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Synthesis and Gene Transfer Activities of Novel Serum Compatible Reducible Tocopherol-Based Cationic Lipids

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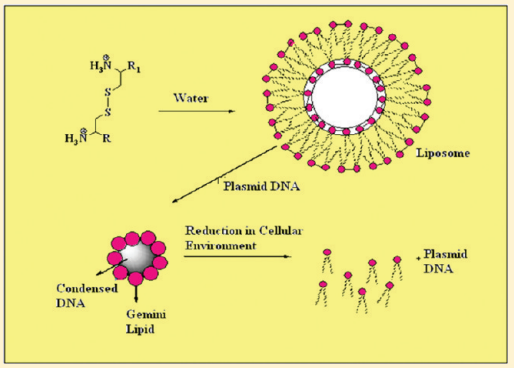
Supporting Information

ABSTRACT: The molecular structure of the cationic lipids greatly influences their transfection efficiency. High transfection efficiencies of tocopherol-based simple monocationic transfection lipids with hydroxylethyl headgroups were recently reported by us (Kedika, B., et al. *J. Med. Chem.* **2011**, *54* (2), 548−561). Toward enhancing the transfection efficiency of tocopherol-based lipids, we have synthesized two tocopherol-based dicationic lipids (**1** and **2**) using simple cystine in the headgroup region. The efficiency of tocopherol-based lipids (**1** and **2**) were compared with nontocopherol-based lipids (**3** and **4**) with cystine in the headgroup region. We report also a comprehensive structure−activity relationship study that identified tocopherol-based gemini cationic lipid **1** is a better transfecting agent than its monomeric lipid counterpart **2** and two other nontocopherol-based gemini cationic lipids (**3** and **4**). The transfection efficiency of lipid **1** was also greater than that of commercial formulation in HepG2 cell lines. A major characteristic feature of this investigation is that serum does not inhibit the transfection activity of tocopherol-based lipids (**1** and **2**) in general and in particular lipid **1** which is found to be highly serum-compatible even at higher concentrations of serum when compared to its monomeric counterpart lipid **2** and the other two control lipid analogues **3** and **4**.



KEYWORDS: *gene delivery, cationic liposome, lipoplexes, reducible tocopherol-based cationic amphiphiles, lipofection, DNA release*

INTRODUCTION

The primary challenge for gene therapy is to develop a method that delivers a therapeutic gene (transgene) to selected cells where proper gene expression can be achieved. The ideal gene delivery vectors should satisfy three major criteria in gene delivery, namely, (1) protect the transgene against degradation by nucleases in intercellular matrices, (2) bring the transgene across the plasma membrane, release the content into the cytoplasm and finally into the nucleus of target cells, and (3) have no detrimental effects.

Viral vectors are able to mediate gene transfer with high efficiency and the possibility of long-term gene expression.[1−3] However, the limitation in the size of the transgene that recombinant virus can carry safety concerns, namely, acute immune response, immunogenicity, and insertion mutagenesis and issues related to the production of viral vectors have imposed serious limits on their use. Methods of nonviral gene delivery have been explored using synthetic vectors.[4−7] Cationic liposomes are one of the more promising nonviral systems for used in gene therapy.[8,9] Since Felgner et al. in 1987 first reported[10] on cationic liposome-mediated gene delivery, hundreds of new cationic lipids have been developed.[11−18] A number of structure−activity studies have demonstrated that the gene delivery efficiencies of cationic amphiphiles crucially depend upon the molecular architectures of the hydrophobic group,[19,20] the nature of polar head groups,[21,22] and the nature of backbone[23] as well as on the nature of linker and spacer functionalities used in covalent tethering of the polar headgroups and the nonpolar tails of cationic amphiphiles.[24,25]

In our ongoing program of designing efficient novel cationic transfection lipids, we recently demonstrated the potential of novel tocopherol-based monocationic lipids for use in liposomal gene delivery.[26] The influence of minor backbone structural variations on gene transfer efficacies of tocopherol-based monocationic lipids has also been recently reported by us.[23] Toward enhancing the transfection efficiency of these novel tocopherol-based lipids further through an increase in the charge on the headgroup of tocopherol-based cationic lipids and to facilitate the release of liposomal content into the cell cytoplasm, we have designed and synthesized two novel tocopherol-based lipids (i) gemini cationic lipid **1** having a cystine headgroup with two tocopherol units as anchoring groups and (ii) cationic lipid **2** having a cystine headgroup with one tocopherol unit as an anchoring group. Disulfide-containing gemini surfactants[27−31] proved to be superior to others in its efficient release of DNA inside the cell cytoplasm after reduction of the disulfide linker by the intracellular glutathione pool. As inefficient release of DNA from lipoplex into the cell cytoplasm is believed to be one of the major

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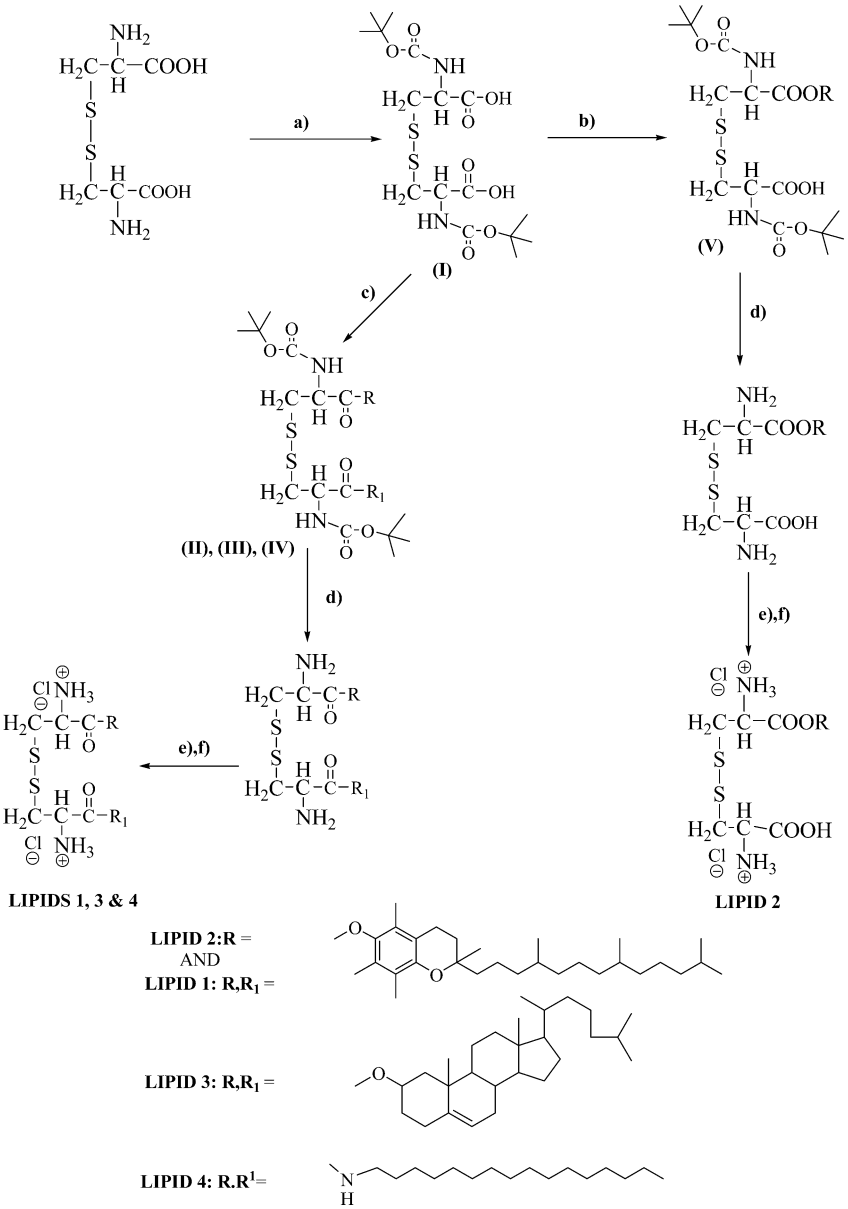
Scheme 1. Synthesis of Lipids 1−4[a]



[a]Reagents: (a) butoxycarbonyl (BOC) anhydride, 1N NaOH, rt, 16 h; (b) tocopherol, *N*,*N*-dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), dichloromethane (DCM), rt, 24 h; (c) tocopherol or cholesterol or hexadecylamine, DCC or (1-ethyl-3-1,3-dimethylaminopropyl) carbodiimide hydrochloride (EDCi), DMAP, DCM, rt, 24 h; (d) trifluoroacetic acid (TFA), dry DCM, rt overnight; (e) 10% HCl, rt, 6 h; (f) Amberlyst anion exchange resin.

impeding factors behind the generally poor transfection efficacies of cationic lipids, we have exploited the reducible nature of the disulfide linker strategy in designing of novel tocopehrol-based gemini cationic lipid (**1**) with cystine as the headgroup. To probe the role of tocopherol on the transfection efficiency, the transfection efficiency of these novel tocopherol-based lipids (**1** and **2**) are compared with the synthesized control gemini cationic lipids (i) with cholesterol as the anchoring groups and cystine as the headgroup (lipid **3**) and (ii) with aliphatic chain C_{16} as the anchoring groups and cystine as the headgroup (lipid **4**).

EXPERIMENTAL PROCEDURES

General Procedures and Materials. Mass spectral data were acquired by using a commercial LCQ ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with an electrospray ionization (ESI) source. ^{1}H NMR spectra were recorded on a Varian FT 300 MHz spectrometer. *α*-Tocopherol, cholesterol, and hexadecylamine were purchased from Sigma Co. *p*-CMV-SPORT-*β*-gal plasmid was a generous gift from Indian Institute of Chemical Technology (IICT; Hyderabad, India). Lipofectamine was purchased from Invitrogen Life Technologies (Grand Island, NY, USA). Cell culture media, fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), polyethylene glycol 8000, and *o*-nitrophenyl-*β*-D-galactopyranoside were purchased from Sigma (St. Louis, MO, USA). Nonylphenylpoly(ethylene glycol) (NP-40), antibiotics, and agarose were purchased from Hi-media (Mumbai, India). 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dio-

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leoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) were purchased from Fluka (Buchs, Switzerland). Unless otherwise stated all other reagents are of analytical grade purchased from local commercial suppliers and 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, Mumbai, India; 60−120 mesh). Elemental analyses were performed by combustion procedure using a Perkin-Elmer 2400 series II CHNS analyzer. All of the synthesized lipids (**1**−**4**) showed more than 95% purity.

Synthesis. Lipids **1**−**4** were synthesized following the strategies depicted in Scheme 1. ^{1}H NMR and mass spectra for all of the intermediates, as well as the final lipids **1**−**4**, are provided in the Supporting Information. The final lipids **1**−**4** were further characterized by the elemental analysis data (Supporting Information).

