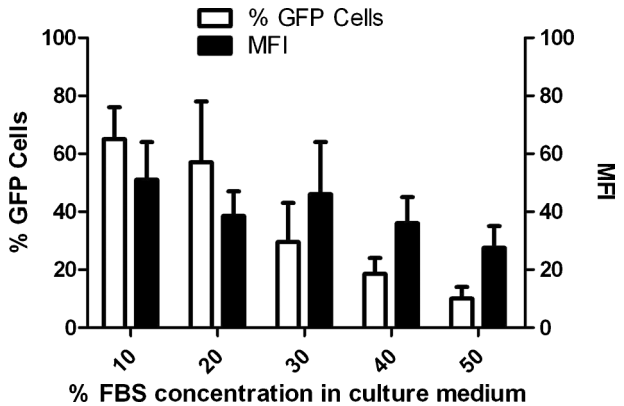


Fig. 8 Effect of variation of the amount of DNA on the gene transfection efficiency of gemini lipid HG-5/DOPE (1 : 2 mol ratio) formulation at N/P ratio of 0.5.

between cationic ammonium headgroups, possessing hydroxyethyl moieties, have been accomplished. Each of these new gemini lipids and their monomeric counterpart formed stable lipid suspensions in water. These gemini lipids in the presence of helper lipid, DOPE showed a significant enhancement in gene transfection activities as compared to their monomeric lipid counterpart. With increase in length of the spacer from trimethylene [-$(CH_2)_3$-] to pentamethylene [-$(CH_2)_5$-], the transfection efficiency increases, whereas further increase in the spacer length to dodecamethylene [-$(CH_2)_{12}$-] leads to a decrease in the transfection activity. All the gemini lipids were more effective than their monomeric counterpart "H-M". Gemini lipid HG-5 was found to be the best among all the formulations and showed better MFI compared to Lipofectamine 2000, one of the best commercially available transfecting agents. HG-5 afforded ~20% more cell viability and under optimized conditions showed more than two-fold higher MFI and comparable transfection efficiency in comparison to Lipofectamine 2000 in presence of 10% serum.

Thus it was possible to reduce cytotoxicity, enhance the transfection efficiency and serum stability significantly by optimizing the distance (spacer chain length in gemini lipid) between two cationic charges present on the lipid monomeric units of gemini which also possess hydroxyethyl groups. These gemini lipids could be synthesized conveniently incorporating desired variations at the molecular level.

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 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 or on a Micro-Mass ESI-TOF spectrometer or Shimadzu table-top GC-MS or ESI-MS (HP1100LC-MSD). Infra-red (IR) spectra were recorded on

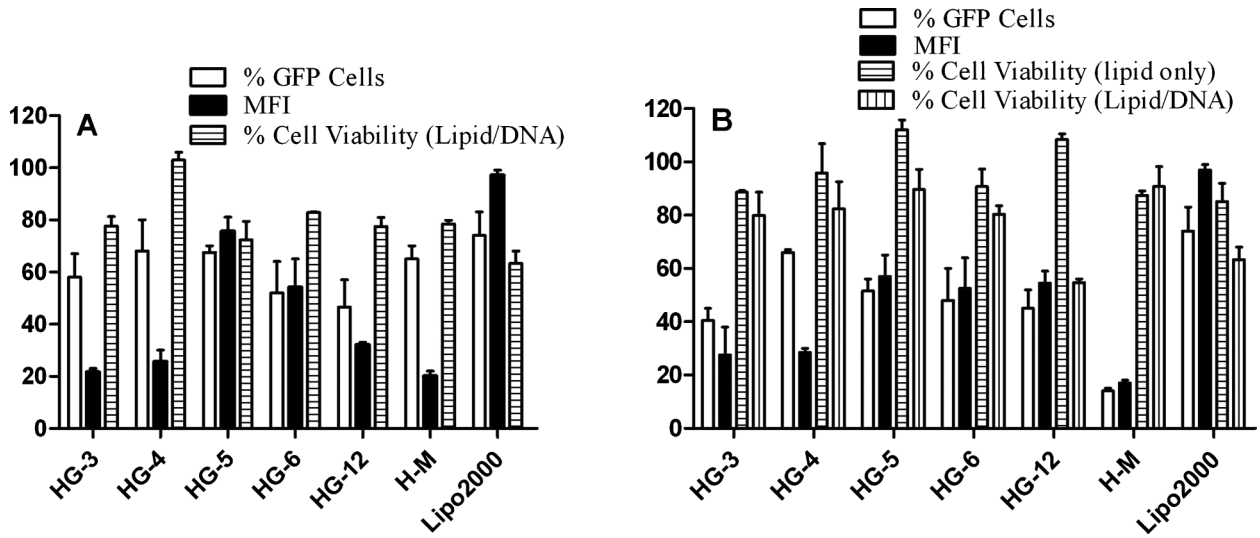


Fig. 9 Transfection efficiency as well as %-cell viability of gemini lipids. Transfection efficiencies were obtained using optimized lipid: DOPE formulations at optimal N/P ratios in absence and presence of serum in comparison to monomeric lipid based formulation and Lipofectamine 2000, while %-cell viabilities were obtained using optimized lipid: DOPE formulations at optimized N/P ratios. (A) In absence of serum and (B) in the presence of serum. Concentrations of DNA = 0.8 μg/well. Data are expressed as the number of transfected cells and MFI as obtained from flow cytometry analysis.

