Novel Series of Non-Glycerol-Based Cationic Transfection Lipids for Use in Liposomal Gene Delivery[1,†]

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A novel series of nontoxic and non-glycerol-based simple monocationic transfection lipids containing one or two hydroxyethyl groups directly linked to the positively charged nitrogen atom were synthesized. The in vitro transfection efficiencies of these new liposomal gene delivery reagents were better than that of lipofectamine, a widely used transfection agent in cationic lipid-mediated gene transfer. The most efficient transfection formulation was observed to be a 1:1:0.3 mol ratio of DHDEAB (*N*,*N*-di-*n*-hexadecyl-*N*,*N*-dihydroxyethylammonium bromide):cholesterol:HDEAB (*N*-*n*-hexadecyl-*N*,*N*-dihydroxyethylammonium bromide) using a DHDEAB-to-DNA charge ratio (+/−) of 0.3:1. Observation of good transfection at charge ratios lower than 1 suggests that the amphiphile−DNA complex may have net negative charge. Our results reemphasize the important point that in cationic lipid-mediated gene delivery, the overall charge of the lipid−DNA complex need not always be positive. In addition, our transfection results also imply that favorable hydrogen-bonding interactions between the lipid headgroups and the cell surface of biological membranes may have some role for improving the transfection efficiency in cationic lipid-mediated gene delivery.

Introduction

In gene therapy, patients carrying identified defective genes are supplemented with the copies of the corresponding normal genes.[1,2] Many gene delivery reagents (also known as transfection vectors) including retrovirus,[3] adenovirus,[4] positively charged polymers and peptides,[5−7] and cationic amphiphilic compounds[8,9] are currently being used as carriers of genes in combating the hereditary diseases by gene therapy. Reproducibility, low cellular and immunological toxicities, and ease in preparation and administration associated with cationic transfection lipids are increasingly making them the transfection vector of choice in gene therapy.

Since the first reports[8] on cationic liposome-mediated gene delivery by Felgner et al. in 1987, an upsurge of global interest has been witnessed in synthesizing efficient cationic transfection lipids.[10−29] Many of the reported liposomal transfection vectors, e.g. DOTMA,[8] DMDHP,[18] DMRIE,[25] and DOTAP,[29] have a common element in their molecular structures: namely, the presence of a glycerol backbone. Interestingly, among the glycerol-based cationic transfection lipids, the polar headgroup domains of the most efficient lipids, such as DMRIE[25] and DMDHP,[18] contain one or two hydroxyethyl groups directly linked to the positively charged nitrogen atoms. Development of efficient non-glycerol-based liposomal transfection lipids has been reported, e.g. DC-Chol synthesized by Huang and co-workers[28] and the long chain alkyl acyl carnitine esters recently designed by Szoka and colleagues.[13] These non-glycerol-based liposomal gene delivery reagents have no hydroxyethyl groups present in their polar headgroup regions. Except for the patented report by Nantz et al. on the development of a 1,4-diaminobutane-based dicationic transfection lipids,[21] detailed investigations on the transfection efficiencies of non-glycerol-based monocationic liposomal transfection vectors containing hydroxyethyl groups directly attached to the positively charged nitrogen atoms have not been reported. Herein, we report on the synthesis and remarkably high in vitro transfection efficiencies of a novel series of simple, nontoxic and non-glycerol-based transfection lipids **1**−**5** (Chart 1) containing hydroxyethyl group(s) directly attached to the positively charged quaternized nitrogen atom.

Results and Discussion

Chemistry. The key structural elements common to all the transfection lipids **1**−**5** (Chart 1) described in the present investigation include: (a) the presence of a hydrophobic group either directly linked to the positively charged nitrogen atom or linked to the positively charged nitrogen via an ester group, (b) the presence of at least one hydroxyethyl group directly linked to the positively charged nitrogen atom, and (c) the absence of glycerol backbone in the molecular architecture of the monocationic amphiphiles. The details of the synthetic procedures for all the novel transfection lipids shown in Chart 1 are described in the Experimental Section.

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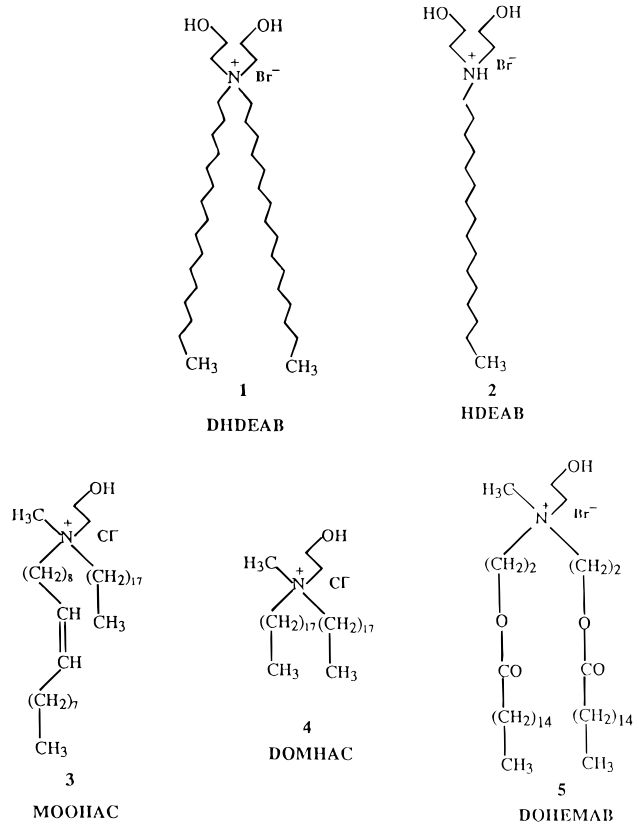
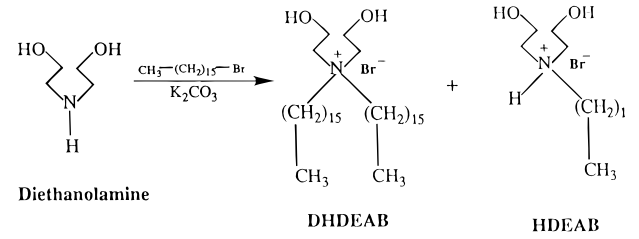
Chart 1. Structures of New Transfection lipids