Synthesis of *N,N*-Di-*tert*-butyloxycarbonyl-L-cystine (I, Scheme 1). To a solution of 2.4 g (10 mmol) of L-cystine in 22 mL of 1N NaOH, 4.36 g (20 mmol) of BOC anhydride was added dropwise for two hours. The resulting solution was left stirring at room temperature for 16 h. The reaction mixture was washed with hexane adjusted to pH 2 with a saturated solution of potassium bisulfate, extracted with ethylacetate (4 × 20 mL), dried over anhydrous sodium sulfate, and filtered. The filtrate was concentrated with a rotary evaporator to yield 2.63 g (yield 59.77%, R_f = 0.2, 10% methanol in chloroform) of *N,N*-di-*tert*-butyloxycarbonyl-L-cystine as a pure compound. ^{1}H NMR (300 MHz, $CDCl_3$): δ/ppm 1.45−1.48 [s, 18H, C(CH_3)$_3$], 2.9−3.4 [dd, 4H, S−CH_2], 4.2−4.4 [t, 2H, S−CH_2−CH], 6.2−6.4 [br, NH]. ESI-MS: *m/z*: 463 [M^+ + Na] for $C_{16}H_{28}N_2O_8S_2^+$.

Synthesis of the Ditocopherol Ester of Cystine, DTEC (Lipid 1, Scheme 1). *Step a: Synthesis of Ditocopherol Ester of N,N-Di-tert-butyloxycarbonyl-L-cystine, Intermediate II, Scheme 1.* Sequentially 0.515 g (2.5 mmol) of *N,N*-dicyclohexylcarbodiimide (DCC), 0.06 g of 4-dimethylaminopyridine (DMAP), and 0.5 g (1.13 mmol) of *N,N*-di-BOC-L-cystine were added to a solution of 1.07 g (2.48 mmol) of α-tocopherol in 15 mL of anhydrous dichloromethane (DCM). The resulting solution was left for stirring at room temperature overnight. The precipitate formed was filtered off, and the solution was evaporated in vacuum to dryness. The residue was dissolved in 20 mL of ethyl acetate and washed with water (2 × 20 mL). The organic layer was dried over anhydrous sodium sulfate and evaporated the solvent. Column chromatographic purification of the residue using 60−120 mesh size silica and 2−3% (v/v) ethylacetate in hexane as eluent afforded 0.65 g (yield = 45.45%, R_f = 0.8, 10% ethylacetate in hexane) of intermediate II as a yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ/ppm 0.8−0.9 [m, 24H, CH−CH_3 tocopheryl], 1.0−1.4 [m, 36H, (CH_2)$_9$ tocopheryl], 1.45 [s, 6H, CH_3-2 tocopheryl], 1.46 [s, 18*H*-BOC], 1.6−1.9 [m, 4H CH_2-3 tocopheryl], 1.96 [s, 6H, CH_3-5 tocopheryl], 2.00 [s, 6H, CH_3-8 tocopheryl], 2.08 [s, 6H,CH_3-7 tocopheryl], 2.60 [t, 4H, CH_2-4 tocopheryl], 3.2−3.6 [dd, 4H, CH_2-cystine], 4.8−5.0 [t, 2H, CH-cystine]. ESI-MS: *m/z*: 1265 [M^+ + 1] for $C_{74}H_{124}N_2O_{10}S_2^+$.

*Step b: Synthesis of DTEC (Lipid **1**, Scheme 1).* To a solution of 0.5 g (0.39 mmol) of ester obtained in step a in 2 mL of anhydrous DCM, 2 mL of trifluoroacetic acid (TFA) was added, and the resulting mixture was stirred at room temperature for 1 h. TFA-DCM was completely removed with a stream of nitrogen gas. Column chromatographic purification of the residue (using 60−120 mesh size silica and 1−2% (v/v) methanol in chloroform as the eluent) followed by chloride ion exchange in Amberlyst A-26 anion exchange resin using methanol as the eluent afforded lipid **1** as yellow oil (0.365 g, 86.90% yield, R_f = 0.7, 5% methanol in chloroform). ^{1}H NMR (300 MHz, $CDCl_3$): δ/ppm 0.7−0.9 [m, 24H, CH-CH_3 tocopheryl], 1.0−1.4 [m, 36H, (CH_2)$_9$ tocopheryl], 1.45−1.6 [m, 6H, CH_3-2 tocopheryl], 1.7−1.9 [m, 4H CH_2-3 tocopheryl], 1.96 [s, 6H, CH_3-5 tocopheryl], 2.00 [s, 6H, CH_3-8 tocopheryl], 2.1 [s, 6H, CH_3-7 tocopheryl], 2.60 [t, 4H, CH_2-4 tocopheryl], 3.2−3.6 [m, 4H, CH_2-cystine], 4.1−4.4 [m, 2H, CH-cystine]. ESI-MS: *m/z*: 1066 [M^+ − 1] for $C_{64}H_{110}N_2O_6S_2^+$. Elemental analysis: Calculated: %N: 2.62, %C: 71.99, %H: 10.38, %S: 6.01. Observed: %N: 2.60, %C: 71.95, %H: 10.35, %S: 6.04.

Synthesis of the Monotocopherol Ester of Cystine, MTEC (Lipid 2, Scheme 1). *Step a: Synthesis of Intermediate N,N-Di-tert-butyloxycarbonyl-L-cystine-monotocopherol Ester, Intermediate V, Scheme 1.* Samples of 0.28 g (1.36 mmol) of DCC, 0.03 g of DMAP, anhydrous DCM, and 0.5 g (1.13 mmol) of *N,N*-di-BOC-L-cystine were sequentially added to a solution of 0.58 g (1.34 mmol) of α-tocopherol in 15 mL of anhydrous dichloromethane (DCM). The reaction and workup were carried out following the same procedure as given in step a of the synthesis of lipid **1**. Silica gel column chromatographic purification of the residue using 60−120 mesh size silica and 8−10% (v/v) ethylacetate in hexane as eluent afforded 0.46 g (yield = 47.91%, R_f = 0.4, 20% ethylacetate in hexane) of intermediate V as yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ/ppm 0.8−0.9 [m, 12H, CH−CH_3 tocopheryl], 1.1−1.4 [m, 18H, (CH_2)$_9$ tocopheryl], 1.45 [s, 3H, CH_3-2 tocopheryl], 1.45 [s, 18*H*-BOC], 1.7−1.9 [m, 3H CH_2-3 tocopheryl], 1.96 [s, 3H, CH_3-5 tocopheryl], 2.00 [s, 3H, CH_3-8 tocopheryl], 2.12 [s, 3H, CH_3-7 tocopheryl], 2.58−2.62 [t, 4H, CH_2-4 tocopheryl], 3.2−3.55 [dd, 4H, CH_2-cystine], 4.9−5.0 [t, 2H, CH-cystine]. ESI-MS: *m/z*: 876 [M^+ + Na] for $C_{45}H_{76}N_2O_9S_2^+$.

*Step b: Synthesis of MTEC (Lipid **2**, Scheme 1).* A portion of 0.3 g (0.35 mmol) of ester obtained in step a of the synthesis of lipid **2** was taken and followed the procedure as given in step b of the synthesis of lipid **1** to yield lipid **2** as yellow oil. Column chromatographic purification of the residue (using 60−120 mesh size silica and 3−4% (v/v) methanol in chloroform as the eluent) followed by chloride ion exchange in Amberlyst A-26 anion exchange resin using methanol as the eluent afforded lipid **2** as a yellow oil (0.2 g, 86.95% yield, R_f = 0.6, 10% methanol in chloroform). ^{1}H NMR (300 MHz, DMSO): δ/ppm 0.8−0.9 [m, 12H, CH−CH_3 tocopheryl], 1.0−1.4 [m, 18H, (CH_2)$_9$ tocopheryl], 1.45−1.55 [m, 3H, CH−CH_3] 1.7−1.9 [m, 2H CH_2-3 tocopheryl], 1.96 [s, 3H, CH_3-5 tocopheryl], 1.98 [s, 3H, CH_3-8 tocopheryl], 2.1 [s, 3H, CH_3-7 tocopheryl], 2.65 [t, 2H, CH_2-4 tocopheryl], 3.2−3.55 [dd, 4H, CH_2-cystine], 4.8−5.0 [t, 2H, CH-cystine]. ESI-MS: *m/z*: 653 [M^+-1] for $C_{35}H_{62}N_2O_5S_2^+$. Elemental analysis: Calculated: %N: 4.28, %C: 64.18, %H: 9.54, %S: 9.79. Observed: %N: 4.22, %C: 64.12, %H: 9.50, %S: 9.71.

Synthesis of Dicholesterol Ester of Cystine, DCEC (Lipid 3, Scheme 1). *Step a: Synthesis of Intermediate N,N-Di-tert-butyloxycarbonyl-L-cystine−Dicholesterol Ester III, Scheme 1.* Samples of 15 mL of anhydrous DCM, 0.515 g (2.5 mmol) of DCC, 0.06 g of DMAP, and 0.5 g (1.13 mmol) of *N,N*-di-BOC-L-cystine were sequentially added to a solution of 0.965 g (2.5 mmol) of cholesterol in DCM. The reaction and workup was carried out following the same procedure as given

in step a of the synthesis of lipid **1**. Silica gel column chromatographic purification of the residue using 60−120 mesh size silica and 2−3% (v/v) ethylacetate in hexane as eluent afforded 0.98 g (yield = 73.68%, R_f = 0.8, 10% ethylacetate in hexane) of intermediate (III) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ/ppm 0.55−2.40 [m, 86H, cholesterol skeleton], 1.46 [s, 18*H*-BOC], 3.1−3.2 [m, 2H, $H_3\alpha$ (chol)], 3.2−3.4 [m, 4H, CH_2-cystine], 4.6−4.8 [bm, 2H, C*H*-cystine], 5.3−5.4 [2H, H_6 (chol)]. ESI-MS: *m*/*z*: 1200 [M^+ + Na] for $C_{70}H_{116}N_2O_8S_2^+$.

*Step b: Synthesis of DCEC (Lipid **3**, Scheme 1).* A portion of 0.500 g (0.42 mmol) of ester obtained in step a of synthesis of lipid **3** was taken and followed the procedure as given in step b of the synthesis of lipid **1** to yield lipid **3** as a white solid. Column chromatographic purification of the residue (using 60−120 mesh size silica and 1−2% (v/v) methanol in chloroform as the eluent) followed by chloride ion exchange in Amberlyst A-26 anion exchange resin using methanol as the eluent afforded lipid **3** as a white solid (0.38 g, 92.68% yield, R_f = 0.7, 10% methanol in chloroform). ^{1}H NMR (300 MHz, $CDCl_3$): δ/ppm 0.55−2.40 [m, 86H, cholesterol skeleton], 3.1−3.2 [m, 2H, $H_3\alpha$ (chol)], 3.3−3.5 [m, 4H, CH_2-cystine], 4.7−4.9 [m, 2H, C*H*-cystine], 5.3−5.4 [2H, H_6 (chol)]. ESI-MS: *m*/*z*: 979 [M^+ + 1] for $C_{70}H_{116}N_2O_4S_2^+$. Elemental analysis: Calculated: %N: 2.86, %C: 73.57, %H: 10.50, %S: 6.55. Observed: %N: 2.84, %C: 73.59, %H: 10.45, %S: 6.58.