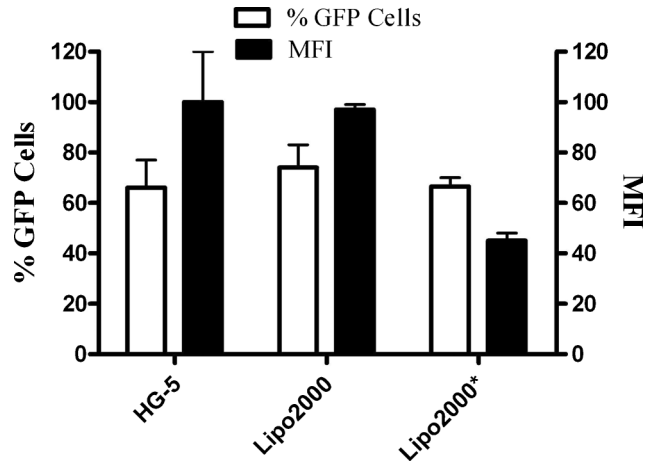


Fig. 10 Comparison of transfection efficacies of gemini HG-5 based formulation and two commercial transfecting agents. Transfection data were obtained using optimized HG-5 : DOPE formulation at optimized N/P ratios in presence of serum in comparison to Lipofectamine 2000. 'Lipo2000′ represents data obtained from cytometric analysis when transfection was performed using 'optiMEM' medium during lipoplex incubation with cells according to the protocol of the manufacturer. 'Lipo2000*' represents data obtained from cytometric analysis when experiment was performed in presence of 10% FBS as in case of HG-5. (A) In the absence of serum and (B) in presence of serum. Concentrations of DNA = 0.8 μg/well. Data are expressed as the number of transfected cells and MFI as obtained from flow cytometry analysis.

a JASCO FT-IR 410 spectrometer using KBr pellets or neat. All compounds were synthesized as described below and were characterized fully by their melting point, ^{1}H-NMR, ^{13}C-NMR, mass spectra and elemental analysis.

Synthesis. Cholest-5-en-3β-tosylate (1). To an ice-cooled solution of cholesterol (5.0 g, 0.013 mol) in dry pyridine (5 mL) and dry chloroform (5 mL), *p*-toluene sulfonyl 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. This solution was concentrated using rotary evaporator to leave a residue. The residue was recrystallized using chloroform and methanol to afford cholest-5-en-3β-tosylate (1) as a white solid, 6.49 g, (0.012 mmol, 92.8% yield). Mp: 72–75 °C. ^{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), 5.3 (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). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ 11.77, 18.66, 19.11, 21.59, 22.52, 22.78, 23.77, 24.20, 27.96, 28.16, 28.58, 31.70, 31.81, 35.71, 36.12, 36.30, 36.83, 38.84, 39.46, 39.61, 42.25, 49.86, 56.05, 56.59, 82.15, 123.68, 127.58, 129.69, 134.65, 138.79, 144.34. ESI-MS: 563.1 ($M + Na^+$). Anal. ($C_{34}H_{52}O_3S$) Calcd. C, 75.51; H, 9.69; S, 5.93; found. C, 75.23; H, 9.33; S, 5.80.

Cholest-5-en-3β-oxyethan-2-ol (2). Chlolest-5-en-3β-tosylate (1) (3.5 g, 6.5 mmol) was taken in 10 mL of 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 saturated aq. $NaHCO_3$ solution (50 mL), water (50 mL), and saturated brine solution (50 mL), and dried over anhydrous Na_2SO_4. Finally, the organic solvent was removed *in vacuo* and the product, cholest-5-en-3β-oxyethan-2-ol (2), was purified by column chromatography over silica gel using a mixture of petroleum ether and ethyl acetate. This afforded a white solid, 2.3 g, (5.3 mmol, 85% yield). Mp: 97–98 °C. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 0.67 (s, 3H), 0.85–2.35 (m, 41H), 3.20 (m, 1H), 3.57–3.6 (t, 2H, J = 4.5 Hz), 3.72 (t, 2H, J = 4.5 Hz), 5.34 (d, 1H, J = 4.5 Hz). ^{13}C ($CDCl_3$, 75 MHz): δ 11.84, 18.70, 19.35, 21.06, 22.54, 22.79, 23.82, 24.27, 28.00, 28.21, 28.41, 31.57, 31.90, 35.77, 36.18, 36.85, 37.17, 39.10, 39.51, 39.78, 42.31,