Scheme 1. Synthesis of DHDEAB and HDEAB

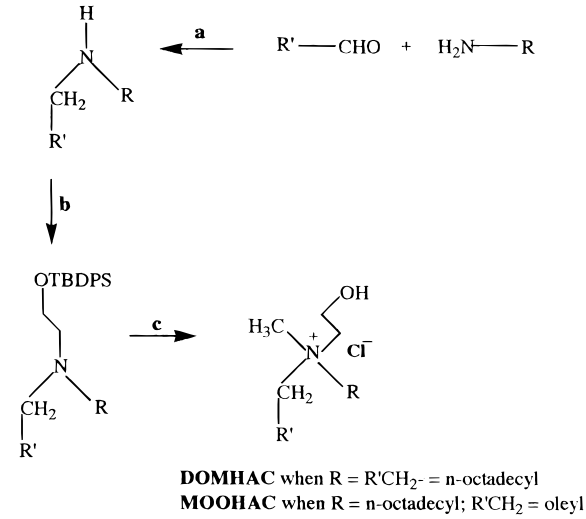


As delineated in Schemes 1−3, the chemistries involved in preparing these new lipids are straightforward. However, given the high transfection efficiencies of these nontoxic hydroxyethyl-containing cationic transfection lipids, the overall yields need to be improved in the future.

Scheme 1 outlines the one-step synthetic procedure for preparing DHDEAB and HDEAB. Diethanolamine was initially refluxed with *n*-hexadecyl bromide in the presence of potassium carbonate in methanol. The resulting intermediate tertiary amine (*N*-*n*-hexadecyldiethanolamine, not isolated) was then refluxed in a mixed solvent containing 80:15:5 (v/v) acetonitrile:ethyl acetate:methanol. Finally, column chromatographic purification of the product mixture afforded pure DHDEAB and HDEAB.

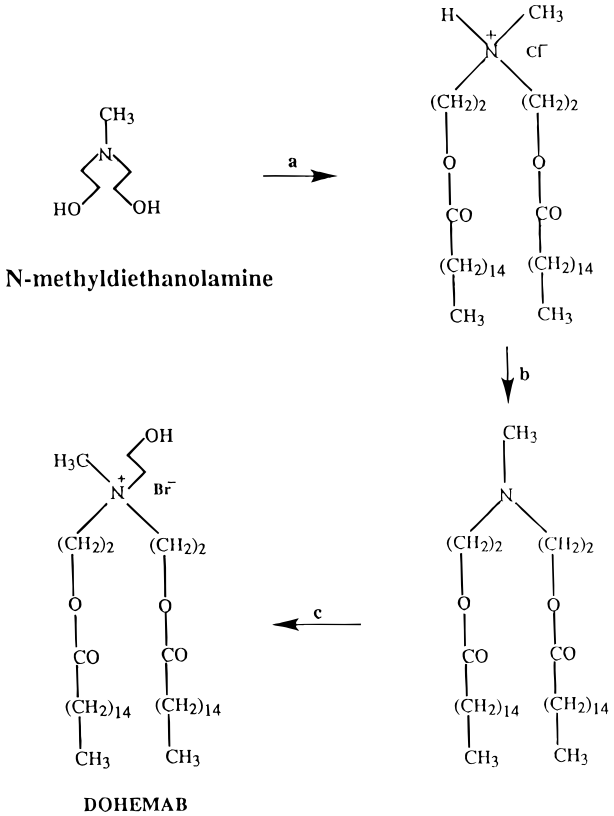
The steps used in synthesizing MOOHAC and DOMHAC (Scheme 2) include: (a) coupling the appropriate aliphatic saturated or unsaturated aldehyde with the appropriate long chain aliphatic amine followed by reduction of the resulting imine to obtain the corresponding secondary amine, (b) conversion of the secondary amine obtained in step (a) to *N*-hydroxyethyl-*N*,*N*-dialkylamine (tertiary amine) by reacting with the

Scheme 2. Synthesis of MOOHAC and DOMHAC[a]



[a] Reagents: (a) anhyd $MgSO_4$ (1.0 equiv)/DCM, $NaBH_4$ (2.0 equiv)/DCM/MeOH; (b) Br-$(CH_2)_2$-OTBDPS (1 equiv)/K_2CO_3 (1.1 equiv)/ethyl acetate; (c) TBAF (2.5 equiv)/THF, MeI (huge excess)/$CHCl_3$/MeOH, Amberlyst A-26 chloride ion-exchange resin.

Scheme 3. Synthesis of DOHEMAB[a]



[a] Reagents: (a) *n*-hexadecanoyl chloride (2.2 equiv)/DMF; (b) 1.0 M aq NaOH/DCM (biphasic system); (c) 2-bromoethanol (1.5 equiv)/85 °C/4 h.

hydroxyl-protected 2-bromoethanol followed by removal of the hydroxyl protecting group, and (c) quaternizing the tertiary amine obtained in step (b) with excess of methyl iodide followed by chloride ion-exchange chromatography of the resulting intermediate quaternary amphiphilic iodide.

