Influence of Minor Backbone Structural Variations in Modulating the in Vitro Gene Transfer Efficacies of α-Tocopherol Based Cationic Transfection Lipids

ARTICLE *in* BIOCONJUGATE CHEMISTRY · DECEMBER 2011

Impact Factor: 4.51 · DOI: 10.1021/bc2004395 · Source: PubMed

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Influence of Minor Backbone Structural Variations in Modulating the in Vitro Gene Transfer Efficacies of α-Tocopherol Based Cationic Transfection Lipids

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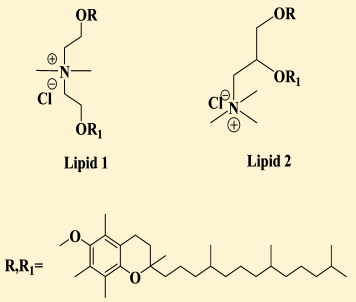
Supporting Information

ABSTRACT: Toward probing the influence of backbone structural variation in cationic lipid mediated gene delivery of α-tocopherol based lipids, two novel α-tocopherol based lipids **1** and **2** have been designed and synthesized. The only structural difference between the cationic amphiphiles **1** and **2** is the backbone structure, where lipid **1** has a non-glycerol backbone and lipid **2** has a glycerol backbone. The lipids **1** and **2** showed contrasting transfection efficiencies: lipid **1** showed high gene transfer efficacy in multiple cultured animals cell lines, whereas lipid **2** is transfection incompetent. In summary, the present findings demonstrate that in the case of α-tocopherol based lipids even minor structural variations like backbone can profoundly influence size, DNA binding characteristics, cellular uptake, and consequently gene delivery efficacies.

Structures of Lipids 1 and 2 containing the same hydrophobic region, same head group and linker group functionality but differ only in the backbone region.



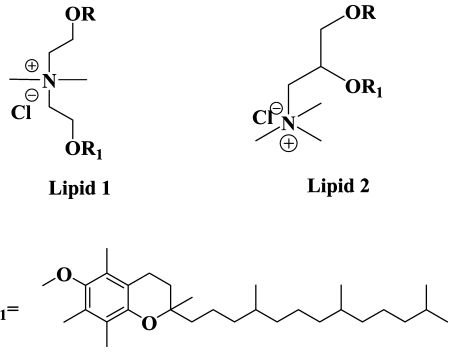
INTRODUCTION

To develop an efficient gene therapeutic approach, design of safe and efficient gene delivery reagents is a prerequisite. Cationic liposomes are one of the more promising nonviral systems for use in gene therapy.[1−18] Since the pioneering development of glycerol backbone-based cationic transfection lipid (*N*-[1-(2,3-dioleyloxy)propyl])-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) in 1987,[19] design and syntheses of a plethora of more efficient cationic transfection lipids, including our own,[20] have been reported. The number of commercially available cationic lipid-based transfection kits are the fruits of such intense global efforts in developing safe and efficient cationic lipids for use in gene therapy. The molecular structures of most of these commercially available cationic transfection lipids contain glycerol as the backbone. With a view to address how important the presence of glycerol backbone in the molecular architectures of many commercially available cationic transfection lipids is, several groups have succeeded in developing a highly efficient series of non-glycerol-based novel transfection lipids.[21−29] The in vitro transfection efficiencies of some of these new cationic lipids developed are better than the most extensively used commercially available glycerol-based cationic amphiphiles used in transfecting cells.[21−29]

In our ongoing program on designing efficient novel cationic transfection lipids, we recently demonstrated the potential of novel α-tocopherol based monocationic lipids for use in liposomal gene delivery.[20] Understanding the structural parameters of cationic amphiphiles which can influence their gene transfer properties is important for designing efficient liposomal gene delivery reagents. Investigations into the role of various molecular-level modifications in different synthetic lipids on their membrane properties and further influence their gene transfer efficiencies are reported.[30−34] To our knowledge, the importance of structure of the backbone group linking the hydrophobic tail and hydrophilic head of cationic lipid is an unexplored area in cationic lipid gene delivery. To this end, with a view to understand the effect of structural variation in backbone on the transfection efficiencies of α-tocopherol based cationic amphiphiles, we have designed and synthesized two novel α-tocopherol based cationic lipids (**1** and **2**; Chart 1), wherein lipid **1** is with nonglycerol backbone and lipid **2** is with glycerol backbone. Findings from the transfection studies and DNA binding studies demonstrate that lipid **1** is more stable and highly transfecting when compared to lipid **2**. The fluorescence microscopic experiments reveal that lipid **1** has higher uptake by the cells and greater expression of reporter gene when compared to lipid **2**. Taken together, the present findings demonstrate that even minor structural variations like backbone in α-tocopherol based cationic amphiphiles can profoundly influence DNA binding, size, cellular uptake, and consequently gene delivery efficacies of cationic liposomes.

Received: August 11, 2011
Revised: October 7, 2011
Published: October 11, 2011

Chart 1. Structure of Lipids 1 and 2



EXPERIMENTAL SECTION

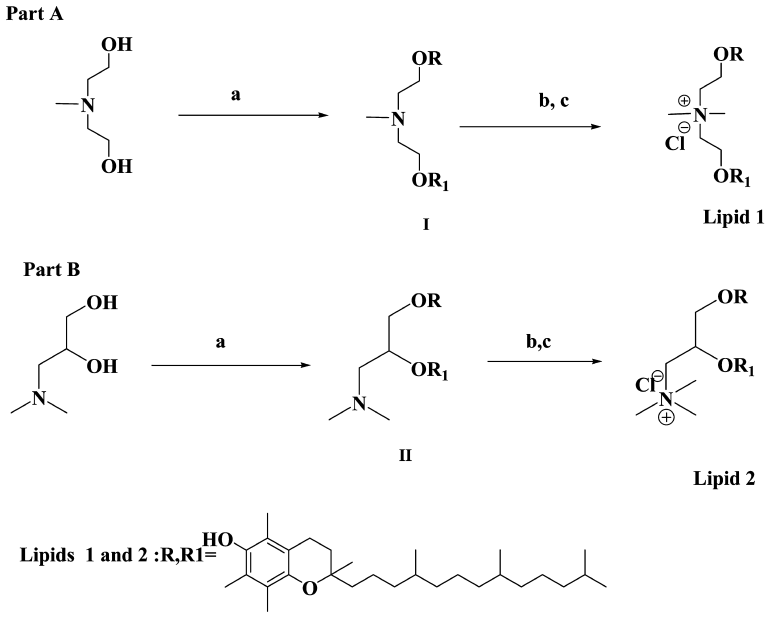
General Procedures and Reagents. Mass spectral data were acquired by using a commercial LCQ ion trap mass spectrometer (ThermoFinnigan, SanJose, CA, USA) equipped with an ESI source. ^{1}H NMR spectra were recorded on a Varian FT 300 MHz NMR spectrometer. *N*-Methyl diethanol amine and *N*,*N*-dimethyl l,2,3-propane diol were purchased from Sigma Co. *p*-CMV-SPORT-*β*-gal plasmid, *α*5GFP plasmid, and Rhodamine-PE were generous gift from IICT (Indian Institute of Chemical Technology), Hyderabad. LipofectAmine-2000 was purchased from Invitrogen Life Technologies (USA). Cell culture media, fetal bovine serum, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), poly(ethylene glycol) 8000, *o*-nitrophenyl-*β*-D-galactopyranoside were purchased from Sigma, St. Louis, USA. NP-40, antibiotics, and agarose were purchased from Hi-media, India. DOPC was purchased from Fluka (Switzerland). Unless otherwise stated, all the other reagents purchased from local commercial suppliers were of analytical grades and were used without further purification. The progress of the reaction 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). Elemental analyses were performed by combustion procedure using Perkin-Elmer 2400 series II CHNS analyzer. Both the synthesized lipids **1** and **2** showed more than 95% purity.

