Covalent Grafting of Common Trihydroxymethylaminomethane in the Headgroup Region Imparts High Serum Compatibility and Mouse Lung Transfection Property to Cationic Amphiphile

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Clinical success of cationic transfection lipids in nonviral gene therapy continues to remain critically dependent on the use of serum compatible cationic amphiphiles efficient in delivering genes into our body cells. To this end, we demonstrate that covalent grafting of simple Tris-base component of the widely used biological Tris buffer in the headgroup region is capable of imparting high serum compatibility and intravenous mouse lung transfection properties to cationic amphiphile.

Introduction

Clinical success of gene therapy critically depends on the use of efficient reagents to deliver the correct copy of the malfunctioning genes into our body cells. Contemporary gene delivery reagents ("transfection vectors") can be classified into two major types: viral and nonviral. The viral vectors, owing to their natural ability to infect cells, are remarkably efficient in transfecting our body cells. However, viral vectors are potentially capable of generating replication competent virus through various recombination events with the host genome. They also can induce adverse immunogenic responses and can lead to insertional mutagenesis through random integration into the host genome. For example, the first fatality in gene therapy clinical trial involving the use of viral transfection vector was attributed to an inflammatory reaction to an adenovirus vector.[1] Ectopic chromosomal integration of viral DNA has been demonstrated to disrupt expression of a tumor-suppressor gene or to activate an oncogene.[2,3] Contrastingly, a multitude of distinguished favorable clinical features of cationic liposomes, including their least immunogenic characters, robust manufacture, easy preparation and handling techniques, ability of delivering large DNA, etc., are increasingly making them the nonviral transfection vectors of choice in gene therapy.[4] However, the instability of the injected cationic lipid/DNA complexes in biological fluids (containing serum proteins and high salt concentration) remains a major impeding factor in systemic setting.

In general, excess of cationic lipids are used in preparing lipid/DNA complexes so that the overall positive charge of the resulting lipoplexes ensures efficient endocytotic cellular uptake by the negatively charged biological cell surfaces. Unfortunately, excess positive charges of the lipoplexes also favor their nonspecific electrostatic interactions with negatively charged hydrophobic serum albuminate proteins, cellular components such as low-density lipoproteins and macroglobulins, and myriads of other negatively charged systemic molecules.[5–8] Such nonspecific interactions promote promiscuous binding of the transfection complexes to biological surfaces and other systemic molecules at the cost of compromised lipofection. This is why many reported strategies on nonviral systemic transfection[9–15] including our own[16] have been centered around development of serum compatible cationic lipids or improved formulations of known cationic amphiphiles and DNA. Ensuring clinical success of cationic liposomes in gene therapy continues to remain critically dependent on the use of serum compatible cationic lipids. To this end, we report herein on a highly serum compatible novel cationic transfection lipid containing a Tris[a] functionality in its polar headgroup region (Tris-lipid **1**, Scheme 1) for intravenous mouse lung transfection. Our findings demonstrate that covalent grafting of a simple base component of the widely used biological Tris buffer in the headgroup region can impart high serum compatibility and intravenous mouse lung transfection properties to cationic amphiphile.

Results and Discussion

Tris has been used previously in the design of efficient cationic transfection lipids.[17–21] In the design of these previously reported Tris-based cationic transfection lipids, the polar amino functionality of Tris was covalently coupled to the α-carboxyl groups of various amino acids or peptides via an amide bond and the nonpolar hydrophobic tails are attached to the hydroxyl functionalities of the Tris linker via formation of an ester or an ether bond.[17–21] Taking the impressive gene transfer properties of many previously reported cationic amphiphiles with hydroxyalkyl headgroups[22–26] into account, we envisaged that design of a cationic lipid containing three hydroxymethyl functionalities of Tris in its headgroup region may be rewarding. With such a rationale, in the present investigation we have designed and synthesized Tris-lipid **1** (Scheme 1). Three simple steps synthesis of Tris-lipid **1** are shown in Scheme 1A. Tris, upon treatment with 2 equiv of 1-bromo-*n*-hexadecane in dimethyl sulfoxide at 100 °C for 16 h in the presence of 2.2 equiv of potassium carbonate followed by usual workup, afforded the tertiary amine intermediate **I** (Scheme 1A).

