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Design, Synthesis, and inVitro Transfection Biology of Novel Tocopherol Based Monocationic Lipids: A Structure−Activity Investigation

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Herein, we report on the design, synthesis, and in vitro gene delivery efficacies of five novel tocopherol based cationic lipids (**1**−**5**) in transfecting CHO, B16F10, A-549, and HepG2 cells. The in vitro gene transfer efficiencies of lipids (**1**−**5**) were evaluated by both β-galactosidase reporter gene expression and inverted fluorescent microscopic experiments. The results of the present structure−activity investigation convincingly demonstrate that the tocopherol based lipid with three hydroxyl groups in its headgroup region showed 4-fold better transfection efficiency than the commercial formulation. The results also demonstrate that these tocopherol based lipids may be targeted to liver. Transfection efficiency of all the relevant lipids was maintained even when the serum was present during the transfection conditions. The results indicated that the designed systems are quite capable of transferring the DNA into all four types of cells studied with low or no toxicity.

Introduction

The success of gene therapy mainly depends on the development of efficient and safe vectors for gene delivery. Basically, two approaches have been adapted to introduce naked DNA into cells using viral and nonviral vectors. Development of nonviral vectors has been one of the primary areas of research because of several significant factors, viz., greater carrier capacity, safety, ease of large-scale production, stability, potential to incorporate targeting ligands, and unlimited vector size. However, these synthetic delivery systems are less efficient than viral gene transfection vectors.[1−3] Among nonviral vectors, cationic liposomes and cationic polymer based vectors are investigated mostly to improve the transfection efficiency. Although the cationic polymers can condense DNA efficiently, exhibit better stability, and easily control polyplexes properties compared with those of lipoplexes, cationic polymers usually display higher cytotoxic and immunogenic profiles than cationic lipids[4,5] by virtue of "templating" features of the polymeric backbone. Since the first reports,[6] on cationic liposome-mediated gene delivery by Felgner et al. in 1987, an upsurge of global interest has been witnessed in synthesizing efficient cationic transfection lipids.[7−17]

Most of the cationic lipid formulations studied for gene delivery are glycerol[18] or cholesterol based[19] or aliphatic-hydrocarbon-tail based[10−15] cationic transfection lipids. Clearly, design and synthesis of new lipid system with alternative structural types are crucial for the development of potent synthetic vectors for gene delivery. The molecular structure of cationic lipids is an important parameter that controls their DNA complexation and gene transfection activity. Tocopherol (vitamin E), being a natural amphiphilic molecule, consists of a group of isoprenoid compounds of plant origin that were described initially as essential micronutrients for normal fertility in rats.[20] Subsequent studies have established a wide range of functions to it, especially the RRR-tocopherol stereoisomer, the most biologically active form.[21,22] Besides its well documented role as a free radical chain-breaking antioxidant, α-tocopherol can also modulate directly cellular signaling pathways, viz., protein kinase C, leading to diverse biological responses in different cell types.[23] Many analogues of α-tocopherol are proved to be efficient antiproliferative agents in a wide range of cell types.[24−26] Tocopherol has been used for drug delivery with significant success.[27−29] In addition, cationic lipid self-assemblies formulations including tocopherol were used for targeted gene delivery.[30−33] In the present work, the tocopherol moiety is introduced in the cationic lipid backbone and its targeting efficacy is assessed. Toward this, novel cationic lipids with tocopherol (vitamin E) as an anchoring group are designed. It is demonstrated that some of these tocopherol based lipids exhibit higher transfection efficiency and low cytotoxicities.

Taking the impressive gene transfer properties of many previously reported cationic amphiphiles with oxyethylene units at linkages[34,35] and hydroxyalkyl head groups[12,14,36−39] into account, it is envisaged that design of tocopherol based cationic lipids with varying number of hydroxylethyl groups in the headgroup region and oxyethylene unit as linkage may be rewarding. In this direction, the present work reports the synthesis and characterization of a series of five cationic lipids (**1**−**5**) with tocopherol as the hydrophobic tail region, oxyethylene units at linkages, and varying numbers of hydroxy ethyl groups directly attached to the positively charged nitrogen atom in the headgroup region.

Results and Discussion

The present work illustrates the synthesis of lipids **1**−**5** and their physicochemical characteristics. The results of in vitro

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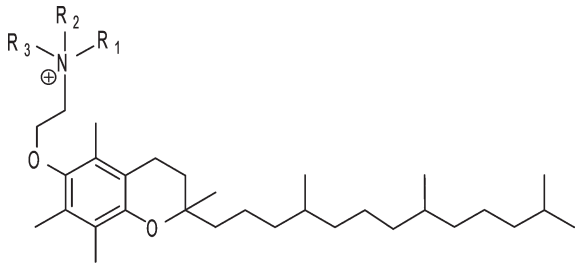
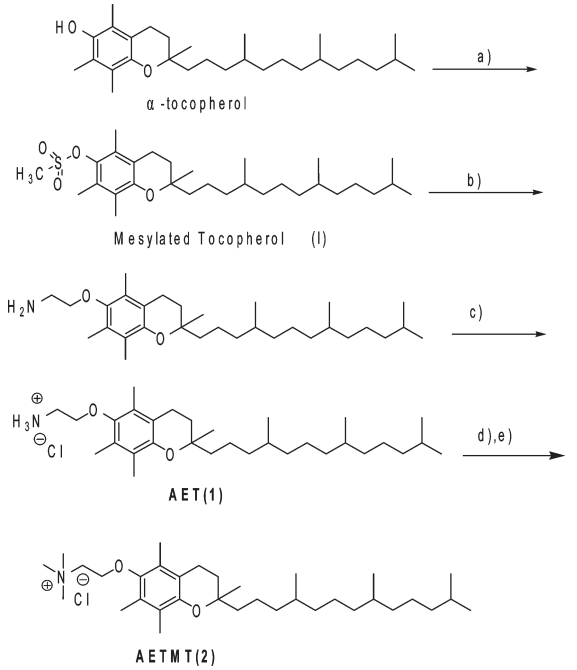
Chart 1. Structure of Lipids **1-5**



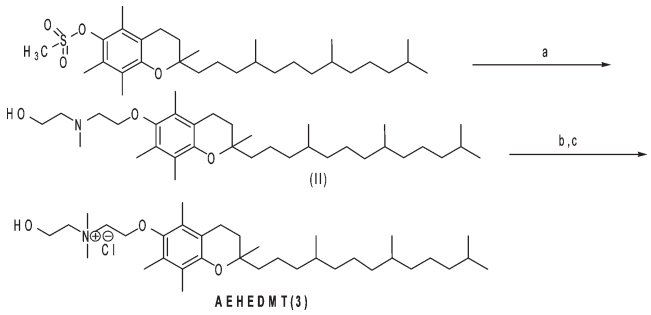
Scheme 1. Synthesis of AET and AETMT (Lipids **1** and **2**)[a]



[a] Reagents: (a) CH_3SO_2Cl, pyridine, DMAP, dry DCM, room temp, 24 h; (b) ethanolamine, KOH, toluene, 48 h; (c) HCl, methanol, overnight; (d) MeI, K_2CO_3, room temp, 4 days; (e) Amberlyst anion exchange resin.

transfection experiments performed on four different types of cell lines to assess the transfection properties of lipids **1**−**5** and control lipid **6** based lipoplexes 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 and serum compatibility in four types of cell lines for lipids **1**−**5** is also reported. A control lipid **6** (Scheme 4) was designed and synthesized in order to investigate whether the favorable hydrogen-bonding interactions between the biological membrane components/DNA and hydroxyl functionalities present in the headgroup region of tocopherol based lipids could play a significant role in stabilizing lipoplexes and improving transfection efficiency of the lipids. In this lipid **6**, three hydroxyl groups of lipid **5** were replaced with three methoxy groups. The stability, transfection efficiencies, and the cellular uptake of lipoplexes of lipids **1**−**5** are compared with the control lipid **6**.