Synthesis. Synthetic routes for preparing cationic lipids **1** and **2** are shown schematically in parts A and B, Scheme 1, respectively. Details of the synthetic procedures, purifications, and spectral characterizations of lipids **1** and **2**, as well as all their synthetic intermediates, shown in Scheme 1 are described below.

Synthesis of Lipid ***1*** *(Scheme 1 Part A). Synthesis of Tertiary Amine (Intermediate I Scheme 1 Part A).* A mixture of *N*-methyl diethanolamine (0.5 g, 4.19 mmol) and potassium hydroxide (~4.0 g, 7.13 mmol) was taken in 10 mL of toluene in a two-necked round-bottomed flask fitted with a Dean−Stark apparatus. The reaction mixture is refluxed for two hours to remove the water as azeotropic mixture. To the reaction mixture, mesylated *α*-tocopherol (4.69 g, 9.22 mmol) (prepared as reported earlier[18]) is added and refluxed at 80 °C for 48 h. The solvent is evaporated and dried. The residue was taken in ethyl acetate (100 mL), washed with water (2 × 100 mL), dried over anhydrous magnesium sulfate, and filtered. The organic solvent is evaporated on a rotary evaporator. The column chromatographic purification of the resulting residue using 60−120 mesh size silica gel and eluting with 3−4% ethyl acetate (v/v) in hexane afforded 1.7 g, (42.92% yield, R_f = 0.8 in 10% ethyl acetate/hexane) of the intermediate tertiary amine.

^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.75−0.95 [m, 24H, CH-C$\underline{H}_3$ tocopheryl], 1.55 [s, 6H, C$\underline{H}_3$-2 tocopheryl], 1.00−1.4 [m, 36H -(C$\underline{H}_2$)$_9$ tocopheryl], 1.8−1.9 [m, 4H, C$\underline{H}_2$-3 tocopheryl], 1.96−1.98 [s, 3H, N-C$\underline{H}_3$], 2.05 [s, 6H, C$\underline{H}_3$-5 tocopheryl], 2.15 [s, 6H, C$\underline{H}_3$-8 tocopheryl], 2.20 [s, 6H, C$\underline{H}_3$-7 tocopheryl], 2.55−2.6 [t, 4H, C$\underline{H}_2$-4 tocopheryl], 3.4−3.6[m,

Scheme 1. Synthesis of Lipids 1 and 2



Reagents: a) Mesylated tocopherol, KOH, Toulene, 48 h b) Methyl Iodide, K_2CO_3, rt, 3 days c) Amberlyst anion exchange resin

dx.doi.org/10.1021/bc2004395 | *Bioconjugate Chem.* 2011, 22, 2581−2592

4H, N-C$\underline{H}_2$-CH$_2$-O-], 3.8−3.9 [m, 4H, N-CH$_2$-C$\underline{H}_2$-O-]. ESIMS: m/z 945 [M$^+$+1] for $C_{63}H_{109}NO_4^+$.

Synthesis of N,N-di[(O-α-deoxyα-tocopherol)-ethyl]-N,N-dimethylammonium chloride (lipid **1** *Scheme 1 Part A).* The intermediate tertiary amine obtained in step (a) of synthesis of lipid **1** (0.5 g, 0.52 mmol) was taken in a 25 mL round-bottomed flask and huge excess of methyl iodide (6 mL) was added to it. After stirring the reaction mixture at room temperature for 48 h, the solvent was removed on a rotary evaporator. The column chromatographic purification of the resulting residue using 60−120 mesh size silica and 2−3% (v/v) methanol in chloroform as eluent afforded the title compound as a quaternary iodide salt (0.4 g, 78.89% yield, R_f = 0.7, 10% methanol/chloroform). Finally, the pure title lipid **1** was obtained by subjecting the quaternized ammonium iodide salt to "repeated chloride ion exchange chromatography" using Amberlyst A-26 chloride anion exchange column and 60 mL of chloroform as eluent. ^{1}H NMR (300 MHz, CDCl$_3$) δ ppm 0.8−0.9 [m, 24H, CH-C$\underline{H}_3$ tocopheryl], 1.45 [s, 6H, C$\underline{H}_3$-2 tocopheryl], 1.00−1.4 [m, 36H -(C$\underline{H}_2$)$_9$ tocopheryl], 1.7−1.9 [m, 4H, C$\underline{H}_2$-3 tocopheryl], 2.05 [s, 6H, C$\underline{H}_3$-5 tocopheryl], 2.15 [s, 6H, C$\underline{H}_3$-8 tocopheryl], 2.20 [s, 6H, C$\underline{H}_3$-7 tocopheryl], 2.55−2.6 [t, 4H, C$\underline{H}_2$-4 tocopheryl], 3.7−3.8 [s, 6H, N-C$\underline{H}_3$], 4.1−4.2 [m, 4H, N-C$\underline{H}_2$-CH$_2$-O-], 4.35−4.45 [m, 4H, N-CH$_2$-C$\underline{H}_2$-O-]. ESIMS (lipid **1**): m/z 959 [M$^+$] for $C_{64}H_{112}NO_4^+$. Calculated: %N, 1.41; %C, 77.25; %H, 11.35. Observed: %N, 1.46; %C, 77.54; %H, 11.61.

Synthesis of Lipid **2** *(Scheme 1 Part B). Synthesis of Tertiary Amine (Intermediate II Scheme 1 Part B).* The intermediate tertiary amine is synthesized by taking a mixture of 3-(dimethylamino) propane-1,2-diol (0.5 g, 4.19 mmol), potassium hydroxide (∼4.0 g, 7.13 mmol), and mesylated α-tocopherol (4.69 g, 9.22 mmol) and following the same procedure as given in the synthesis of lipid **1**. The column chromatographic purification of the resulting residue using 60−120 mesh size silica gel and eluting with 4−5% ethyl acetate (v/v) in hexane afforded 2.0 g (50.50% yield R_f = 0.6, 10% ethyl acetate in hexane) of the intermediate tertiary amine. ^{1}H NMR (300 MHz, CDCl$_3$) δ ppm 0.8−0.9 [m, 24H, CH-C$\underline{H}_3$ tocopheryl], 1.55 [s, 6H, C$\underline{H}_3$-2 tocopheryl], 1.00−1.4 [m, 36H -(C$\underline{H}_2$)$_9$ tocopheryl], 1.7−1.9 [m, 4H, C$\underline{H}_2$-3 tocopheryl], 1.94−1.96 [s, 6H, N-C$\underline{H}_3$], 2.05 [s, 6H, C$\underline{H}_3$-5 tocopheryl], 2.15 [s, 6H, C$\underline{H}_3$-8 tocopheryl], 2.20 [s, 6H, C$\underline{H}_3$-7 tocopheryl], 2.55−2.6 [t, 4H, C$\underline{H}_2$-4 tocopheryl], 2.95−3.05 [m, 2H, CH$_2$-OR-CH-OR-C$\underline{H}_2$-N-(CH$_3$)$_2$], 4.0−4.1 [m, 1H, CH$_2$-OR-C$\underline{H}$-OR-CH$_2$-N-(CH$_3$)$_2$], 4.1−4.2 [m, 2H, C$\underline{H}_2$-OR-CH-OR-CH$_2$-N-(CH$_3$)$_2$]. ESIMS: m/z 944 [M$^+$] for $C_{63}H_{108}NO_4^+$.