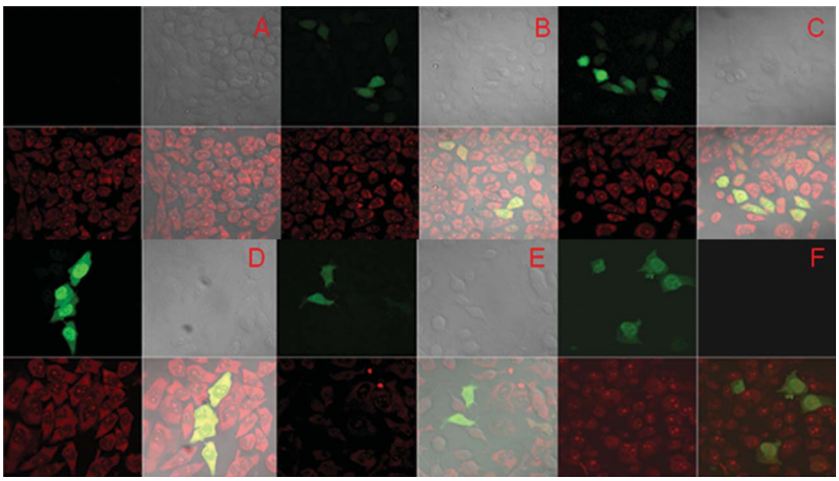


Fig. 11 Confocal images of pEGFP-C3 transfected HeLa cells stained with PI. (A) Untreated HeLa Cells; (B) HeLa Cells treated with HG-5 in absence of serum (–FBS–FBS), (0.8 μg DNA/well); (C) treated with HG-5 in absence of serum (–FBS–FBS), (1.2 μg DNA/well); (D) treated with HG-5 in presence of serum (–FBS+FBS), (1.2 μg DNA/well); (E) treated with Lipo2000* with serum (–FBS+FBS) and (F) treated with Lipo2000 without serum (–FBS–FBS).

50.18, 56.16, 56.76, 62.08, 68.92, 121.71, 140.71. ESI-MS: 453.2 ($M + Na^+$). Anal. ($C_{29}H_{50}O_2$) Calcd. C, 80.87; H, 11.70; found. C, 80.69; H, 11.56.

Cholest-5-en-3β-oxyethane tosylate (3). To an ice-cooled solution of cholest-5-en-3β-oxyethan-2-ol (2) (2.2 g, 5.1 mmol) in dry pyridine (5 mL) and dry chloroform (5 mL), *p*-toluenesulfonyl 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 (3), was purified by column chromatography over silica gel using a mixture of petroleum ether and ethyl acetate to furnish 2.68 g of the product (3) (7.04 mmol, 90.0% yield). Mp: 79–83 °C. ^{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, J = 4.5 Hz), 4.13–4.16 (t, 2H, J = 4.5 Hz), 5.31 (d, 1H, J = 4.5 Hz), 7.32–7.35 (d, 2H, J = 8.1 Hz), 7.79–7.82 (d, 2H, J = 8.1 Hz). ^{13}C ($CDCl_3$, 75 MHz): δ 11.86, 18.51, 19.02, 21.72, 22.67, 22.93, 23.62, 24.09, 27.86, 28.06, 28.50, 31.56, 31.94, 35.55, 36.06, 36.41, 36.75, 38.71, 39.28, 39.66, 42.09, 49.94, 55.74, 56.17, 56.73, 60.78, 82.08, 123.84, 127.71, 129.86, 134.79, 138.87, 144.51. ESI-MS: 607.4 ($M + Na^+$). Anal. ($C_{36}H_{56}O_4S$) Calcd. C, 73.93; H, 9.65; S, 5.48; found. C, 73.68; H, 9.48; S, 5.41.