Scheme 2. Synthesis of AEHEDMT (Lipid **3**)[a]



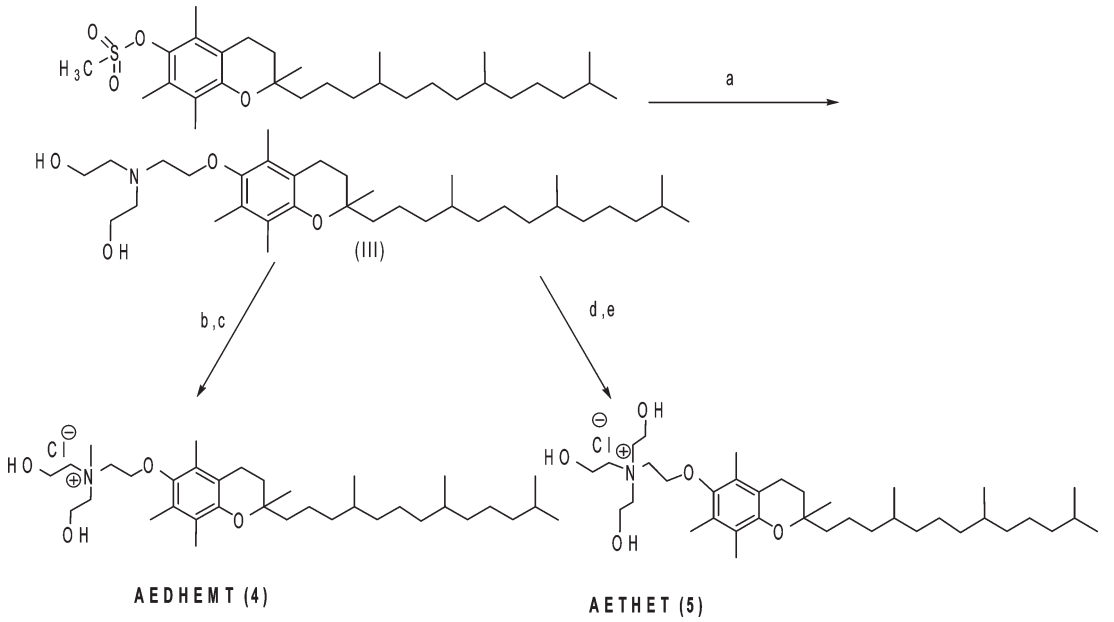
[a] Reagents: (a) *N*-methyldiethanolamine, toluene, 48 h reflux; (b) MeI, K_2CO_3, room temp, 4 days; (c) Amberlyst anion exchange resin.

Chemistry. The key structural elements common to all of the transfection lipids **1**−**5** (Chart 1) described herein include (a) the presence of α-tocopherol as the hydrophobic group, (b) the presence of oxyethylene group as the linker group, and (c) the absence or presence of hydroxyl ethyl groups in the headgroup region. The details of the synthetic procedures for all the novel transfection lipids shown in Chart 1 are described in the Experimental Section. As outlined in Schemes 1−3, the chemistries involved in preparing these new lipids are straightforward. However, given their remarkable high transfection efficiencies, the overall yields of these transfection lipids need to be improved in the future. Cationic lipids **1**−**5** were synthesized by the reaction of the common starting material mesylated tocopherol (prepared from α-tocopherol in a single step as outlined in Scheme 1) with various substituted ethanol amines to yield the intermediate tertiary amines. The resulting tertiary amine intermediates upon quaternization with excess methyl iodide or bromoethanol followed by chloride ion exchange over Amberlyst-26 yielded the lipids **1**−**5** (Scheme 1−3). Structures of all the synthetic intermediates and final lipids shown in Schemes 1−3 are confirmed by ^{1}H NMR and molecular ion peaks in their ESI mass spectra. The final lipids are also characterized by the elemental analysis data, as described in the Experimental Section.

Nanosizes and Global Surface Charges of the Lipoplexes. Toward physicochemically characterizing the present lipids, the nanosizes and the global surface charges of the liposomes and lipoplexes of all the five lipids **1**−**5** and DOPC[a] as colipid were measured using a dynamic laser light scattering instrument equipped with ζ -sizing capacity. These measurements were made across the lipid/DNA charge ratios of 0.3:1 to 9:1 in the presence of Dulbecco's modified Eagle's medium (DMEM). It is observed that the nanosizes of lipoplexes prepared from both the transfection efficient and the incompetent lipids similarly increased with increasing lipid/DNA charge ratios within the range of 300−1000 nm (Table 1). Such larger sizes for lipoplexes prepared in DMEM were also reported previously for DOTAP−oligonucleotide complexes.[40] The reported physicochemical characteristics of

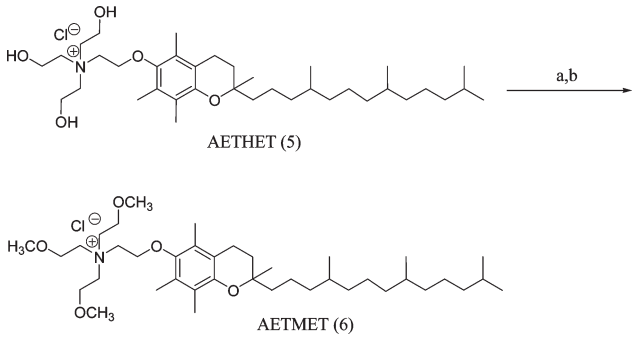
[a] Abbrevations: AET, *O*-aminoethyl-α-tocoherol; AETMT, *O*-aminoethyl[*N*,*N*,*N*-trimethyl]-α-tocopherol; AEHEDMT, *O*-aminoethyl-[*N*-hydroxyethyl,*N*,*N*-dimethyl]-α-tocopherol; AEDHEMT, *O*-aminoethyl-[*N*,*N*-di(hydroxyethyl),*N* methyl]-α-tocopherol; AETHET, *O*-aminoethyl-[*N*,*N*,*N*-tri(hydroxyethyl)]-α-tocopherol; AETMET, *O*-aminoethyl-[*N*,*N*,*N*-tri(methoxyethyl)]-α-tocopherol; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycerol-3-phosphoethanolamine; DMEM, Dulbecco's modified Eagle's medium; DMAP, 4-(dimethylamino)pyridine; FBS, fetal bovine serum; PBS, phosphate-buffered saline; ONPG, *o*-nitrophenyl-β-D-galactopyranoside.

Scheme 3. Synthesis of AEDHEMT and AETHET (Lipids **4** and **5**)[a]



[a] Reagents: (a) triethanolamine, KOH, toluene, 48 h reflux; (b) MeI, K_2CO_3, room temp, 4 days; (c) Amberlyst anion exchange resin; (d) bromoethanol, reflux, 4 days; (e) Amberlyst anion exchange resin.

Scheme 4. Synthesis of AETMET (Control Lipid **6**)[a]



[a] Reagents: (a) NaH, THF, MeI, room temp, 12 h; (b) Amberlyst anion exchange resin.

the cationic liposomes and oligonucleotide complexes[41] show that the larger sizes of the lipoplexes prepared in cell growth medium may originate from aggregation and fusion of liposomes after adding them to culture medium. The results reveal that the apparent lipoplex sizes at a given ±charge ratio did not vary by a maximum of 20−50% (Table 1). Thus, lipoplex sizes are unlikely to play any key role in imparting superior transfection properties to the tocopherol based lipids. The global surface potentials of lipoplexes made from representative lipids **1**−**5** were found to be negative in the presence of DMEM. Thus, surface potentials of lipid/DNA complexes are unlikely to play any key role in modulating transfection efficacies of the tocopherol based cationic lipids studied. The ζ potential is only one among several other factors that govern the uptake of lipoplex. In fact, the literature contains many articles[42,43] on the efficient uptake and delivery of the contents using negative lipoplexes/negative particles.

Transfection Biology. In Vitro Transfection Studies. The relative in vitro gene delivery efficacies of lipids **1**−**5** 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 colipid are summarized in (Figures 1−4). *p*CMV-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**−**5** were compared with that of the commercial formulation and control lipid **6**.

The results of Figures 1−4 summarize several interesting transfection profiles. For the present lipids **1**−**5**, 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). The difference in the transfection efficiencies of lipoplexes in the presence of colipids[44] DOPE and DOPC is perhaps justified as follows. The uptake of the lipoplexes in the presence of DOPE takes place mainly from the fusion of the lipoplexes with the plasma membrane, whereas "endocytosis" facilitates uptake in the presence of DOPC. The highest transfection efficiencies of all the lipids **1**−**5** were found to be across the lipid/DNA ratio of 3:1 and 1:1. In general, lipids **3**, **4**, and **5** with one, two, and three hydroxyl groups in its headgroup region, respectively, were found to be more efficient in transfection when compared to lipids **1** and **2** having no hydroxyl groups in its headgroup region. In particular, among the lipids **3**−**5**, lipid **5** with three hydroxyl groups in its headgroup region is found to show the highest transfection efficiency across all four types of cells studied at the lipid to DNA ratio of 3:1. The lipids **4** and **5** at lipid to DNA ratios 3:1 and 1:1 are equally efficient in transfecting CHO and A-549 cell lines in the presence of colipid DOPC. Under these conditions, these lipids also showed 2-fold better transfection efficiency than the commercial formulation, whereas lipid **3** showed transfection comparable to that of commercial formulation. However, lipids **1**−**5** are not very efficient in transfecting B16F10 cell lines. The lipids **4** and **5** showed transfection efficiencies comparable to that of commercial formulation when DOPC is used as colipid at lipid to DNA ratio of 3:1. In HepG2 cell lines lipid **5** showed almost 4-fold better transfection activity than commercial formulation at lipid to DNA ratio of 3:1 and 2-fold better transfection efficiency at lipid to DNA ratio of 1:1. Lipid **4** also showed almost 3- to

