Design, Synthesis, and Transfection Biology of Novel Cationic Glycolipids for Use in Liposomal Gene Delivery

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The molecular structure of the cationic lipids used in gene transfection strongly influences their transfection efficiency. High transfection efficiencies of non-glycerol-based simple monocationic transfection lipids with hydroxyethyl headgroups recently reported by us (Banerjee et al. *J. Med. Chem.* **1999**, *42*, 4292−4299) are consistent with the earlier observations that the presence of hydroxyl functionalities in the headgroup region of a cationic lipid contributes favorably in liposomal gene delivery. Using simple sugar molecules as the source of multiple hydroxyl functionalities in the headgroup region of the transfection lipids, we have synthesized four novel simple monocationic transfection lipids, namely, 1-deoxy-1-[dihexadecyl(methyl)-ammonio]-D-xylitol (**1**), 1-deoxy-1-[methyl(ditetradecyl)ammonio]-D-arabinitol (**2**), 1-deoxy-1-[dihexadecyl(methyl)ammonio]-D-arabinitol (**3**) and 1-deoxy-1-[methyl(dioctadecyl)ammonio]-D-arabinitol (**4**), containing hydrophobic aliphatic tails and the hydrophilic arabinosyl or xylose sugar groups linked directly to the positively charged nitrogen atom. Syntheses, chemical characterizations, and the transfection biology of these novel transfection lipids **1**−**4** are described in this paper. Lipid **1**, the xylosyl derivative, showed maximum transfection on COS-1 cells. All the lipids showed transfection with cholesterol as colipid and not with dioleoylphosphatidylethanolamine (DOPE). Radioactive quantitation of free and complexed DNA combined with ethidium bromide exclusion measurements suggest that though nearly 70% of the DNA exists as complexed DNA, the DNA may not have condensed as was observed with other cationic lipids. Presence of additional (more than two) hydroxyl functionalities in the headgroup of the cationic lipids appears to have improved the transfection efficiency and made these lipids less cytotoxic compared to two-hydroxyl derivatives.

Introduction

Delivering genes into cells or tissues is an extremely promising therapeutic tool to alleviate morbidity in clinically challenging genetic diseases and cancers. In the area of gene therapy, the focus of an intensely pursued current research is centered around development of efficient viral[1] and nonviral transfection vectors.[2−10] Among nonviral vectors, cationic liposome-mediated transfection holds considerable promise for its (a) reproducibility and simplicity in preparation, (b) nonimmunogenic nature, and (c) efficiency in forming stable injectable complexes even with large DNA.[9] A wide variety of cationic lipids have been synthesized and tested for their transfection efficiencies as ingenious formulations with success.[8] Despite a considerable knowledge base on the transfection biology of cationic lipids, the critical factors influencing the transfection efficiency of cationic lipids are still elusive.[11]

A number of physical properties of the lipid−DNA complexes were studied in an effort to correlate them with the transfection efficiency. The size, surface potential,[12] DNase sensitivity,[13] density centrifugation,[14] ethidium bromide exclusion,[12,15] circular dichroism,[16] fusogenic ability of the complexes,[13,14] electron microscopy,[17] and X-ray diffraction of the lipid−DNA complexes[18] were investigated to understand the cell biological basis of transfection. Broadly, these studies made the following observations: (a) at 1:1 charge ratio of lipid to DNA, i.e., at charge neutrality, the ζ potential of the complex becomes positive and the particle size increases abruptly; (b) cationic lipids condense the DNA; (c) cationic liposomes that have fluid membranes help transfection; (d) cone-shaped colipids facilitate endosomal escape; and (e) the biology of the cell has strong bearing on the transfection efficiency.

Transfection efficiency strongly depends on the cell type. The factors capable of modulating transfection efficiencies include (a) adsorption of the lipid−DNA complex to the cell surface;[11,19] (b) uptake of the complex, mainly by endocytosis;[19] (c) escape of DNA from the endosomes;[20] (d) the half-life of DNA in the cytoplasm;[21] and (e) entry of the DNA into the nucleus.[22] The molecular architecture of the cationic lipids is likely to influence the first three of these factors. Since Felgner et al.[23] reported their first cationic liposome-mediated gene delivery in 1987, there has been a considerable

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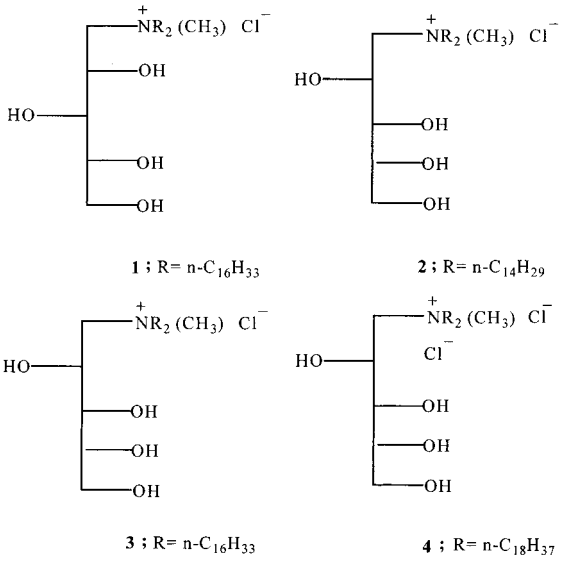
Chart 1



increase in design and syntheses of efficient nonviral cationic transfection vectors.[4,7,24−44] Interestingly, many of these reported cationic transfection lipids, such as DOTMA, DMDHP, DMRIE, DOTAP, etc., have a common element in their molecular structure, namely, the presence of a glycerol backbone.[8] Among these glycerol-based transfection lipids, the most efficient lipids, such as DMRIE and DMDHP, contain one or two hydroxyethyl groups directly attached to the positively charged nitrogen atoms. Recently, we reported, for the first time, a series of highly efficient non-glycerol-based simple monocationic transfection lipids in which the most promising lipid was demonstrated to be DHDEAB, containing two hydroxyethyl functionalities directly attached to the positively charged nitrogen atom.[41] The promising transfection efficiency of DHDEAB motivated us to design and synthesize non-glycerol-based cationic amphiphiles containing more than two hydroxyl functionalities in the headgroup regions and investigate their transfection efficiencies. Herein, we report the design, syntheses, and transfection biology of four novel non-glycerol-based cationic glycolipids, namely, 1-deoxy-1-[dihexadecyl(methyl)ammonio]-D-xylitol (**1**), 1-deoxy-1-[methyl(ditetradecyl)ammonio]-D-arabinitol (**2**), 1-deoxy-1-[dihexadecyl(methyl)ammonio]-D-arabinitol (**3**), and 1-deoxy-1-[methyl(dioctadecyl)ammonio]-D-arabinitol (**4**) containing simple monosaccharide units as the source of multiple hydroxyl functionalities in the headgroup region.

Results

Chemistry. The key structural elements common to all the transfection lipids **1**−**4** described herein include (a) the presence of hydrophobic group directly linked to the positively charged nitrogen atom, (b) the presence of either an arabinosyl or a xylosyl pentose sugar group attached covalently to the positively charged nitrogen atom, and (c) the absence of glycerol backbone in the molecular architectures of the monocationic glycolipids. The details of the synthetic procedures for all the novel transfection lipids shown in Chart 1 are described in the Experimental Section. As outlined in Schemes 1 and 2, the chemistries involved in preparing these new lipids are straightforward. However, given their remarkably high transfection efficiencies, the overall yields of these transfection lipids need to be improved in future.