Synthesis of DOHEMAB (Scheme 3) essentially consists of: (a) reacting *n*-hexadecanoyl chloride with *N*-methyldiethanolamine to obtain the hydrochloride

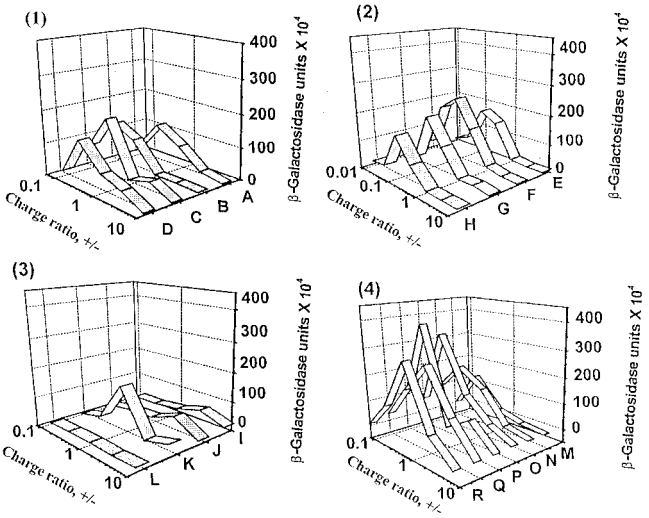


Figure 1. Transfection efficiencies of (1) DOMHAC, (2) MOOHAC, (3) DOHEMAB, and (4) DHDEAB on COS-1 cells. The transfection efficiencies of the four lipids were tested by varying both the charge ratio (*X*-axis) and cholesterol (*Z*-axis). The following mole ratios of cholesterol:cationic lipids were used in the *Z*-axis: 0.2:1 (A, E, I, M); 0.4:1 (B, F, J, N); 0.6:1 (C, G, K, O); 1:1 (D, H, L, P); 1.2:1 (Q); 1.5:1 (R). In each well of a 24-well plate a fixed amount of plasmid DNA (0.3 μg) was used to complex with 0.1−9 nmol of cationic lipid to vary the charge ratio (+/−) from 0.1 to 9.

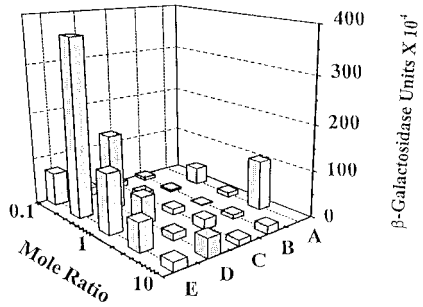


Figure 2. Transfection efficiencies of DHDEAB and DDAB with cholesterol and DOPE as helper lipids on COS-1 cells. The charge ratios and amount of DNA used are as in Figure 1. Lipofectamine (A) was used for comparision. DHDEAB (C, E) and DDAB (B, D) were used in combination with DOPE (B, C) and cholesterol (D, E) at a mole ratio of 1:1. The *X*-axis is given in mole ratio ([cationic amphiphile]/[DNA]) instead of charge ratio for comparing the lipofectamine with the cationic amphiphiles on the same scale. The charge ratio and the mole ratio are same for our cationic amphiphiles (since they carry one charge per molecule) unlike lipofectamine carrying five positive charges for one molecule.

salt of the di-*O*-acylated intermediate, (b) neutralizing the hydrochloride salt obtained in step (a) with alkali, and (c) quaternizing the resulting tertiary amine obtained in step (b) with 2-bromoethanol.

Biology. The transfection efficiencies of the cationic amphiphiles **1**−**5** (Chart 1) were tested in COS-1 cells using pCH110 plasmid carrying a β-galactosidase reporter gene under the control of RSV promoter. Initially we tested the transfection efficiencies of all the novel transfection lipids using the widely used auxilliary lipid DOPE. All the amphiphiles with DOPE showed very poor transfection. Interestingly enough, the amphiphiles **1** and **3**−**5** showed remarkable transfection efficiencies with varying amounts of cholesterol as helper lipid (Figure 1). Amphiphile **2** did not show any transfection even with cholesterol as helper lipid at any ratio, probably because of the single acyl chain, which might interfere with the proper formation of a bilayer. Amphiphiles **4** and **5** showed the highest transfection efficiency in the presence of 60 mol % cholesterol (with respect to the cationic lipid), whereas **3** showed highest efficiencies in the presence of 40 mol % of cholesterol. The transfection efficiencies of the amphiphiles **3**−**5** having a single hydroxyethyl group in the headgroup regions were poorer than that of amphiphile **1**. Amphiphile **1** with two hydroxyethyl functionalities directly linked to the positively charged nitrogen atom in combination with an equimolar amount of cholesterol was clearly the most efficient transfection lipid among all the lipids tested (Figure 1). Among amphiphiles **3**−**5** with single hydroxyethyl groups directly linked to the positively charged nitrogen atom, amphiphile **3** was the most efficient one (Figure 1). The transfection efficiency of the most efficient amphiphile DHDEAB was observed to be 2−3 times more in COS-1 cells than that of lipofectamine, one of the most widely used commercially available transfection lipids (Figure 2).

An interesting observation with these non-glycerol-based hydroxyethyl headgroup amphiphiles was that the optimal transfection efficiencies were in most cases observed with formulations containing lipid-to-DNA charge ratios (+/−) less than 1 (Figure 1). Amphiphiles **1** and **3** were most efficient at lipid-to-DNA charge ratios of 0.3:1 and 0.1:1, respectively (Figure 1). Formulations with less than 1 lipid-to-DNA charge ratios for optimal transfection have previously been reported for cationic lipids with hydroxyethyl groups directly attached to the positively charged nitrogen atom.[21,25] However, in liposomal gene delivery it is generally believed that the overall positive charge of the cationic lipid−DNA complex plays a key role in their interaction with the negatively charged biological membranes. Thus, the remarkable efficiencies of the presently described transfection complexes prepared using lipid-to-DNA charge ratios significantly less than 1 (Figures 1 and 2) convincingly indicate that the overall charge of the lipid−DNA complex for efficient gene delivery need not be necessarily positive. The positive charge may be important for condensation of DNA or/and improve the uptake of the complex by the cells. Since the plasmid DNA has to interact with a variety of environments and membranes before it is expressed in the nucleus, higher expression of plasmid complexed with the novel hydroxyethyl group containing amphiphiles outlined here suggests that the plasmid longevity is enhanced on its route to nucleus. Thus, the function of the positive charge on lipids in cationic lipid-mediated gene delivery is still open to investigation. An important point deserves to be emphasized at this point of discussion. The charge ratios of the present work refer to the charge ratios of the lipid-to-DNA used in preparing the transfection complex, and these may or may not be the net charge of the resulting complexes.