Cholest-5-en-3β-oxyethane bromide (4). To a solution of cholest-5-en-3β-oxyethane tosylate (3) (1.75 g, 3.54 mmol) in dry DMF, lithium bromide (460 mg, 5.3 mmol) was added. The solution was refluxed under N_2 atmosphere at 70 °C for 7 h. Solvent was evaporated in vacuum. The crude product was dissolved in ethyl acetate (50 ml) and the organic layer was washed first with water (3 × 10 ml), and then with brine (saturated NaCl) (20 ml). Organic solvent was evaporated to get a yellowish solid. The product, cholest-5-en-3β-oxyethane bromide (4) was isolated as a pure material upon column chromatography over silica gel using a mixture of petroleum ether and ethyl acetate. This furnished a white solid (4), 1.23 g, (2.49 mmol, 95% yield). Mp: 70–73 °C. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 0.67 (s, 3H), 0.75–1.51 (m, 34H), 1.69–1.96 (m, 5H), 2.12–2.32 (m, 2H), 3.15 (m, 1H), 3.35–3.39 (t, 2H, J = 6.0 Hz), 3.69–3.73 (t, 2H, J = 6.0 Hz), 5.33 (d, 1H, J = 4.5 Hz). ^{13}C ($CDCl_3$, 75 MHz): δ 11.89, 18.56, 19.42, 21.14, 22.61, 22.83, 23.78, 24.21, 27.24, 28.07, 28.18, 28.39, 31.60, 31.85, 35.70, 36.23, 36.77, 37.14, 39.16, 39.47, 39.84, 42.26, 50.22, 56.11, 62.03, 68.89, 121.75, 140.75. ESI-MS: 515.6 ($M + Na^+$). Anal. ($C_{29}H_{49}BrO$) Calcd. C, 70.56; H, 10.01; found. C, 70.19; H, 9.88.

Cholest-5-en-3β-oxyethan-*N*-methyl-*N*-2-hydroxyethyl-amine (5). *N*-Methylethanolamine (25 mg, 0.33 mmol) was dissolved in dry CH_3CN in a round bottom flask. Cholest-5-en-3β-oxyethane tosylate (3) (293 mg, 0.5 mmol) dissolved in dry CH_3CN (4 mL) was carefully added to the N-methylethanolamine solution. The reaction mixture was refluxed at 80 °C for 24 h. 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 aq. $NaHCO_3$ (25 mL), water (25 mL), and then saturated brine solution (25 mL). Organic solvent was evaporated to get a yellowish solid. The residue was purified by column chromatography over silica gel using a mixture of $CHCl_3$ and MeOH to afford a yellowish solid, (5) 237 mg, (0.49 mmol, 97% yield). Mp: 67–70 °C. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 0.67 (s, 3H), 0.85–2.2 (m, 41H), 2.27 (s, 3H), 2.38 (m, 1H), 2.47–2.51 (t, 4H, J = 6.0 Hz), 3.11–3.18 (m, 1H), 3.54–3.59 (t, 2H, J = 6.0 Hz), 3.69–3.74 (t, 2H, J = 6.0 Hz), 5.34 (d, 1H, J = 4.5 Hz). ^{13}C ($CDCl_3$, 75 MHz): δ 11.87, 18.74, 19.40, 21.12, 22.63, 22.69, 23.77, 24.19, 28.06, 28.27, 28.50, 31.48, 31.99, 35.65, 36.05, 36.92, 37.09, 39.23, 39.68, 39.67, 42.56, 50.09, 50.74, 52.64, 56.29, 56.61, 61.94, 69.03, 121.64, 140.57. ESI-MS: 488.5 ($M + H^+$). Anal. ($C_{32}H_{57}NO_2$) Calcd. C, 78.79; H, 11.78; N, 2.87; found. C, 78.54; H, 11.58; N, 2.68.

Cholest-5-en-3β-oxyethane-*N*,*N*-dimethyl-*N*-2-hydroxyethyl ammonium bromide (H-M). *N*,*N*-Dimethylethanolamine (29 mg, 0.33 mmol) was dissolved in dry ethanol and taken in a screw-top pressure tube. Cholest-5-en-3β-oxyethane bromide (4)

(247 mg, 0.5 mmol) dissolved in dry MeOH (2 mL) was added to the *N*,*N*-dimethylamine solution. The pressure tube containing the reaction mixture inside was screw-capped and heated at 80 °C for 48 h. Then it was cooled and the solvent was removed by rotary evaporation. Crude reaction mixture was then crystallized repeatedly from a mixture of MeOH and ethyl acetate to afford a white solid (H-M) in 81% of yield 236 mg, (0.41 mmol). Mp: 164–168 °C. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 0.67 (s, 3H), 0.85–2.35 (m, 41H), 3.2 (m, 1H), 3.67 (s, 6H), 3.82–3.92 (m, 6H), 4.15 (s, 2H), 5.36 (d, 1H, J = 4.5 Hz). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ 11.6, 19.1, 20.7, 22.7, 23.2, 24.7, 27.3, 27.7, 28.2, 29.6, 30.4, 31.9, 35.8, 36.1, 37.2, 37.8, 39.7, 39.9, 44.0, 50.8, 52.5, 56.5, 58.3, 64.7, 65.4, 65.8, 67.7 80.6, 122.2, 140.1. ESI-MS: 503.1 ($M^+ - Br^-$). Anal. ($C_{33}H_{60}O_2NBr\cdot 0.5H_2O$) Calcd. C, 66.98; H, 10.39; N, 2.37; found. C, 67.28; H, 10.45; N, 2.06.