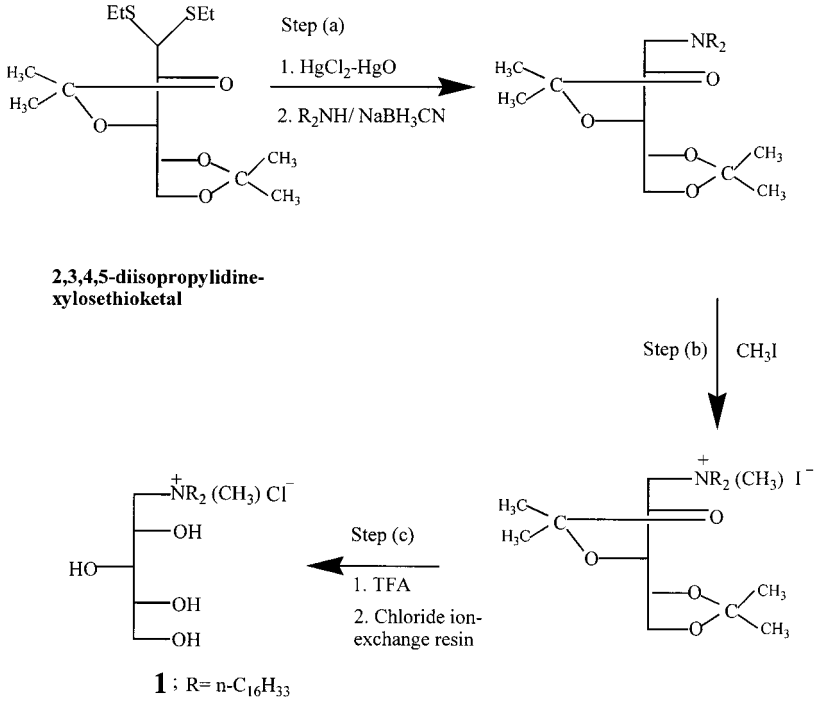
Scheme 1 outlines the general synthetic strategies adopted for preparing lipid **1.** The steps involved were (a) reacting 2,3,4,5-diisopropylidinexylose thioketal with mercuric chloride−mercuric oxide to deprotect the thioketal group selectively to aldehyde, converting the resulting aldehyde to the intermediate iminium compound by treating with the appropriate secondary amine, followed by in situ reduction of the resulting iminium compound with sodium cyanoborohydride to produce the diisopropylidine derivative of the intermediate tertiary amine; (b) treating the diisopropylidine derivative of the tertiary amine prepared in step a with methyl iodide; and (c) sequential treatment of the quaternized ammonium iodide intermediate obtained in step b with trifluoroacetic acid and chloride ion exchanger resin, respectively.

Synthesis of lipids **2**−**4** (Scheme 2) essentially consists of similar steps as outlined in Scheme 1 for the synthesis of lipid **1** except with the use of thioketal- and diisopropylidine-protected arabinose as the starting material. Highly exchangeable protons of the hydroxyl functionalities in the sugar headgroups caused severe line broadening of the peaks in the ^{1}H NMR spectra of all the final glycolipids. However, the molecular ion peaks were observed mostly as the base peaks in LSIMS-HRMS or FABMS as described in the Experimental Section. Given the overall mild reaction conditions employed in our method, stereo-integrities of the sugar skeletons in lipids **1**−**4** are likely to be preserved throughout the reaction sequences.

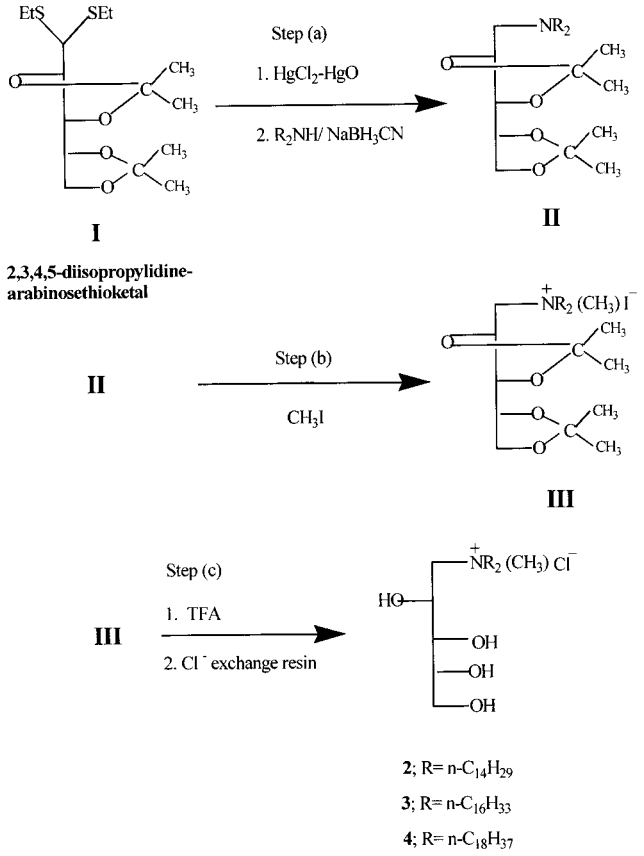
Transfection. The transfection efficiencies of cationic lipids **1**−**4** were tested in COS-1 cells with pCMV-β-gal plasmid, carrying the β-galactosidase reporter gene. Since cationic transfection lipids work efficiently with colipids, preferably DOPE or cholesterol, we prepared lipid formulations containing the cationic lipids **1**−**4** with both of these colipids at 1:1 mole ratios. The transfection efficiencies of the eight formulations, i.e., **1**−**4** with either DOPE or cholesterol, were tested on COS-1 cell lines along with DHDEAB (Figure 1). All the four lipids **1**−**4** showed higher reporter gene activity with cholesterol as colipid compared to DOPE. Lipids **1**−**4** without any colipids showed transfection efficiency equal to or lower than transfection obtained with DOPE as colipid (Figure 1). The charge ratios between 1 and 3 (lipid/DNA) produced maximal reporter gene activity. Above 3:1 charge ratio and up to 9:1, the transfection efficiency was poor as seen by the decrease in β-gal activity (Figure 1). Lipofectin gave values lower than 5 units (10^{-4}) at comparable charge. Lipoplexes with charge ratios above 3 were poor in transfecting. In our earlier studies, DHDEAB was compared with Lipofectin and LipofectAmine and found to be equal or better than these extensively used commercial formulations.[41] In the present study we have compared the transfection efficiencies of the four lipids **1**−**4** with that of DHDEAB. Lipid **1** gave the best results with cholesterol as colipid. Lipid **3** containing a palmityl hydrophobic chain was better among the arabinose headgroup lipids **2**−**4**.

Formation of DNA−Lipid Complexes and Sensitivity of the Complexes to DNase I. The binding

Scheme 1. Synthesis of Lipid **1**



Scheme 2. Synthesis of Cationic Glycolipids **2**−**4**



of lipids **1**−**4** to DNA was evaluated by quantitating both free DNA and DNA bound in the complex. DNA−lipid complexes were made and resolved by agarose gel electrophoresis in order to separate the bound and free DNA. Ethidium bromide (EtBr) staining to visualize the DNA in a lipid−DNA complex is complicated; hence, solvent extraction and centrifugation were used to separate and quantitate the free DNA from bound DNA.[12,14,47] Several reports used EtBr staining as a method of viewing the free DNA, which permits quantitation of the free DNA.[12,17] To quantitate both bound and free DNA in electrophoresis, we have used radioactive plasmid DNA. This approach is simple since the separation and quantitation of free and complexed DNA does not involve any additional steps and is highly reproducible. The agarose gel after electrophoresis was exposed to a Fuji phosphorimager and the radioactive bands were qunantitated. Nick-translated radioactive pCMV-β-gal DNA was complexed with lipids **1**−**4** with cholesterol as colipid at varying lipid−DNA charge ratios (agarose gels containing radioactive bands of complexed and free DNA after electrophoresis are shown in Figure S1 of the Supporting Information). In these studies lipid preparations with DOPE as colipid was not included since the transfection with DOPE was poor. Free plasmid was well resolved from the positively charged complexes that were retarded and retained in the well. The intensity of the band in the well was maximum at the 3:1 lipid/DNA charge ratio, indicating the formation of a positively charged complex that does not migrate in an electrophoretic field. Correspondingly, the amount of free DNA migrates at the same position as the free (control) DNA, lane 1, albeit with decreased band intensity. The radioactive bands in the gel were quantitated by densitometry and the percentages of free DNA in lipid−DNA complexes with four lipids (with cholesterol as the colipid) are shown in Figure 2. The amount of free DNA was maximum at low charge ratios and lower at high charge ratios. Densitometric quantitation indicates that at 3:1 charge ratio nearly 70% of the DNA exists as lipid−DNA complex with all the four lipids **1**−**4** and DOTAP, a lipid well-known for its remarkable DNA-condensing ability (hence used as a control in Figure 2). The rank order of lipids in terms of binding strength was **3** = DOTAP < **4** < **2** < **1** at