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

	size (nm) for lipid/DNA (molar ratio) of				
lipid	1:0	0.3:1	1:1	3:1	9:1
1	177.1 ± 3.8	396.8 ± 28.7	489.2 ± 119.7	658.3 ± 20.5	1000.6 ± 21.8
2	177.6 ± 36.2	278.9 ± 63.2	292.7 ± 7.0	628.2 ± 159.8	826.0 ± 278.9
3	289.7 ± 21.5	416.4 ± 83.7	445.9 ± 24.2	507.8 ± 65.2	709.9 ± 87.4
4	255.3 ± 73.2	370.3 ± 3.9	296.8 ± 72.5	562 ± 16.7	1056.4 ± 54.3
5	208.7 ± 94.7	302.8 ± 1.9	258.9 ± 56.2	879.5 ± 121.1	863.4 ± 251.3
	ζ potential (mV) for lipid/DNA (molar ratio) of				
	1:0	0.3:1	1:1	3:1	9:1
1	13.9 ± 2.3	−8.3 ± 6.8	−6.5 ± 12.4	−29.3 ± 1.8	−12.0 ± 5.2
2	15.6 ± 5.5	−3.9 ± 3.1	−6.5 ± 1.3	−5.9 ± 2.6	- 3.3 ± 2.0
3	8.9 ± 1.3	−8.0 ± 3.0	−5.9 ± 2.3	−12.9 ± 3.4	−2.3 ± 0.5
4	14.9 ± 2.9	−4.6 ± 2.6	−15.5 ± 5.9	−8.2 ± 3.1	3.2 ± 1.4
5	22.8 ± 2.3	−8.7 ± 2.5	−3.7 ± 5.3	−12.4 ± 3.8	−0.3 ± 19.4

[a] Sizes and ζ potentials were measured by laser light scattering technique using Zetasizer 3000A (Malvern Instruments, U.K). Values shown are the averages obtained from 3 (size) and 10 (ζ potential) measurements.

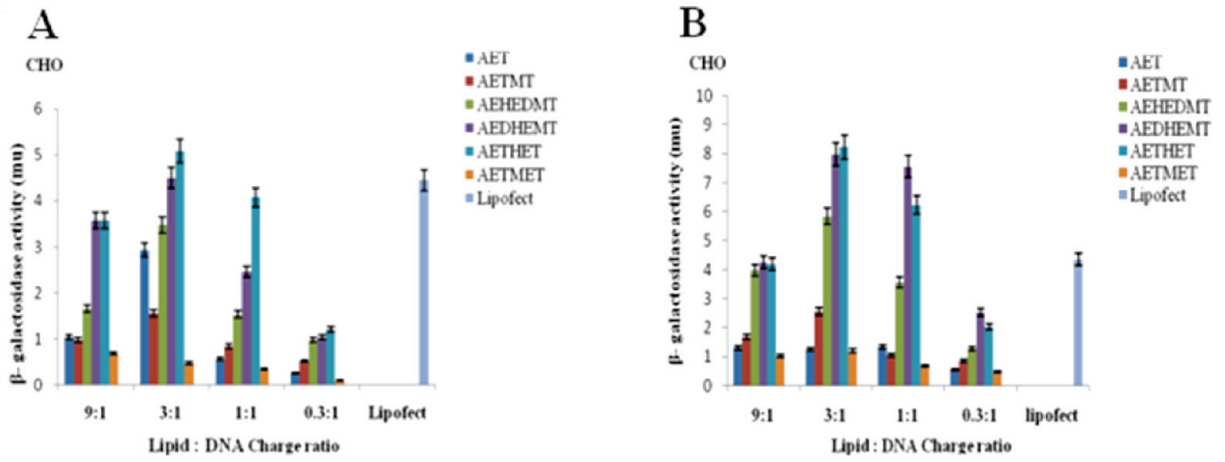


Figure 1. Transfection efficiencies of lipids **1**−**5** and control lipid **6** in CHO cells with DOPE (A) and DOPC (B) as colipid (the concentration of each lipid and colipid is 0.5 mol, respectively). The transfection efficiencies of the lipids were compared to that of commercial formulation. Transfection experiments were performed as described in the text. All the lipids were tested on the same day, and the data presented are the average of 3 experiments performed on 3 different days.

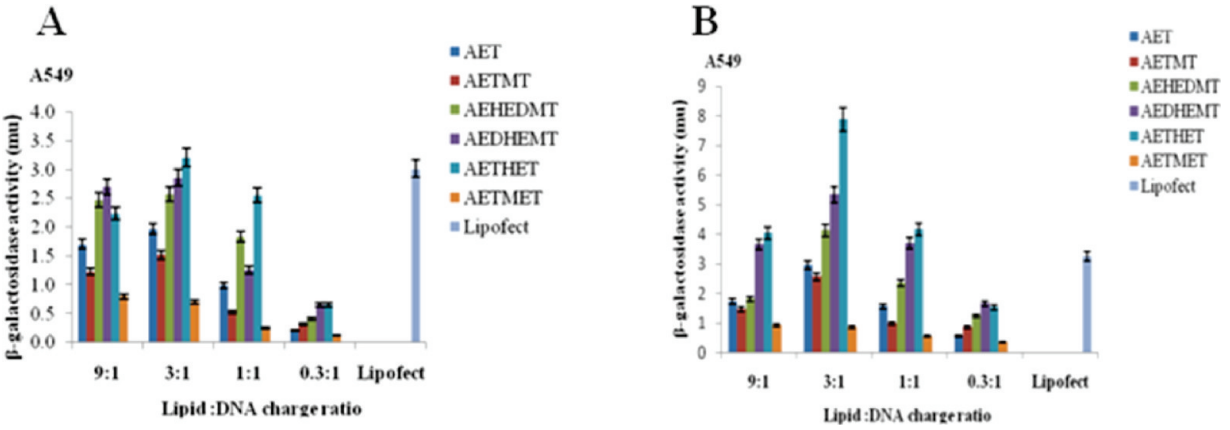


Figure 2. Transfection efficiencies of lipids **1**−**5** and control lipid **6** in A-549 cells with DOPE (A) and DOPC (B) as colipid (the concentration of each lipid and colipid is 0.5 mol, respectively). The transfection efficiencies of the lipids were compared to that of commercial formulation. Transfection experiments were performed as described in the text. All the lipids were tested on the same day, and the data presented are the average of 3 experiments performed on 3 different days.

4-fold higher transfection efficiency than commercial formulation at 3:1 lipid/DNA ratio. The higher transfection efficiencies of the lipids **4** and **5** are expected because of the presence of hydroxyl alkyl groups in the headgroup region as reported earlier.[12,14,36−39] The results also demonstrate increased transfection efficiencies with increasing number of hydroxyalkyl groups in the headgroup region (lipid **5** is shown to be the highest transfection efficient lipid of lipids

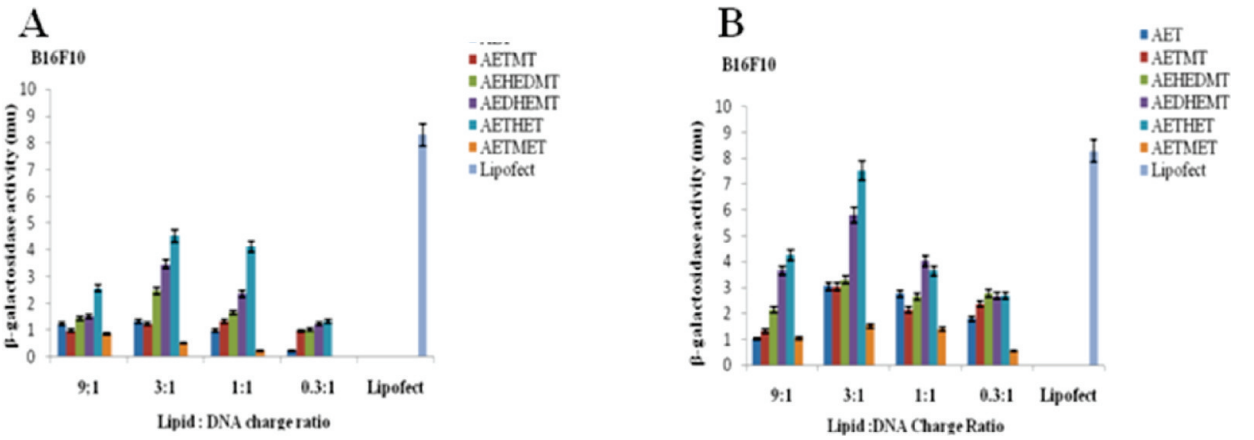


Figure 3. Transfection efficiencies of lipids **1**−**5** and control lipid **6** in B16F10 cells with DOPE (A) and DOPC (B) as colipid (the concentration of each lipid and colipid is 0.5 mol, respectively). The transfection efficiencies of the lipids were compared to that of commercial formulation. Transfection experiments were performed as described in the text. All the lipids were tested on the same day, and the data presented are the average of 3 experiments performed on 3 different days.