Amphiphile **2** with only one aliphatic chain in the hydrophobic tail did not show any transfection either in pure form or in combination with any helper lipids (data not shown). However, at 0.3 charge ratio of DHDEAB to DNA, amphiphile **2** when used at 30 mol % with respect to DHDEAB modestly enhanced the

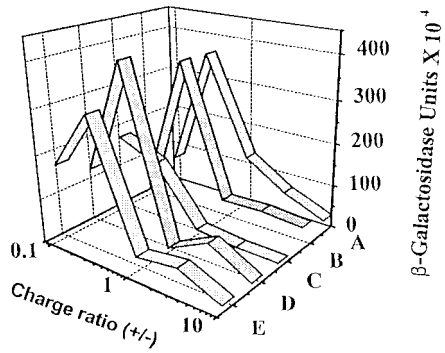


Figure 3. Transfection efficiency of DHDEAB:cholesterol (1:1 mol ratio) with HDEAB (2) on COS-1 cells. HDEAB, which carries a single hydrophobic chain, was added at a mole ratio of 0 (A), 0.15 (B), 0.20 (C), 0.30 (D), and 0.5 (E) with respect to DHDEAB. The charge ratios on the *X*-axis are based on the charge of DHDEAB only.

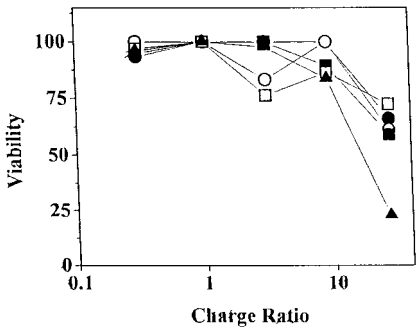


Figure 4. Cytotoxicities of cationic amphiphiles on COS-1 cells. The amphiphiles DOMHAC (filled circles); MOOHAC (open circles), DOHEMAB (filled squares), and DHDEAB (open squares) were tested in combination with cholesterol at a 1:1 mol ratio. DHDEAB:cholesterol:HDEAB (1:1:0.5) mole ratio was also tested (filled triangles).

transfection efficiency of DHDEAB (Figure 3). Use of higher mol % of **2** with respect to DHDEAB and higher charge ratios of DHDEAB to DNA (i.e. >0.3) did not improve the transfection further (Figure 3). Given that the single-chain micelle-forming surfactants are known to destroy the bilayer structures of liposomes, the observed modest increase in the transfection efficiency of DHDEAB in the presence of 30 mol % HDEAB is intriguing. Clearly, detailed investigations using a host of known transfection lipids and varying the mole percent of HDEAB need to be carried out to ensure possible future use of HDEAB as a new helper lipid.

The cytotoxicities (Figure 4) of the amphiphiles as 1:1 amphiphile:cholesterol preparations were tested in COS-1 cells by using reduction of MTT.[30] The cytotoxicity assays were performed in identical conditions as those in transfection experiments. In most cases, the cell viabilities were more than 80% up to 9 nmol of lipid, which is the highest concentration of the lipid used in transfections. Low cytotoxicities of the amphiphiles and good transfection efficiencies indicate that the formulations may be used in a variety of cell lines.

Toward understanding any key role played by the hydroxyethyl headgroups of the present transfection lipids, we have compared the transfection efficiency of the most efficient amphiphile DHDEAB with that of DDAB having two methyl groups directly linked to nitrogen instead of two hydroxyethyl groups. Transfection results shown in Figure 2 demonstrate that in the presence of an equimolar amount of cholesterol as the auxiliary lipid, DDAB was 2−3 times less efficient than DHDEAB. Interestingly, both DHDEAB and DDAB were also observed to show their optimal transfection efficiencies at the lipid-to-DNA charge ratio of 0.3:1 (Figure 2). Such modestly improved transfection efficiency of DHDEAB compared to that of DDAB (Figure 2) implies that the presence of hydroxyethyl functionalities in the headgroup regions of the monocationic non-glycerol-based amphiphiles possibly may have some role in enhancing the transfection efficiencies. Similar observations were made earlier with glycerol-based amphiphiles such as DMRIE, DORIE, DORI, and DMDHP, with one or two hydroxyethyl groups linked to the nitrogen atom,[18,25] and in the case of 1,4-diaminobutane-based dicationic transfection lipid.[21] Perhaps, hydrogen-bonding interactions with the cell surface of biological membranes play some role for transfection lipids containing hydroxyethyl groups in their headgroup structures. Previous reports have also indicated that the enhanced transfection efficiencies of glycerol-based cationic lipids containing polar hydroxyethyl headgroups may originate from improved interactions of such functionalized cationic lipids or lipid−DNA complexes with cellular membranes via hydrogen bonding.[18,25] However, given the modestly (2−3 fold) enhanced transfection efficiency of DHDEAB compared to that of DDAB with no hydroxyalkyl functionalities in the headgroup regions (Figure 2), the role of hydrogen-bonding interactions between the lipid headgroups and the cell surface of biological membranes is not likely to be a key issue in cationic lipid-mediated gene delivery. In sharp contrast to most other reported transfection results, transfection capabilities of both DHDEAB and DDAB were observed to be virtually lost when used in combination with DOPE as the helper lipid (Figure 2). It is worth mentioning here that the commercially available DDAB-containing transfection reagent lipofectamine also contains DOPE as the auxiliary lipid. However, to our knowledge, except for the present comparative study (Figure 2), the relative transfection efficiencies of DDAB−cholesterol and DDAB−DOPE combinations have not been reported so far.