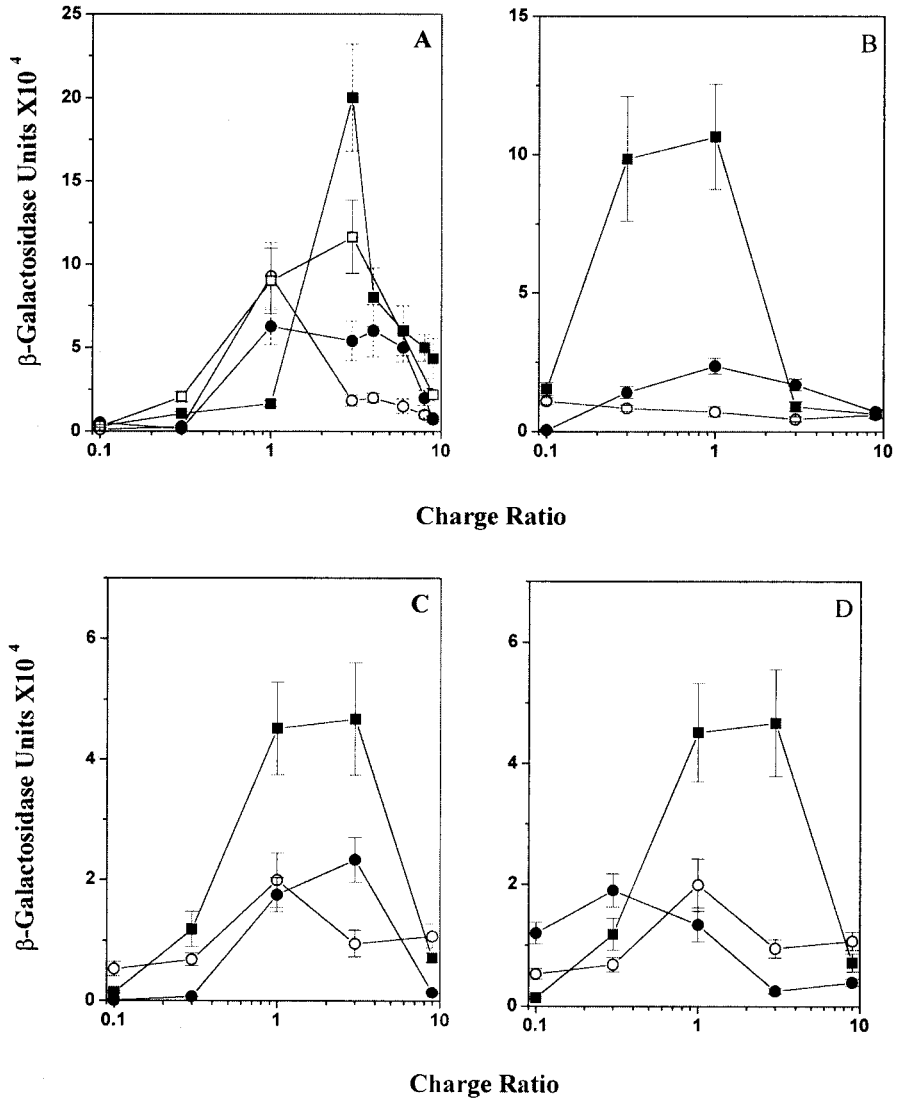


Figure 1. Transfection efficiencies of lipids **1**−**4** on COS-1 cells without any colipid (○), with cholesterol as colipid (■), and with DOPE (●) as colipid. Units of β-galactosidase activity were plotted against the varying lipid to DNA (±) charge ratios. The *o*-nitrophenol formation (micromoles of *o*-nitrophenol produced per minute) was converted to units by use of a standard curve obtained with pure (commercial) β-galactosidase. **(**A**)** Lipid **1**; **(**B**)** lipid **2**; **(**C**)** lipid **3**; **(**D**)** lipid **4**. The transfection efficiency of DHDEAB (□) was compared with lipid **1**. All the lipids were tested on the same day and the data presented are averages of three experiments ($n = 3$). DNA (0.3 μg) was complexed with lipid at various charge ratios in a volume of 50 μL for 30 min and added to cells after the medium was diluted to 100 μL. The incubation of lipoplex with cells was allowed for 30 min before addition of another 100 μL of DMEM medium. The reporter gene activity was assayed after 48 h by lysing the cells with NP40 (0.5%) containing PBS.

50% free DNA. With lipids **2** and **4**, maximum transfection was seen at a charge ratio of 1:1, where only 25% and 5% of DNA exists as lipid−DNA complex, respectively. A large amount of added DNA exists as free and uncomplexed. These data suggest that the transfection-competent complex formation was very much less in percentage; when viewed in combination with uptake studies, it appears that nearly 50% complexed DNA enters the cell.

When the accessibility of DNA in the lipid−DNA complex was probed with DNase I, maximum protection was observed at charge ratios of 3:1 and some protection was also seen at 1:1 charge ratios (Figure 3). At lower lipid to DNA charge ratios the free DNA was highly susceptible to degradation by DNase I and was digested to a large extent in the time period of incubation. Control DNA, in the absence of the lipid (Figure 3, lane 2), was also completely digested within 10 min. DNA present as a complex with lipid was undigested and remains in the well. Lipid **4** was least protective among the four lipids to DNase I treatment. We observed some undigested free DNA (e.g., lanes 4, 5, 8, and 12 in Figure 3). The reaction of DNase I was stopped with EDTA (30 mM) and then the agarose gel was run. EDTA, besides inhibiting the DNase I activity, could interact with the cationic lipids and destabilize the lipid−DNA complex. The DNA released after interaction of EDTA with the lipid−DNA complex could be seen as free DNA. The ability of EDTA to destabilize the complex was confirmed by independently observing free DNA bands at 3:1 charge ratio with increasing amounts of added EDTA in the absence of DNase I treatment (data not shown).

Ethidium Bromide Titration of the Lipid−DNA Complex. Intercalation-induced fluorescence increase and competition with cationic lipids to bind to DNA has

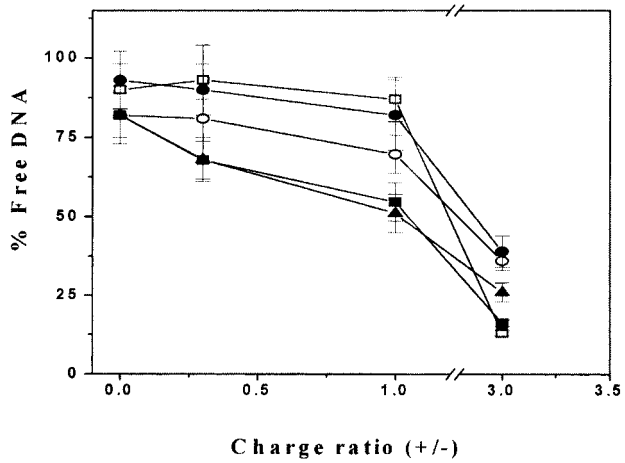


Figure 2. Percent of free DNA in lipid−DNA complexes prepared with four lipids (with cholesterol as colipid) and DOTAP. Agarose gels were scanned and quantitated by phosphorimager. By considering the total lane intensity as 100%, the free and complexed DNA percentages were calculated and replotted. Free DNA (percent) was plotted against charge ratio for lipids **1** (●), **2** (○), **3** (■), **4** (□), and DOTAP (▲) (n = 2).