Synthesis of N-([1-(2,3-dideoxyα-tocopherol)propyl])-N,N,N-trimethylammonium chloride (lipid **2** *Scheme 1 Part B).* The intermediate tertiary amine obtained in step a of synthesis of lipid **2** (0.5 g, 0.52 mmol) was quaternized using methyliodide. The quaternization is carried out following the procedure given in the synthesis of lipid **1** that yields the title compound as a quaternary iodide salt (0.43 g, 84.81% yield, R_f = 0.7, 10% methanol/chloroform). Finally, the pure title lipid **2** was obtained by subjecting the quaternized ammonium iodide salt to "repeated chloride ion exchange chromatography" using Amberlyst A-26 chloride ion exchange column and 60 mL of chloroform as eluent. ^{1}H NMR (300 MHz, CDCl$_3$) δ ppm 0.7−0.9 [m, 24H, CH-C$\underline{H}_3$ tocopheryl], 1.55 [m, 6H, C$\underline{H}_3$-2 tocopheryl], 1.00−1.4 [m, 36H -(C$\underline{H}_2$)$_9$ tocopheryl], 1.7−1.85 [m, 4H, C$\underline{H}_2$-3 tocopheryl], 2.05 [s, 6H, C$\underline{H}_3$-5 tocopheryl], 2.15 [s, 6H, C$\underline{H}_3$-8 tocopheryl], 2.20 [s, 6H, C$\underline{H}_3$-7 tocopheryl], 2.55−2.6 [t, 4H, C$\underline{H}_2$-4 tocopheryl], 3.8−3.9 [s, 9H, N-C$\underline{H}_3$], 4.15−4.25 [dd, 2H, CH$_2$-OR-CH-OR-C$\underline{H}_2$-N-(CH$_3$)$_3$], 4.3−4.45 [dd, 2H, C$\underline{H}_2$-OR-CH-OR-CH$_2$-N-(CH$_3$)$_3$], 4.7−4.8 [m, 1H, CH$_2$-OR-C$\underline{H}$-OR-CH$_2$-N-(CH$_3$)$_3$]. ESIMS (lipid **1**): m/z 959 [M$^+$] for $C_{64}H_{112}NO_4^+$. Calculated: %N, 1.41; %C, 77.25; %H, 11.35. Observed: %N, 1.74; %C, 77.05; %H, 11.05.

Cells and Cell Culture. B16F10, CHO, A-549, MCF-7, and HepG2 cell lines were procured from the National Centre for Cell Sciences (NCCS), Pune, India. Cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 50 μg/mL penicillin, 50 μg/mL streptomycin, and 20 μg/mL kanamycin in a humidified atmosphere containing 5% CO_2.

Preparation of Liposomes. The cationic lipid and the colipid (DOPC) in 1:2 mol ratio were dissolved in a mixture of chloroform and methanol (1:1) in a glass vial. The solvent was removed with a thin flow of moisture-free nitrogen gas and the dried lipid film 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 overnight. Liposomes were vortexed for 1−2 min to remove any adhering lipid film and sonicated in a bath sonicator for 2−3 min at room temperature to produce multilamellar vesicles (MLV). MLVs were then sonicated in an ice bath until clarity using a Branson 450 sonifier at 100% duty cycle and 25 W output power for 1−2 min to give a clear, translucent solution. These resulting clear aqueous liposomes were used in forming lipoplexes.

Preparation of Plasmid DNA. *p*CMV-SPORT-β-gal plasmid was amplified in DH5α strain of *Escherichia coli*, isolated by the alkaline lysis procedure, and finally purified by PEG-8000 precipitation as described previously.[35] The purity of plasmid was checked by A_{260}/A_{280} ratio (around 1.9) and 1% agarose gel electrophoresis.

Transfection Biology. Cells were seeded at a density of 10 000 (for B16F10 and MCF-7) and 15 000 cells (for CHO, A-549, and HepG2) per well in a 96-well plate 18−24 h before the transfection. Then, 0.3 μg (0.91 nmol) of plasmid DNA was complexed with varying amounts of lipids in plain DMEM medium (total volume made up to 100 μL) for 30 min. The charge ratios were varied from 0.3:1 to 9:1 over these ranges of lipids. Just prior to transfection, cells plated in the 96-well plate were washed with PBS (2 × 100 μL) followed by the addition of lipoplexes. After 4 h of incubation, 100 μL of DMEM with 20% FBS 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 of incubation. The cells were washed twice with PBS (100 μL each) and lysed in 50 μL 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 7.3), and 2 mM magnesium chloride] to the lysate in a 96-well plate. Absorbance of the product *ortho*-nitrophenol at 405 nm was converted to β-galactosidase units by using a calibration curve constructed using pure commercial β-galactosidase enzyme. Each transfection experiment was repeated 3 times on 3 different days. The transfection values reported were average values from three replicate transfection plates assayed on three different days. The values of β-galactosidase units in replicate plates assayed on the same day varied by less than 20%.

Transfection Biology in the Presence of Serum. Cells were seeded at a density of 10 000 (for B16F10 and MCF-7) and 15 000 cells (for CHO, A-549, and HepG2) per well in a 96-well plate 18−24 h before the transfection. Then, 0.3 μg (0.91 nmol) of plasmid DNA was complexed with lipids (**1** and **2**) in DMEM medium in the presence of increasing concentrations of added serum (10−50% v/v and total volume made up to 100 μL using plain DMEM medium) for 30 min. The lipid/DNA charge ratio of these lipoplexes was maintained at 9:1, at which both lipids showed their highest transfection ability in all five types of cells used for transfection (CHO, A-549, B16F10, HepG2, and MCF-7). The remaining experimental procedure and determination of β-galactosidase activity per well are similar to that reported for the in vitro transfection experiments.

Transfection Using α5GFP Plasmid. For α5GFP pDNA expression experiment, 25 000−30 000 cells per well were seeded in 24-well plates (Corning Inc., Corning, NY) for 12 h in 300 μL of growth medium such that the well became 30−50% confluent at the time of transfection. Liposomes of lipids **1** and **2** were complexed with α5GFP expressing *p*DNA (1 μg/well) at 9:1 lipid/DNA charge ratio in plain DMEM (total volume made up to 100 μL) for 30 min. The complexes were then diluted with 200 μL DMEM and added to the cells. After 4 h of incubation, DMEM was removed and cells were supplemented with complete medium. The cells were incubated for 24 h. Cells were washed with PBS (100 μL) and fixed with 3.8% paraformaldehyde in PBS at room temperature for 10 min. The green fluorescent cells expressing α5GFP were detected under an inverted fluorescence microscope (Nikon, Japan).

Cellular Uptake Studies by Inverted Fluorescence Microscopy. Cells were seeded at a density of 10 000 cells/well in a 96-well plate usually 18−24 h prior to the treatment in 200 μL of growth medium such that the well became 30−50% confluent at the time of transfection. *p*CMV-SPORT-β-gal DNA (0.3 μg of DNA diluted to 50 μL with serum-free DMEM media) was complexed with rhodamine-PE labeled cationic liposomes (diluted to 50 μL with DMEM) of lipids **1** and **2** using 9:1 lipid to DNA charge ratio. The cells were washed with PBS (1 × 200 μL), then treated with lipoplexes, and incubated at a humidified atmosphere containing 5% CO_2 at 37 °C. After 4 h of incubation, the cells were washed with PBS (3 × 200 μL) to remove the dye and fixed with 3.8% paraformaldehyde in PBS at room temperature for 10 min. The red fluorescent cells were detected under an inverted fluorescence microscope (Nikon, Japan).

Toxicity Assay. Cytotoxicity of lipids **1** and **2** was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay as described earlier.[36] The cytotoxicity assay was performed in 96-well plates by maintaining the same ratio of number of cells to amount of cationic lipid, as used in the transfection experiments. MTT was added 24 h after the addition of cationic lipids to the cells followed by 3 h of incubation. Results were expressed as percentage viability = [A_{540} (treated cells) − background/A_{540} (untreated cells) − background] × 100.

DNA-Binding Assay. The DNA binding ability of cationic lipids **1**and **2** was assessed by conventional gel retardation assay on a 1% agarose gel (prestained with ethidium bromide) across the varying lipid/DNA charge ratios of 0.3:1 to 9:1. *p*CMV-SPORT-β-gal (0.30 μg) was complexed with varying amounts of cationic lipids in a total volume of 20 μL of DMEM and incubated at room temperature for 20−25 min. Four microliters of 6× loading buffer (0.25% bromophenol blue in 40% (w/v) sucrose in H_2O) was added to it, and 20 μL of the resultant solution was loaded in each well. The samples were electrophoresed at 80 V for 45 min and the DNA bands were visualized in the gel documentation unit.