Synthesis of Dihexadecylamide of Cystine, DHDAC (Lipid 4, Scheme 1). *Step a: Synthesis of Intermediate N,N-Di-tert-butyloxycarbonyl-L-cystine−Dihexadecylamide IV, Scheme 1.* To a solution of 0.60 g (2.5 mmol) of hexadecylamine in 20 mL of anhydrous DCM 0.484 g (2.52 mmol) of (1-ethyl-3-1,3-dimethylaminopropyl) carbodiimide hydrochloride (EDCi) and 0.0.6 g of DMAP are added followed by the addition of 0.5 g (1.13 mmol) of *N*,*N*-di-BOC-L-cystine. The resulting solution was left stirring at room temperature for overnight. The solution was evaporated in vacuum to dryness. The residue was extracted with ethyl acetate (3 × 20 mL) and washed with water (2 × 20 mL). The organic layer was dried over anhydrous sodium sulfate and evaporated the solvent. Silica gel column chromatographic purification of the residue using 60−120 mesh size silica and 4−6% (v/v) ethylacetate in hexane as eluent afforded 0.410 g of (yield = 40.63%, R_f = 0.4, 10% ethylacetate in hexane) intermediate IV as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ/ppm 0.9 [t, 6H, CH_2-CH_3],1.29 [m, 52H, $(CH_2)_{13}-CH_3$], 1.45 [s, 18*H*, boc], 1.55 [t, 4H, $NH-CH_2-CH_2$], 2.8−3.0 [dd, 4H, $NH-CH_2$], 3.2−3.4 [dd, 4H, CH_2-cystine], 4.7 [m, 2H,C*H*-cystine. ESI-MS: *m*/*z*: 910 [M^+ + Na] for $C_{48}H_{94}N_4O_6S_2^+$.

*Step b: Synthesis of DHDAC (Lipid **4**, Scheme 1).* A portion of 0.300 g (0.33 mmol) of ester obtained in step a of the synthesis of lipid **4** was taken and followed the procedure as given in step b of the synthesis of lipid **1** to yield lipid **4** as a white solid. Column chromatographic purification of the residue (using 60−120 mesh size silica and 2−3% (v/v) methanol in chloroform as the eluent) followed by chloride ion exchange in Amberlyst A-26 anion exchange resin using methanol as the eluent afforded lipid **4** as a white solid (0.200 g, 86.95% yield, R_f = 0.6, 10% methanol in chloroform). ^{1}H NMR (300 MHz, $CDCl_3$): δ/ppm 0.8−0.9 [t, 6H, CH_2-CH_3], 1.2−1.4 [s, 52H, $(CH_2)_{13}-CH_3$], 1.55 [t, 4H, $NH-CH_2-CH_2$], 2.7−2.85 [dd, 4H, $NH-CH_2$], 3.1−3.4 [dd, 4H, CH_2-cystine], 3.7 [m, 2H, C*H*-cystine]. ESI-MS: *m*/*z*: 710 [M^+ + Na] for $C_{38}H_{78}N_4O_2S_2^+$. Elemental analysis: Calculated: % N: 8.15, %C: 66.42, %H: 11.44, %S: 9.33. Observed: %N: 8.14, % C: 66.39, %H: 11.48, %S: 9.29.

Cells and Cell Culture. B16F10 (human melanoma cancer cells), CHO (Chinese hamster ovary), A-549 (human lung carcinoma cells), and HepG2 (human hepatocarcinoma) 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% FBS, 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 or DOPE) in a 1:2 mole 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. A portion of 1 mL 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 to 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.

Plasmid DNA. pCMV-SPORT-β-gal plasmid was amplified in a DH5α strain of *Escherichia coli*, isolated by the alkaline lysis procedure, and finally purified by PEG-8000 precipitation as described previously.[32] The purity of plasmid was checked by A_{260}/A_{280} ratio (around 1.9) and 1% agarose gel electrophoresis.

Zetapotential (ξ) 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, UK). The sizes were measured in DMEM with a sample refractive index of 1.59 and a viscosity of 0.89. The system was calibrated by using the 200 ± 5 nm polystyrene polymer (Duke Scientific Corps., 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.

Gel Retardation Assay. The DNA binding ability of the cationic lipids **1**−**4** was assessed by a conventional gel retardation assay on a 1% agarose gel (prestained with ethidium bromide) across the varying lipid/DNA charger ratios of 0.3:1 to 9:1 pCMV-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. A total of 4 μL of 6× loading buffer (0.25% bromophenol blue in 40% (w/v) sucrose in H_2 O) 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 ethylenediaminetetraacetic acid (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 a 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.

Monitoring Glutathione-Induced DNA Release from DNA−Liposome Complexes. One microgram of plasmid DNA was dissolved in 10 μL of 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.4). Cationic liposomes with lipids **1** and **3** which showed the highest transfection efficacy were added to the pDNA solution to obtain final lipid/DNA (±) charge ratios of 3:1 and 1:1 at which they showed their higher levels of transfection. After the complexes were incubated for 30 min at room temperature, 5 μL of 50 mM of glutathione in 10 mM HBS (pH 7.4) was added to the mixture to reach a final 10 mM concentration of glutathione. The mixtures were incubated at 37 °C for 2 h. Released DNA was visualized by 1% agarose gel electrophoresis.

DNA Release. The DNA release experiment is carried out by monitoring ethidium bromide exclusion from the lipoplexes of lipids **1**−**4**. DNA−lipid complexes of lipids **1**−**4** with different charge ratios, that is, 9:1 to 0.3:1, using 10 mM HEPES buffer were prepared the same as described in the transfection experiment. These complexes were incubated for 30 min at room temperature with ethidium bromide (4 μg). To this intercalated ethidium bromide complex mixture 10 μL of 50 mM glutathione in 10 mM HEPES buffer (pH 7.4) is added to reach a final concentration of 10 mM. The mixtures were incubated at 37 °C for up to 2 h, and the DNA release was monitored at specified time intervals as shown in Figure 4 by an increase in EtBr fluorescence. The percent fluorescence was calculated considering the fluorescence value in the absence of lipid as 100.

Transfection Biology. Cells were seeded at a density of 10 000 (for B16F10) and 15 000 cells (for CHO, A-549, and HepG2) per well in a 96-well plate 18−24 h before the transfection. Plasmid DNA, 0.3 μg (0.91 nmol), was complexed with varying amounts of lipids (to give ± charge ratios of 0.3:1, 1:1, 3:1, and 9:1) in plain DMEM medium (total volume made up to 100 μL), for 30 min. Immediately prior to transfection, cells plated in the 96-well plate were washed with phosphate-buffered saline (PBS, 2 × 100 μL), followed by the addition of lipoplexes. After 4 h of incubation, the medium was changed to 10% complete medium. The medium was again replaced with 10% complete medium after 24 h, and the reporter gene activity was estimated after 48 h. The cells were washed twice with PBS (2 × 100 μL each) and lysed in 50 μL of lysis buffer [0.25 M Tris-HCl (pH 8.0) and 0.5% NP-40]. 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 *o*-nitrophenyl-β-D-galactopyranoside (ONPG), 0.2 M sodium phosphate (pH 7.3), and 2 mM magnesium chloride] to the lysate in a 96-well plate. The 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 three times on three different days. The transfection values reported here are average values from three replicate transfection plates assayed on three different days. The error bars in the figures represent the standard error of these three replicate experiments. 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 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**−**4**) in DMEM medium in the presence of increasing concentrations of added serum (10−50% v/v and total volume made up to 100 μL) for 30 min. The lipid/DNA charge ratio of these lipoplexes was maintained as 3:1 and 1:1 (Figures 9 and 10), at which all of the lipids showed their highest transfection ability in all four types of cells used for transfection (CHO, A-549, B16F10, and HepG2). 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 the α5GFP *p*DNA expression experiment, 25 000−30 000 per well cells 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**−**4** were complexed with α5GFP expressing *p*DNA (1 μg/well) at 3:1 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 allowed for 24 h incubation. 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. pCMV-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**−**4** using a 3:1 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).

Cellular Uptake Assay. 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. pCMV-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**−**4** using a 3:1 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). The fluorescence was measured by a microplate fluorescent reader (FLX 800, Bio-Tek Instruments Inc., USA) using filter sets for red channels. The fluorescence of lipoplexes with the same amount of cell lysates (in a total volume of 100 μL) were also measured and considered as total or 100% fluorescence. The percentage uptake was calculated using the formula:

% uptake = 100 × (fluorescence intensity of the fluorescence lipoplex treated cell lysate − background)/(fluorescence intensity of lipoplex added to the cells − background).

Toxicity Assay. Cytotoxicities of the lipids **1**−**4** were assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay as described earlier.[33] The cytotoxicity assay was performed in 96-well plates by maintaining the ratio of number of cells to the amount of cationic lipid, the same as that used in the transfection experiments. Briefly, the cells were incubated with lipoplexes for 3 h followed by the addition of 100 μL of DMEM containing 20% FBS, and 10 μL MTT (5 mg/mL in PBS) was added after 24 h of transfection. After 3−4 h of incubation at 37 °C, the medium was removed, and 100 μL of DMSO:methanol (50:50, v/v) was added to the cells. The absorbance was measured at 550 nm, and results were expressed as percent viability = [A_{540} (treated cells) − background/A_{540} (untreated cells) − background] × 100.

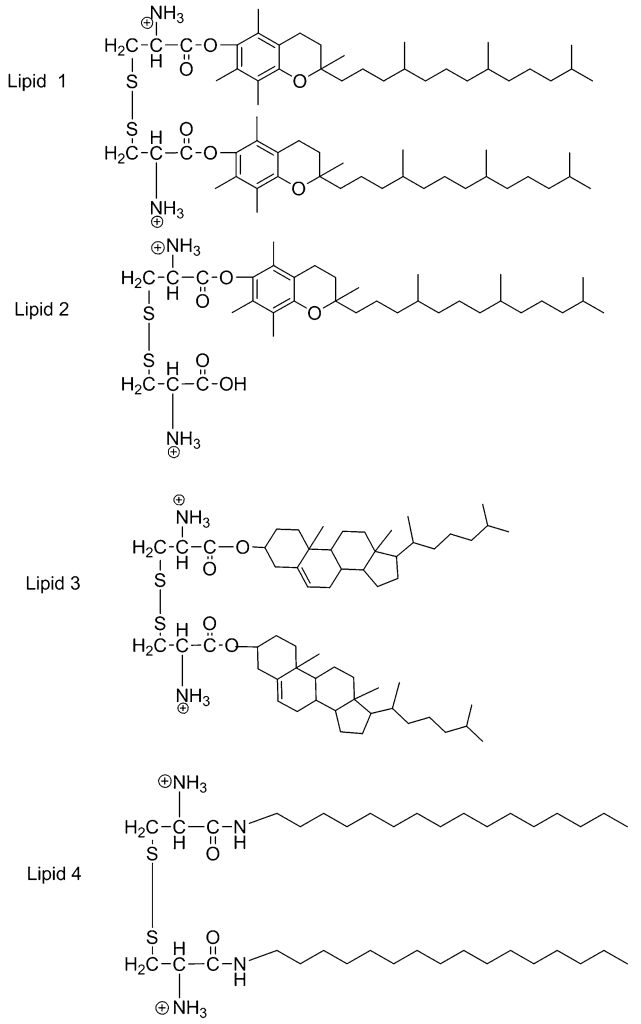
Statistical Analysis. Data were expressed as mean ± standard error. Statistical analyses were performed using Student's *t* test for comparison of means. A probability of less than 0.005 was considered to be statistically significant.