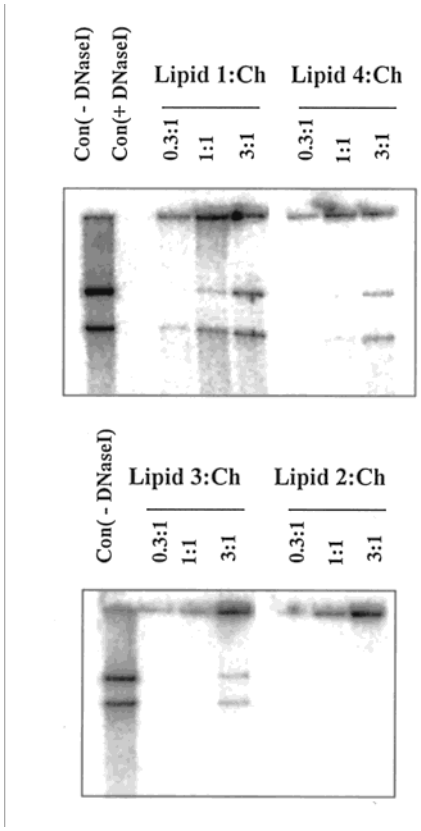


Figure 3. Sensitivity of lipid−DNA complexes to DNase I. The lipid−DNA complex was treated with DNase I as indicated in the Experimental Section and electrophoresed. The details of treatment are indicated at the top of each lane.

made EtBr an excellent tool to study lipid−DNA interactions. To investigate the interaction of lipids **1**−**4** with DNA, we have also titrated the EtBr−DNA complex with increasing amounts of cationic lipids. The data in Figure 4 shows that lipids **1**−**4** interact poorly with DNA, as seen by their relatively poor ability to exclude ethidium bromide from DNA when compared to DOTAP. The decrease in EtBr fluorescence at charge ratios

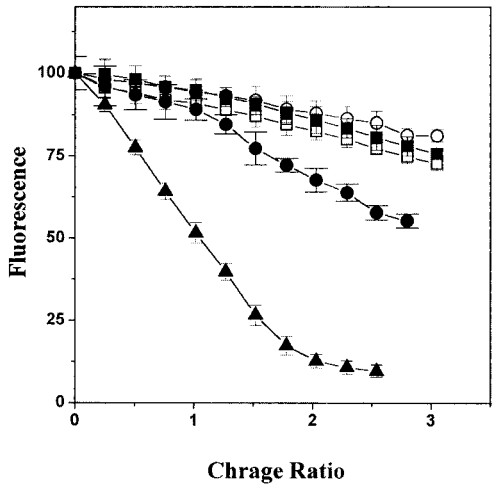


Figure 4. EtBr exclusion from lipid−DNA complex. Decrease in fluorescence of EtBr was used to assess the interaction of DNA with lipids **1** (●), **2** (○), **3** (■), **4** (□), and DOTAP (▲) (n = 2). DNA−EtBr complex was titrated with increasing amounts of lipid. The order of addition of lipid or EtBr to DNA did not alter the observed profiles. Fluorescence in the absence of lipid was taken to be 100%.

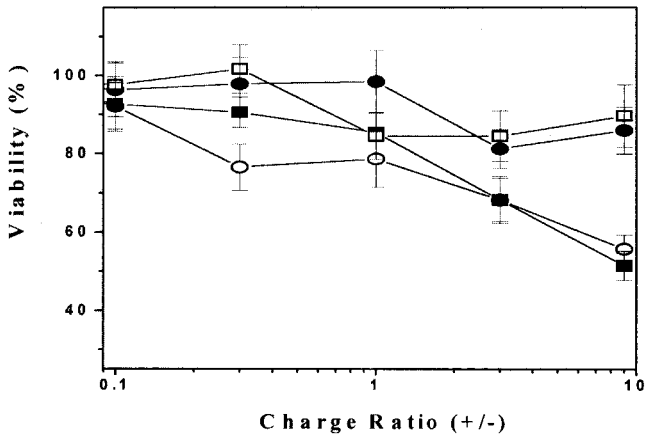


Figure 5. Cytotoxicity (viability) of lipids **1**−**4** on COS-1 cells. Toxicity of lipids **1** (●), **2** (○), **3** (■), and **4** (□) was assesssed in COS-1 cells by the MTT assay. The absorption obtained with reduced formazon with cells in the absence of lipids was taken to be 100%. The data presented are averages of three independent experiments (n = 3).

of 3:1 was less than 20% with lipids **2**−**4** and about 40% with lipid **1**, whereas the presence of 1.5:1 charge ratio DOTAP decreased 90% of the EtBr fluorescence. EtBr data when viewed in combination with the data obtained with radioactive DNA (Figure 2) indicate that, although at 3:1 charge ratio nearly 75% of the DNA exists as a complex and is retained in the loading well, the DNA may not be condensed, which is essential for squeezing out the intercalated EtBr from the DNA.

Cytotoxicity. An MTT-based viability assay was performed to assess the cytotoxicity of the four lipid formulations at various charge ratios with COS-1 cells. The lipid−DNA complexation and treatment were identical to transfections. Lipids **1** and **4** showed the least cytotoxicity even at 9:1 charge ratio (Figure 5). For lipids **1**−**4** at 3:1 charge ratio, the toxicity varied from 15% to 25% of the treated cells. At comparable charge ratios the two-hydroxyl derivatives showed higher cytotoxicity than the lipids **1**−**4**.[41]

Discussion

The considerable promise shown by hydroxyethyl headgroup-containing cationic lipids reported by us[41] and others[28,33] has prompted us to investigate the usefulness of cationic lipids with additional headgroup hydroxyl functionalities for improving cell transfections. In our earlier report,[41] DHDEAB [*N*,*N*-di-(*n*-hexadecyl)-*N*,*N*-di(hydroxyethyl)ammonium bromide] was shown to be the most promising among the non-glycerol-based simple monocationic transfection lipids we synthesized and tested. Similarly, the DORIE[28] series has shown considerable promise and was commercialized. In the present work, toward assessing the transfection efficiencies of non-glycerol-based monocationic transfection lipids with multiple hydroxyl functionalities in the headgroup region, we have used 5-carbon sugars, arabinose and xylose, as the source of four hydroxyl groups. Lipids **2**−**4** were designed with arabinose as the common headgroup with varying aliphatic hydrocarbon chain lengths (C-14, C-16, and C-18), whereas lipid **1** contains xylose as the headgroup with two C-16 saturated aliphatic hydrocarbon chains. Among the four lipids, lipid **1** showed the highest transfection efficiency. The reporter gene activity with lipid **1** was twice that of DHDEAB. Lipid **2** was equal to DHDEAB in transfection efficiency.