DNase I Sensitivity Assay. Briefly, in a typical assay, pCMV-β-gal (0.6 μg) was complexed with varying amounts of the cationic lipids (using the indicated lipid/DNA charge ratios of 0.3:1 to 9:1) in a total volume of 20 μL in DMEM and incubated at room temperature for 30 min on a rotary shaker. Subsequently, the complexes were treated with 5 μL of DNase I (at a final concentration of 1 μg/mL) in the presence of 20 mM $MgCl_2$ in a final volume of 50 μL and incubated for 20 min at 37 °C. The reactions were then halted by adding EDTA (to a final concentration of 50 mM) and incubated at 60 °C for 10 min in a water bath. The aqueous layer was washed with 50 μL of phenol/chloroform mixture (1:1, v/v) and centrifuged at 10 000 rpm for 5 min. The aqueous supernatants were separated, loaded (15 μL) on a 1% agarose gel, and electrophoresed at 80 V for 2 h. The DNA bands were visualized with ethidium bromide staining.

Zeta Potential (ζ) and Size Measurements. The sizes and the surface charges (zeta potentials) of liposomes and lipoplexes were measured by photon correlation spectroscopy and electrophoretic mobility on a Zeta sizer $3000HS_A$ (Malvern, U.K.). The sizes were measured in Dulbecco's modified Eagle's medium (DMEM) with a sample refractive index of 1.59 and a viscosity of 0.89 cP. The system was calibrated by using the 200 nm ± 5 nm polystyrene polymer (Duke Scientific Corp., Palo Alto, CA). The diameters of liposomes and lipoplexes were calculated by using the automatic mode. The zeta potential was measured using the following parameters: viscosity, 0.89 cP; dielectric constant, 79; temperature, 25 °C; *F*(Ka), 1.50 (Smoluchowski); maximum voltage of the current, V. The system was calibrated by using DTS0050 standard from Malvern. Measurements were done 10 times with the zero-field correction. The potentials were calculated by using the Smoluchowski approximation.

RESULTS AND DISCUSSION

The present work illustrates the synthesis of lipids **1** and **2** and their physicochemical characteristics. The results of in vitro transfection experiments performed on five different types of cell lines to assess the transfection properties of lipoplexes of lipids **1** and **2** are reported. In addition, the inverted fluorescent microscope experiments in support of the results obtained in in vitro transfection studies are reported. A study of cytotoxicity in all the cell lines studied for transfection and serum compatibility of lipids **1** and **2** are also reported.

Chemistry. Toward probing the influence of the backbone structural variation in gene delivery efficacies of α-tocopherol based cationic amphiphiles, we designed and synthesized lipids **1** and **2** (Scheme 1) such that the two lipids architecturally differ only in the backbone functionality (non-glycerol and glycerol backbone). Lipid **1** was synthesized by reacting *N*-methyldiethanolamine with mesylated α-tocopherol to yield an intermediate followed by quarternization using methyl iodide and chloride ion exchange chromatography over Amberlyst anion exchange resin (Scheme 1, part A). To prepare lipid **2**, *N*,*N*-dimethyl-l,2,3-propanediol was reacted with mesylated α-tocopherol to yield tertiary amine as intermediate (Scheme 1, part B). The tertiary amine intermediate upon quarternization

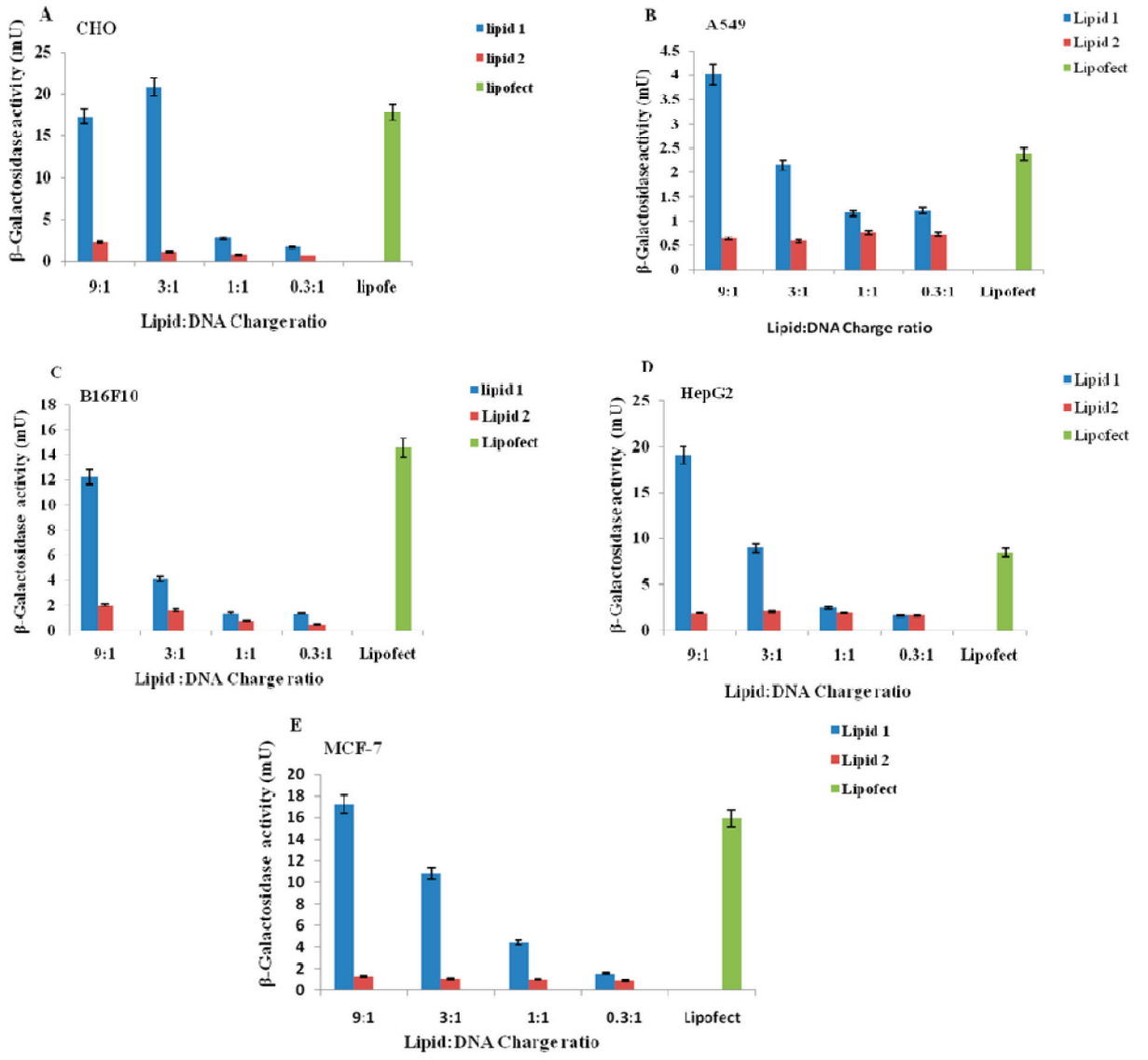


Figure 1. In vitro gene delivery efficiencies of lipids **1** and **2** in CHO (A), A549 (B), B16F10 (C), HepG2 (D), and MCF-7 (E) cell lines. Units of *β*-galactosidase activity were plotted against the varying lipid-to-DNA charge ratios (9:1−0.3:1). The transfection efficiencies of the lipids were compared to that of commercial formulation. Transfection experiments were performed as described in the text. Both lipids were tested on the same day, and the data presented are the average of three experiments performed on three different days.

with excess methyl iodide followed by chloride ion exchange over Amberlyst anion exchange resin afforded the target lipid **2** (Scheme 1, part B). The structures of all the intermediates (Scheme 1) and of the final target lipids **1** and **2** were confirmed by ^{1}H NMR and mass spectra. The purity of the final lipids were confirmed by elemental (C, H, N) analysis.