Nantz et al.[33] have recently shown that the electron-withdrawing headgroup functionalities diminish the transfection activities of glycerol-based cationic lipids. This report implies that the mechanism for the enhanced transfection efficiencies observed with the presently described hydroxyethyl headgroup-containing cationic amphiphiles is not based on inductive activation of the ammonium ions and indirectly emphasizes the possible role of favorable hydrogen-bonding interactions in the case of the present amphiphiles. The in vivo transfection efficiencies and in vivo cytotoxicities of the cationic amphiphiles **1**−**5** are currently being evaluated.

Conclusions

We have synthesized a novel series of nontoxic and non-glycerol-based simple monocationic liposomal transfection lipids containing one or two hydroxyethyl groups directly linked to the positively charged nitrogen atom. The in vitro transfection efficiency of DHDEAB, the

most efficient transfection lipid described herein, is better than that of lipofectamine, one of the most widely used transfection vectors in cationic lipid-mediated gene transfer. Unlike most of the reported liposomal transfection studies, cholesterol instead of DOPE is needed to be used as the helper lipid with the presently described amphiphiles. Interestingly, the most efficient formulations contained cationic lipid-to-DNA charge ratios of 0.3:1. Such improved transfection efficiencies with lower lipid-to-DNA ratios have also been previously observed for the 1,4-diaminobutane-based dicationic amphiphile, *N*,*N*,*N*′,*N*′-tetramethyl-*N*,*N*′-bis(hydroxyethyl)-2,3-bis(oleoyloxy)-1,4-butanediammonium iodide.[21] Thus, our results reemphasize the important point that in cationic lipid-mediated gene delivery, the overall charge of the lipid−DNA complex need not always be positive. In addition, our transfection results also imply that favorable hydrogen-bonding interactions between the lipid headgroups and the cell surface of biological membranes may have some role for improving the transfection efficiency in cationic lipid-mediated gene delivery. However, the transfection results delineated in the present investigation also indicate that such hydrogen-bonding interactions are not likely to be a key controlling parameter in liposomal transfection.

Experimental Section

General Procedures and Materials. The high-resolution mass spectrometric (HRMS) analyses were performed on a Micromass AUTOSPEC-M mass spectrometer (Manchester, U.K.) with OPUS V3.1X data system. Data were acquired by liquid secondary ion mass spectrometry (LSIMS) technique using *m*-nitrobenzyl alcohol as the matrix. LSIMS analyses were 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. Melting point determinations were performed using a Fisher-Johns appratus and are uncorrected. Elemental analyses (C, H, N) were performed at the microanalytical Laboratories of Central Salt and Marine Chemical Research Institute, Bhavnagar, Gujarat, India, and National Chemical Laboratory, Poona, India. Unless otherwise stated, all reagents were purchased from local commercial suppliers and were used without further purification. The progress of the reactions was monitored by thin-layer chromatography using 0.25-mm silica gel plates. Column chromatography was performed with silica gel (Acme Synthetic Chemicals, India; finer than 200 and 60−120 mesh). COS-1 cell line (SV 40 transformed African green monkey kidney, ATCCCRL 1650) was obtained from ATCC, MD. *o*-Nitrophenyl β-D-galactopyranoside, cholesterol, β-galactosidase, and MTT were purchased from Sigma Co. DOPE was purchased from Avanti Polar Lipids. Lipofectamine was purchased from Life Technologies Ltd.

Synthesis of *N*,*N*-Di-*n*-hexadecyl-*N*,*N*-dihydroxyethylammonium Bromide (DHDEAB, amphiphile 1) and *N*-*n*-Hexadecyl-*N*,*N*-dihydroxyethylammonium Bromide (HDEAB, amphiphile 2). A mixture of diethanolamine (1 g, 9.5 mmol), *n*-hexadecyl bromide (2.9 g, 9.5 mmol), and potassium carbonate (1.44 g, 10.5 mmol) was refluxed in methanol for 48 h. Methanol was removed on a rotatory evaporator and the residue was extracted with chloroform (3 × 100 mL). The combined chloroform extract was filtered repeatedly (3 times) to remove potassium carbonate. Chloroform was removed from the filtrate on a rotary evaporator and the residue was taken in 50 mL 80:15:5 (v/v) acetonitrile:ethyl acetate:methanol. One equivalent of *n*-hexadecyl bromide (2.9 g, 9.5 mmol) was added and the mixture was refluxed for 56 h. At this point, an additional equivalent of potassium carbonate (1.44 g, 10.5 mmol) was added and refluxing was continued for another 30 h. Finally, one more equivalent of *n*-hexadecyl bromide (2.9 g, 9.5 mmol) was added and the mixture was refluxed for 24 h. The solvent was removed on a rotary evaporator and the residue was extracted with chloroform (100 mL). Potassium carbonate was filtered from the chloroform extract and the chloroform was removed on a rotary evaporator. The residue was dissolved in ethyl acetate (30 mL) with the help of little methanol (0.3−0.5 mL) and the resulting solution of the product mixture showed three spots (with R_f = 0.9, 0.8, and 0.7) on thin layer chromatography (TLC) using 30:70 (v/v) methanol:chloroform as the TLC developing solvent. The solution was kept at 4 °C for 36 h. The precipitate that appeared at this point was predominantly the compound with R_f = 0.9 which was further purified by silica gel chromatography using finer than 200 mesh size silica and eluting with 2−5% methanolic (by volume) chloroform. The NMR and HRMS of this column purified product (yield 470 mg) showed it to be an *O*-*n*-hexadecyl derivative of DHDEAB that did not show any gene transfection activity. The mother liquor was concentrated and dry-packed with 60−120 mesh size silica. The dry packed residue was loaded on a 60−120 mesh size silica column and was eluted first with ethyl acetate and then with chloroform to wash off unreacted *n*-hexadecyl bromide and diethanolamine. The compounds with R_f = 0.8 and 0.7 were then isolated as a mixture by changing the eluent to 90:10 (v/v) chloroform:methanol. The isolated product mixture was finally dry-packed with finer than 200 mesh size silica and the dry-packed mixture was loaded on a finer than 200 mesh size silica column. The title amphiphiles **1** and **2** (with R_f = 0.8 and 0.7, respectively) were finally isolated in pure forms by eluting the dry-packed column carefully with chloroform containing 2−5% methanol (by volume). The yields of the purified amphiphile **1** (DHDEAB) and amphiphile **2** (HDEAB) were respectively 8% (500 mg, 0.787 mmol, white solid, mp 68−70 °C) and 12.7% (500 mg, 1.2 mmol, white solid, mp 64−65 °C).