Lipids **2**−**4** with arabinose as the headgroup and containing different lengths of hydrophobic chains demonstrated good ability to deliver genes into the cells. A myristoyl derivative of the cationic lipid (**2**) showed maximum transfection. Myristoyl derivatives of several other classes of cationic lipids have also been reported before. Among them, the myristoyl derivatives of im1-[2-(acyloxy)ethyl]-2-alkyl(alkenyl)-3-(2-hydroxyethyl)-imidazolinium chloride have given higher in vitro transfection in CV-1 cells compared to oleoyl and palmitoyl derivatives.[4] Szoka and co-workers similarly reported the highest transfection efficiences of the myristoyl analogue of carnitines.[37] However, no strict dependency on chain length was observed in transfection of CV-1 cells in the study reported by Wang et al.[37] The 2,3-di(alkyloxy)propyl quaternary derivative of myristoyl was better compared to oleoyl, palmitoyl, and stearoyl derivatives in transfecting the COS-7 cells.[38] The transfection efficiency of the caproyl derivative of glycine betaines was better than those of lauroyl and myristoyl derivatives in transfecting the HT 29 cells.[39] High L_β to L_α transition temperatures of the lipids would render the membrane stiff and may not allow the fluidity required for lipid mixing events in endosomal escape or in any other fusogenic events the complex may be involved.[39] Among the saturated acyl chains, in general, shorter chains, less than palmitic acid, seem to help. Mannosylated and galactosylated cholesterol derivatives are capable of cell-specific delivery of genes (into macrophages and liver cells, respectively) and the uptake could be inhibited by either mannose or galactose.[48,49] Lipidic glycosides with aminoalkyl pendant groups showed good gene delivery and low toxicity to HeLa and 3T3 cells.[50] The lipid presented in this study were not designed with any surface receptor in consideration; however, it would be interesting to assess how far these 5-carbon lipids are similar to 6-carbon lipids in specific cell uptake, e.g., utilizing asialoglycoprotein receptor for gene transfer in liver cells.[48]

Earlier we have demonstrated that for non-glycerol-based simple monocationic lipids, a derivative containing two hydroxyethyl headgroups was better than a single-hydroxyl derivative[41] Hydroxyl derivatives on different backbones have also been shown to be very promising, viz., DMRIE/DORIE, DOTIM, etc.[28] The present study demonstrates that transfection lipids with four hydroxyls in the headgroup area are better than transfection lipids containing two hydroxyalkyl headgroups on COS-1 cells. On the basis of lipid hydration and geometric considerations, factors such as a small polar domain or close headgroup association and unsaturated acyl chains were considered to promote fusion.[33] Lipids used in this study, based on chemical structure, are expected to be more cylindrical in shape given their large polar heads and saturated chains. Though the interaction with the DNA was shown to be weak by EtBr exclusion, their transfection ability was very high. No clear understanding of the dependency of in vitro transfection efficiency on the structure of cationic lipid is yet available.[8]

To quantitate the undigested DNA in DNase I protection experiments, the lipid−DNA complex was detergent-solubilized or extracted by organic solvents. These methodologies require careful optimizations. Similarly, to assess the extent of association of DNA with the lipid, sucrose or dextran gradients were used.[12,14,47] In this study, we have used radiolabled DNA, obtained by nick translation, to visualize the free and complexed DNA, which does not involve any extraction or fractionation procedures. Densitometric quantitation provides a convenient method to evaluate the process of DNA and lipid complexation. Lipids **3** and **1,** which showed good transfections, differ in their ability to bind DNA as assessed by electrophoresis (Figure 2). The poor ability to cause exclusion of EtBr from DNA by the four lipids even at 3:1 charge ratios (Figure 4) indicates that the lipids **1**−**4** do not induce the conformational changes required for EtBr exclusion. It has been proposed that cationic lipids must be sufficiently neutralizing the negative charges of DNA to induce a cooperative collapse in the DNA structure.[17] Lipids used in this study do not induce such cooperative collapse of DNA, even at 3:1 charge ratio. However, the extent of complexation as seen by electrophoresis (Figure 2) suggests nearly 60−80% of the DNA exists as a complex at 3:1 charge ratio, which was DNase I-insensitive. These observations suggest that the DNA was ensheathed by the lipid bilayers but that this did not cause condensation of DNA. Similar lack of condensation was observed in lipid−DNA interactions by binding of dye TOPRO-1, a DNA intercalating dye.[47] Transfection studies, when compared to lipofectin or DHDEAB, indicate higher transfection, although the binding of lipid to DNA as assessed by EtBr exclusion was weaker than that of lipofectin or DHDEAB. Tight lipid−DNA complexes may not be more efficient in transfection than weaker lipid−DNA complexes as suggested.[8] An ideal lipid formulation should facilitate maximal uptake of DNA by the cell and also should dissociate from DNA inside the cell.

As a colipid DOPE was ineffective compared to cholesterol. DOPE, a H_{II} phase-preferring lipid at physi-

ological temperatures, was considered to help the fusogenic ability of the complex.[51] The fusogenic abilities of DOPE are not always required to obtain efficient transfection. Cholesterol enhances the transfection efficiency of all the four lipids used in this study and also of DHDEAB, the two-hydroxyethyl-containing transfection lipid reported by us.[41] Cholesterol has also been reported as a preferred colipid in transfection formulations in vivo.[52] Biophysical characteristics of DOTAP/DOPE and DOTAP/cholesterol show that particle diameter, ζ potential, and amount of adsorbed protein were less with cholesterol as colipid.[53]

Conclusions

In summary, we have developed an efficient and novel series of non-glycerol-based cationic glycolipids for use in in vitro liposomal gene delivery. Similar to dihydroxyl derivatives, the four lipids reported in this study, based on xylose or arabinose headgroups, transfect with cholesterol but not with DOPE as colipid. The present synthetic protocols should, in principle, be applicable in synthesizing various glycosylated non-glycerol-based structural analogues of lipids **1**−**4** for use in targeted gene delivery. The transfection efficiencies of these new lipids, when used in combination with cholesterol as a colipid, were observed to be higher than that of DHDEAB, which was shown by us[41] to be more transfection efficient than LipofectAmine, one of the widely used cationic transfection lipids. Structural studies on lipid−DNA complexes of these four cationic lipids demonstrate that the formation of the complex was not strong and does not condense the DNA. However, the transfection efficiencies were higher than those lipids that form strong complexes with the DNA.

Experimental Section

General Procedures and Materials. The high-resolution mass spectrometric (HRMS) analysis were performed on a Micromass AUTOSPEC-M mass spectrometer (Manchester, U.K.) with an OPUS V3.1X data system. Data were acquired by the liquid secondary ion mass spectrometry (LSIMS) technique with *m*-nitrobenzyl alcohol as the matrix. LSIMS analysis was performed in the scan range 100−1000 amu at the rate of 3 scans/s. ^{1}H NMR spectra were recorded on a Varian FT 200 MHz, Bruker 300 and 500 MHz. Unless otherwise stated, all reagents were purchased from local commercial suppliers and were used without further purification. Final purification of the transfection lipids **1**−**4** was carried out by semipreparative reverse-phase HPLC (on a Whatman, Partisil 10 ODS-3 column). The LC system consisted of a Shimadzu model LC10A. The progress of the reactions was monitored by thin-layer chromatography on 0.25 mm silica gel plates. Column chromatography was performed with silica gel (Acme Synthetic Chemicals, India, finer than 200 and 60−120 mesh). Elemental analyses (C, H, N) were performed at the microanalytical laboratory of Central Salt and Marine Chemical Research Institute, Bhavnagar, Gujarat, India. The hydrophobic secondary amines namely, *N*,*N*-di-(*n*-tetradecyl)amine, *N*,*N*-di-(*n*-hexadecyl)amine, and *N*,*N*-di-(*n*-octadecyl)amine used in step a of Schemes 1 and 2 for the syntheses of the lipids **1**−**4** were prepared by the same synthetic procedure as described below for the synthesis of *N*,*N*-di-(*n*-hexadecyl)amine. Lipids **1**−**4** were synthesized by the synthetic procedure outlined in Schemes 1 and 2. As a representative of the procedure detail, synthesis of the lipid **3** is delineated below. pCMV.SPORT-β-gal, cell culture media, and fetal calf serum were purchased from Gibco−BRL, Rockville, MD. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), poly(ethylene glycol) 8000, Trizma base, DNase I, calf thymus DNA, NP-40, ethidium bromide, antibiotics, agarose, *o*-nitrophenyl β-D-galactopyranoside were purchased from Sigma, St. Louis, MO. T_4 DNA polymerase and the nick translation buffer were purchased from New England Biolabs Ltd., U.K. Radiolabeled DNA was purified on Promega DNA purification columns. dNTP stocks and DNA molecular weight markers were purchased from Bangalore Genei, Bangalore, India. [α-^{32}P]dATP was purchased from Bhabha Atomic Research Centre, Bombay, India. Dioleoyl-3-(trimethylammonium)propane (DOTAP), cholesterol, and dioleoylphosphatidylethanolamine (DOPE) were purchased from Avanti Polar Lipids, Alabaster, AL. COS-1 cell line (SV 40 transformed African green monkey kidney, ATCC CRL 1650) was obtained from ATCC. Diisopropylidine dithioethyl derivatives of arabinose and xylose were obtained as generous gifts respectively from Dr. P. K. Sasmal and Dr. P. Chand of the Indian Institute of Chemical Technology. Unless otherwise stated, all reagents were purchased from local commercial suppliers and were used without further purification.