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[a] Abbreviations: Tris, trihydroxymethylaminomethane; Chol, cholesterol; DCM, dichloromethane; THF, tetradydrofuran; DMEM, Dulbecco's modifid Eagle's medium; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; FBS, fetal bovine serum; ONPG, *o*-nitrophenyl- β-D-galactopyranoside; PBS, phosphate buffered saline.

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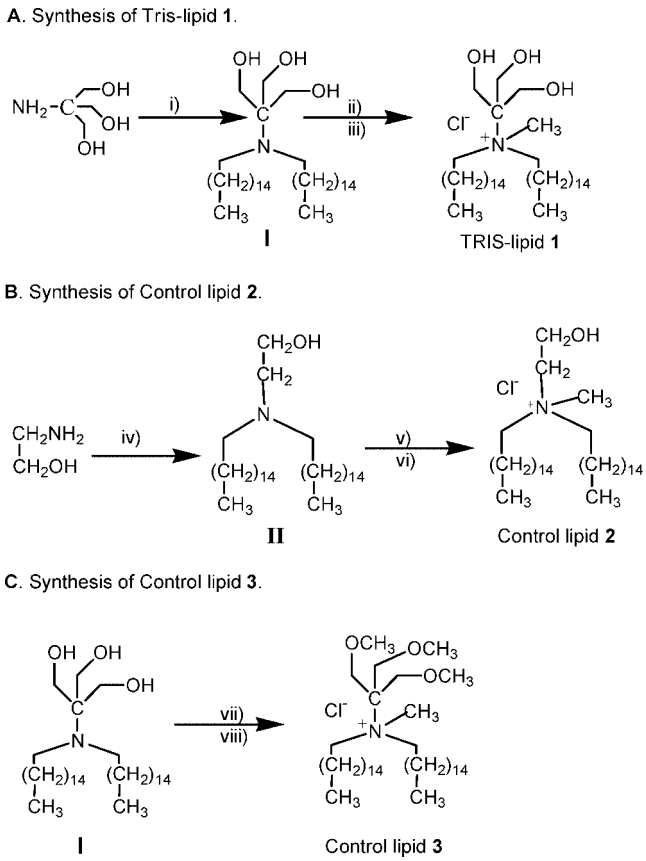
Scheme 1. Synthesis of Tris-Lipid **1** and Control Lipids **2** and **3**[a]



[a] Reagents: (i) *n*-$C_{16}H_{33}Br$ (two equiv), DMSO, K_2CO_3 (2.2 equiv), 100 °C, 16 h; (ii) excess CH_3I, room temp, overnight; (iii) chloride ion exchange over Amberlyst A-26; (iv) *n*-$C_{16}H_{33}Br$ (2 equiv), DMSO, K_2CO_3 (4 equiv), 80 °C, 16 h; (v) excess CH_3I, room temp; (vi) chloride ion exchange over Amberlyst A-26; (vii) dry THF, NaH, excess MeI, 0 °C to room temp, overnight; (viii) chloride ion exchange over Amberlyst A-26.