^{1}H NMR of DHDEAB (200 MHz, $CDCl_3$): δ/ppm = 0.9 [t, 6H, CH_3-$(CH_2)_n$-]; 1.20−1.5 [m, 52H, -$(CH_2)_{13}$-]; 1.55−1.85 [m, 4H, $(HOCH_2$-$CH_2)_2N^+(CH_2$-CH_2-$)_2$]; 3.4−3.6 [m, 4H, $(HOCH_2$-$CH_2)_2N^+(CH_2$-CH_2-$)_2$]; 3.65−3.80 [m, 4H, $(HOCH_2$-$CH_2)_2N^+$-$(CH_2$-CH_2-$)_2$]; 4.05−4.2 [m, 4H, $(HOCH_2$-$CH_2)_2N^+(CH_2$-CH_2-$)_2$]; 4.75− 4.95(m, 2H, -OH). HRMS (LSIMS) *m*/*z*: calcd (for $C_{36}H_{76}NO_2$ the 4°-ammonium ion, 100%) 554.5876, found 554.5899. Anal. ($C_{36}H_{76}BrNO_2$) C, H, N.

^{1}H NMR of HDEAB (200 MHz, $CDCl_3$): δ/ppm = 0.9 [t, 3H, CH_3-$(CH_2)_n$-]; 1.15−1.5 [m, 26H, CH_3-$(CH_2)_{13}$-]; 1.75−1.85 [m, 2H, $(HOCH_2$-$CH_2)_2N^+(CH_2$-CH_2-$)_2$]; 3.2−3.35 [m, 2H, $(HOCH_2$-$CH_2)_2N^+(CH_2$-CH_2-$)_2$]; 3.35−3.5 [m, 4H, $(HOCH_2$-$CH_2)_2N^+(CH_2$-CH_2-$)_2$]; 4.0−4.15 [m, 4H, $(HOCH_2$-$CH_2)_2N^+(CH_2$-CH_2-$)_2$]. HRMS (LSIMS) *m*/*z*: calcd (for $C_{20}H_{44}NO_2$ the 4°-ammonium ion, 100%) 330.3372, found 330.3346. Anal. ($C_{20}H_{44}BrNO_2$) C, H, N.

Synthesis of *N*-Methyl-*N*-*n*-octadecyl-*N*-oleyl-*N*-hydroxyethylammonium Chloride (MOOHAC, amphiphile 3). Step (a). 100 mL dry dichloromethane was cooled to 0 °C and to the cold dichloromethane solution were added 1.9 g of oleyl aldehyde (7.14 mmol), 1.92 g of stearylamine (7.14 mmol), and 0.86 g of anhydride magnesium sulfate (7.14 mmol). The mixture was kept under stirring for 3 h while the temperature of the stirred mixture raised 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 to the cold solution, 0.54 g sodium borohydride (14.0 mmol) was added. The solution was kept stirred for 4 h during which time the temperature of the reaction mixture gradually raised to room temperature. The reaction mixture was taken in 100 mL chloroform and washed with water (2 × 100 mL); the chloroform layer was dried over anhydride magnesium sulfate and filtered. Chloroform was removed from the filtrate on a rotary evaporator and column chromatographic (using 60−120 mesh size silica) purification of the residue using 20−50% ethyl acetate in pet ether as the eluent afforded 2.34 g (64% yield) of the desired intermediate secondary amine, namely, *N*-oleyl-*N*-*n*-octadecylamine.

Step (b). A mixture of 0.95 g (1.83 mmol) of *N*-oleyl-*N*-*n*-octadecylamine obtained in step (a) and 0.67 g (1.83 mmol) of 2-bromoethyl *tert*-butyldiphenylsilyl ether (prepared conventionally by the reaction between 2-bromoethanol and *tert*-butyldiphenylsilyl chloride in the presence of triethylamine and *N*,*N*-dimethylaminopyridine) was refluxed in ethyl acetate in presence of anhydrous potassium carbonate (0.28 g, 2.01 mmol) for 48 h. The reaction mixture was taken in 100 mL chloroform, washed with water (2 × 100 mL), dried over anhydrous magnesium sulfate, and filtered. Chloroform was removed from the filtrate on a rotary evaporator. Silica gel column chromatographic purification of the resulting residue using 60−120 mesh size silica and 3−4% ethyl acetate (by volume) in pet ether as the eluent afforded 0.69 g (47% yield) of the intermediate tertiary amine, namely the *O*-*tert*-butyldiphenylsilyl derivative of *N*-2-hydroxyethyl-*N*-oleyl-*N*-*n*-octadecylamine. The *tert*-butyl-diphenylsilyl protecting group of this intermediate tertiary amine (0.67 g, 0.84 mmol) was conventionally removed by stirring with tetrabutylammonium fluoride (2.1 mmol, 2.1 mL of 1 M tetrabutylammonium fluoride solution in tetrahydrofuran) in dry tetrahydrofuran (cooled to 0 °C) for 4 h during which the temperature of the reaction mixture gradually raised to room temperature. The usual workup and silica gel column purification (as described above in detail for isolating pure protected intermediate except that the eluent used in this case was 7−10% ethyl acetate in pet ether) of the product mixture afforded 0.32 g (67.5% yield) of pure deprotected tertiary amine, namely, *N*-oleyl-*N*-*n*-octadecyl-*N*-2-hydroxyethylamine.