Synthesis of *N*,*N*-Di-(n-hexadecyl)amine. Dry dichloromethane (100 mL) was cooled to 0 °C and 5.5 g of *n*-hexadecylaldehyde (22.9 mmol), 5.5 g of *n*-hexadecylamine (22.9 mmol), and 2.75 g of anhydrous magnesium sulfate (22.9 mmol) were added. The mixture was kept under stirring for 3 h, and the temperature of the reaction mixture was allowed to rise gradually from 0 °C to room temperature. The magnesium sulfate was filtered from the reaction mixture and the filtrate was diluted with 50 mL of methanol. The diluted dichloromethane/methanol solution was cooled to 0 °C and 4.35 g of sodium borohydride (115 mmol) was added. The solution was stirred for 4 h and the temperature of the reaction mixture was gradually raised to room temperature. The reaction mixture was then taken up in 100 mL of chloroform and washed with water (2 × 100 mL), and the chloroform layer was dried over anhydrous magnesium sulfate and filtered. Chloroform was removed from the filtrate on a rotary evaporator, and column chromatographic (using 60−120 mesh size silica gel) purification of the residue with 20−50% ethyl acetate in petroleum ether as the eluent afforded 3.30 g (30% yield) of pure *N*,*N*-di-(*n*-hexadecyl)amine.

^{1}H NMR of *N*,*N*-di-(n-hexadecyl)amine (200 MHz, $CDCl_3$): δ (ppm) = 0.85 [t, 6H, $CH_3(CH_2)_n$-]; 1.15−1.35 [m, 56H, -$(CH_2)_{14}$-]; 1.4−1.6 [m, 4H, $N^+(CH_2CH_2\text{-})_2$]; 2.55 [t, 4H, $N^+(CH_2\text{-}CH_2\text{-})_2$].

Synthesis of 1-Deoxy-1-[dihexadecyl(methyl)ammonio]-D-arabinitol (3): Step a. The thioketal derivative of 2,3,4,5-diisopropylidine arabinose (Scheme 2; 1 g, 2.97 mmol) was deprotected by refluxing in 25 mL of 80/20 acetone/water (v/v) with 2.43 g (8.9 mmol) of mercuric chloride and 2.4 g (8.9 mmol) of red mercuric oxide for strictly 1.5 h. The inorganic materials were filtered and the residue was washed with chloroform (2 × 20 mL). Solvents from the combined filtrate were evaporated and the residue was taken up in chloroform (40 mL). The chloroform extract was sequentially washed with 1 N aqueous potassium iodide solution (2 × 30 mL) and water (2 × 30 mL). The combined aqueous solution was extracted with chloroform (2 × 30 mL), and this chloroform extract (2 × 30 mL) was added to the first chloroform extract (40 mL). The combined chloroform extract was dried over anhydrous sodium sulfate and filtered, and the chloroform from the filtrate was evaporated in a rotary evaporator below 35 °C. The crude aldehyde residue (636 mg, 2.97 mmol) was taken up in 10 mL of dichloromethane, and 1.55 mg (3.2 mmol) of *N*,*N*-di-(*n*-hexadecyl)amine was added to the solution. After the reaction was stirred for 0.5 h under nitrogen in room temperature, sodium cyanoborohydride 410 mg (2.97 mmol) was added. The reaction mixture was stirred at room temperature for 30 h under nitrogen. Removal of the solvent followed by silica gel column chromatographic purification of the resulting residue with 60−120 mesh size silica and 98/2 (v/v) hexane/acetone as the eluent finally afforded 780 mg of the intermediate tertiary amine (white gummy liquid, intermediate **II** in Scheme 2, where R= *n*-$C_{16}H_{33}$, R_f 0.8 in 80/20 hexane/acetone as the TLC developing solvent) in 40% yield.

^{1}H NMR (200 MHz, $CDCl_3$) of intermediate tertiary amine (**II**, Scheme 2, R = *n*-$C_{16}H_{33}$): δ (ppm) = 0.9 [t, 6H, $CH_3(CH_2)_n$-]; 1.20−1.40 [m, 52H, -$(CH_2)_{13}$-]; 1.4−1.6 [m, 16H, $COC(CH_3)_2$-OC, $N(CH_2CH_2\text{-})_2$]; 2.3−2.6 [m, 5H, -$CH^1H^2N(CH_2CH_2\text{-})_2$]; 2.8 [dd, 1H, -$CH^1H^2N(CH_2CH_2\text{-})_2$]; 3.55 [t, 1H, $(O)CH_2CH(O)$-$CH(O)CH(O)CH_2N$ $(CH_2CH_2\text{-})_2$]; 3.8−4.2 [m, 4H, $(O)CH_2CH(O)$-$CH(O)CH(O)CH_2N$ $(CH_2CH_2\text{-})_2$].

FABS (LSIMS) *m/z* 679 [M^+] (calcd for $C_{43}H_{85}NO_4$, 38%,).

Step b. The intermediate tertiary amine (**II**, Scheme 2, R= *n*-$C_{16}H_{33}$) (210 mg, 0.3 mmol) prepared in step a was reacted with excess methyl iodide (7 mL) for 3 h at room temperature. The residue after evaporation of the excess methyl iodide was column-purified on 60−120 mesh size silica gel. The column was eluted sequentially with 5% acetone/hexane and pure chloroform to remove the relatively less polar impurities. The pure quaternized ammonium iodide salt (intermediate **III** in Scheme 2 where R= *n*-$C_{16}H_{33}$, R_f 0.6 in 10/90 methanol/chloroform) was finally eluted with 10% methanol in chloroform (250 mg, 98% yield).

^{1}H NMR (200 MHz, $CDCl_3$) of the diisopropylidine quaternary ammonium iodide (intermediate **III** in Scheme 2 where R = *n*-$C_{16}H_{33}$): δ (ppm) = 0.9 [t, 6H, $CH_3(CH_2)_n$-]; 1.20−1.40 [m, 52H, -$(CH_2)_{13}$-]; 1.4 and 1.5 [s, 12H, $\{COC(CH_3)_2OC\}_2$]; 1.65−1.95 [m, 4H, $N^+(CH_2CH_2\text{-})_2$]; 3.55 [s, 3H, CH_3N^+]; 3.55−3.90 [m, 7H, $(O)CH^1H^2CH(O)CH(O)CH(O)CH_2N^+(CH_2CH_2\text{-})_2$]; 3.85−4.05 [m, 1H, $(O)CH^1H^2CH(O)CH(O)CH(O)CH_2N^+(CH_2$-$CH_2\text{-})_2$]; 4.05−4.25 [m, 2H, $(O)CH_2CH(O)CH(O)CH(O)CH_2N^+$-$(CH_2CH_2\text{-})_2$]; 4.35−4.50 [m, 1H, $(O)CH_2CH(O)CH(O)CH(O)$-CH_2N^+].