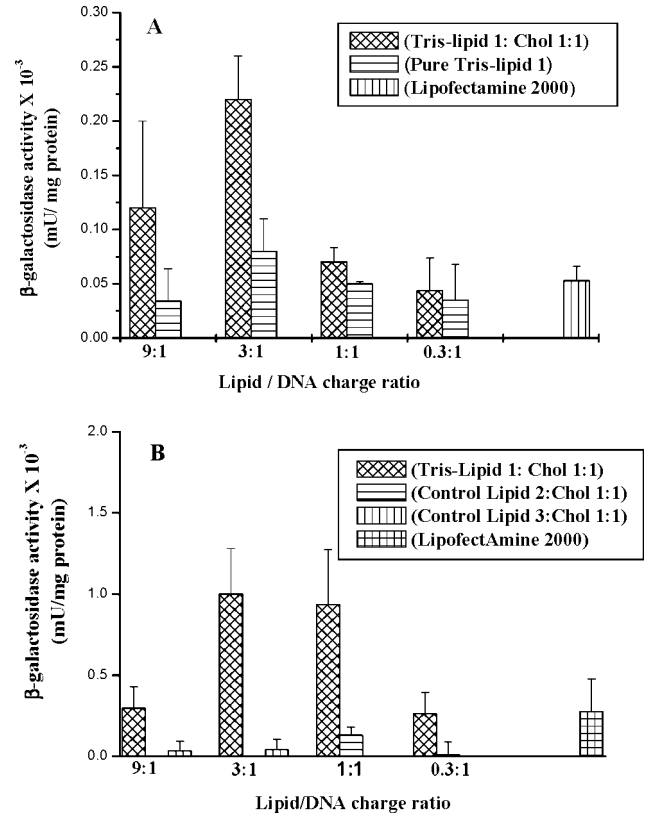


Figure 1. In vitro gene transfer efficacies of Tris-lipid **1** (in presence of equimolar cholesterol as colipid and without colipid) in A549 cells in absence of serum using *p*-CMV-*β*-gal-SPORT reporter gene across varying lipid/DNA charge ratios (A) and comparative in vitro gene transfer efficiencies of Tris-lipid **1** and the control lipids **2** and **3** in representative CHO cells (B). The transfection efficiencies of the Tris-lipid **1** were compared to those of commercially available lipofectamine-2000. The transfection values shown are the average of triplicate experiments performed on the same day. Details of transfection experiments are as described in the text.

Intermediate **I** upon quaternization with excess methyl iodide followed by chloride ion exchange chromatography over Amberlyst A-26 afforded the target Tris-lipid **1** (Scheme 1A). To address the importance of the Tris headgroup in imparting gene transfer properties to Tris-lipid **1**, we synthesized the control lipid **2** (*N*,*N*-di-*n*-hexadecyl-*N*-methyl-*N*-2-hydroxyethylammonium chloride) in which the same two *n*-hexadecyl hydrophobic tails as those in the Tris-lipid **1** were retained but the Tris headgroup of lipid **1** was changed to an ethanolamine headgroup (Scheme 1B). In addition, to gain mechanistic insights into whether favorable hydrogen-bonding interactions between the biological membrane components and the hydroxyl functionalities present in the polar headgroup region of the Tris-lipid **1** could play an important role behind the transfection efficiency of the Tris-lipid **1**, we designed and synthesized a new control lipid **3** (Scheme 1C) in which the three hydroxyl functionalities of Tris-lipid **1** were replaced with three methoxy groups.

We evaluated the in vitro gene transfection properties of Tris-lipid **1** using the *p*CMV-SPORT-*β*-Gal reporter gene under the control of a CMV promoter in multiple cultured cells including A-549, HepG2, MCF-7, and CHO cells. Figure 1A summarizes the relative efficacies of Tris-lipid **1** in combination with equimolar amounts of cholesterol as colipid (cholesterol was found to be a more efficacious colipid than the commonly used colipid DOPE; data not shown) in transfecting the representative A-549 cells across the varying lipid/DNA charge ratios of 0.3:1 to 9:1. Notably, the in vitro gene transfer efficacies of Tris-lipid **1** in combination with an equimolar amount of cholesterol were found to be superior to that of lipofectamine-2000, a widely used commercially available liposomal transfection kit, and the maximum transfection efficacies of Tris-lipid **1** were observed at lipid/DNA charge ratios of 3:1 and 1:1 (Figure 1A). Interestingly, the optimal in vitro gene transfer efficacies of Tris-lipid **1** alone (i.e., without help from any auxiliary lipid) were also found to be comparable to that of lipofectamine-2000 in A-549 cells (Figure 1A). Tris-lipid **1** (with equimolar cholesterol and without cholesterol colipid) were found to be similarly efficacious in transfecting HepG2, MCF-7, and CHO cells (Figure S12 of Supporting Information). The comparative transfection efficacy profiles of the Tris-lipid **1** and the control lipids **2** and **3** were evaluated in representative CHO cells across the lipid/DNA charge ratios 0.3:1 to 9:1 (Figure 1B). Importantly, the in vitro gene transfer efficiencies of Tris-lipid **1** were found to be remarkably higher than that of control lipid **2** lacking the Tris group in its headgroup region (Figure 1B). Such severely compromised transfection efficiencies of the control lipid **2** is consistent with a dominant role played by the Tris headgroup in imparting good transfection properties to Tris-lipid **1**.