Step (c). Quaternization of 0.12 g (0.21 mmol) of *N*-oleyl-*N*-*n*-octadecyl-*N*-2-hydroxyethylamine obtained in step (b) was carried out in 5 mL of 5:4 chloroform:methanol (v/v) at room temperature for overnight using huge excess of methyl iodide (4 mL). The solvents were removed on a rotary evaporator and silica gel column chromatographic purification of the residue using 60−120 mesh size silica and 3−4% methanol (by volume) in chloroform afforded the quaternary iodide salt, 0.1 g (66.5% yield), of the title amphiphile. Finally, 0.08 g of pure title amphiphile **3** (waxy white solid) in 91.9% yield was obtained by subjecting the quaternized ammonium iodide salt (0.1 g, 0.14 mmol) to repeated (4 times) chloride ion-exchange chromatography, each time using a freshly generated Amberlyst A-26 chloride ion-exchange column and about 80 mL of chloroform as the eluent. All the isolated intermediates gave spectroscopic data in agreement with their assigned structures shown in Scheme 3. Thus, the title amphiphile **3** was prepared in 3 steps with an overall yield of 12.4%.

^{1}H NMR of MOOHAC (200 MHz, $CDCl_3$): δ/ppm = 0.89 [t, 6H, $C\underline{H}_3$-$(CH_2)_n$-]; 1.20−1.45 [m, 52H, -$(C\underline{H}_2)_{13}$-]; 1.60−1.80 [br, 4H, $C\underline{H}_3(HOCH_2$-$CH_2)N^+(CH_2$-$C\underline{H}_2$-$)_2$]; 1.90−2.10 (m, 4H, -$C\underline{H}_2$-CH=CH-$C\underline{H}_2$-); 3.35 [s, 3H, $C\underline{H}_3(HOCH_2$-$CH_2)N^+(CH_2$-$C\underline{H}_2$-$)_2$)]; 3.41−3.58 [br, 4H, $CH_3(HOC\underline{H}_2$-$CH_2)N^+(C\underline{H}_2$-$CH_2$-$)_2$]; 3.65−3.80 [br, 2H, $CH_3(HOCH_2$-$C\underline{H}_2)N^+(CH_2$-$CH_2$-$)_2$]; 4.07−4.12 [br, 2H, $CH_3(HOC\underline{H}_2$-$CH_2)N^+(\overline{C}H_2$-CH_2-$)_2$]; 5.32 (m, 2H, -CH_2-$C\underline{H}$=$C\underline{H}$-CH_2-). HRMS (LSIMS) *m*/*z*: calcd (for $C_{39}H_{80}$-NO, the 4°-ammonium ion, 100%) 578.6239, found 578.6233. Anal. ($C_{39}H_{80}ClNO\cdot1.5H_2O$) C, H, N.

Synthesis of *N*-methyl-*N*,*N*-di-*n*-octadecyl-*N*-hydroxyethylammonium Chloride (DOMHAC, amphiphile 4). The title amphiphile **4** (white solid, mp 83−85 °C) was synthesized from *n*-octadecyl aldehyde and stearylamine following the same synthetic procedure for preparing the amphiphile **3** (MOOHAC) as described above with an overall yield of 7.1%. All the isolated intermediates gave spectroscopic data in agreement with their assigned structures shown in Scheme 3.

^{1}H NMR of DOMHAC (200 MHz, $CDCl_3$): δ/ppm = 0.89 [t, 6H, $C\underline{H}_3$-$(CH_2)_n$-]; 1.20−1.45 [m, 60H, -$(C\underline{H}_2)_{13}$-]; 1.60−1.80 [br, 4H, $C\underline{H}_3(HOCH_2$-$CH_2)N^+(CH_2$-$C\underline{H}_2$-$)_2$]; 3.35 [s, 3H, $C\underline{H}_3$-$(HOCH_2$-$CH_2)N^+(CH_2$-CH_2-$)_2$)]; 3.41−3.58 [br, 4H, $CH_3(HOC\underline{H}_2$-$CH_2)N^+(C\underline{H}_2$-$CH_2)_2$]; 3.70−3.80 [br, 2H, $CH_3(HOCH_2$-$C\underline{H}_2)N^+$-$(CH_2$-CH_2-$)_2$]; 4.07−4.13 [br, 2H, $CH_3(HOC\underline{H}_2$-$CH_2)N^+(CH_2$-CH_2-$)_2$]. HRMS (LSIMS) *m*/*z*: calcd (for $C_{39}H_{82}NO$, the 4°-ammonium ion, 100%) 580.6388, found 580.6396. Anal. ($C_{39}H_{82}ClNO\cdot0.5H_2O$) C, H, N.

Synthesis of *N*,*N*-Di[*O*-hexadecanoyl]hydroxyethyl-*N*-hydroxyethyl-*N*-methylammonium Bromide (DOHEMAB, amphiphile 5). Step (a). Di-*O*-palmitoylation of *N*-methyldiethanolamine was effected following a published protocol.[31] Briefly, *N*-methyldiethanolamine (1 g, 8.39 mmol) was reacted with palmitoyl chloride (5.075 g, 18.5 mmol) in 10 mL dry *N*,*N*-dimethylformamide at 0 °C and the temperature was gradually raised to room temperature within a period of 4 h. The resulting hydrochloride salt of *N*-methyl-di-*O*-palmitoylethanolamine was filtered, crystallized from 20 mL of dry ether. Finally, recrystallization from 20 mL 5:15 (v/v) methanol:ethyl acetate afforded 3.8 g of the pure hydrochloride intermediate (Scheme 4) in 68% yield.