HRMS (LSIMS) *m/z* calcd (for $C_{44}H_{88}NO_4$ the 4°-ammonium ion, 100%) 694.6713; found 694.6720.

Step c. The diisopropylidine deprotection of the diisopropylidine-protected quaternized ammonium iodide salt (Scheme 2, R = *n*-$C_{16}H_{33}$) obtained in step b was accomplished by stirring the quaternized salt (130 mg, 0.16 mmol) in 5 mL of neat trifluoroacetic acid (TFA) at 55 °C for 48 h. Excess TFA was evaporated and the residue was loaded over a chloride ion-exchange resin. The title compound **3**, 103 mg, waxy, semisolid, 100%, R_f 0.25 in 10/90 methanol/chloroform) was eluted with methanol. The eluted compound was further purified by treatment with charcoal in hot methanol. The final HPLC purification of the title compound was carried out on a semipreparative reverse-phase column with 95/5 methanol/water at a flow rate of 5 mL/min (UV detector was set at 219 nm). Pure **3** was obtained in an overall yield of 39.2%.

HRMS **(**LSIMS**)** *m/z* calcd **(**for $C_{38}H_{80}NO_4$ the 4° ammonium ion, 80%) 614.6087; found 614.6045. Anal. ($C_{38}H_{80}ClNO_4$·$1.0H_2O$) C, H, N.

Synthesis of 1-Deoxy-1-[methyl(ditetradecyl)ammonio]-D-arabinitol (2). The title lipid **2** (waxy, semisolid) was synthesized following the same procedure as described above for synthesizing lipid **3** but with di-(*n*-tetradecyl)amine with an overall yield of 40%. All the isolated intermediates gave spectroscopic data in agreement with their assigned structures shown in Scheme 2.

^{1}H NMR (200 MHz, $CDCl_3$) of diisopropylidine quaternary ammonium iodide [intermediate **III** in Scheme 2 where R = *n*-$C_{14}H_{29}$]: δ (ppm) = 0.9 [t, 6H, CH_3-$(CH_2)_n$-]; 1.20−1.40 [m, 44H, -$(CH_2)_{11}$-]; 1.4 and 1.5 [s, 12H, $\{COC(CH_3)_2OC\}_2$]; 1.65−1.95 [m, 4H, $N^+(CH_2CH_2\text{-})_2$]; 3.55 [s, 3H, CH_3N^+]; 3.60−3.90 [m, 7H, $(O)CH^1H^2CH(O)CH(O)CH(O)CH_2N^+(CH_2CH_2\text{-})_2$]; 3.90−4.05 [m, 1H, $(O)CH^1H^2CH(O)CH(O)CH(O)CH_2N^+(CH_2CH_2\text{-})_2$]; 4.10−4.30 [m, 2H, $(O)CH_2CH(O)CH(O)CH(O)CH_2N^+(CH_2CH_2$-$)_2$]; 4.35−4.50 [m, 1H, $(O)CH_2CH(O)CH(O)CH(O)CH_2N^+$].

HRMS (LSIMS) *m/z* calcd (for $C_{34}H_{72}NO_4$ the 4° ammonium ion, 100%) 558.5461; found 558.5448.

Synthesis of 1-Deoxy-1-[methyl(dioctadecyl)ammonio]-D-arabinitol (4). The title lipid **4** (waxy, semisolid) was synthesized following the same synthetic procedure as described above for preparing the lipid **3** but with di-(*n*-octadecyl)amine with an overall yield of 37%. All the isolated intermediates gave spectroscopic data in agreement with their assigned structures shown in Scheme 2.

^{1}H NMR (200 MHz, $CDCl_3$) of diisopropylidine quaternary ammonium iodide [intermediate **III** in Scheme 2 where R = *n*-$C_{18}H_{37}$]: δ (ppm) 0.9 [t, 6H, $CH_3(CH_2)_n$-]; 1.20−1.40 [m, 60H, -$(CH_2)_{15}$-]; 1.4 and 1.5 [s, 12H, $\{COC(CH_3)_2OC\}_2$]; 1.65−1.95 [m, 4H, $N^+(CH_2CH_2\text{-})_2$]; 3.55 [s, 3H, CH_3N^+]; 3.55−3.90 [m, 7H, $(O)CH^1H^2CH(O)CH(O)CH(O)CH_2N^+(CH_2CH_2\text{-})_2$]; 3.85−4.05 [m, 1H, $(O)CH^1H^2CH(O)CH(O)CH(O)CH_2N^+(CH_2CH_2\text{-})_2$]; 4.05−4.25 [m, 2H, $(O)CH_2CH(O)CH(O)CH(O)CH_2N^+(CH_2CH_2$-$)_2$]; 4.35−4.50 [m, 1H, $(O)CH_2CH(O)CH(O)CH(O)CH_2N^+$].

HRMS (LSIMS) *m/z* calcd (for $C_{42}H_{88}NO_4$ the 4° ammonium ion, 100%) 670.6713; found 670.6731. Anal. ($C_{42}H_{88}ClNO_4$·$1.5H_2O$) C, H, N.

Synthesis of 1-Deoxy-1-[dihexadecyl(methyl)ammonio]-D-xylitol (1). As outlined in Scheme 1, the title lipid **1** (waxy semisolid) was synthesized following the same synthetic procedure described above for preparing the lipid **3** but with 2,3,4,5-diisopropylidinexylose thioketal as the starting material with an overall yield of 18.4%. All the isolated intermediates gave spectroscopic data in agreement with their assigned structures shown in Scheme 1.

^{1}H NMR (200 MHz, $CDCl_3$) of diisopropylidine quaternary ammonium iodide intermediate [Scheme 1, R = *n*-$C_{16}H_{33}$]: δ (ppm) = 0.9 [t, 6H, $CH_3(CH_2)_n$-]; 1.20−1.40 [m, 52H, -$(CH_2)_{13}$-]; 1.40−1.60 [m, 12H, $\{COC(CH_3)_2OC\}_2$]; 1.60−1.95 [m, 4H, $N^+(CH_2CH_2\text{-})_2$]; 3.45 [s, 3H, -$CH_2(CH_3)N^+(CH_2CH_2\text{-})_2$] 3.50−3.70 [m, 5H, -$CH^1H^2N^+(CH_2CH_2\text{-})_2$]; 3.90−4.00 [m, 2H, (O)-$CH^1H^2CH(O)CH(O)CH(O)CH^1H^2N^+(CH_2CH_2\text{-})_2$]; 4.10−4.20 [m, 1H, $(O)CH^1H^2CH(O)CH(O)CH(O)CH_2$]; 4.40−4.50 [m, 1H, (O)-$CH_2CH(O)CH(O)CH(O)CH_2$]; 4.55−4.70 [m, 2H, $(O)CH_2CH$-$(O)CH(O)CH(O)CH_2N^+$].

HRMS (LSIMS) *m/z* calcd (for $C_{38}H_{80}NO_4$ the 4° ammonium ion, 100%) 614.6087; found 614.6104. Anal. ($C_{38}H_{80}ClNO_4$·$1.5H_2O$) C, H, N.

Preparation of Plasmid DNA. pCMV-β-gal plasmid DNA was prepared by the alkaline lysis procedure and purified by PEG-8000 precipitation according to published procedures.[45] The plasmid preparations showing a value of A_{260}/A_{280} more than 1.8 were used.