General method for the synthesis of Gemini Lipids. A solution of cholest-5-en-3β-oxyethan-*N*-methyl-*N*-2-hydroxyethylamine (5) (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, each reaction mixture was cooled and the solvent was evaporated to furnish a crude solid. The solid was repeatedly washed with ethyl acetate to remove any of the unreacted cholest-5-en-3β-oxyethan-*N*-methyl-*N*-2-hydroxyethyl amine (5) and the residue was finally subjected to repeated crystallizations from a mixture of MeOH and ethyl acetate. This furnished a white solid in each case and the product yields ranged from *ca.* 40% to 50%. The purity of each lipid was ascertained from TLC; the R_f values ranged from 0.2 to 0.3 in (10 : 1) $CHCl_3$/MeOH. All the new gemini lipids were fully characterized by melting point, IR, ^{1}H-NMR, ^{13}C-NMR, mass spectrometry, and CHN analysis. Pertinent spectroscopic and analytical data are given below.

Lipid HG-3. Mp: 202–204 °C. IR (neat) (cm^{-1}): 3434, 2926, 2858, 1469, 1355, 1274 and 1078. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 0.68 (s, 6H), 0.85–2.36 (m, 84H), 3.35 (br s, 8H), 3.69 (br s, 12H) 3.88 (br s, 8H), 5.3 (d, 2H, J = 4.5 Hz). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ 11.7, 18.4, 19.1, 21.3, 22.4, 22.8, 23.9, 27.3, 27.5, 28.0, 28.6, 31.1, 31.5, 32.5, 36.0, 36.6, 37.0, 38.1, 39.5, 39.7, 43.1, 49.8, 56.1, 57.5, 61.6, 62.8, 64.5, 65.1, 79.4, 122.6, 139.9. ESI-MS: 508.4 (M^{+2}/2). Anal. ($C_{67}H_{120}Br_2N_2O_4$) Calcd. C, 68.30; H, 10.27; N, 2.38; found. C, 68.34; H, 9.95; N, 2.49.

Lipid HG-4. Mp: 211–215 °C. IR (neat) (cm^{-1}): 3436, 2937, 2859, 1461, 1346, 1281 and 1087. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 0.67 (s, 6H), 0.85–2.33 (m, 86H), 3.28 (m, 2H), 3.36 (br s, 6H), 3.76 (br s, 12H) 3.87–3.96 (br m, 8H), 5.34 (d, 2H, J = 4.5 Hz). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ 11.5, 18.6, 19.3, 21.6, 22.3, 22.9, 23.5, 27.7, 27.9, 28.02, 28.7, 31.0, 31.7, 32.3, 36.3, 36.5, 37.2, 38.4, 39.3, 39.8, 43.4, 50.1, 56.5, 57.2, 61.3, 62.5, 64.7, 65.3, 79.9, 122.9, 139.3. ESI-MS: 515.4 (M^{+2}/2). Anal. ($C_{68}H_{122}Br_2N_2O_4\cdot 1.5H_2O$) Calcd. C, 67.03; H, 10.34; N, 2.30; found. C, 66.87; H, 10.41; N, 2.21.

Lipid HG-5. Mp: 219–224 °C. IR (neat) (cm^{-1}): 3429, 2936, 2869, 1456, 1361, 1283 and 1080. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 0.67 (s, 6H), 0.85–2.3 (m, 88H), 3.24 (m, 2H), 3.34 (br s, 6H), 3.69–3.77 (br m, 12H) 3.86–3.95 (br m, 8H), 5.32 (d, 2H, J = 4.5 Hz). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ 11.9, 18.7, 19.1, 21.8, 22.2, 22.5, 24.1, 27.4, 27.7, 28.3, 28.9, 30.7, 31.8, 32.5, 36.2, 36.7, 37.4, 38.0, 39.2, 39.9, 43.6, 49.4, 56.5, 57.9, 61.1, 62.8, 64.2, 65.7, 80.2, 121.9, 139.7. ESI-MS: 522.4 (M^{+2}/2). Anal. ($C_{69}H_{124}Br_2N_2O_4\cdot 2H_2O$) Calcd. C, 66.75; H, 10.39; N, 2.26; found. C, 66.49; H, 10.12; N, 2.37.