RESULTS

Chemistry. The key structural elements common to all of the transfection lipids (**1**−**4**) described herein include (a) the presence of cystine as the hydrophilic headgroup, (b) the presence of ester or amide group as the linker group, and (c) the presence of one or two tocopherols (lipids **2** and **1**) or cholesterols (lipid **3**) or aliphatic long chains (lipid **4**) as the hydrophobic tail region. Structures of all four lipids are reported in Chart 1. The details of the synthetic procedures for all of the novel transfection lipids shown in Scheme 1 are described in the Experimental Section. As outlined in Scheme 1 the chemistry involved in preparing these new lipids is straightforward. The lipids **1**−**4** were synthesized by esterification or amidification of the common starting material, *N*,*N*-Di-BOC-L-cystine (prepared from L-cystine in a single step as outlined in Scheme 1) with tocopherol or cholesterol or aliphatic amines. The resulting ester or amide intermediates upon deprotection of a BOC group using TFA in DCM followed by chloride ion exchange over Amberlyst-26 anion exchange resin afforded lipids **1**−**4** (Scheme 1). Structures of all synthetic intermediates and final lipids shown in Scheme 1 were confirmed by ^{1}H NMR and by the molecular ion peaks in their ESI mass. The final lipids were also characterized by elemental analysis data as described in the Experimental Section.

Liposomal Formation. Liposomes could be conveniently prepared from a mixture of lipid with varying amounts of DOPE and DOPC as colipids. Out of various combinations of mole ratios of lipid to colipid (0.5:0.5, 1:1, 1:2, 1:3, and 1:4) tried, it is found that a 1:2 molar ratio of lipid and colipid (DOPE or DOPC) is found to form optically transparent suspensions. Liposomes were prepared under sterile conditions and were sonicated for 5 min at room temperature before transfection experiments. The vesicular suspensions were sufficiently stable, and no precipitation was observed within 3 months if stored at 4 °C.

Chart 1. Structures of Lipids 1−4



Nanosizes and Global Surface Charges of the Lipoplexes. Toward physicochemical characterization of the lipids (**1**−**4**), the nanosizes and the global surface charges of the liposomes and lipoplexes of all four lipids (**1**−**4**) prepared were measured. The sizes and surface charges of these lipids (**1**−**4**) were measured using a dynamic laser light scattering instrument equipped with a ζ-sizing capacity across the lipid/DNA charge ratios of 0.3:1 to 9:1 in the presence of DMEM. The liposomes formed from all of the gemini lipids (**1**, **3**, and **4**) ranged from 100 to 150 nm in size. It is found that all of the gemini lipid-based liposomes of lipids (**1**, **3**, and **4**) were generally found to be smaller in size as compared to the liposomes of monomeric lipid **2**, which is found to 180 ± 30 nm in size (Table 1). This property of smaller sizes of gemini lipid-based liposomes and lipoplexes when compared to their monomeric counterparts is reported earlier.[29] The smaller sizes of the gemini lipid-based lipoplexes may be due to their highly ordered and compacted structures. The higher gene transfer efficacies observed for the

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

	lipid/DNA (molar ratio)				
lipid	1:0	0.3:1	1:1	3:1	9:1
			Size (nm)		
lipid 1	116.7 ± 1.6	270.3 ± 3.9	315.9 ± 24.2	407.8 ± 65.2	530.1 ± 119.2
	(160.2 ± 2.3)	(450.6 ± 24.9)	(506.7 ± 77.0)	(630.8 ± 65.3)	(729.1 ± 39.7)
lipid 2	210.6 ± 26	456.3 ± 15.8	752.9 ± 28.5	940.1 ± 12.7	1021.5 ± 45.9
	(265.9 ± 42.9)	(558.9 ± 10.2)	(712.5 ± 78.6)	(1248.9 ± 56.9)	(1638.5 ± 113.8)
lipid 3	123.5 ± 5.6	332.0 ± 11.3	338.5 ± 4.8	490.8 ± 8.4	658.8 ± 24.8
	(182.6 ± 21.8)	(518.7 ± 12.6)	(654.8 ± 24.8)	(705.2 ± 29.1)	(940.7 ± 10.7)
lipid 4	136.5 ± 2.8	373.7 ± 3.7	404.5 ± 9.8	632.8 ± 25.6	698.5 ± 56.8
	(223.5 ± 63.4)	(553.2 ± 12.9)	(692.4 ± 45.8)	(725.4 ± 56.4)	(1000.6 ± 21.8)
			Zeta Potential (mV)		
lipid 1	28.6 ± 6.2	−18.9 ± 0.3	−25.9 ± 4.5	−27.8 ± 2	−15.8 ± 1.1
lipid 2	22.8 ± 3.2	−32.5 ± 2.3	−35.8 ± 0.6	−35.6 ± 2.5	−29.3 ± 0.2
lipid 3	16.8 ± 5.5	−15.5 ± 5.9	−29.3 ± 1.8	−16.5 ± 0.2	−12.9 ± 0.5
lipid 4	19.3 ± 2.3	−28.5 ± 1	−45.8 ± 0.8	−23.5 ± 0.1	−21.8 ± 6.3

[a]Sizes of liposomes and lipoplexes using DOPC and DOPE (values within parentheses) as colipid and ξ potentials using DOPC as colipid were measured by a laser light scattering technique using a Zetasizer 3000A (Malvern Instruments, U.K). Values shown are the averages obtained from three (size) and ten (zeta potential) measurements.

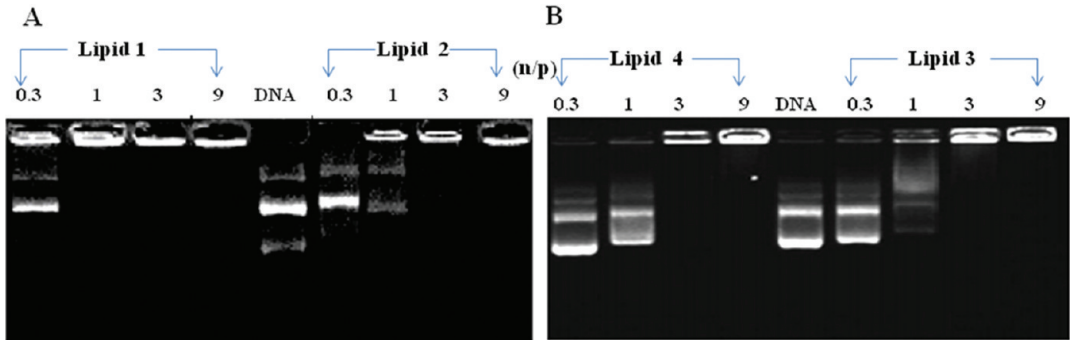

Figure 1. (A and B) Electrophoretic gel patterns for lipoplex of lipids **1**−**4** in gel retardation assay. The lipid/DNA charge ratios are indicated at the top of each line. The details of the treatment are as described in the text.

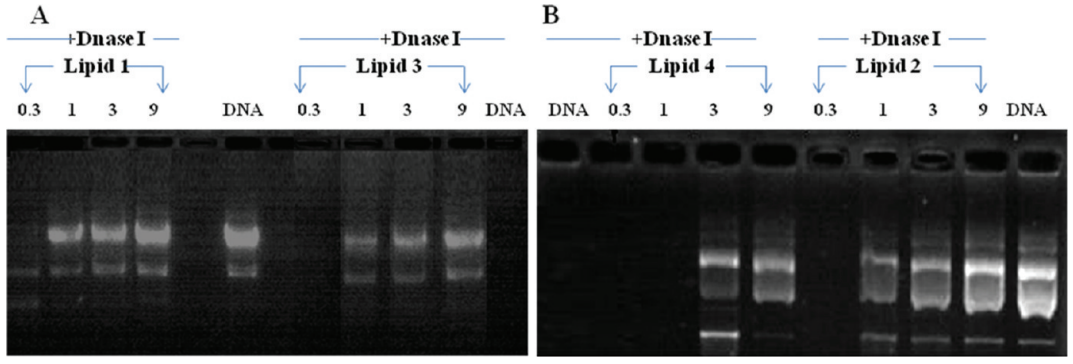

Figure 2. Electrophoretic gel patterns for lipoplex-associated DNA in DNase I sensitivity assay. A and B refer to lipid **1**−**4**. The lipid/DNA charge ratios are indicated at the top of each lane. The details of the treatment are as described in the text.

gemini lipids when compared to their monomeric lipids is also attributed to their smaller sizes which helps in the enhanced entry of the lipoplexes inside the cells through endocytotic pathway. The sizes of the lipoplexes prepared using colipids DOPE and DOPC (Table 1) clearly demonstrate that in general the lipoplexes prepared using DOPC as colipid are smaller in size compared to the lipoplexes prepared using DOPE as a colipid.

Lipid/DNA Binding Interactions and DNase I Sensitivities. The electrostatic binding interactions between the plasmid DNA and all of the lipids mentioned (**1**−**4**) were measured by both conventional electrophoretic gel retardation assays and DNase I sensitivity assays. The electrophoretic gel patterns in simple gel retardation assay (Figure 1) revealed some interesting features. All of the lipids in general were capable of completely inhibiting the electrophoretic mobility of plasmid DNA from lipoplexes prepared at high lipid/DNA charge ratios of 9:1 and 3:1 (Figure 1). At lipid/DNA charge ratios of 1:1, only lipid **1** exhibited optimal binding ability, and all of the other lipids exhibited poor DNA-binding properties.

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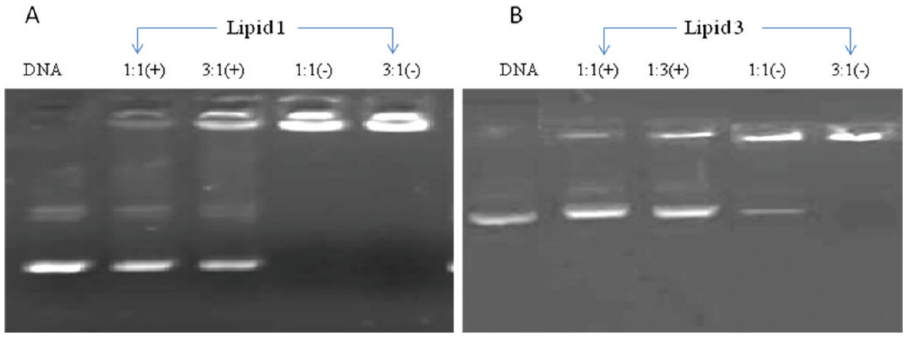

Figure 3. Electrophoretic gel retardation depict gel patterns observed with lipoplexes in the absence of glutathione, and lipoplexes in the presence of 10 mM glutathione of lipids **1** and **3**. The lipid/DNA charge ratios used in gel retardation assays are indicated at the top of each lane. The details of the treatment are as described in the text.