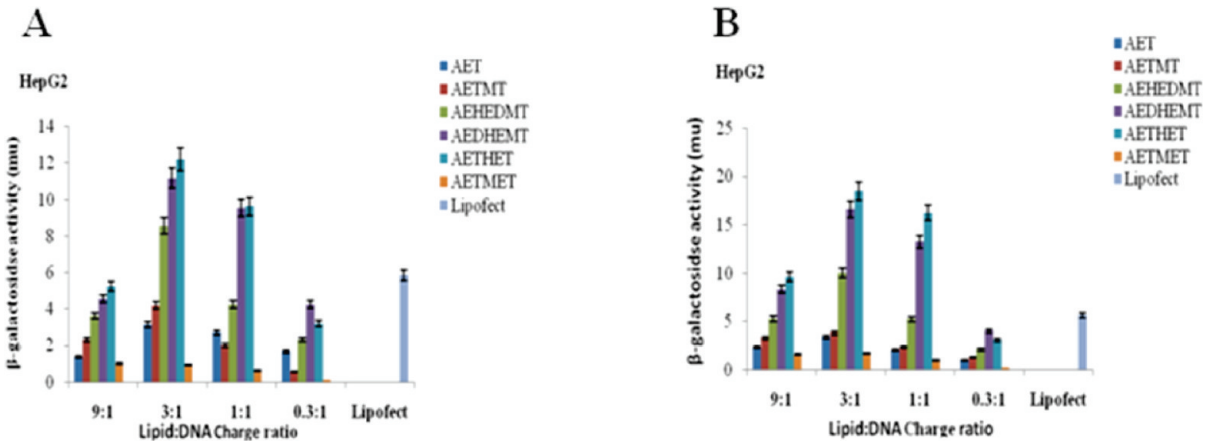


Figure 4. Transfection efficiencies of lipids **1**−**5** and control lipid **6** in HepG2 cells with DOPE (A) and DOPC (B) as colipid (the concentration of each lipid and colipid is 0.5 mol, respectively). The transfection efficiencies of the lipids were compared to that of commercial formulation. Transfection experiments were performed as described in the text. All the lipids were tested on the same day, and the data presented are the average of 3 experiments performed on 3 different days.

1−**5** studied). The control lipid **6** in which the three hydroxyl groups of lipid **5** were replaced with three methoxy groups showed minimal transfection when compared to lipids **1**−**5** in all four types of cell lines studied. Another significant observation from the transfection results is that lipid **1** is found to be better transfection efficient than lipid **2**. This may be attributed to the fact that lipid **1** is more sensitive toward the lower pH values, which helps in the faster release of DNA in the cell cytoplasm as reported earlier.[45−47]

In the reporter gene assay, the in vitro gene transfer efficiencies of lipids **1**−**5** in general are found to be higher in HepG2 cells when compared to the other three types of cancer cells, i.e., CHO, B16F10, A-549. The lipids **1**−**5** showed very low transfection profiles in B16 F10 cells. This higher transfection profile of tocopherol based lipids in HepG2 cells is expected because of the presence of TTP (tocopherol-transfer protein) in liver cells which helps in the transfer of tocopherol and its analogues to HepG2 cells as reported.[48−51] Further, gene delivery to hepatocytes bears 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 cationic lipids to efficiently transfect liver cells can be exploited in the future to design liver targeted tocopherol based gene delivering vectors. The role of TTP for lipids **1**−**5** needs to be further investigated through in vivo studies and receptor mediated gene delivery.

Toxicity Studies. MTT-based cell viabilities of lipids **1**−**5** 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 transfection efficient lipids (**3**−**5**) and transfection inefficient lipids **1** and **2** were found to be remarkably high (more than 85%) particularly up to the lipid/DNA charge ratios of 3:1 (Figure 5). Thus, the contrasting in vitro gene transfer efficacies of lipids **1**−**2** and **3**−**5** (Figures 1−4) are unlikely to originate from varying cell cytotoxicities of the lipids.

Lipid−**DNA Binding Interactions and Lipoplex Sensitivities to DNase I.** Toward initial characterization of the present lipoplexes, the electrostatic interactions between the plasmid DNA and cationic liposomes as a function of lipid/DNA charge ratios were determined by conventional electrophoretic gel retardation assay and DNase I sensitivity assays. The corresponding electrophoretic gel patterns observed in the gel retardation assay for lipoplexes formed from lipids **1**−**5** are shown in (Figure 6). All of the lipids **1**−**5** were

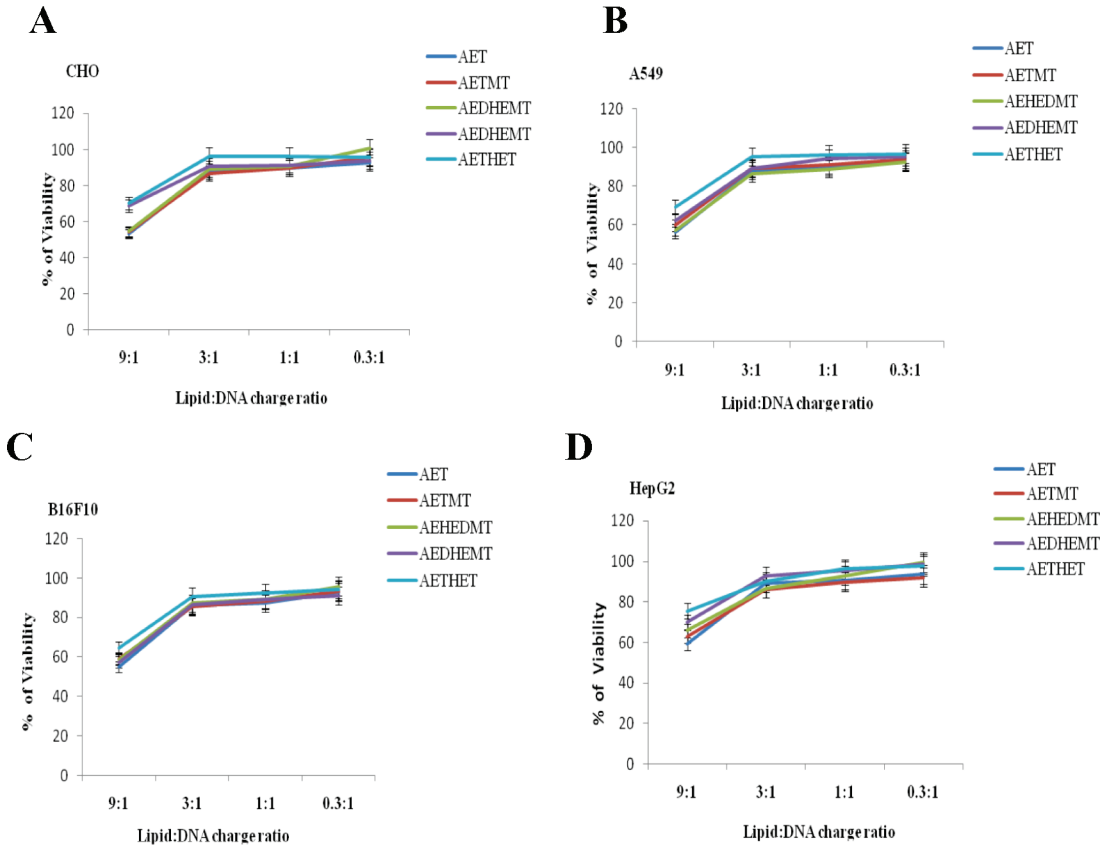


Figure 5. Representative percent cell viabilities of lipids **1**−**5** in CHO (A), A549 (B), B16F10 (C), and HepG2 (D) cells using MTT assay. The absorption obtained with reduced formazan with cells in the absence of lipids was taken to be 100. The toxicity assays were performed as described in the text. The data presented are the average values of three independent experiments (n = 3).

found to be capable of completely inhibiting the electrophoretic mobility of plasmid DNA from lipoplexes prepared at high lipid/DNA charge ratios of 9:1 (Figure 6). However, at lower lipid/DNA charge ratios, it was observed that a lesser amount of free DNA is present in the lipoplex of lipid **3** when compared to lipoplexes of **1** and **2** and optimal amount of free DNA was found in lipoplexes made from lipids **4** and **5**. Thus, the lipoplexes of lipids **4** and **5** having optimally strong lipid−DNA interactions may effectively facilitate the release of plasmid DNA in the cytoplasm after entering into the cell cytoplasm. This eventually shows better transfection efficiency of the lipoplexes of lipids **4** and **5** than the lipids **1**, **2**, and **3**.