Transfection Biology. *In Vitro Transfection Studies.* Reporter gene expression assay was used in evaluating the in vitro gene delivery efficacies of lipids **1** and **2** in five cultured mammalian cells including A-549 (human lung carcinoma cells), B16F10 (murine melanoma cells), CHO (Chinese hamster ovary cells), HepG2 (human hepatocarcinoma), and MCF-7 (human breast adenocarcinoma cells) using *p*-CMV-SPORT-*β*-gal plasmid DNA as the reporter gene encoding the enzyme *β*-galactosidase across the lipid/DNA charge ratios of 9:1−0.3:1. Despite the presence of glycerol and nonglycerol backbone being the only structural differences between lipids **1** and **2**, only lipid **1** is competent in delivering reporter gene into all the five cell lines particularly at higher charge ratios, that is, at 3:1 and 9:1 (Figure 1, parts A−E).

Lipid **1** showed its higher gene delivery efficacies at lipid/DNA charge ratio of 9:1 in A-549, B16F10, HepG2, and MCF-7 cells (Figure 1, parts B−E, respectively) and at lipid/DNA charge ratio of 3:1 in CHO cells (Figure 1, part A). In contrast, lipid **2** turned out to be essentially incompetent in delivering genes into any of these five cells across the entire lipid/DNA charge ratios of 9:1−0.3:1 (Figure 1, parts A−E). Interestingly, lipid **1** is found to be comparable with or better than the commercially available formulation at specified lipid/DNA charge ratios in all cell lines except in B16 F10 type in which it is found to be less transfecting compared to commercial formulation. Lipid **1** is found to be one and half times better in transfecting A549 and nearly two and half times better in transfecting HepG2 type of cells compared to commercial formulation. Thus, the relative transfection profiles of lipids **1** and **2** summarized in parts A−E of Figure 1 demonstrate that the structural variation in the backbone region of *α*-tocopherol

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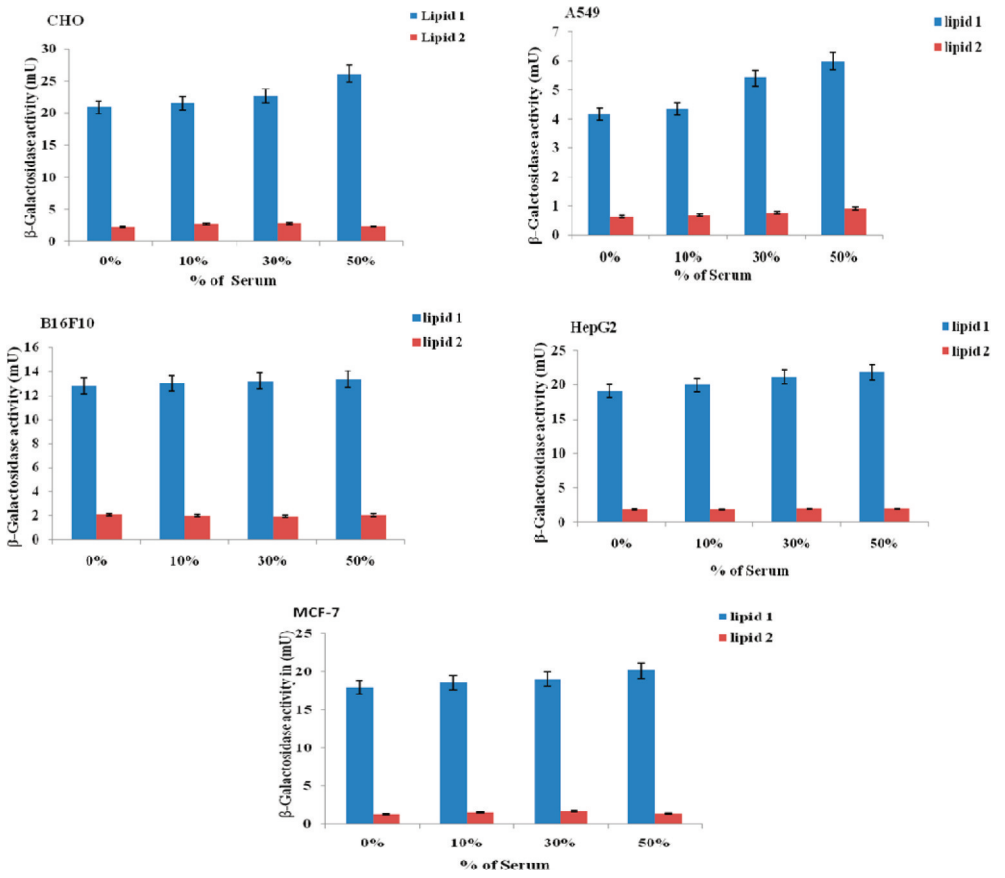

Figure 2. Transfection efficacies of the cationic lipids **1** and **2** in the presence of increasing concentrations of serum added. In vitro transfection efficiencies of lipid/DNA complexes prepared using *p*CMV-*β*-gal-SPORT reporter gene at lipid/DNA charge ratio of 9:1 were evaluated in the presence of increasing concentrations of serum added in CHO (A), A-549 (B), B16F10 (C), HepG2 (D), and MCF-7 (E) cell lines. Both the lipids were tested on the same day, and the data presented are the average of three experiments performed on three different days.

based cationic amphiphiles is a crucial parameter capable of profoundly influencing gene transfer efficacies of *α*-tocopherol based cationic amphiphiles.

Serum Compatibility. The gene transfer efficacies of cationic amphiphiles, in general, are evaluated either in complete absence of serum or in the presence of only 10% (v/v) added serum as disclosed in many prior reports.[23,29,37−41] However, the clinical success of cationic transfection lipids depends on their serum compatibility. The in vitro gene transfer efficacies of cationic amphiphiles usually get adversely affected in the presence of serum.[42−49] Such serum incompatibility of cationic transfection lipids is believed to begin via adsorption of negatively charged serum proteins onto the positively charged cationic liposome surfaces preventing their efficient interaction with cell surface and/or internalization.[50−52]

Toward probing the serum compatibility of the lipids **1** and **2**, in vitro gene transfer efficacies for the lipids **1** and **2** were evaluated across lipid/DNA charge ratio 9:1 at which both lipids showed their higher transfection ability in all five types of cell lines used for transfection (CHO, A-549, B16F10, MCF-7, and HepG2) in the presence of increasing concentrations of serum added (10−50%, v/v) (Figure 2). Interestingly, for reasons not clear at this stage of investigation, the in vitro gene transfer efficacy of lipid **1** is found to be increasing in the presence of up to 50% added serum while that of lipid **2** is found to be unaffected by concentration of serum added (Figure 2). Thus, the transfection efficiency of lipid **1** is found to be highly serum-compatible even at higher levels of added serum.

α5GFP Plasmid Transfection. The relative transfection efficacies of lipids **1** and **2** were also evaluated by the in vitro transfection studies using *α*5GFP (a plasmid DNA encoding green fluorescence protein) in representative HepG2 and A549 (Figure 3, I and II) cells using lipoplexes with 9:1 lipid/DNA charge ratio (the charge ratio where the lipid **1** shows higher transfection in both types of cell lines). After washing the cells with phosphate buffer saline, live HepG2 and A549 cells were viewed under an inverted fluorescence microscope. The numbers of fluorescently labeled cell lines are found to be more when the cells were treated with lipoplexes of lipid **1** than that of lipoplexes of lipid **2** (Figure 3).