A mechanistic origin behind the high gene transfer properties of the Tris-lipid **1** could be related to the enhanced cellular uptake of the Tris-lipid **1**/DNA lipoplexes mediated by favorable hydrogen-bonding interactions between the hydroxyl headgroups of the Tris-lipid **1** and hydrogen bond forming cell surface

molecules. To test this hypothesis, we synthesized the control lipid **3** (Scheme 1C) in which the three hydroxyl groups of the Tris-lipid **1** are substituted with methoxy groups and we evaluated its in vitro gene transfer efficacy in representative CHO cells. In sharp contrast to the high transfection efficiency of Tris-lipid **1**, the control lipid **3** was found to be transfection incompetent (Figure 1B). Such severely compromised transfection property of the control lipid **3** supports our hypothesis that high gene transfer property of Tris-lipid **1** might be related to favorable hydrogen-bonding interactions between the three hydroxyl groups of the Tris-lipid **1** and cell surface components.

A major impediment retarding the clinical success of cationic transfection lipids is that the gene delivery efficiencies of cationic lipids often get adversely affected in presence of serum.[4–7,27,28] To this end, Templeton et al. succeeded in developing a serum stable formulation of bilamellar invaginated vesicles (BIVs) of 1,2-bis(oleoyloxy)-3-(trimethylamino)propane (DOTAP) and cholesterol (colipid) for efficiently entrapping large amounts of DNA between two bilamellar invaginated vesicles.[29,30] The in vivo efficacies of BIVs were subsequently tested in small and large animal models for lung,[31] breast,[32] and pancreatic cancers.[33] Crook et al. demonstrated that DOTAP/cholesterol formulation is capable of transfecting cells with high efficiency in the presence of up to 80% of added serum.[34] To evaluate the serum compatibility of Tris-lipid **1**, we measured its in vitro gene transfer efficacies in a representative COS-1 cell in the presence of increasing amounts of added serum. Since Tris-lipid **1** showed its optimal transfection efficacies at lipid/DNA charge ratios of 3:1 in all four cells (Figure 1 and Supporting Information Figure S12), the serum compatibility experiments were conducted at 3:1 lipid/DNA charge ratios. The in vitro gene transfer efficacies of Tris-lipid **1** in the presence of up to 50% added serum were enhanced by about 2- to 3-fold compared to serum-free conditions (Figure 2A; the relative gene transfer efficiency of Tris-lipid **1** in serum free conditions has been depicted as 1 by the extreme left bar). Within the range of 50–90% added serum, the gene transfer efficiencies of Tris-lipid **1** were found to be comparable or higher than that of Tris-lipid **1** in serum free conditions (Figure 2A). Thus, Tris-lipid **1** is highly serum compatible in the presence of up to 90% added serum. Recently, Garinot et al. has demonstrated that "bunch shaped" amphiphilic polyether branched molecules when inserted into cationic liposome/DNA complexes impart enhanced surface charge shielding characteristics to the lipoplexes.[13] The significantly prolonged circulation times of such "bunch shaped" amphiphilic polyether branched molecules containing lipoplexes presumably originate from such enhanced surface charge shielding characteristics, which in turn efficiently inhibited the nonspecific interactions between the lipoplexes and the blood components.[13] The highly serum compatible transfection characteristics of the Tris-lipid **1** may be related to similar enhanced surface charge shielding of the Tris-lipid **1**/DNA complexes induced by the three hydroxyl headgroups.

The enhanced gene transfer efficiencies of Tris-lipid **1** in the presence of added serum (Figure 2A) are contrary to a recent report on dramatic decrease in transfection efficiencies of di-C14-amidine/DNA lipoplex in the presence of plasma.[5] In this report Tandia et al.[5] studied the role of purified individual human plasma components in modulating the in vitro gene transfer properties of their di-C14-amidine/protamine/p-CMV-luc lipoplex in lung endothelial cell. They have shown that in addition to total plasma protein, individual plasma protein components such as the lipoproteins HDL and LDL strongly inhibit transfection efficacies of lipoplex whereas fibrinogen significantly enhanced its transfection property.[5] This prior report on the net transfection inhibition by plasma proteins and the serum