The optimal stability of lipoplexes formed by lipids **4** and **5** is 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 7A−C summarizes results of such DNase I protection experiments for lipoplexes prepared from the representative cationic lipids **1**−**5** 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 **1** and **2** were found to be significantly less compared to those associated with transfection efficient lipids **3**−**5** across the lipid/DNA charge ratios of 3:1 to 0.3:1. Thus, from the findings in the conventional gel mobility shift (Figure 6) and DNase I sensitivity assays (Figure 7), although the lipoplex of lipid **3** exhibits strong lipid−DNA interactions among the lipoplexes of **3**−**5**, the lipoplexes of lipids **4** and **5** demonstrate better transfection activity. This is consistent with the assumption that optimally strong lipid−DNA binding interactions in the lipids **4** and **5** and DNA complexes perhaps play an important role behind their high transfection efficacies among the presently described cationic lipids. The stability of lipoplexes of lipids **3**−**5** may be attributed to the favorable hydrogen-bonding interactions between DNA and the hydroxyl functionalities present in the polar headgroup region of lipids **3**−**5**. This is further strengthened by the design and synthesis of a control lipid **6** carried out by us in which the hydroxyl functional groups in the headgroup region of lipid **5** have been replaced with methoxy functional groups. The gel retardation assay (Figure 6) and sensitivity of the lipoplexes of lipid **6** upon treatment with DNase I (Figure 7) showed that the lipoplexes formed by the lipid **6** are less stable when compared to the lipids **3**−**5**.

The release efficiency of plasmid DNA from the lipoplexes of lipids **4** and **5** is studied in the presence of anionic lipid (results are not shown) using gel retardation assay and sensitivity of the lipoplexes upon treatment with DNase I using gel electrophoresis.[52,53] The results reveal that the anionic lipid studied in this experiment can displace the ionic interaction between plasmid DNA and the cationic lipids **4** and **5**. 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**−**5**, thereby releasing plasmid DNA into the cell cytoplasm.

Cellular Expression of GFP. The transfection results revealed that lipids **1**−**5** in all four types of cells lines (B16F10,

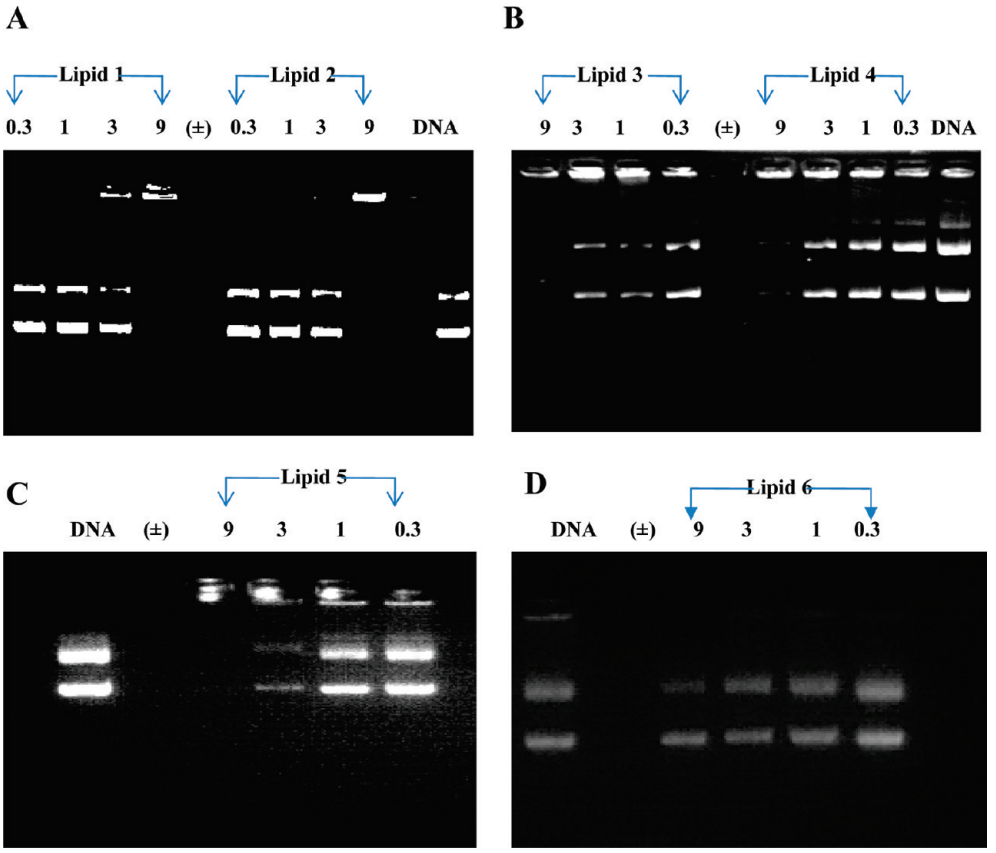


Figure 6. (A−D) Electrophoretic gel patterns for lipoplex-associated DNA in gel retardation assay for lipids **1**−**5** and control lipid **6**. The lipid/DNA charge ratios are indicated at the top of each line. The details of the treatment are as described in the text.

CHO, A-549, and HepG2) showed their maximum transfection efficiencies at lipid/DNA charge ratio of 3:1. At this juncture, it is necessary to investigate whether the DNA expression attains its maximum value at this given charge ratio. Toward this end, the representative CHO cells were treated with lipoplexes of lipids **1**−**5** containing a α5 GFP plasmid DNA encoding green fluorescent protein at lipid/DNA charge ratio of 3:1. The corresponding cellular expression of GFP in CHO cells was monitored subsequently by inverted fluorescent microscope. These microscopic cellular expressions demonstrate that maximum expression of GFP in CHO cells was obtained with lipoplexes of lipid **5** at the given lipid/DNA charge ratio (Figure 8). Lipoplexes of lipid **4** also showed better expression of GFP (Figure 8) at this charge ratio when compared to lipids **1**−**3**, while those of lipids **1** and **2** showed minimum cellular expression of GFP. These results are found to be consistent with the transfection profile of lipids **1**−**5**.

Cellular Uptake Assay and Cellular Uptake Observed under Inverted Microscope. Careful inspection of the transfection results in all four types of cell lines (B16F10, CHO, A549, and HepG2) revealed that lipids **1**−**5** showed their maximum transfection efficiencies at a lipid/DNA charge ratio of 3:1. In order to verify whether the maximum uptake occurs at this given charge ratio, CHO cell lines were treated with lipoplexes comprising *p*CMV-SPORT-*β*-gal plasmid DNA and rhodamine-PE labeled liposome of lipids **1**−**5** across the lipid/DNA charge ratio of 3:1. The CHO cell lines were also treated with the lipoplexes of control lipid **6** in a similar fashion. Cellular uptake of rhodamine labeled lipoplexes was quantified using microplate fluorescent reader (Figure 9). It was also observed under an inverted fluorescent microscope (Figure 10). The findings in these cellular uptake study showed that maximum cellular uptake of lipoplexes in CHO cells was obtained with lipoplexes of lipids **4** and **5** at the given lipid/DNA charge ratio. Thus, these findings in the cellular uptake experiments (Figures 9 and 10) support the notion that the varying transfection profiles of lipids **1**−**5** 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 modulating the transfection efficiencies of the presently described tocopherol based cationic lipids.

These findings in the cellular uptake experiments (Figures 9 and 10) also suggest that the presence of hydroxyl groups in the headgroup region imparts higher uptake of lipoplexes of lipid **5** among the series of lipids studied. On the other hand, the lipoplexes of control lipid **6**, in which the three hydroxyl functionalities in the headgroup region of the lipid **5** have been replaced with three methoxy functional groups, showed very minimal uptake of lipoplexes (Figures 9 and 10). This insinuates possible favorable hydrogen-bonding interactions between the biological membrane components and hydroxyl functionalities in the polar headgroup region of the lipid **5**. This could augment the cellular uptake of lipoplexes and hence showed the highest transfection efficiency. Thus, the degree of cellular uptake appears to play an important role in modulating the transfection efficiencies of the presently described tocopherol based cationic lipids.