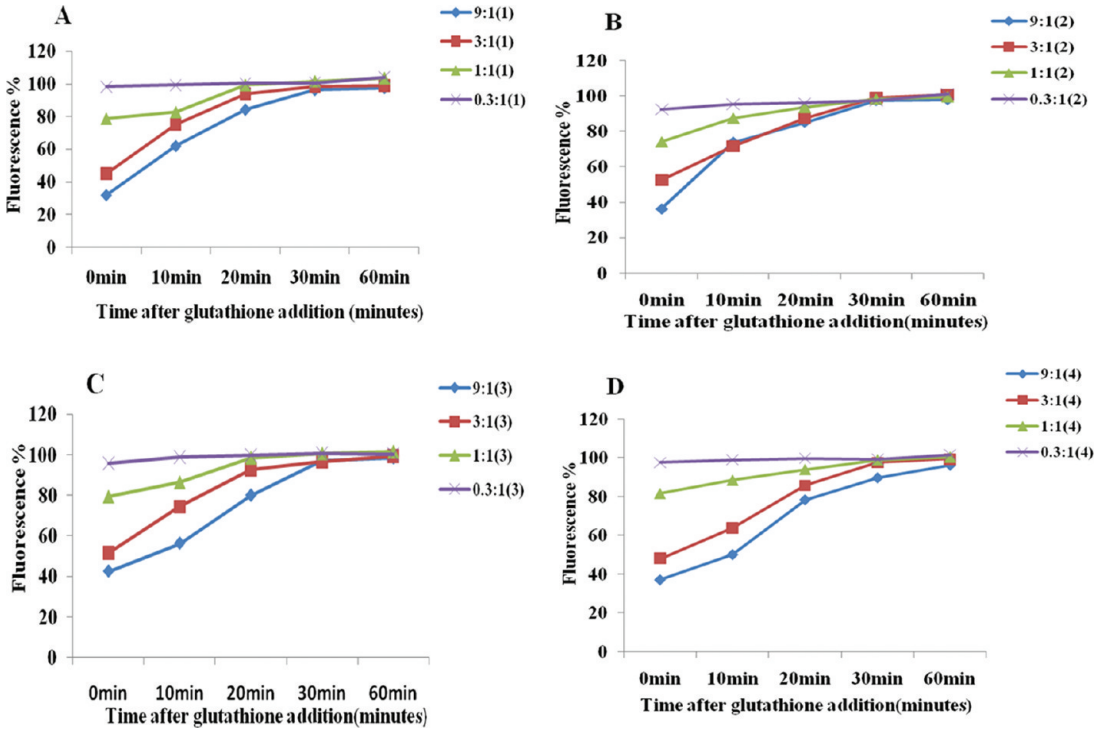

Figure 4. Kinetics of DNA release from complexes of DNA and lipids was assessed by residual EtBr fluorescence increase. A–D refer to lipids **1**–**4** with charge ratios from 0.3:1 to 9:1. Complexes were incubated with 10 mM of glutathione. An increase in fluorescence values was observed on increasing the incubation time from 0 to 60 min. The details of the experiment are as described in the text.

At lipid/DNA charge ratios lower than 1:1 all of the lipids showed poor DNA binding (Figure 1).

With a view to obtaining insights into the accessibilities of the lipoplex associated DNA to DNase I, DNase I sensitivity assays were carried out across the lipid/DNA charge ratios of 9:1 to 0.3:1. The electrophoretic gel patterns observed in the DNase I sensitivity assays are in agreement with the results obtained from gel retardation assay. After the free DNA digestion by DNase I, the total DNA (both digested and inaccessible DNA) was separated from the lipid and DNase I (by extracting with organic solvent) and loaded onto a 1% agarose gel. The band intensities of inaccessible and therefore undigested DNA associated with transfection incompetent lipids **2** and lipid **4** were found to be significantly less compared to those associated with transfection efficient lipids **1** and **3** across the lipid/DNA charge ratios of 3:1 to 0.3:1 (Figure 2).

Monitoring Glutathione-Induced DNA Release from DNA–Liposome Complexes. With a view to gain insight into whether the disulfide linker of the presently designed cystine headgroup-based lipids is reducible by glutathione, we also performed a representative gel retardation assay in presence of 10 mM glutathione using lipoplexes of lipids **1** and **3** (proved to be higher transfecting lipids among the present series) with charge ratios of 3:1 and 1:1 at which they reported their higher transfection results. Consistent with the rationale of the disulfide linker strategy, significant DNA was released from lipoplexes of both of the lipids when incubated for 2 h in the presence of 10 mM glutathione (Figure 3).

DNA Release under a Reducible Environment. Results from electrophoretic gel retardation assay experiments demonstrate that the presently studied disulfide lipids are able to release DNA in the cytoplasm by intracellular glutathione pool. To confirm the enhanced DNA release from reducible cationic lipids **1**–**4** upon reduction, further DNA-release experiments were performed. Release of DNA due to complex dissociation is measured by EtBr fluorescence.

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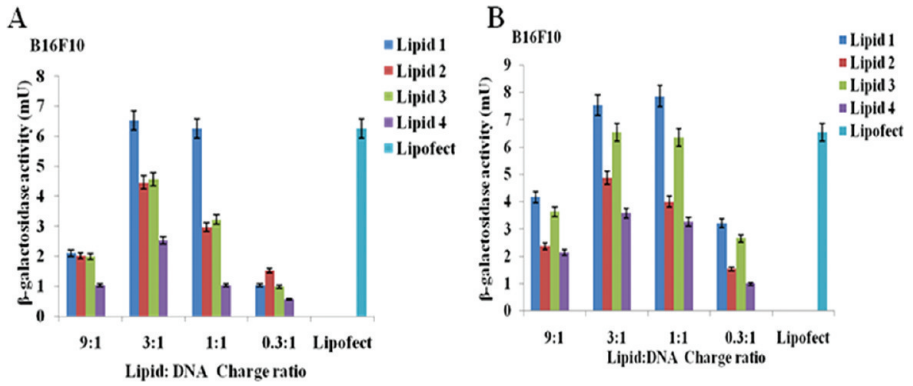

Figure 5. Transfection efficiencies of lipids **1**–**4** in B16F10 cells with DOPE (A) and DOPC (B) as colipids (at a 1:2 mole ratio of lipid to colipid). The transfection efficiencies of the lipids were compared to that of commercial formulation. Transfection experiments were performed as described in the text. All lipids were tested on the same day, and the data presented are the average of three experiments performed on three different days. The error bar represents the standard error. The difference in the data obtained is statistically significant in all charge ratios ($P < 0.005$).

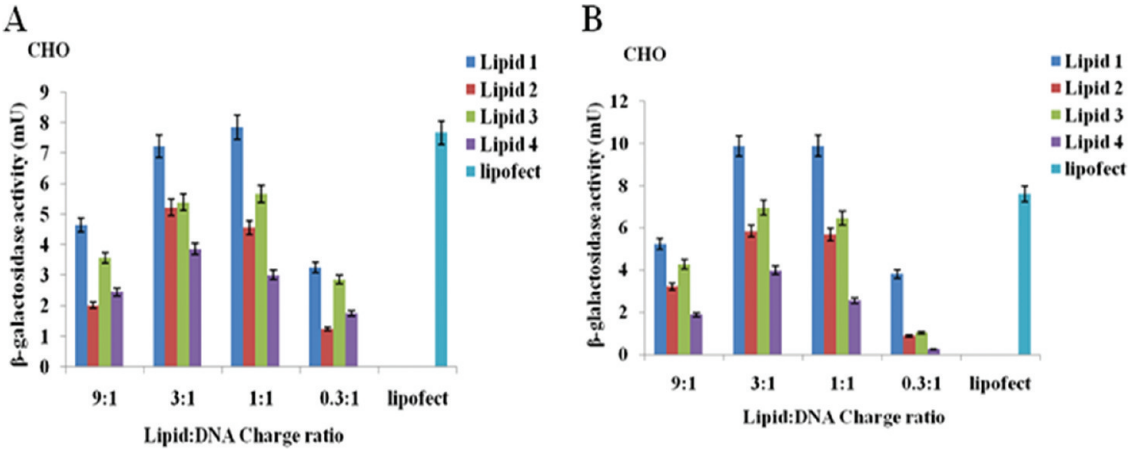

Figure 6. Transfection efficiencies of lipids **1**–**4** in CHO cells with DOPE (A) and DOPC (B) as colipids (at a 1:2 mole ratio of lipid to colipid). The transfection efficiencies of the lipids were compared to that of commercial formulation. Transfection experiments were performed as described in the text. All lipids were tested on the same day, and the data presented are the average of three experiments performed on three different days. The error bar represents the standard error. The difference in the data obtained is statistically significant in all charge ratios ($P < 0.005$).

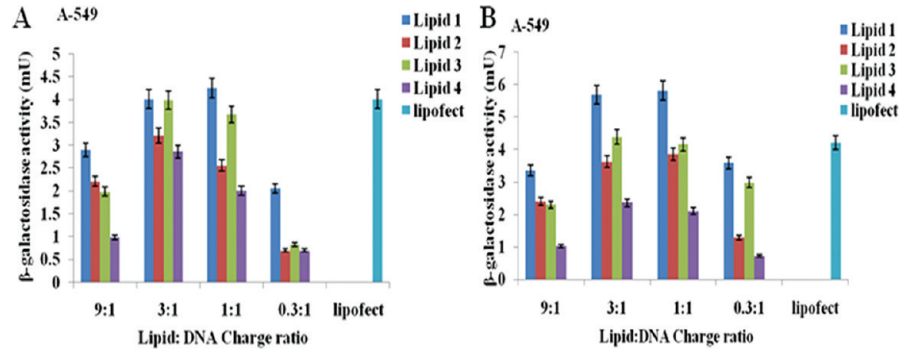

Figure 7. Transfection efficiencies of lipids **1**–**4** in A-549 cells with DOPE (A) and DOPC (B) as colipids (at a 1:2 mole ratio of lipid to colipid). The transfection efficiencies of the lipids were compared to that of commercial formulation. Transfection experiments were performed as described in the text. All lipids were tested on the same day, and the data presented are the average of three experiments performed on three different days. The error bar represents the standard error. The difference in the data obtained is statistically significant in all charge ratios ($P < 0.005$).

Glutathione was added to the mixture of EtBr and lipid/DNA complexes of different charge ratios so that the final concentration of glutathione was 10 mM. The complexes were then incubated at 37 °C for 2 h, and the extent of dissociation of lipid/DNA complex was monitored with the increase of fluorescence because of exclusion of EtBr from the complex at various time intervals as shown in Figure 4. The results demonstrate that the lipid/DNA complexes of all of the lipids studied showing a similar trend of enhanced release of DNA from their lipoplexes in the presence of glutathione with the time of incubation. Nearly 90% of the DNA is released from the lipoplexes of lipids **1**–**4** in just 20 min when incubated with 10 mM glutathione (Figure 4). It is observed that after

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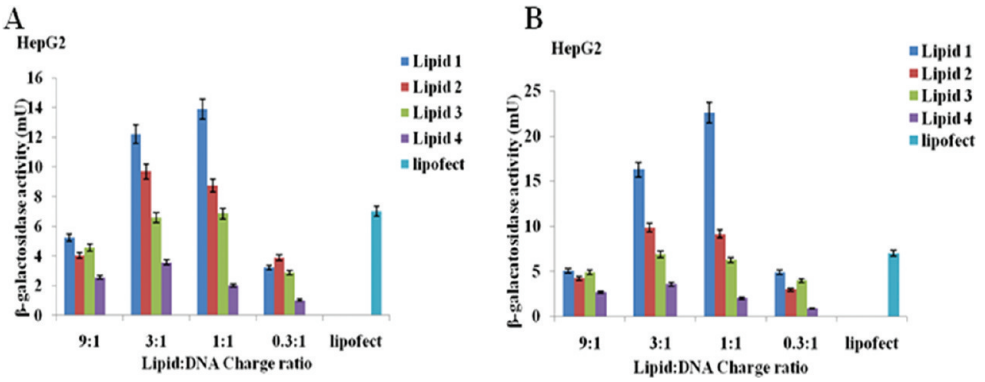

Figure 8. Transfection efficiencies of lipids **1**–**4** in HepG2 cells with DOPE (A) and DOPC (B) as colipids (at a 1:2 mole ratio of lipid to colipid). The transfection efficiencies of the lipids were compared to that of commercial formulation. Transfection experiments were performed as described in the text. All lipids were tested on the same day, and the data presented are the average of three experiments performed on three different days. The error bar represents the standard error. The difference in the data obtained is statistically significant in all charge ratios ($P < 0.005$).