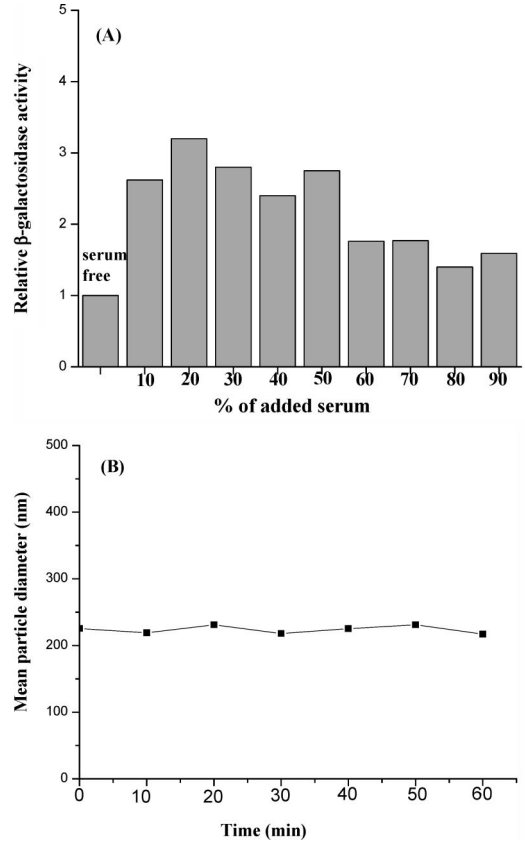


Figure 2. Serum compatibility and colloidal stabilities of the Tris-lipid **1**/DNA complexes. In vitro transfection efficiencies of Tris-lipid **1** using *p*-CMV-*β*-gal-SPORT reporter gene at lipid/DNA charge ratio of 3:1 were evaluated in the presence of increasing concentrations of added serum in representative COS-1 cell (A). Colloidal stabilities of the Tris-lipid **1**/DNA complex (with 3:1 lipid/DNA charge ratio) in transfection media (B) were evaluated by measuring the sizes of the lipoplexes using dynamic light scattering technique as described in the text.

compatible (up to 90% added serum, Figure 2A) transfection properties of Tris-lipid **1** described in the present study support the notion that the overall effect of plasma/serum proteins on the transfection efficiencies of a given lipoplex is likely to be dependent on the nature of lipids and the cells to be transfected. Importantly, the transfection profile of Tris-lipid **1** summarized in Figure 2A demonstrates that covalent grafting of the simple base component of the widely used biological Tris buffer in the headgroup region is capable of imparting serum compatible gene transfer properties to cationic amphiphiles. Future studies probing the influence of individual serum components in modulating the transfection properties of Tris-lipid **1** are likely to throw more meaningful mechanistic insights into the origin of the enhanced transfection properties of Tris-lipid **1** in the presence of serum. The sizes of the lipoplexes made from Tris-lipid **1** and equimolar cholesterol (measured by dynamic laser light scattering instrument, Malvern, U.K.) were found to vary within 240–290 nm across the entire lipid/DNA charge ratios of 0.3:1 to 9:1 (data not shown). Dynamic light scattering analysis performed every 10 min on the Tris-lipid **1** lipoplexes (containing 3:1 lipid/DNA charge ratios) prepared in the presence of 10% fetal bovine serum revealed that the lipoplex sizes remains practically unaffected (220–240 nm) in the presence of transfection medium (Figure 2B). This size range (220–240 nm) remained the same even after 4 h (data not shown). Clearly, the serum compatible gene delivery efficiencies and the size characteristics summarized in Figure 2 indicated that the colloidal stabilities of the lipoplexes prepared

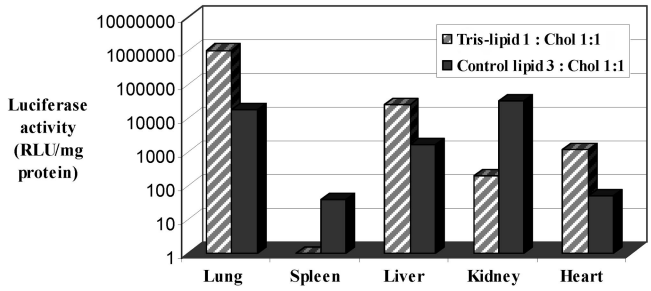


Figure 3. In vivo transfection profiles of Tris-lipid **1** and control lipid **3**. Each mouse was intravenously injected with 70 μg of *p*-CMV-luc complexed with Tris-lipid **1** or control lipid **3** at lipid/DNA charge ratio of 8:1. Luciferase activities were measured 8 h postinjection as described in the text. Values are the mean values ($n = 4$). Details of in vivo experiments are as described in the text.

from Tris-lipid **1** are unlikely to be adversely affected under systemic settings.