Transfection Biology in Presence of Serum. Generally the gene transfer efficacies of libraries pertaining to the novel

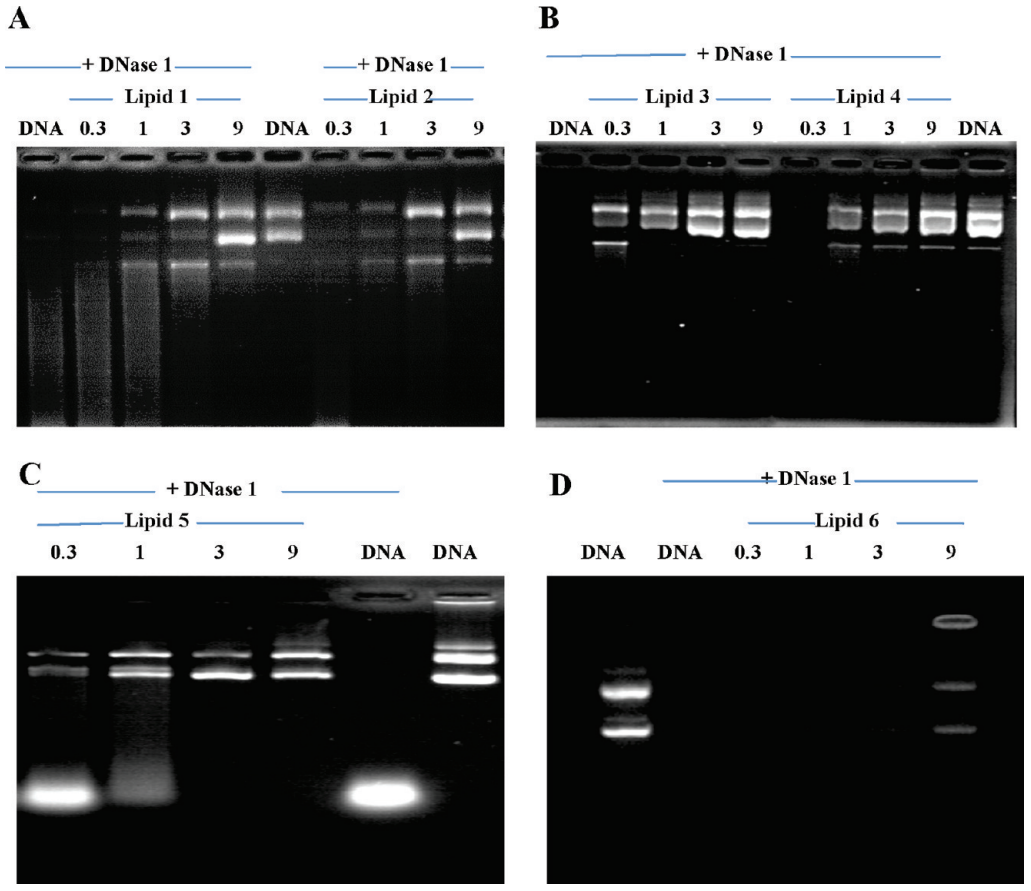


Figure 7. (A−D) Electrophoretic gel patterns for lipoplex-associated DNA in DNase I sensitivity assay for lipids **1**−**5** and control lipid **6**. The lipid/DNA charge ratios are indicated at the top of each lane. The details of the treatment are as described in the text.

cationic amphiphiles are evaluated either in the absence of added serum or in the presence of only 10% (v/v) serum as reported in many prior investigations.[12,15,37,54−58] However, serum incompatibility still remains one of the major setbacks retarding clinical success of cationic transfection lipids. The high in vitro gene transfer efficacies of cationic amphiphiles are often found to be adversely affected in the presence of serum.[59−66] The usual serum incompatibility of cationic transfection lipids is believed to begin via adsorption of negatively charged serum proteins onto the positively charged cationic liposome surfaces. This prevents their efficient interaction with cell surface and/or internalization.[67−69] Clearly, evaluation of gene transfer efficacies across a range of lipid/DNA charge ratios in multiple cultured cells in the presence of increasing concentrations of added serum is needed for obtaining meaningful systemic potential of any in vitro efficient cationic transfection lipid. In this direction, a detailed serum-compatibility study was carried out for lipids **1**−**5** across lipid/DNA charge ratio 3:1 at which all the lipids showed their highest transfection ability in all four types of cells used for transfection (CHO, A-549, B16F10, and HepG2) in the presence of increasing concentrations of added serum (10−50%, v/v). The in vitro gene transfer efficacies of lipids **1**−**5** were found to be unaffected in the presence of serum of up to 10% of added serum as demonstrated through Figure 11.

Lipid **5** was found to be the highest serum compatible throughout the serum concentrations, and lipid **4** also showed better serum compatibility among lipids **1**−**4**. The transfection efficiencies of lipids **4** and **5** are found to be improved on increasing the percentage of serum added. The highly serum compatible transfection characteristics of lipids **4** and **5** (up to 50%) may be related to enhanced surface charge shielding of the lipid/DNA complexes induced by the multiple hydroxyl functionalities in their polar headgroup regions.

Conclusions

In summary, an efficient and novel series of tocopherol based cationic lipids was developed to be used in in vitro liposomal gene delivery. DOPC is found to be an efficient colipid when compared to DOPE. The transfection efficiencies of some of these new lipids, when used in combination with DOPC, were observed to be higher than that of commercial formulation. The higher transfection profiles of some of these lipids are further confirmed using inverted microscopic experiments. Structural investigation studies of these lipids clearly demonstrate the differences in transfection profiles. However, there is a necessity of further investigation on the targetability of these lipids to the liver.

Experimental Section

General Procedures and Materials. Mass spectral data were acquired by using a commercial LCQ ion trap mass spectrometer (ThermoFinnigan, SanJose, CA, U.S.) equipped with an ESI source. ^{1}H NMR spectra were recorded on a Varian FT 300 MHz NMR spectrometer. α-Tocopherol was purchased from Sigma Co. *p*-CMV-SPORT-β-gal plasmid, α5GFP plasmid, and rhodamine-PE were generous gifts from IICT (Indian Institute of Chemical Technology, Hyderabad, India). Lipofectamine-2000 was purchased from Invitrogen Life Technologies

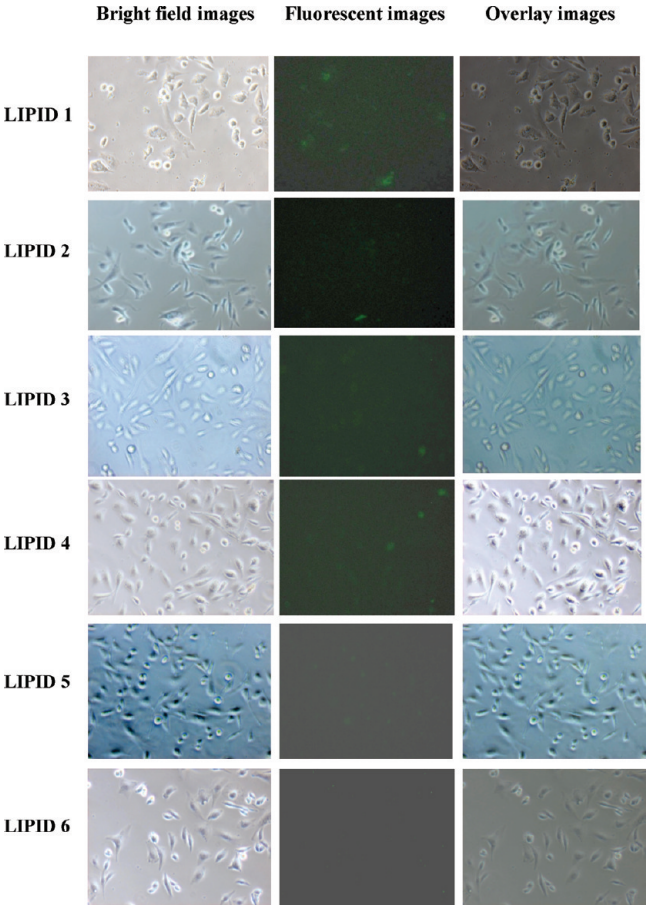


Figure 8. Cellular expression of GFP. Inverted microscopic images of CHO cells transfected with lipoplexes of lipids **1**−**5** and the control lipid **6** prepared at the highest in vitro transfection lipid/DNA charge ratios of 3:1: bright field images; fluorescent images; overlay images. The details of the experiments are as described in the text.