Lipid HG-6. Mp: 223–226 °C. IR (neat) (cm^{-1}): 3442, 2944, 2870, 1460, 1347, 1276 and 1077. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 0.67 (s, 6H), 0.85–2.32 (m, 90H), 3.16–3.24 (m, 2H), 3.36 (br s, 6H), 3.6–3.69 (br m, 12H), 3.82–3.92 (br m, 8H), 5.34 (d, 2H, J = 4.5 Hz). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ 11.6, 18.6, 19.7, 21.2, 22.5, 22.9, 23.7, 27.6, 27.8, 28.4, 28.7, 31.5, 31.9, 32.5, 36.2, 36.5, 37.3, 38.0, 39.3, 39.6, 43.3, 49.6, 56.0, 57.4, 61.3, 62.7, 64.3, 65.4, 79.9, 122.4, 139.9. ESI-MS: 529.4 (M^{+2}/2). Anal. ($C_{70}H_{126}Br_2N_2O_4$) Calcd. C, 68.94; H, 10.41; N, 2.30; found. C, 68.69; H, 10.23; N, 2.44.

Lipid HG-12. Mp: 230–233 °C. IR (neat) (cm^{-1}): 3432, 2945, 2872, 1454, 1353, 1282 and 1079. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 0.67 (s, 6H), 0.85–2.30 (m, 102H), 3.18–3.26 (m, 2H), 3.32 (br s, 6H), 3.64–3.72 (br m, 12H), 3.84–3.9 (br m, 8H), 5.38 (d, 2H, J = 4.5 Hz). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ 11.6, 18.8, 19.6, 21.6, 22.7, 23.1, 23.8, 27.0, 27.4, 28.4, 28.8, 30.7, 31.8, 32.3, 36.4, 36.7, 37.4, 38.5, 39.4, 39.7, 43.5, 49.6, 56.5, 57.9, 61.9, 62.4, 64.7, 65.5, 79.7, 122.7, 139.4. ESI-MS: 571.5 (M^{+2}/2). Anal. ($C_{76}H_{138}Br_2N_2O_4$) Calcd. C, 70.02; H, 10.67; N, 2.15; found. C, 69.82; H, 10.43; N, 2.36.

Liposome preparation. Cationic liposomes were prepared either from monomeric or gemini lipid alone or from its mixture with DOPE at specified ratios. For the mixed liposome preparation, individual solution of lipids in chloroform was taken in different molar ratios of DOPE and gemini lipids in Wheaton glass vials. For pure lipid vesicle preparation, only a given lipid was dissolved in chloroform and taken in Wheaton glass vials. After mixing the organic solvent was evaporated by rotating the vials under N_2 stream, which afforded a thin layer of a lipid or lipid mixtures on the bottom wall of the Wheaton glass vials. Traces of organic solvents were removed under high vacuum condition. The resulting dry thin lipid layer was hydrated at 4 °C for 10–12 h with freshly autoclaved water (Milli-Q). Buffered water was added in such a way that the final lipid concentration in the suspension was maintained at 0.5 mg ml^{-1}. To assume optimal hydration, the lipid layer was subjected to freeze-thaw cycles from 0 °C to 60 °C several times to furnish multi-lamellar vesicles (MLVs). Subsequent bath sonication of MLVs at 60 °C for 20 min then afforded unilamellar liposomes. Cationic lipid or lipid-DOPE suspensions were prepared and kept under sterile condition. Formulations were found to be stable when stored at 4 °C.

Plasmid DNA. Plasmid pEGFP-c3 (Clontech, USA) which encodes for an enhanced green fluorescence protein (GFP) under a CMV promoter was employed for transfection studies. It was amplified in Escherichia coli (DH10α). Plasmid pEGFP-c3 has anti kanamycin gene which allows transformed E. coli to grow selectively in kanamycin containing bacterial culture medium (conc. 10 μg ml^{-1}). Amplified pEGFP-c3 was extracted 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 DNA was estimated spectroscopically by measuring the absorption

spectrum at 260 nm. The plasmid preparations showing a value of $OD_{260}/OD_{280} > 1.8$ were used for further investigation.

***In vitro* cell culture.** HeLa cells (Human cervical cancer cell) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma) supplemented with centrifuged 10% fetal bovine serum (FBS) in T25 culture flasks (Nunc, Denmark) and were incubated at 37 °C in a 99% 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 Dulbecco's phosphate buffer saline (DPBS, pH 7.2). Non-synchronized cells were used for all the experiments.