Cellular Uptake Study. Results from in vitro transfection experiments demonstrate that lipid **1** is efficient in transfecting multiple cultured cell lines, whereas lipid **2** is incompetent in transfecting in any of these cell lines. Thus, in order to probe the role of cellular uptake behind the contrasting transfection profiles of lipids **1** and **2** the cellular uptake experiments were performed. The representative A549 and MCF-7 cell lines were treated with lipoplexes comprising *p*CMV-SPORT-*β*-gal plasmid DNA and rhodamine-PE labeled liposomes of lipids **1** and **2** with lipid/DNA charge ratio 9:1. The number of A549 and MCF-7 cell lines labeled red are found to be relatively more when treated with lipoplexes of lipid **1** against lipid **2** from the cellular uptake experiment (Figure 4). Thus, these results of cellular uptake experiments (Figures 4) support the

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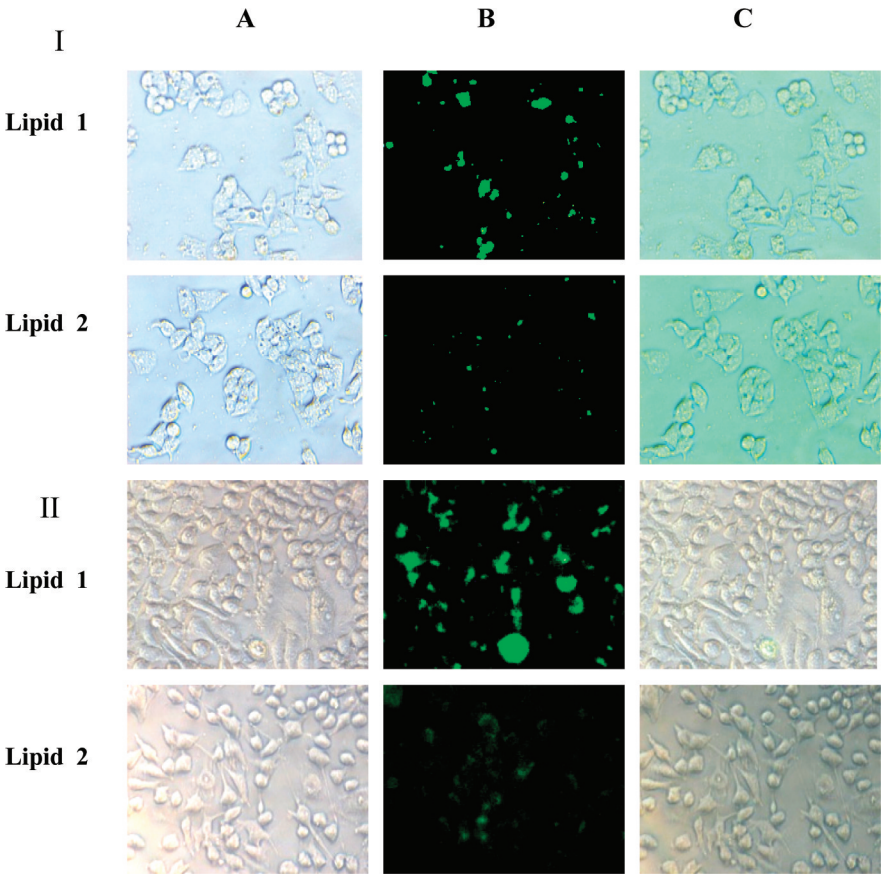

Figure 3. Inverted microscopic images of representative HepG2 (I) and A549 (II) cell lines transfected with lipoplexes of lipids (**1** and **2**) and α5GFP expressing plasmid DNA. Lipid/DNA charge ratios in all the lipoplexes were maintained at 9:1 (A) bright field images, (B) fluorescent images, and (C) overlay images. The details of inverted fluorescence microscopic experiments are described in the text.

notion that the varying transfection profiles of lipids **1** and **2** could be attributed to the cellular uptake variations of the respective lipids. This reemphasizes the supposition that the degree of cellular uptake plays an important role in contrasting transfection profiles of lipids **1** and **2**.

Lipid/DNA Binding Assay. The electrostatic binding interactions between the plasmid DNA and the cationic lipids **1** and **2** at varying lipid/DNA charge ratios were measured by conventional electrophoretic gel retardation assay and DNase I sensitivity assays (Figures 5 and 6). The corresponding electrophoretic gel patterns observed in the gel retardation assay for lipoplexes formed from lipids **1** and **2** are shown in (Figure 5). The gel patterns revealed striking contrasting DNA binding profiles of lipids **1** and **2**. Careful observation of the experimental results unveil that lipid **1** was capable of completely inhibiting electrophoretic mobility of plasmid DNA when lipoplexes were prepared at higher lipid/DNA charge ratios of 9:1 and 3:1 (Figure 5). In contrast, the lipid **2** was found to be completely inefficient in binding DNA even at this higher lipid/DNA charge ratios. At lower lipid/DNA charge ratios (1:1 and 0.3:1), the amount of free DNA associated with lipid **2** is found to be higher when compared to lipid **1** (Figure 5). The stability of lipoplexes formed by lipids **1** and **2** was further confirmed by monitoring the sensitivities of the lipoplexes upon treatment with DNase I. After the free DNA digestion by DNase I, the total DNA (both the digested and inaccessible DNA) was separated from lipid (by extracting with organic solvents) and loaded on a 1% agarose gel. Figure 6 summarizes the results of such DNase I protection experiments for lipoplexes prepared from the cationic lipids **1** and **2** across the entire lipid/DNA charge ratios of 9:1 to 0.3:1. The band intensities of inaccessible and therefore undigested DNA associated with transfection incompetent lipids **2** is found to be significantly less compared to those associated with transfection efficient lipid **1** across the lipid/DNA charge ratios of 9:1 to 0.3:1. Thus, the findings in the conventional gel mobility shift (Figure 5) and DNase I sensitivity assays (Figure 6) together are consistent with the supposition that strong lipid−DNA binding interactions in the lipoplexes perhaps play important role behind the higher transfection efficacies of lipid **1**.

The results of gel retardation assay and sensitivity of the lipoplexes upon treatment with DNase I in the presence of anionic lipid[53,54] (Supporting Information) reveal that the anionic lipid studied in this experiment can displace the ionic interaction between plasmid DNA and the cationic lipids **1** and **2**. It can be deduced from this result that the anionic molecules present in the cell can also displace the ionic interactions between plasmid DNA and the cationic lipids **1** and **2**, thereby releasing plasmid DNA into the cell cytoplasm.

Toxicity Studies. MTT-based cell viability assays were performed in all five types of cell lines used for transfection (CHO, A-549, B16F10, MCF-7, and HepG2) treating with lipoplexes of lipids **1** and **2** across the range of lipid/DNA charge ratios (9:1−0.3:1) used in the actual transfection experiments. Cell viabilities of both the lipids **1** and **2** in all five types of cell lines are found to be remarkably high (more than 85%) particularly up to the lipid/DNA charge ratios of 3:1