Findings in the conventional gel mobility shift and DNase I sensitivity assays indicated the presence of strong lipid−DNA binding interactions in the Tris-lipid **1**/DNA complexes at high lipid/DNA charge ratios of 9:1 and 3:1 (electrophoretic gel patterns for this standard assays are shown in Figure S2 of Supporting Information). MTT-based cellular cytotoxicity assays revealed the equally noncytotoxic nature of the lipoplexes prepared with Tris-lipid **1** and control lipid **3** across the lipid/DNA charge ratios of 9:1–0.3:1 (Figure S14 of Supporting Information). However, the control lipid **2**/DNA lipoplexes were found to be significantly more cytotoxic than the lipoplexes prepared from Tris-lipid **1** and the control lipid **3** (Figure S14 of Supporting Information). Inspired by all these promising features (namely, high serum compatibilities, strong DNA-binding characteristics, and noncytotoxic nature) of the Tris-lipid **1**, we evaluated its systemic potential by intravenously injecting the Tris-lipid **1**/*p*-CMV-luc lipoplexes in mice. Since Tris-lipid **1** showed its optimal in vitro gene transfer efficacies mostly in combination with equimolar amount of cholesterol (Figure 1), its in vivo potentials were also measured using equimolar amounts of cholesterol as colipid. Initial experiments using lipoplexes of varying lipid/DNA (+/−) charge ratios (4:1–16:1) revealed 8:1 as the optimal in vivo charge ratio (data not shown). Transgene expressions in all the in vivo experiments were monitored 8 h postinjection. Importantly, transgene expression in lung was found to be 2–3 orders of magnitude higher than in kidney, heart, and liver (Figure 3). Consistent with the in vitro findings (Figure 1A), the control lipid **3** was found to be significantly less efficient (by almost 2 orders of magnitude) than the Tris-lipid **1** in transfecting mouse lung, and lipids **1** and **3** were found to be significantly less efficient in transfecting the other organs (Figure 3). Intravenous injection of 5% aqueous glucose solution of naked plasmid DNA failed to transfect mice body organs to any significant extent (data not shown). Needless to mention, the preclinical formulation parameters need to be further optimized (for instance, transgene expression in the present study gradually diminished after 24 h postinjection; data not shown) before evaluating the clinical potentials of Tris-lipid **1** for transfecting lungs of human cystic fibrosis patients with CFTR gene.

Conclusions

In conclusion, the present findings convincingly demonstrate that covalent grafting of the simple base component of the widely used biological Tris buffer in the headgroup region is capable of imparting remarkable serum compatibility and lung transfection properties to cationic amphiphile. Given its simple structure, nontoxic nature, remarkable serum compatibility, and intravenous lung transfection properties, Tris-lipid **1** is likely to find future applications in nonviral gene therapy of inherited lung diseases. The findings described herein sets the stage for future structure–activity investigations aimed at further improving the systemic potentials of cationic amphiphiles with Tris headgroups.