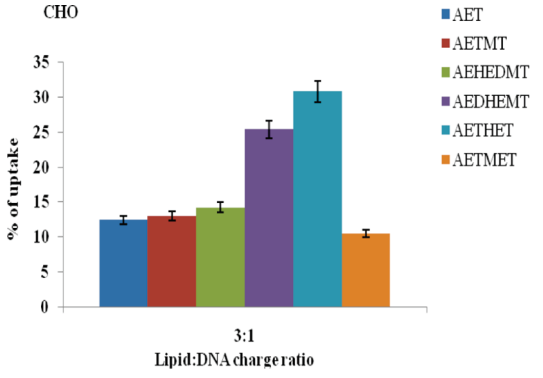


Figure 9. Cellular uptake of rhodamine labeled lipoplexes. The fluorescence of lysates of CHO cells transfected with rhodamine labeled lipoplexes of lipids **1**−**5** and the control lipid **6** was measured by a microplate fluorescent reader (FLX 800, Bio-Tek instruments Inc., U.S.) using filter sets 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.

(U.S.). Cell culture media, fetal bovine serum, 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, U.S.). NP-40, antibiotics, and agarose were purchased from Hi-media, India. DOPE and DOPC was purchased from Fluka (Switzerland). Unless otherwise stated, all the other reagents purchased from local commercial suppliers were of analytical grade 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. All the synthesized lipids (**1**−**6**) showed more than 95% purity.

Synthesis of *O*-Mesyl-α-tocopherol (I, Scheme 1). To a solution of α-tocopherol (4.0 g, 9.0 mmol) in 10 mL of dry DCM were added methanesulfonyl chloride (2.12 g, 18 mmol), pyridine (1.46 g, 18 mmol), and a catalytic amount of DMAP. The reaction mixture was stirred at room temperature overnight. The solvent was evaporated in vacuum to dryness. The residue was dissolved in 25 mL of ethyl acetate and washed twice with 2 × 20 mL of copper sulfate solution to remove any excess pyridine. The organic layer was dried on anhydrous sodium sulfate, the solvent was evaporated, and the sample was purified by column chromatography, eluting with 2−3% (v/v) ethyl acetate in *n*-hexane to obtain 4.0 g (yield 85.10%, R_f = 0.4, 10% ethyl acetate in hexane) of *O*-mesytyl-α-tocopherol. ^{1}H NMR (300 MHz, $CDCl_3$) δ/ppm 0.8−0.90 [m, 12H, CH−CH_3 tocopheryl], 1.00−1.4 [m,18H, −(CH_2)$_9$ tocopheryl], 1.56 [s, 3H, CH_3-2 tocopheryl], 1.6−1.9 [m, 3H, CH−CH$_3$ tocopheryl], 2.05 [s, 3H, CH_3-5 tocopheryl], 2.19 [s, 3H, CH_3-8 tocopheryl], 2.22 [s, 3H, CH_3-7 tocopheryl], 2.55−2.6 [t, 2H, CH_2-4 tocopheryl], 3.22 [s, 3H, SO_2−CH_3].

Synthesis of *O*-Aminoethyl-α-tocoherol, AET (Lipid 1, Scheme 1). A mixture of ethanolamine (0.5 g, 8.18 mmol) and potassium hydroxide (∼5.1 g, 90.9 mmol) taken in 10 mL of toluene in a two-necked round bottomed flask fitted with a Dean−Stark apparatus is refluxed for 2 h to remove the water as azeotrope mixture. To the reaction mixture, mesylated tocopherol (4.16 g, 8.18 mmol) is added and refluxed at 80 °C for 48 h. The reaction mixture was taken into ethyl acetate (100 mL), washed with water (2 × 100 mL), dried over anhydrous magnesium sulfate, and filtered. Ethyl acetate is removed from the filtrate on a rotary evaporator. The column chromatographic purification of the resulting residue using 60−120 mesh size silica gel and eluting with 1−2% methanol (v/v) in chloroform afforded 2.4 g (63.15% yield R_f = 0.4, 5% methanol in chloroform) of the tertiary amine. Then 0.5 g (1.05 mmol) of purified tertiary amine was dissolved in 5 mL of (1:1 v/v) a mixture of chloroform and methanol, and 1 mL of 1 N HCl was added at 0 °C. The resulting solution was left stirred at room temperature for overnight. Excess HCl was removed by flushing with nitrogen to give the title compound as a hydrochloride salt. Column chromatographic purification using 60−120 mesh size silica gel and 3−4% (v/v) methanol/chloroform as eluent followed by chloride ion exchange chromatography using Amberlyst A-26 chloride ion-exchange resin afforded lipid **1** as a dark yellow liquid (0.92 g, yield 92%, R_f = 0.2, 5% methanol in chloroform). ^{1}H NMR (300 MHz, $CDCl_3$) δ/ppm 0.85−0.90 [m, 12H, CH−CH_3 tocopheryl], 1.35 [s, 3H, CH_3-2 tocopheryl], 1.00−1.4 [m, 18H, −(CH_2)$_9$ tocopheryl], 1.7−1.8 [m, 2H, CH_2-3 tocopheryl], 2.05 [s, 3H, CH_3-5 tocopheryl], 2.15 [s, 3H, CH_3-8 tocopheryl], 2.18 [s, 3H, CH_3-7 tocopheryl], 2.55−2.6 [t, 2H, CH_2-4 tocopheryl], 3.1−3.3 [t, 2H, N−CH$_2$−CH_2−O−], 3.7−3.9 [m, 2H, N−CH_2−CH$_2$−O−], 8.3−8.4 [s, 3H,NH_3−CH$_2$−CH$_2$−O−]. ESIMS (lipid **1**), *m*/*z*: 474 **[M$^+$] for $C_{31}H_{56}NO_2^+$. Elemental analysis, calculated: % N, 2.95; % C, 78.42; % H, 11.89. Observed: % N, 2.93; % C, 78.41; % H, 11.81.

Synthesis of *O*-Aminoethyl [*N*,*N*′,*N*′′-Trimethyl]-α-tocopherol, AETMT (Lipid 2, Scheme 1). The intermediate tertiary amine obtained in the synthesis of lipid **1** (1.0 g, 2.10 mmol) was taken in a 25 mL round-bottomed flask, and a huge excess of methyl

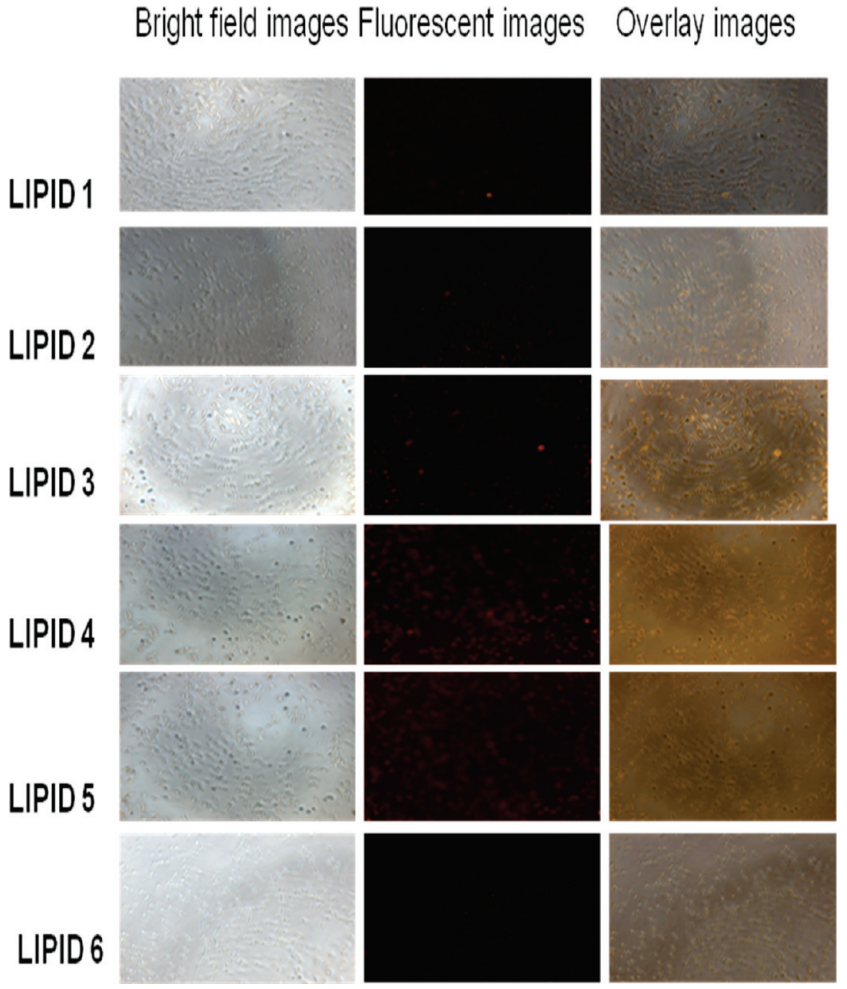


Figure 10. Cellular uptake of rhodamine labeled lipoplexes, shown by inverted microscopic images of CHO cells transfected with rhodamine labeled lipoplexes of lipids **1**−**5** and the control lipid **6** prepared at the highest in vitro transfection lipid/DNA charge ratios of 3:1: bright field images; fluorescent images; overlay images. The details of the experiments are as described in the text.