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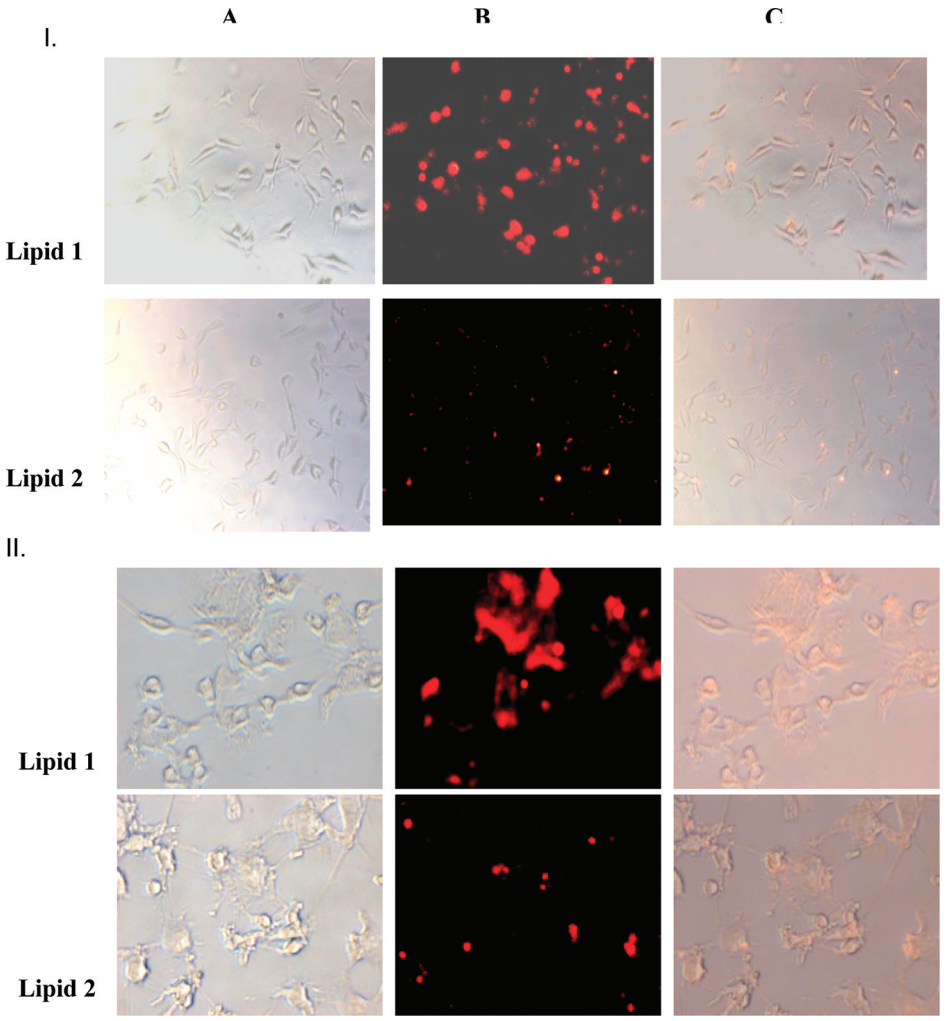

Figure 4. Cellular uptake of Rhodamine labeled lipoplexes. Inverted microscopic images of A549 (I) and MCF-7 (II) cell lines transfected with rhodamine labeled lipoplexes of lipids **1** and **2** prepared at higher in vitro transfection lipid/DNA charge ratios of 9:1 (A) bright field images, (B) fluorescent images, and (C) overlay images. The details of the experiments are described in the text.

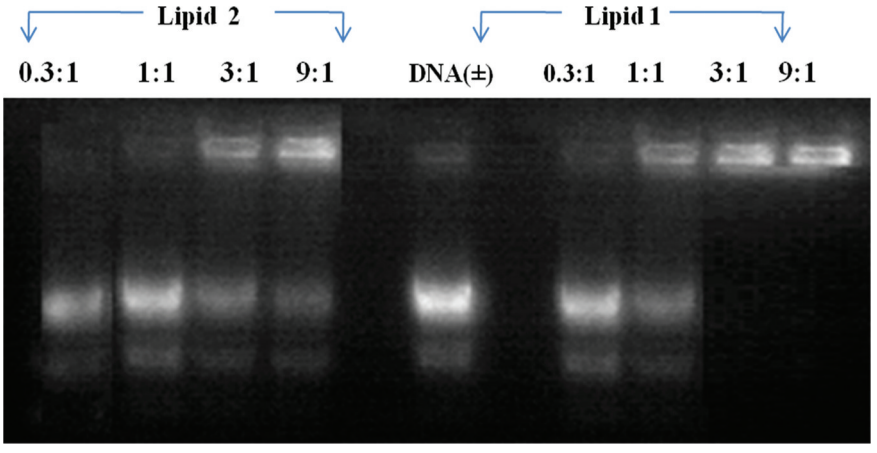

Figure 5. Electrophoretic gel patterns for lipoplex-associated DNA in gel retardation assay. The lipid/DNA charge ratios are indicated at the top of each lane. The details of the gel experiment are as described in the text.

(Figure 7). Except in CHO cell lines, in all the other cell lines the cell viabilities of both lipids **1** and **2** are found to be $\gtrsim$80% at higher charge ratio of 9:1. Thus, the contrasting in vitro gene transfer efficacies of the lipids **1** and **2** are unlikely to originate from varying cell cytotoxicities of the lipids.

Nanosizes and Global Surface Charges of the Lipoplexes. To gain insight into the liposomal physical properties, sizes, and surface potentials of the liposomes of lipids **1** and **2** (prepared in DI water) and their lipoplexes (prepared in DMEM) were measured using dynamic laser light scattering technique

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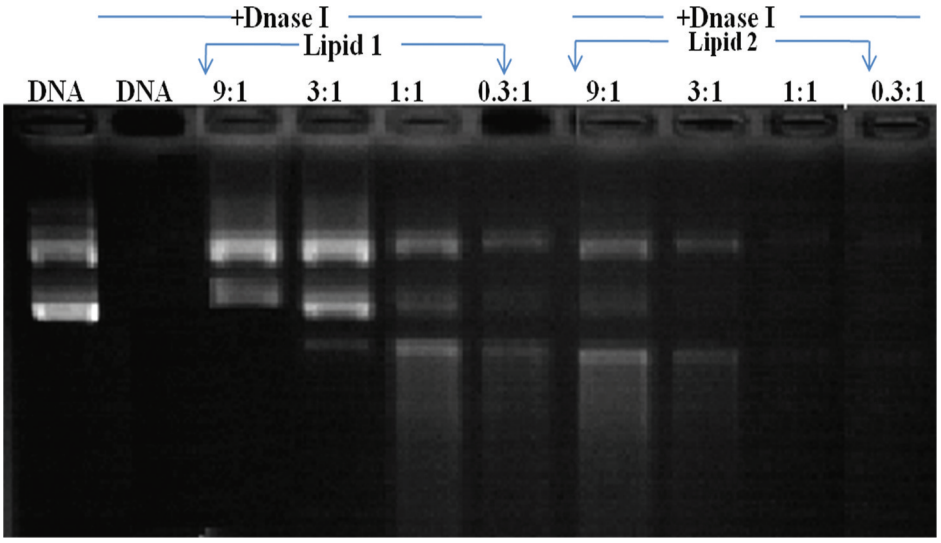

Figure 6. Electrophoretic gel patterns for lipoplex-associated DNA in DNase I sensitivity assay. The lipid/DNA charge ratios are indicated at the top of each lane. The details of the treatment are as described in the text.