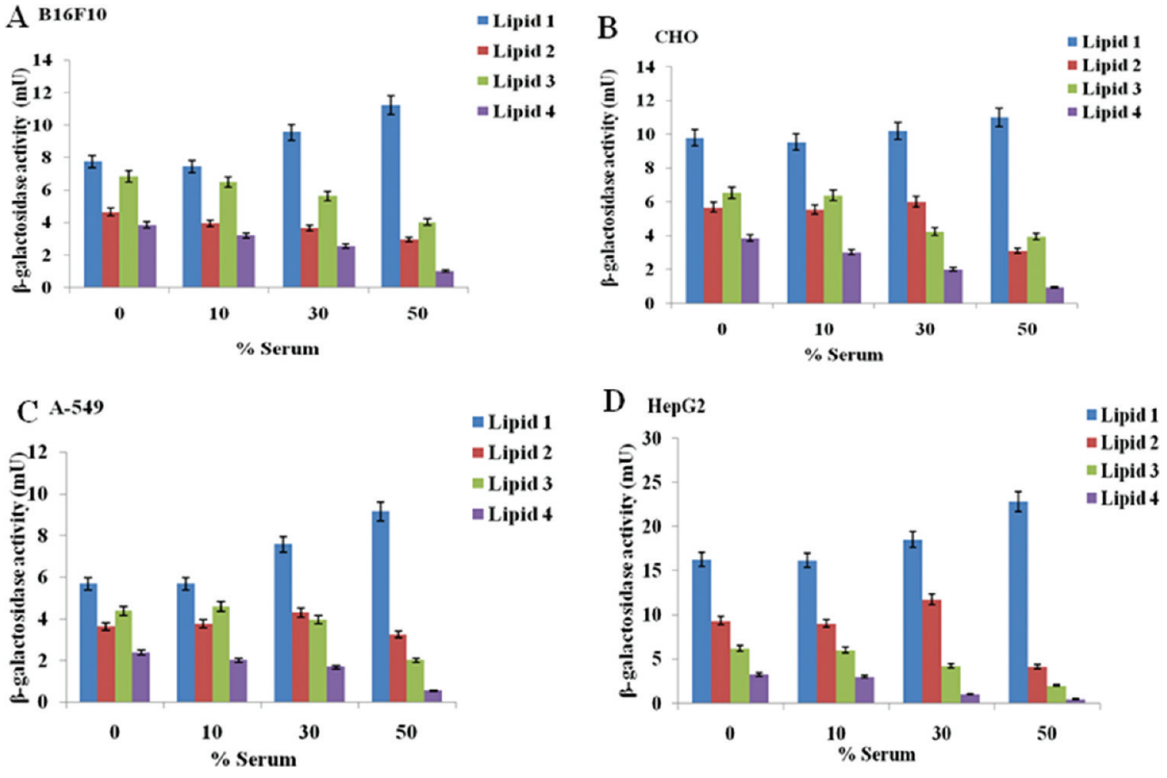

Figure 9. Transfection efficacies of the cationic lipids **1**–**4** in the presence of increasing concentrations of added serum. In vitro transfection efficiencies of lipid/DNA complexes prepared using the pCMV-*β*-gal-SPORT reporter gene at a lipid/DNA charge ratio of 3:1 were evaluated in the presence of increasing concentrations of added serum in B16F10 (A), CHO (B), A-549 (C), and HepG2 (D) types of cells. The error bar represents the standard error. The difference in the data obtained is statistically significant in all charge ratios ($P < 0.005$).

incubation for 1 h the release of DNA reached to a saturation point of almost 100%.

Transfection Biology. *In Vitro Transfection Studies.* The relative in vitro gene delivery efficacies of tocopherol-based lipids (**1** and **2**) and control lipids (**3** and **4**) in CHO, B16F10, A-549, and HepG2 cells across the lipid/DNA charge ratios of 9:1 to 0.3:1 using both DOPE and DOPC as colipids are summarized in Figures 5–8. pCMV-SPORT-*β*-gal plasmid DNA was used as the reporter gene across the lipid/DNA charge ratios of 9:1 to 0.3:1. The transfection efficiencies of the lipids (**1**–**4**) were compared with that of the commercial formulation. The results of Figures 5–8 summarize several interesting transfection profiles of synthesized lipids **1**–**4**. For the present lipids **1**–**4** the in vitro transfection profiles were found to be remarkably influenced by the colipid used in liposomal preparation. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was found to be a more efficacious colipid than 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) for all of the lipids in all four types of cell lines studied. In general, the highest transfection efficiencies of all of the lipids **1**–**4** were found to be across the lipid/DNA charge ratios of 3:1 and 1:1. The cationic lipid **1** with two tocopherol units and cystine headgroup having gemini surfactant characters is found to be a better transfection efficient when compared to lipid **2** with one tocopherol unit and cystine headgroup in all four types of cells studied. This property of gemini lipids to be more efficient than

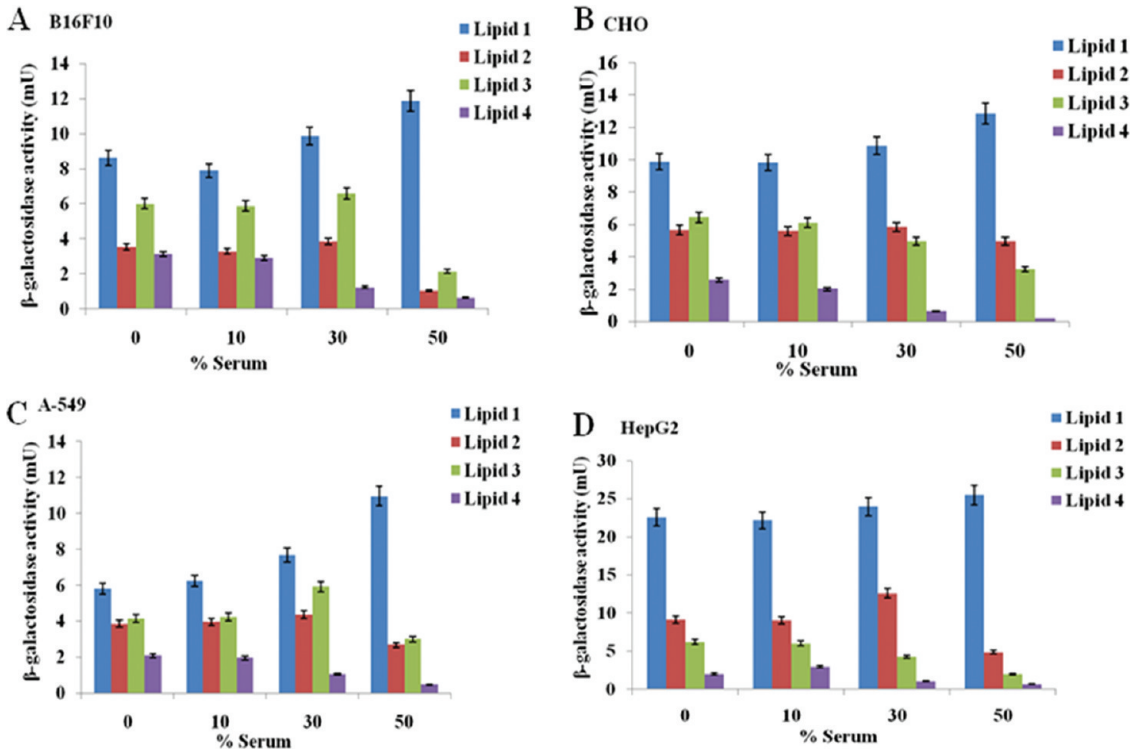

Figure 10. Transfection efficacies of the cationic lipids **1**−**4** in the presence of increasing concentrations of added serum. In vitro transfection efficiencies of lipid/DNA complexes prepared using the pCMV-*β*-gal-SPORT reporter gene at a lipid/DNA charge ratio of 1:1 were evaluated in the presence of increasing concentrations of added serum in B16F10 (A), CHO (B), A-549 (C), and HepG2 (D) types of cells. The error bar represents standard error. The difference in the data obtained is statistically significant in all charge ratios ($P < 0.005$).

their monomeric counterpart has been reported earlier.[34,35] The results also demonstrate that the gemini cationic lipid **1** is a better transfection efficient than the gemini cationic lipid **3** with two cholesterol units and cystine headgroup in all four types of cells studied. The lipid **1** is found to be the highest gene transfection efficient lipid among the present series of the lipids at 3:1 and 1:1 lipid/DNA charge ratios in all four types of cell lines and a better transfection efficient than the commercial formulation in HepG2 cell lines. It is also observed that transfection efficiency of lipid **1** with DOPC as colipid is slightly better than the commercial formulation in all other three type of cell lines studied (B16F10, CHO, and A-549). In the case of DOPE as colipid, the transfection efficiency of lipid **1** is comparable to that of commercial formulation. The lipid **4** was found to be the least efficient transfection in all four types of cell lines at all charge ratios studied.

In the reporter gene assay, the in vitro gene transfer efficiencies of lipids **1** and **2** with two and one tocopherol units, respectively, in general are found to be higher in HepG2 cells when compared to other three types of cancer cells, that is, CHO, B16F10, and A-549.

Transfection Biology in the Presence of Serum. One of the serious limitations of the cationic lipid-mediated gene delivery, especially involving *in vivo* trials, is that the transfection is inefficient particularly in the presence of serum proteins. Transfections in the absence of serum even for *in vitro* experiments are also cumbersome due to the increased toxicity of cationic lipid formulations. To have better potential especially in gene therapy, it is important to develop lipids that are capable of delivering and expressing an external gene inside a cell in the presence of serum.[36,37] To have a clear understanding of the effect of the serum in the present set of cationic lipid-mediated gene delivery, we performed transfections with our optimized lipid formulations in the presence of serum, in all four types of cells used for in vitro transfection experiments using 3:1 and 1:1 lipid/DNA charge ratios at which all of the lipids reported their higher transfection results. As DOPC proved to be a better colipid when compared to DOPE for the present series of cationic lipids as evidenced by their in vitro transfection experiments, all of the formulations used in serum compatibility studies are with DOPC as a colipid.

In all four types of cells studied at lower percentages of serum, that is, up to 10% of serum added the lipids **1**−**4** in general showed slight differences in their transfection efficiencies at 3:1 as well as at 1:1 charge ratios (Figures 9 and 10). It is observed that lipids **1** and **3** showed a slightly better transfection efficiency when compared to lipids **2** and **4** up to 10% of serum added.