Step (b). The recrystallized hydrochloride salt (200 mg) was stirred for 5 min in a dichloromethane (10 mL)/1.0 M aqueous NaOH (10 mL) biphasic system. The top aqueous layer was discarded and the lower organic layer was washed with water (2 × 50 mL), dried over anhydrous sodium sulfate, and filtered and dichloromethane was removed on a rotary evaporator. Pure *N*-methyl-di-*O*-palmitoylethanolamine (0.14 g, 0.22 mmol, 74.1% yield) was purified from the residue by silica gel column chromatography using 60−120 mesh size silica and 98:2 (v/v) chloroform:methanol as the eluent.

Step (c). Quaternization of *N*-methyl-di-*O*-palmitoylethanolamine was effected by reacting the purified tertiary amine (0.14 g, 0.22 mmol) with 1.5 equiv of neat 2-bromoethanol (0.063 g, 0.34 mmol) at 85 °C for 4 h. The quaternized title amphiphile salt **5** was crystallized from the residue using 10 mL 2:8 (v/v) benzene:*n*-pentane. Silica gel column chromatographic purification of the resulting crystal using 60−120 mesh size silica and 4:96 (v/v) methanol:chloroform as the eluent finally afforded 0.04 g of the pure title amphiphile **5** (white solid, mp 85−87 °C) in 16.5% yield. All the isolated intermediates (shown in Scheme 4) gave spectroscopic data in agreement with their assigned structures. Thus, the title amphiphile **5** was prepared in 3 steps with an overall yield of 8.3%.

^{1}H NMR of DOHEMAB (200 MHz, $CDCl_3$): δ/ppm = 0.88 [t, 6H, $C\underline{H}_3$-CH_2-$C_{13}H_{26}$-]; 1.20−1.40 [m, 48H, -$(C\underline{H}_2)_n$-]; 1.50−1.68 [m, 4H, $CH_3(HOCH_2$-$CH_2)N^+(CH_2$-CH_2-O-$\overline{C}O$-CH_2-$C\underline{H}_2$-$)_2$]; 2.21−2.40 [2t, 4H, $CH_3(HOCH_2$-$CH_2)N^+(CH_2$-CH_2-O-$\overline{C}O$-$C\underline{H}_2$-CH_2-$)_2$]; 3.43 [s, 3H, $C\underline{H}_3(HOCH_2$-$CH_2)N^+(CH_2$-CH_2-O-CO-$C\underline{H}_2$-CH_2-$)_2$]; 3.50−4.30 [m, 9H, $CH_3(\underline{H}OC\underline{H}_2$-$C\underline{H}_2)N^+(C\underline{H}_2$-$CH_2$-O-CO-$CH_2$-$CH_2$-$)_2$]; 4.51−4.58 [br, t, 4H, $C\underline{H}_3(HOC\underline{H}_2$-$CH_2)N^+(CH_2$-$C\underline{H}_2$-O-CO-$CH_2$-$CH_2$-$)_2$]. HRMS (LSIMS) *m*/*z*: calcd (for $C_{39}H_{79}NO_5$, the 4°-ammonium ion, 100%) 641.5958, found 641.5907. Anal. ($C_{39}H_{79}BrNO_5$) C, H, N.

Liposome Preparation. The mixture of cationic amphiphiles and cholesterol in the appropriate ratio were dissolved in chloroform in a glass vial. The 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. 1 mL of autoclaved 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 sonicating the MLV placed in an ice bath for 3−4 min until clarity using a Branson 450 sonifier at 100% duty cycle and 25-W output power.

Preparation of Plasmid DNA. pRSV-β-Gal plasmid DNA was prepared by alkaline lysis procedure and purified by PEG-8000 precipitation according to Maniatis and co-workers.[32] The plasmid preparations showing OD_{260}/OD_{280} more than 1.8 were used.

Transfection Assay. COS-1 cells were seeded at a density of 50 000 cells/well in a 24-well plate 18 h before the transfection. 0.3 μg of plasmid was complexed with varying amount of lipid (0.1−9.0 nmol) in 25 μL of plain DMEM medium for 30 min. The charge ratio would vary from 0.1:1 to 9:1 (+/−) over this range of the lipid. The complex was diluted to 200 μL with plain DMEM and added to the wells. After 3 h of

incubation, 200 μL of DMEM with 10% FCS was added to the cells. The medium was changed after 24 h and the reporter gene activity was estimated after 48 h. The cells were washed twice with PBS and lysed in 100 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 of ONPG, 0.2 M sodium phosphate, pH 7.15, and 2 mM magnesium chloride) to 50 μL of lysate in a 96-well plate. Absorption at 405 nm was converted to β-galactosidase units by using the calibration curve obtained each day using pure commercial β-galactosidase enzyme. The values of β-galactosidase units in replicate plates assayed on the same day varied by less than 20%. The transfection efficiency values shown in Figures 1−3 are the average values from two replicate transfection plates assayed on the same day. Each transfection experiment was repeated 3 times on 3 different days, and the day-to-day variation in average transfection efficiency values for identically treated two replicate transfection plates was approximately 2-fold and was dependent on the cell density and conditions of the cells.

Toxicity Assay. Cytotoxicity of amphiphiles was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay as described earlier.[30] The assay is performed in 96-well plates by maintaining the ratio of number of cells to amount of cationic amphiphile constant in cytotoxicity and transfection experiments. MTT was added 3 h after adding the cationic amphiphile to the cells. Results were expressed as percent viability = [OD_{540}(treated cells) − background]/[OD540(untreated cells) − background] × 100.

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