Experimental Section

General Procedures and Reagents. The electron spray ionization (ESI) mass spectrometry analysis was performed on a LCQ ion trap mass spectrometer (Thermo Fischer). ^{1}H NMR spectra were recorded on Varian FT 200 and Bruker FT 300 MHz instruments. 1-Bromohexadecane and Amberlyst A-26 chloride ion-exchange resin were purchased from Lancaster (Morecambe, U.K.). Unless otherwise stated, all reagents were purchased from local commercial suppliers and were used without further purification. Column chromatography was performed with silica gel (Acme Synthetic Chemicals, India, 60–120 mesh). Lipofectamine-2000, cell culture mediam and fetal calf serum were purchased from GibcoBRL, Rockville,MD. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), polyethylene glycol 8000, NP-40, antibiotics, agarose, *o*-nitrophenyl- β-D-galactopyranoside were purchased from Sigma, St.Louis, MO. DNA molecular weight markers were purchased from Bangalore Genei, Bangalore, India. Cholesterol and DOPE were purchased from Avanti Polar, AL. Unless otherwise stated, all reagents were purchased from local commercial suppliers and were used without further purification. Purity of the Tris-lipid **1** and the control lipids **2** and **3** were determined by analytical HPLC in two different mobile phases. Peaks were detected by UV absorption at 210 nm. Lipids **1**−**3** showed more than 98% purity (HPLC chromatograms and HPLC conditions are in Supporting Information).

Synthesis of *N*,*N*-Di-*n*-hexadecyl-*N*-methyl-*N*-(trihydroxymethyl)methylammonium Chloride (Tris-Lipid 1, Scheme 1A). In a 100 mL round-bottomed flask, a mixture of 1-bromohexadecane (4.84 g, 16 mmol), Tris (1g, 8 mmol), and potassium carbonate (2.45 g, 176 mmol) was heated at 100 °C in 10 mL of dimethyl sulfoxide for 16 h. The mixture was taken in dichloromethane (50 mL) and washed with water (5 × 50 mL). The dichloromethane portion was dried over sodium sulfate and concentrated on a rotary evaporator. Column chromatographic purification of the residue using silica gel (60–120 mesh size) and 2–3% methanol in chloroform (v/v) as the eluent afforded the tertiary amine intermediate **I** (Scheme 1A) as a yellowish white solid (1.5 g, 31% yield, R_f = 0.5, 90:10 chloroform/methanol, v/v): ^{1}H NMR (200 MHz, $CDCl_3$) δ/ppm 0.9 [t, 6H, $CH_3(CH_2)_{13}-$], 1.20–1.5 [m, 52H, $CH_3(CH_2)_{13}-$], 1.55–1.85 [m, 4H, $CH_3(CH_2)_{13}CH_2CH_2)_2N-$], 2.70–2.80 [m, 4H, $(CH_3(CH_2)_{13}CH_2CH_2)_2N-$], 3.60–3.80 [s, 6H, $(HOCH_2)_3CN-$].

In a 25 mL round-bottomed flask, a mixture of the tertiary amine intermediate **I** obtained above and excess methyl iodide (1 mL) was stirred at room temperature overnight. Methyl iodide was removed by rotatory evaporator, and column chromatographic purification of the residue was performed using 60–120 mesh size silica gel and 5–6% methanol in chloroform as the eluent. The title lipid **1** ($R_f = 0.3$, 10% methanol in chloroform) was obtained as a white solid (150 mg, 75% yield) upon removal of the eluent followed by chloride ion exchange chromatography over Amberlyst A-26 resin: ^{1}H NMR (300 MHz, $CDCl_3$) δ/ppm 0.9 [t, 6H, $(CH_3CH_2)_n-$], 1.20–1.5 [m, 52H, $(CH_2)_{13}-$], 1.65 [m, 4H, $(HOCH_2)_3CN^+(CH_3)(CH_2CH_2-)_2$], 3.10 [t, 4H, $(CH_2OH)_3CN^+(CH_3)(CH_2CH_2-)_2$], 3.8 [s, 6H, $(HOCH_2)_3CN^+(CH_3)(CH_2CH_2-)_2$], 4.1[s, 3H, $(HOCH_2)_3CN^+(CH_3)$ $(n\text{-}C_{16}H_{33})_2$]; ESIMS m/z 585 $[M + 1]^+$ (for $C_{37}H_{78}NO_3$, the 4°-ammonium ion, 100%).

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Supporting Information Available: Details of syntheses of the Control lipids **2** and **3**, details of the preparations of liposomes and plasmid DNA, physico-chemical characterizations of liposomes and lipoplexes and the details of the in vitro and in vivo experiments are provided. ^{1}H NMR and mass spectra, HPLC and transfection profiles, electrophoretic gel patterns, and percent cell viabilities. This material is available free of charge via the Internet at http://pubs.acs.org.

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