Many of the lipids which proved to be serum compatible at lower concentrations are reported to be adversely affected at higher percentages of serum concentrations.[38−45] Any given cationic lipid transfection efficiency should be unaffected even at the higher percentages of serum concentration if it is intended for in vivo transfection purpose. To understand whether the presently designed series of cationic lipids will be capable of in vivo applications we designed to study their transfection profiles in presence of higher concentrations of serum, that is, 30% and 50% of serum in all four types of cells used for in vitro transfection experiments. It is observed that the lipids **3** and **4** showed a decrease in transfection efficiency on moving from lower to higher percentages of serum at both 3:1 and 1:1 charge ratios (Figures 9 and 10). The monomeric lipid **2** showed an increase in transfection up to 30% of serum added and from 30 to 50% of serum concentrations the

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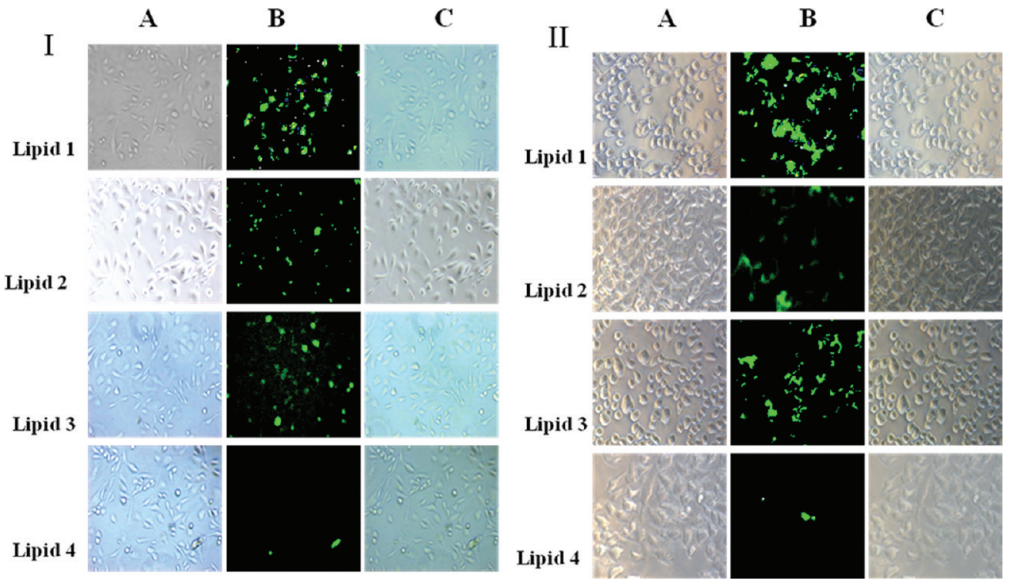

Figure 11. Inverted microscopic images of representative CHO (I) and A549 (II) cell lines transfected with lipoplexes of lipids (**1**−**4**) and α5GFP expressing plasmid DNA. Lipid/DNA charge ratios in all of the lipoplexes were maintained at 3:1: (A) bright field images, (B) fluorescent images, and (C) overlay images. The details of inverted fluorescence microscopic experiments are described in the text.

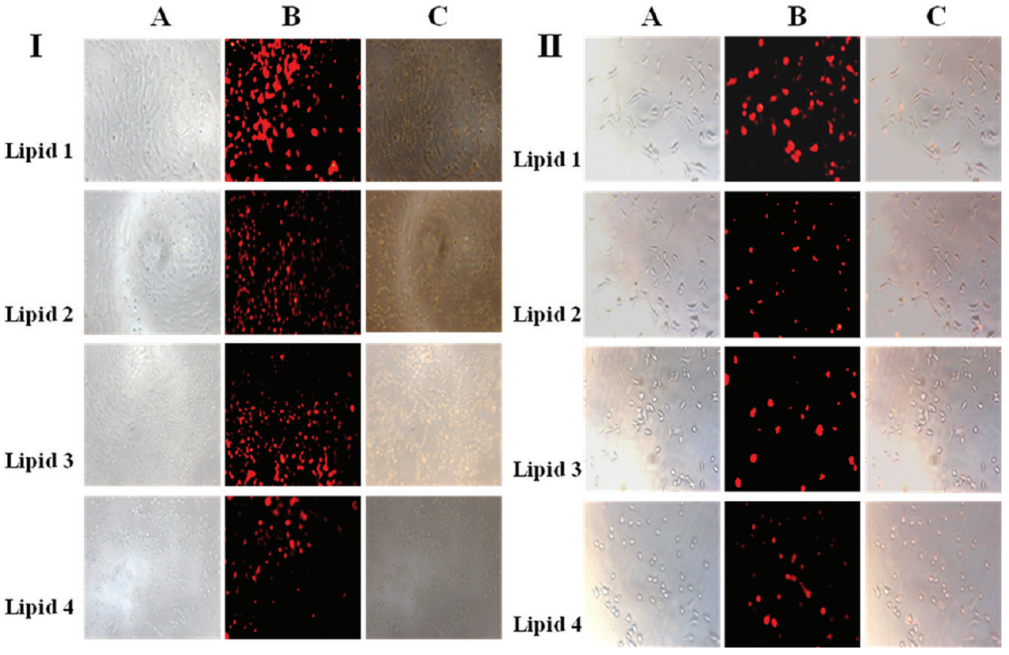

Figure 12. Cellular uptake of rhodamine-labeled lipoplexes. Inverted microscopic images of CHO (I) and A549 (II) cell lines transfected with rhodamine-labeled lipoplexes of lipids **1**−**4** prepared at higher in vitro transfection lipid/DNA charge ratios of 3:1: (A) bright field images, (B) fluorescent images, and (C) overlay images. The details of the experiments are described in the text.

transfection value decreased greatly in both ratios of lipids tested and in all types of cells studied (Figures 9 and 10). The gemini cationic lipid **1** which proved to be the highest transaction efficient of the present series of lipids in *in vitro* transfection experiments is unaffected across all of the concentrations of serum tested in both 3:1 and 1:1 ratios of lipid/DNA in all four types of cells studied (Figures 9 and 10). The transfection efficiency of lipid **1** observed to be increasing with increasing the serum concentration.

Cellular Expression of GFP. The transfection results revealed that lipids **1**−**4** in all four types of cell lines (B16F10, CHO, A-549, and HepG2) showed their maximum transfection efficiencies at a lipid/DNA charge ratio of 3:1. At this juncture, it is necessary to investigate whether the same transfection profile for lipids **1**−**4** will also be followed for DNA expression at this given charge ratio. Toward this end, the representative CHO and A549 cells were treated with lipoplexes of lipids **1**−**4** containing a α5GFP plasmid DNA encoding green fluorescent protein at a lipid/DNA charge ratio of 3:1. The corresponding cellular expression of GFP was monitored subsequently by inverted fluorescent microscope. It is observed from the results (Figure 11) that lipoplexes of lipids **1** and **3** showed better expression of GFP than lipoplexes of lipids **2** and **4** in both of the cell lines studied. Lipoplexes of lipid **1** were found to exhibit

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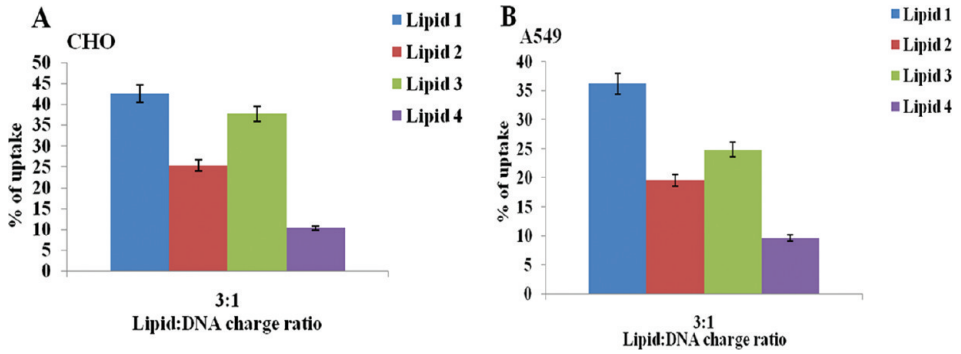

Figure 13. Cellular uptake of rhodamine-labeled lipoplexes. The fluorescence of lysates of CHO and A549 cells transfected with rhodamine-labeled lipoplexes of lipids **1**−**4** was measured by a microplate fluorescent reader (FLX 800, Bio-Tek Instruments Inc., USA) using filter sets for red channels. The percentage uptake was calculated using the formula % uptake = 100× (fluorescence intensity of the fluorescence lipoplex treated cell lysate − background)/(fluorescence intensity of lipoplex added to the cells − background). The details of the experiments are as described in the text.

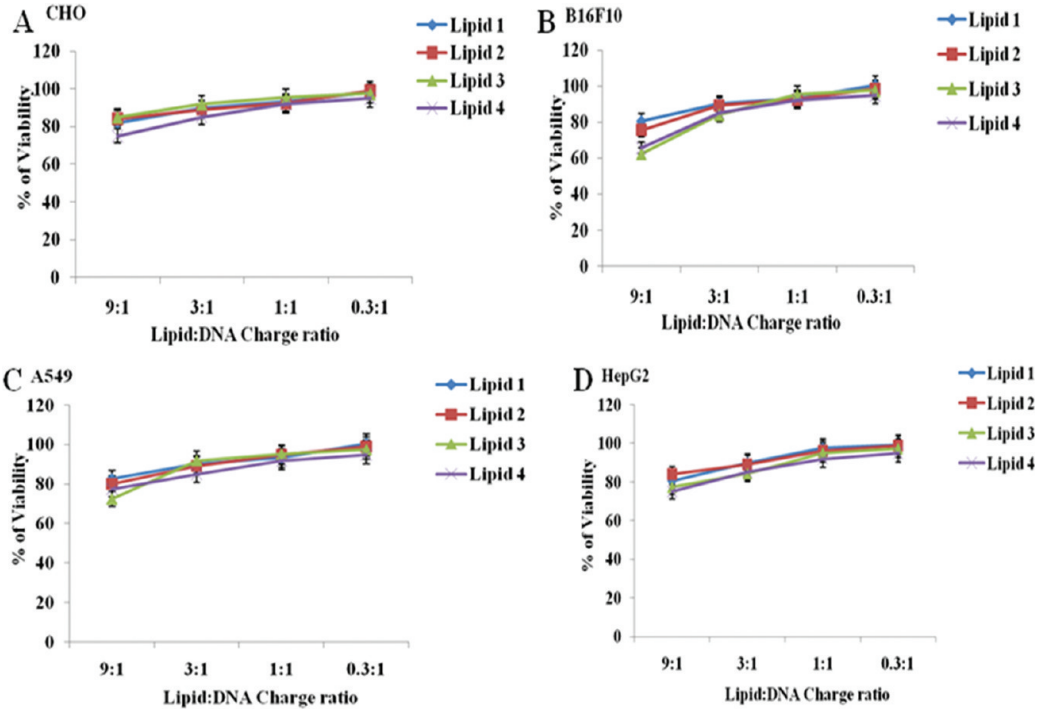

Figure 14. Representative percent cell viabilities of lipids **1**−**4** in CHO (A), B16F10 (B), A549 (C), and HepG2 (D) cells using MTT assay. The absorption obtained with reduced formazan with cells in the absence of lipids was taken to be 100. The toxicity assays were performed as described in the text. The data presented are the average values of three independent experiments (n = 3).

slightly better expression of GFP than lipoplexes of lipid **3**. The expression of GFP is found to be least for lipoplexes of lipid **4**.

Cellular Uptake Assay and Cellular Uptake Observed under an Inverted Microscope. Results from transfection results revealed that in all four types of cell lines (B16F10, CHO, A549, and HepG2) lipids **1**−**4** showed their maximum transfection efficiencies at a lipid/DNA charge ratio of 3:1. To verify whether the maximum uptake occurs at this given charge ratio, CHO and A549 cell lines were treated with lipoplexes comprising pCMV-SPORT-β-gal plasmid DNA and rhodamine-PE labeled liposome of lipids **1**−**4** across the lipid/DNA charge ratio of 3:1. Cellular uptake of rhodamine labeled lipoplexes was observed under an inverted fluorescent microscope (Figure 12). It was also quantified using a microplate fluorescent reader (Figure 13). The results of both quantitative and qualitative cellular uptake experiments reveal that the uptake of lipoplexes of lipid **1** is slightly greater than that of lipoplexes of lipids **1**, **2**, and **3** in both types of cell lines studied.