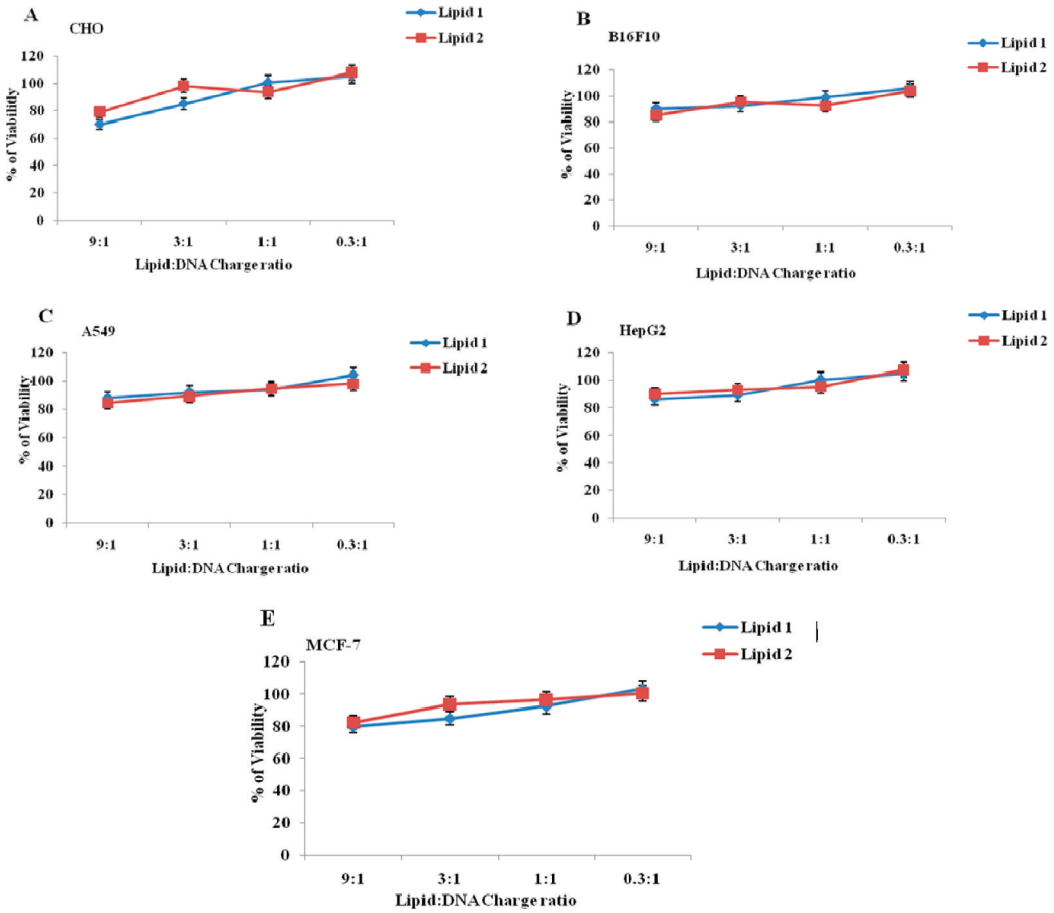

Figure 7. MTT-assay based percent cell viabilities of CHO (A), A-549 (B), B16F10 (C), HepG2 (D), and MCF-7 (E) cell lines treated with lipoplexes of lipids **1** and **2** with lipid/DNA charge ratios 9:1−0.3:1. The cell viability values shown are the average of triplicate experiments performed on same day. The details of the MTT-assay are as described in the text.

(Zetasizer 3000A, Malvern Instruments, U.K.). The sizes and surface potentials of these liposomes and lipoplexes were measured in the absence and in the presence of 10% serum.

Liposomes of lipid **1** were found to be smaller in size compared to liposomes of lipid **2** (Table 1). Lipoplexes of lipid **1** were found to increase from 350 to 800 nm in size as lipid/DNA charge ratios decreased from 9:1 to 0.3:1, whereas lipoplexes of lipid **2** showed steady increase from 600 to 750 nm (Table 1). Significant lipoplex size reduction at higher lipid/DNA charge ratios for lipid **1** can be attributed to the

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Table 1. Hydrodynamic Diameters and Zeta Potentials (ζ) of Lipoplexes[a]

Lipid	Lipid/DNA (molar ratio) 1:0	0.3:1	1:1	3:1	9:1
			Size (nm)		
1	175.4 ± 3.5 (149.2 ± 1.0)	781.7 ± 16.7 (626.1 ± 4.5)	740.3 ± 22.2 (592.7 ± 6.8)	570.6 ± 26.0 (383.9 ± 19.1)	352.6 ± 49.7 (256.3 ± 12.6)
2	253.2 ± 5.3 (219.1 ± 4.4)	739.2 ± 24.1 (529.2 ± 27.1)	706.6.2 ± 9.5 (512.4 ± 11.5)	640.6 ± 18.5 (420.2 ± 1.5)	686.7 ± 2.8 (497.9 ± 10.6)
			Zeta potentials (mV)		
1	22.2 ± 3.1 (18.7 ± 3.5)	−12.5 ± 1.8 (−8.2 ± 4.9)	4.7 ± 3.2 (1.2 ± 0.8)	5.9 ± 2.1 (3.4 ± 1.4)	17.6 ± 5 (13.8 ± 3.2)
2	9.5 ± 6.5 (7.1 ± 2.0)	−14.9 ± 1.8 (−12.8 ± 3.5)	−1.5 ± 1.6 (−5.2 ± 1.5)	5.1 ± 5.0 (1.4 ± 2.1)	8.3 ± 1.0 (6.5 ± 4.0)

[a]Sizes and ζ potentials of liposomes and lipoplexes of lipids **1** and **2** in the absence and in the presence of 10% serum (values within parentheses) were measured by laser light scattering technique using Zetasizer 3000A (Malvern Instruments, U.K). Values shown are the averages obtained from three (size) and ten (zeta potential) measurements.

pronounced DNA condensation efficacies of lipid **1** and hence the higher transfection efficacies of lipid **1** at higher lipid/DNA charge ratios when compared to lipid **2**. Interestingly, the global surface potentials of lipoplexes of lipid **1** were found to be somewhat more positive than those for lipoplexes of lipid **2** in the lipid/DNA charge ratio range 9:1−0.3:1 (Table 1). Results of size and zeta potentials of liposomes and lipoplexes of lipids **1** and **2** in the presence of serum are as shown in the parentheses of Table 1. Significant reduction in size of liposomes and lipoplexes of lipids **1** and **2** was observed. This reduction in size of lipoplexes in the presence of serum was reported earlier.[55] The observed size reduction of lipoplexes in the presence of serum may be the result of a number of factors, including viscosity, serum protein binding, refractive index, and the partial dissociation of the lipoplexes. These observations suggest that the serum-induced decrease in size may not be the primary cause of the higher transfection efficiency of lipid **1** in the presence of serum.

CONCLUSIONS

In summary, the findings described provide the first experimental evidence for the influence of backbone structural variation in liposomal gene delivery of α-tocopherol based cationic lipids. Two α-tocopherol based cationic amphiphiles **1** and **2** were designed and synthesized containing the same hydrophobic region, same headgroup and linker group functionality, but differ only in the backbone region. In vitro findings delineated that lipid **1** showed higher gene transfer efficacies than lipid **2** in multiple cultured mammalian cells including CHO, B16F10, HepG2, MCF-7, and A549. Findings in dynamic laser light scattering studies revealed that lipid **1** significantly condenses DNA compared with lipid **2**. Inverted fluorescence microscopic studies using lipoplexes of lipids **1** and **2** containing green fluorescent protein encoding plasmid DNA and Rhodamine-labeled lipoplexes of lipids **1** and **2** respectively demonstrated significantly higher expression of GFP and higher cellular uptake with lipoplexes of lipid **1**. To conclude, the findings described demonstrate the dramatic influence of backbone structural variation in liposomal gene delivery of α-tocopherol based cationic lipids.

ASSOCIATED CONTENT

Supporting Information

^{1}H NMR spectra of lipids **1** and **2** and intermediates. Mass Spectra of lipids **1** and **2** and intermediates. Elemental analysis data for lipids **1** and **2**. Results and experiment on efficiency of DNA release from the lipoplexes of lipids **1** and **2** in the presence of anionic lipid. Mechanism of the reaction between the mesylated tocopherol and N-methyl diethanolamine or 3-(dimethylamino) propane-1,2-diol. This material is available free of charge via the Internet at http://pubs.acs.org.

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ACKNOWLEDGMENTS

Financial supports for this work from Department of Science and Technology, DST (to P.V. Srilakshmi), Government of India, New Delhi and from National Institute of Technology, Warangal (the doctoral research fellowship to K. Bhavani) are

gratefully acknowledged. We greatly acknowledge Dr. Arabinda Chaudhuri of Division of Lipid Science and Technology, Indian Institute of Chemical Technology, Hyderabad, India, and his group for extending the tissue culture lab facilities to us and also for their immense help in carrying out the transfection experiments.

■ ABBREVATIONS USED:

DOPC, 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine; DMEM, Dulbecco's modified Eagle's medium; DMAP, 4-(dimethylamino)pyridine; FBS, fetal bovine serum; PBS, phosphate-buffered saline; ONPG, *o*-nitrophenyl-*β*-D-galacto-pyranoside.

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