Preparation of Liposomes. Cationic amphiphiles and the colipid (DOPE or cholesterol) in the appropriate ratios were dissolved in chloroform in a glass vial. Chloroform was removed with a thin flow of moisture-free nitrogen and the dried film of lipid left in the vial was then kept under high vacuum for 8 h. One milliliter of sterile deionized water was added to the vacuum-dried lipid film, and the mixture was allowed to swell for 15 h (overnight). The vial was then vortexed for 2−3 min at room temperature and occasionally shaken in a 45 °C water bath to produce multilamellar vesicles (MLV). Small unilamellar vesicles (SUV) were then prepared by sonication of the MLV placed in an ice bath for 3−4 min until clarity was achieved, by use of a Branson 450 sonifier at 100% duty cycle. The formation of SUV was assured by spinning the sonicated preparation for 10 min at 10 000 rpm in a tabletop centrifuge. If a pellet formed, then the sample was sonicated further until a clear solution was obtained.

Nick Translation of Plasmid DNA. Supercoiled plasmid DNA, pCMV-β-gal (7.8 kb), was purified by alkaline lysis followed by precipitation with poly(ethylene glycol).[45] The DNA obtained was resuspended in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). To obtain radiolabeled DNA, pCMV-β-gal plasmid was nick-translated with DNase I and T4 DNA polymerase, in the presence of [α-^{32}P]dATP, dGTP, dCTP, and dTTP. The unincorporated nucleotides were removed with Promega's Wizard DNA clean up system. The nick-translated DNA was finally eluted with TE buffer, analyzed by agarose gel electrophoresis, and shown to have a specific activity of 2.2×10^5cpm/μg of DNA.

Binding of Lipid to DNA and Sensitivity of Lipid−DNA Complex to DNase I. Nick-translated pCMV-β-gal DNA, labeled with [α-^{32}P]dATP, was complexed with cationic lipids at charge ratios varying from 0.3:1 to 3:1 (lipid/DNA) to measure the DNA binding ability and the susceptibility of these complexes to degradation by DNase I. Briefly, in a typical binding assay, 0.15 nmol of DNA, corresponding to 50 ng of

nick-translated DNA, was complexed with the lipids, at indicated charge ratios, in 10 mM phosphate buffer (pH 7.5), in a volume of 15 μL and incubated at room temperature for 30 min on a gyratory shaker. Subsequently, the complexes were treated with DNase I, in the presence of 5 mM $MgCl_2$, at a final concentration of 0.1 μg/0.15 nmol of DNA, and further incubated for 10 min at room temperature. The reactions were halted with EDTA added to a final concentration of 30 mM, and the reaction products, after addition of the tracking dye bromophenol blue, were directly loaded on a 1% agarose gel with TAE buffer and electrophoresed at 100 V for approximately 2 h. The gel was subsequently dried, exposed overnight, and scanned on a Fuji Film phosphorimager (BAS 1800), and the bands were quantitated with Image Gauge software version 8.01.

Exclusion of Ethidium Bromide (EtBr) from DNA by Cationic Lipids. The extent of EtBr binding to the DNA was monitored by changes in the fluorescence. EtBr fluorescence was monitored in a Hitachi 4500 fluorometer by setting the excitation wavelength at 518 nm and the emission wavelength at 585 nm. To 1 mL of TE buffer (pH 8.0) were added 0.78 nmol of DNA and 2.5 nmol of EtBr. The change in fluorescence was monitored after addition of small volumes of lipid to the EtBr−DNA complex. Arbitrary fluorescence values were recorded after sufficient time was allowed for equilibration. The order of addition of EtBr or lipid to DNA did not alter the final value, indicating that the equilibrium does not depend on the order of addition and is attained in minutes. Percent fluorescence was calculated by considering the fluorescence value in the absence of lipid as 100%.

Transfection of Cells. Transfection of the cells was done essentially as described earlier.[41] Briefly, COS-1 cells were seeded at a density of 15 000 cells/well in a 96-well plate 18 h before the transfection. Plasmid (0.3 μg) was complexed with varying amount of lipid (0.05−4.3 nmol) in 13 μL of plain DMEM medium for 30 min. The charge ratios were varied from 0.1:1 to 9:1 (±) over this range of the lipid. The complex was diluted to 100 μL with plain DMEM and added to the wells. After 3 h of incubation, 100 μL of DMEM with 10% FCS was added to the cells. The medium was changed to 10% complete medium after 24 h and the reporter gene activity was estimated after 48 h. The cells were washed twice with PBS and lysed in 50 μL of lysis buffer (0.25 M Tris-HCl, pH 8.0, and 0.5% NP40). Care was taken to ensure complete lysis. The β-galactosidase activity per well was estimated by adding 50 μL of 2× substrate solution (1.33 mg/mL ONPG, 0.2 M sodium phosphate, pH 7.15, and 2 mM magnesium chloride) to the lysate in a 96-well plate. Absorption at 405 nm was converted to β-galactosidase units by use of a calibration curve constructed with pure commercial β-galactosidase enzyme. The values of β-galactosidase units in replicate plates assayed on the same day varied by less than 30%. The transfection efficiency values are the average values from four replicate transfection plates assayed on the same day. Each transfection experiment was repeated three times on different days and the day-to-day variation in average transfection efficiency was found to be within 1-fold. The transfection profiles obtained on different days were identical.

Toxicity Assay. Cytotoxicity of lipids **1**−**4** was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay as described earlier.[46] The cytotoxicity assay was performed in 96-well plates by maintaining a constant ratio of number of cells to amount of cationic lipid, as in transfection experiments. MTT was added 3 h after addition of the cationic lipid to the cells. Results were expressed as percent viability = [A_{540}(treated cells) − background]/[A_{540}(untreated cells) − background] × 100.

Abbreviations

DOTMA, 1,2-dioleyl-3-(*N*,*N*,*N*-trimethylamino)propane chloride; DMDHP, (±)-*N*,*N*-[bis(2-hydroxyethyl)]-*N*-[2,3-bis(tetradecanoyloxy)propyl]ammonium chloride; DMRIE, *N*-[1,2-di(myristyloxy)prop-3-yl]-*N*,*N*-dimethyl-*N*-(hydroxyethyl)ammonium bromide; DOTAP, 1,2-di(oleoyloxy)-3-(trimethylamino)propane; DHDEAB, *N*,*N*-di-(*n*-hexadecyl)-*N*,*N*-di(hydroxyethyl)ammonium bromide; HDEAB, *N*-(*n*-hexadecyl)-*N*,*N*-di(hydroxyethyl)-ammonium bromide; MOOHAC, *N*-methyl-*N*-(*n*-octadecyl)-*N*-oleyl-*N*-(hydroxyethyl)ammonium chloride; DOMHAC, *N*-methyl-*N*,*N*-di-(*n*-octadecyl)-*N*-(hydroxyethyl)ammonium chloride; DOHEMAB, *N*,*N*-di[[(*O*-hexadecanoyl)hydroxy]ethyl]-*N*-(hydroxyethyl)-*N*-methylammonium bromide; DOSPA, 2,3-di(oleyloxy)-*N*-[2-(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-1-propanamminium trifluoroacetate; DDAB, dioctadecyldimethylammonium bromide; DOPE, 1,2-dioleoylpropyl-3-phosphatidylethanolamine.

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Supporting Information Available: Elemental analysis data for lipids **1**, **3,** and **4**; two HPLC chromatograms (with two different mobile phases) for lipid **2**; and Figure S1, showing agarose gel electrophoresis of the susceptibility of DNA in lipid−DNA complexes to DNases with lipids **1**−**4** (with cholesterol as colipid) and DOTAP. This material is available free of charge via the Internet at http://pubs.acs.org.

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