Transfection procedure. To measure the gene delivery efficiency of different lipid formulations, we performed transfection experiments on HeLa cells.[31] Cells, cultured in T25 culture flasks, were trypsinized and plated in 24-well plates as 60 000 cells per well in antibiotic free 10% FBS containing DMEM medium. Cells were grown for 24 h at 99% humidity, 37 °C temperature and 5% CO_2 condition till cell-monolayer gained ~70% confluence. All the experiments were performed using 0.8 μg of DNA per well unless specified otherwise. Working stocks of DNA and lipid formulations were prepared in DMEM. Separately diluted DNA and desired amount of lipid formulations were mixed in a total volume of 200 μl of DMEM and incubated 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. For transfection experiment in the presence of serum (–FBS+FBS), after 30 min of complexation, 200 μl of media possessing 0, 20, 40, 60, 80 and 100% FBS were added to the complexes (final DNA concentration = 12.1 μM) to get final FBS concentration 0, 10, 20, 30, 40 and 50% respectively. Old medium was removed from the wells followed by washing of cells with DMEM. Lipid-DNA complexes in 200 μl media per well were added to the cells. Then plates were incubated for 6 h at ambient condition. Old medium was removed from the wells after 6 h of incubation.

In both the cases, 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 the level of reporter gene expression. GFP expression was examined by fluorescence microscopy and was quantified by flow cytometry analysis. Control transfections were performed in each case using commercially available transfection reagent 'Lipofectamine 2000' based on the standard conditions specified by the manufacturers as well as the protocols as optimized by us. Here, the results obtained with 'Lipofectamine 2000' from manufacturer's protocol are represented as Lipo2000 and the results obtained with 'Lipofectamine 2000' using conditions standardized by us are shown as Lipo2000*. Thus the latter involves complexation of Lipo2000 with plasmid DNA in DMEM with no serum followed by incubation of lipoplex with cells in presence of DMEM containing 10% of FBS. Each experiment was performed in duplicates and results presented are the averages of at least two such independent experiments carried out on two different days. For comparison, transfections using formulations based on monomer (H-M) were performed using optimized lipid : DOPE ratio of 1 : 0 at N/P ratio of 4.0, using 0.8 μg of plasmid DNA and presented in data as 'monomer'.

Flow cytometry and FACS analysis. The reporter gene expression of pEGFP-c3 plasmid examined by fluorescence microscopy at regular intervals and quantified 42 h post-transfection by flow cytometry. After 42 h of transfection, old medium was removed from the wells and cells were thoroughly washed with 100 μl DPBS to remove traces of serum from the cell which prevents cell splitting during trypsinization. Cells were trypsinized with 100 μl of 1% trypsin (EDTA 0.02%, dextrose 0.05%, and trypsin 0.1%). After thorough wetting of cells, trypsin was removed from the wells and cells were incubated at 37 °C in a 99% humidified atmosphere containing 5% CO_2 to provide appropriate condition for cell splitting activity of the enzyme trypsin. Split cells were collected in eppendorf tubes in 200 μl of 5% FBS containing DPBS. Duplicate cultures were pooled and quantified immediately using Becton and Dickinson flow cytometer equipped with a fixed laser source at 488 nm.

The FACS data obtained were analyzed using public domain software WinMDI to eliminate data from cell debris, the dead and clumped cells. Subcellular debris and clumps could be distinguished from single cells by size (estimated by the intensity of low angle forward scatter). The FACS scans were 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. Therefore, the data from dead cells had been eliminated by gating out brightly fluorescent cells.

Cytotoxicity assay. Each cationic lipid formulation, with and without DOPE, was investigated to assess their toxicity level toward HeLa cells in presence of 10% FBS in antibiotic-free media. Toxicity was determined using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide reduction assay (MTT Assay) following literature procedures as described.[32–34] In brief, experiments were performed similar to that employed during transfection procedure in presence of 10% FBS with changes in DNA amounts and cell number per well. Nearly, 15 000 cells per well were plated in 96 well plates (Nunc, Denmark) growing 24 h before transfection experiment. Optimized lipid-DNA complexes were prepared using 0.2 μg of DNA per well, at different charge (N/P) ratios (*i.e.*, 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, 400 μl of 15% FBS containing media were added to 200 μl of complexes (final DNA concentration = 12.1 μM) to get final 600 μl Lipid-DNA complexes possessing 10% FBS. Experiment was performed in triplicates. Old media were removed and cells were incubated with lipoplex suspension, containing 10% FBS, for 6 h. After 6 h of incubation at 37 °C in a 99% humidified atmosphere containing 5% CO_2, lipoplexes were medium was removed and 200 μl DMEM containing 10% FBS was added. After 42 h of incubation, cells were treated with 20 μl (5 mg ml^{-1}) of MTT as per well and further incubated for 4 h. Then the entire media were removed from wells and 200 μl DMSO was added and kept on flat rocker for 10 min to dissolve blue colored formazan crystals. The absorbance was measured using a micro-titer plate reader. The %cell viability was then calculated from readings obtained

from ELISA reader using the formula, %viability = [{A_{590}(treated cells) – background]/[A_{590}(untreated cells) – background}] × 100.