Toxicity Studies. MTT-based cell viabilities of lipids **1**−**4** were evaluated in CHO, B16F10, A-549, and HepG2 cells across the entire range of lipid/DNA charge ratios used in the actual transfection experiments. Cell viabilities of all lipids were found to be remarkably high (more than 85%) particularly up to the lipid/DNA charge ratios of 3:1 (Figure 14). Thus, the contrasting in vitro gene transfer efficacies of the present series of lipids (**1**−**4**) are unlikely to originate from varying cell cytotoxicities of the lipids.

DISCUSSION

In our ongoing program to design efficient novel cationic transfection lipids, we recently demonstrated the potential of novel tocopherol-based monocationic lipids for use in lip-

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osomal gene delivery.[23,26] Toward enhancing the transfection efficiency of these novel tocopherol-based lipids further through an increase in the charge on the headgroup of tocopherol-based cationic lipids and to facilitate the release of liposomal content into the cell cytoplasm, we have designed and synthesized two novel tocopherol-based lipids: (i) gemini cationic lipid **1** having a cystine headgroup with two tocopherol units as anchoring groups and (ii) cationic lipid **2** having a cystine headgroup with one tocopherol unit as an anchoring group. The transfection efficiencies of these lipids are compared with two control gemini lipids **3** and **4** bearing cholesterol and aliphatic chains as anchoring groups, respectively, and cystine as a headgroup.

As demonstrated by the transfection results described above, the gene transfection efficiencies of lipid **1** are found to be better when compared to lipids **2**, **3**, and **4**. Especially in HepG2 cell lines the lipid **1** is found to be the highest transfection efficient among the lipids **1**−**4** and also showed better transfection efficiency than the commercial formulation. To gain instights into the possible origin of such transfection profiles of the lipids **1**−**4**, first we studied the sizes and zeta potentials of liposomes and lipoplexes of lipids **1**−**4** prepared using colipids DOPE and DOPC. The sizes of the lipoplexes prepared using colipids DOPE and DOPC (Table 1) clearly demonstrate that in general the lipoplexes prepared using DOPC as colipid are smaller in size compared to the lipoplexes prepared using DOPE as a colipid. As size greatly influences the transfection properties of cationic lipids, the smaller sizes of the lipoplexes prepared using DOPC as a colipid may be responsible for their higher cellular uptake of DNA, thereby reporting the higher transfection activities when compared to the lipoplexes prepared using DOPE as a colipid. Among the lipoplexes prepared using DOPC as a colipid the lipoplexes of gemini lipid **1** were found to be the smaller in size when compared to lipoplexes of its monomeric lipid **2** and the control analogues **3** and **4**. As all of the lipids are cystine-based, all of the lipids are dicationic; hence the variation in size of lipoplexes is not due to the varying the number of positive charges in the headgroup region. The only difference among lipid **1** and the lipid **2** is the presence of one additional tocopherol moiety in the hydrophobic region, and the present results demonstrate that the presence of this additional tocopherol moiety greatly influences the size of the lipoplex formed and hence thereby the transfection efficiency observed in the in vitro transfection experiments. Both gel retardation (Figure 1) and DNase I sensitivity (Figure 2) assays showed higher binding properties of the present series of lipids up to a 3:1 ratio of lipid/DNA. This may be attributed due to the dicationic charge present on them, which allows for strong DNA binding interactions. The specific feature of lipid **1**, showing greater in vitro transfection efficacy at a lipid/DNA ratio of 1:1 when compared to a lipid/DNA ratio of 3:1 (contrary to other lipids) in all four types of cells studied is expected due to optimal DNA binding capacity of lipid **1** at this charge ratio. The band intensities of lipid **1** at a lipid/DNA ratio of 1:1 is highest among all of the lipids studied (Figure 2). This property of lipid **1** to protect DNA from DNase I even up to lower lipid/DNA ratios further supports the high in vitro gene transfecting ability of lipid **1** and also explains the high serum compatibility of the novel gemini cationic lipid **1**.

To gain insight into whether the cystine group present in the headgroup region is reducible in the cellular environment, gel retardation assay and ethidium bromide exlusion experiments were carried out by incubating the lipoplexes of lipids in 10 mM glutathione. The high cytosolic glutathione concentration, which can reach up to 10 mM in the liver, is also reported to affect intracellular reduction.[46−48] Results from Figures 3 and 4 demonstrate that the present cystine-based lipids (lipids **1**−**4**) which contain a disulfide group can efficiently release DNA. Thus the DNA release study strongly supports that the presently designed cystine-based lipids can efficiently release DNA inside the cell cytoplasm after reduction of the disulfide linker by the intracellular glutathione pool as reported in some earlier reports.[27−31] When such a reducible cystine headgroup is conjugated to two tocopherol units, lipid **1** showed the highest transfection efficiency among the series of lipids **1**−**4** studied.

For the present lipids **1**−**4** the in vitro transfection profiles (Figures 5−8) were found to be remarkably influenced by the colipid used in liposomal preparation. DOPC was found to be a more efficacious colipid than DOPE for all of the lipids in all four types of cell lines studied. Previous studies reveal that DOPE facilitates better transfection than DOPC. The role of DOPE in enhancing the transfection efficiency was attributed to its ability to undergo the transition from bilayer to inverted hexagonal structures, which are known to catalyze the fusion process.[49,50] Several studies have, however, questioned the role of fusion in transfection. No obvious correlation was found between fusion of lipoplexes with cells and transfection efficiency.[51,52] There are reports showing nonfusogenic DOPC-based lipoplexes to be more transfection efficient than fusogenic DOPE-based lipoplexes.[53] The role of helper lipid can vary also depending on the cationic lipid and target cells, rendering the issue more complicated.[54] This higher transfection profiles of lipoplexes of lipids **1**−**4** prepared using DOPC as colipid may also be due to the smaller sizes of these lipoplexes when compared to lipoplexes prepared using DOPE as colipids (Table 1). As uptake of DOPC-based lipoplexes is facilitated through endocytosis, the smaller sizes of lipoplexes prepared using DOPC as colipids further enhances the endocytosis process and thus the transfection efficiencies. The difference in the transfection profiles of lipids **1** and **3** which differ only in a hydrophobic tail group may be due to the smaller size of the lipoplexes prepared from lipid **1** when compared to the lipoplex prepared from lipid **3** and also due to strong DNA binding interactions of lipid **1** at all lipid/DNA charge ratios tested (Figures 1 and 2). Lipid **1** showed optimal binding interactions up to a lipid/DNA charge ratio of 1:1, whereas lipid **3** showed DNA binding interactions only up to a lipid/DNA charge ratio of 3:1. In the reporter gene assay, the in vitro gene transfer efficiencies of lipids **1** and **2** with two and one tocopherol units, respectively, in general, are found to be higher in HepG2 cells when compared to other three types of cancer cells, that is, CHO, B16F10, and A-549. Lipid **1** was found to exhibit 3-fold higher activity at a 1:1 charge ratio and 2-fold higher activity at a 3:1 charge ratio when compared to commercial formulation in HepG2 type of cells (Figure 8). This higher transfection profile of tocopherol-based lipids in HepG2 cells is expected due to the presence of TTP (tocopherol−transfer protein) in liver cells which helps in transfer of tocopherol and its analogues to HepG2 cells as previously reported.[55−58] Further, gene delivery to hepatocytes is of great therapeutic potential because the cells are responsible for the synthesis of a wide variety of proteins that play important biological roles both inside and outside the liver. This property of tocopherol-based gemini cationic lipids to

efficiently transfect liver cells can be exploited in future for designing of liver targeted tocopherol-based gene delivering vectors. The role of TTP in present lipids **1** and **2** needs to be further investigated by in vivo studies and receptor mediated gene delivery. In addition lipid **1** is highly serum compatible. The present observations showed that the monomeric lipid **2** is more serum-compatible than the gemini cationic lipids **3** and **4** (Figures 9 and 10). The results demonstrate that as the concentration of serum is increased from 10% to 50% lipids **3** and **4** show a decrease in transfection efficiency, whereas tocopherol-based lipids **1** and **2** showed increase in the transfection efficiency (especially in HepG2 celllines). The usual serum incompatibility of cationic transfection lipidsis 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. It is reported earlier that tocopherol changes liposomes in a manner similar to cholesterol making them highly resistant to protein-induced disruption. This suppression of protein-induced disruptions is more pronounced with tocopherol than with cholesterol, thus making α-tocopherol containing liposomes as efficient delivery vectors in vivo.[59] Thus, it can be explained that the higher serum compatibility of the gemini cationic lipid **1** and the monomeric lipid **2** is due to the presence of tocopherol which greatly suppresses the protein−induced disruptions compared to cholesterol/aliphatic groups present in lipids **3** and **4**, respectively. The presence of cholesterol in lipid **3** makes it less serum-compatible compared to tocopherol-based lipids **1** and **2** (Figures 9 and 10) even though the lipid **3** is found to be more efficient in its in vitro transfection profile than the monomeric lipid **2** (Figures 5−8). The results of in vitro gene transfer efficacies of lipids **1**−**4** are further emphasized by carrying out microscopic cellular expression experiments using α5 GFP plasmid DNA encoding green fluorescent protein at a lipid/DNA charge ratio of 3:1. The results from microscopic cellular expressions demonstrate that slightly better cellular expression of GFP was obtained with lipoplexes of lipid **1** (Figure 11, I and II) than the lipoplexes of lipid **3** and much better cellular expression of GFP than the lipoplexes of lipids **2** and **4** at the given lipid/DNA charge ratio. These results are found to be consistent with the transfection profile of lipids **1**−**4**. This comparison from the microscopic observation provided a clear evidence for the better transfection profile of lipid **1** relative to other lipids **2**−**4**. The qualitative and quantitative cellular uptake experimental results of lipoplexes of lipids **1**−**4** are also found to be consistent with the transfection profile.

The cell viabilities of the lipids **1**−**4** were found to be remarkably high (more than 85%) particularly up to the lipid/DNA charge ratios of 3:1 (Figure 14). Thus the presently designed novel nontoxic, highly serum-compatible, reducible and transfection efficient gemini cationic lipid **1** can be used further for in vivo applications.

To conclude, two tocopherol-based dicationic lipids (**1** and **2**) and two nontocopherol-based gemini cationic lipids (**3** and **4**) with cystine in the headgroup region have been synthesized and demonstrated the relative transfection efficiencies of these lipids through in vitro gene transfection profiles. The transfection profile of lipids **1**−**4** is further confirmed by the microscopic experiments and quantitative cellular uptake experiment. Lipid **1** with two tocopherol units and cystine in its headgroup region showed the highest transfection efficiency and serum compatibility among the lipids studied, **1**−**4**. Most importantly, the high serum compatibilities of the lipoplexes of lipids **1** make it a promising nonviral transfection vector for the future systemic delivery. Further the property of tocopherol-based gemini cationic lipids to efficiently transfect liver cells can be exploited in the future for the design of liver targeted tocopherol-based gene delivering vectors.

■ ASSOCIATED CONTENT

Supporting Information

^{1}H NMR spectra of lipids **1**−**4** and intermediates; mass spectra of lipids **1**−**4** and intermediates; elemental analysis data for lipids **1**−**4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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