Transfection efficiency as determined from luciferase assay. To quantitate the transfection efficiency of each lipid formulation, both with and without DOPE as well as to obtain optimized N/P charge ratio the lipoplexes were examined using luciferase activity measurements. Experiment performed in this step was very similar to the one performed using GFP based transfection efficiency measurement by FACS analysis. Here it was performed using a single luciferase assay kit provided by Promega (USA), following manufacturer's protocol. Briefly, 60 000 HeLa cells were plated in a 24 well plates 24 h before experiment, so that it became ~70% confluent at the time of experiment. The lipid formulations with or without DOPE at optimized N/P ratios were complexed with pGL-3 in (–FBS–FBS) conditions and incubated with HeLa cells for 6 h at 37 °C and 5% CO_2 condition in 99% humidity condition. After 6 h cells were washed with DMEM media and grown in 500 µl of 10% FBS containing DMEM for ~48 h in standard conditions. After 48 h post transfection, old media were removed from the wells, and cells were washed three times with 200 µl DPBS. To each well 100 µl of cell lysis buffer was then added and the cells were lysed for 30 min in 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 assay, 10 µl of this supernatant was used and 10 µl of luciferase assay substrate (Promega) was used. The lysate and the substrate were both thawed to room temperature 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, USA) in standard single luminescence mode. The measurement was performed for 10 s. A delay of 2 s was given before each measurement. Comparison of the transfection efficiencies of individual lipid formulations were made based on data for luciferase expressed as arbitrary light units (ALU).

Gel electrophoresis. DNA complexation efficiency of lipid formulations was determined by gel electrophoresis. Experiment was performed on 1% agarose gel containing ethidium bromide (20 µg/100 ml) and the gel was run in Tris-acetate-EDTA (TAE) buffer containing ethidium bromide (20 µg/100 ml) electrophoretically. Plasmid DNA (0.2 µg/well) was complexed with lipid formulations at different N/P charge ratios as was done in the case of the transfection experiments. After 30 min of incubation, the complexes were loaded and run electrophoretically. Uncomplexed DNA moved out of the well, but the DNA that was complexed with the cationic lipid remained inside the well[6] as observed under UV light which showed bright fluorescent bands due to the formation of DNA-ethidium bromide complexes.

Confocal fluorescence microscopy. To visualize the gene delivery efficiency of lipid formulations, we performed confocal microscopy on HeLa cells, exactly in the way the transfection experiments were performed.[35] In brief, cells were cultured in T25 culture flasks, trypsinized and plated in 24-well plates, having autoclaved glass slips in wells, as 60 000 cells per glass slip in antibiotic free 10% FBS containing DMEM medium as cells remained on glass slips. Cells were grown for 24 h at 99% humidity, at 37 °C and 5% CO_2 condition till cell-monolayer gained ~70% confluence. The experiments were performed using 0.8 µg and 1.2 µg of DNA per well. Working stocks of DNA and lipid formulations were prepared in plain DMEM. Separately diluted DNA and the desired amount of lipid formulations were mixed in a total volume of 200 µl of plain DMEM and incubated at room temperature for about 30 min to make lipoplexes. For transfection experiment in absence of serum (–FBS–FBS), after 30 min of complexation, 200 µl of plain DMEM media were added to the complexes, while for experiment in presence of 10% serum, 200 µl of 20% FBS containing DMEM were added to the complexes. Old medium was removed from the wells followed by washing of cells with DMEM. Lipid-DNA complexes in 200 µl media per well were added to the cells. Then plates were incubated for 6 h at 37 °C in a 99% humidified atmosphere containing 5% CO_2. In both the cases, at the end of incubation period, medium was removed, cells were washed with DMEM and 500 µl of DMEM containing 10% FBS was added per well. Plates were further incubated for a period of 36 h before checking under confocal microscope. Control transfections were performed in each case by using commercially available transfection reagent 'Lipofectamine 2000′ using conditions specified by the manufacturers, both in absence (–FBS–FBS) and presence (–FBS+FBS) of serum. Further experiment was performed under dark condition. After 36 h of incubation, all the medium was removed from the wells and cells were washed with DPBS buffer properly and carefully to remove all the cell debris without disturbing the monolayer of cells. Then cells were fixed for 10 min with paraformaldehyde using 1 ml of 4% paraformaldehyde in each well. Cells were washed with DPBS for 3 × 10 min. Then cells were kept with 1 ml 0.1% Triton-X-100 for 5 min to increase membrane permeabilization. Again cells were washed with DPBS for 3 × 10 min. Then glass slips having fixed and permeabilizable monolayers of HeLa cells were taken out from the wells and kept on glass slides and incubated for 5 min with 1 µg ml^{-1} of PI (propidium iodide) to specifically stain the nucleus of the cells. Again cells were washed with DPBS for 3 × 10 min to remove extra PI and to reduce over staining. Then vector shield was used to mount the cell possessing glass slips on glass slides and examined under confocal microscope.

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