iodide (6 mL) was added to it. After the reaction mixture was stirred at room temperature for 4 days, the solvent was removed on a rotary evaporator. The column chromatographic purification of the resulting residue using 60−120 mesh size silica and 3−4% (v/v) methanol in chloroform as eluent afforded the title compound as a quaternary iodide salt (0.56 g, 51.85% yield, R_f = 0.7, 10% methanol/chloroform). Finally, the pure title lipid **2** (0.5 g, 89.28% yield) 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 0.8−0.95 [m, 12H, CH−C$\underline{H}_3$ tocopheryl], 1.55 [s, 3H, C$\underline{H}_3$-2 tocopheryl], 1.00−1.4 [m, 18H, −(C$\underline{H}_2$)$_9$ tocopheryl], 1.8−1.9 [m, 2H, C$\underline{H}_2$-3 tocopheryl], 2.05 [s, 3H, C$\underline{H}_3$-5 tocopheryl], 2.15 [s, 3H, C$\underline{H}_3$-8 tocopheryl], 2.20 [s, 3H, C$\underline{H}_3$-7 tocopheryl], 2.55−2.6 [t, 2H, C$\underline{H}_2$-4 tocopheryl], 3.6−3.7 [s, 9H, N−C$\underline{H}_3$], 4.1−4.2 [m, 2H, N−C$\underline{H}_2$−CH$_2$−O−], 4.2−4.3 [m, 2H, N−CH$_2$−C$\underline{H}_2$−O−]. ESIMS (lipid **2**), m/z: 516 [M^+ + 1] for $C_{34}H_{62}NO_2^+$. Elemental analysis, calculated: % N, 2.71; % C, 79.01; % H, 12.09. Observed: % N, 2.74; % C, 79.05; % H, 12.05.

Synthesis of AEHEDMT (Lipid 3, Scheme 2). Step a: Synthesis of Intermediated Tertiary Amine: *O*-Aminoethyl-[*N*-hydroxyethyl,*N*′-methyl]-α-tocopherol (II), Scheme 2. The intermediate tertiary amine is synthesized by taking a mixture of *N*-methyldiethanolamine (0.5 g, 4.19 mmol), potassium hydroxide (∼2.82 g, 50.2 mmol), and mesylated tocopherol (2.13 g, 4.19 mmol) and following the same procedure as given in the synthesis of lipid **1**. The reaction mixture was taken into ethyl acetate (100 mL), washed with water (2 × 100 mL), dried over anhydrous magnesium sulfate, and filtered. Ethyl acetate was removed from the filtrate on a rotary evaporator. The column chromatographic purification of the resulting residue using 60−120 mesh size silica and eluting with 1−2% methanol (v/v) in chloroform afforded 0.98 g (44.14% yield, R_f = 0.9, 10% methanol in chloroform) of the tertiary amine. ^{1}H NMR (300 MHz, $CDCl_3$) δ/ppm 0.8−0.9 [m, 12H, CH−C$\underline{H}_3$ tocopheryl], 1.45 [s, 3H, C$\underline{H}_3$-2 tocopheryl], 1.00−1.4 [m, 18H, −(C$\underline{H}_2$)$_9$ tocopheryl], 1.8−1.9 [m, 2H, C$\underline{H}_2$-3 tocopheryl], 1.95−2.05 [s, 3H, N−C$\underline{H}_3$], 2.05 [s, 3H, C$\underline{H}_3$-5 tocopheryl], 2.15 [s, 3H, C$\underline{H}_3$-8 tocopheryl], 2.20 [s, 3H, C$\underline{H}_3$-7 tocopheryl], 2.75−2.85 [t, 2H, C$\underline{H}_2$-4 tocopheryl], 2.8−3.1 [m, 2H, HO−CH$_2$−C$\underline{H}_2$−N−CH$_2$−CH$_2$−O−], 3.25−3.85 [m, 4H, HO−C$\underline{H}_2$−CH$_2$−N−C$\underline{H}_2$−CH$_2$−O−], 3.9−4.15 [broad, 2H, HO−CH$_2$−CH$_2$−N−CH$_2$−C$\underline{H}_2$−O−]. ESIMS, m/z: 532 [M^+ + 1] for $C_{34}H_{61}NO_3^+$.

Step b: Synthesis of *O*-Aminoethyl-[*N*-hydroxyethyl,*N*′,*N*′′-dimethyl]-α-tocopherol, AEHEDMT (Lipid 3, Scheme 2). The intermediate tertiary amine obtained in step a of the synthesis of lipid **3** (0.5 g, 0.93 mmol) was quaternized using methyl iodide. The quaternization is carried out following the procedure given in the synthesis of lipid **2** that yields the title compound as a quaternary iodide salt (0.26 g 50.68% yield, R_f = 0.6, 10% methanol/chloroform). Finally the pure title lipid **3** (0.23 g, 88.46% yield) was obtained by subjecting the quaternized ammonium iodide salt to "repeated chloride ion exchange

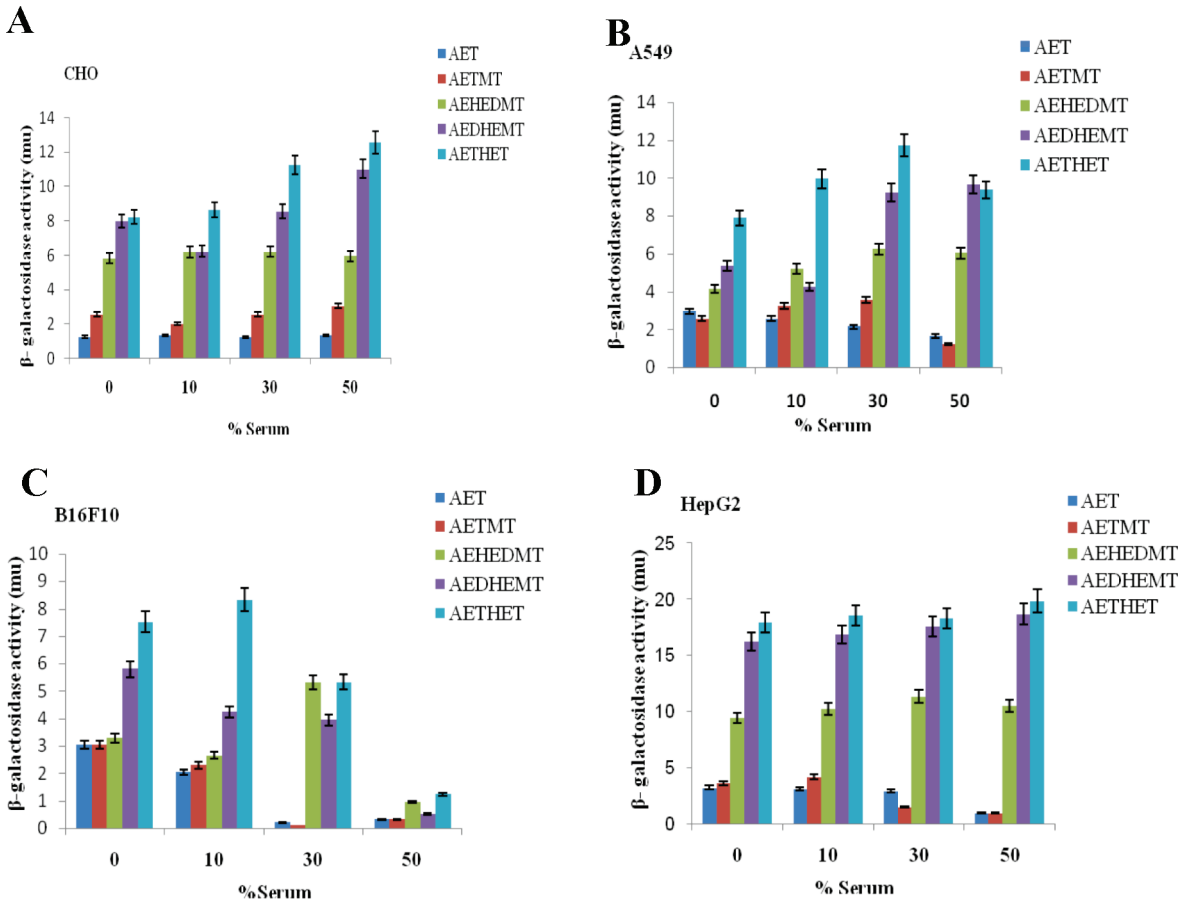


Figure 11. Transfection efficacies of the cationic lipids **1**−**5** in the presence of increasing concentrations of added serum. In vitro transfection efficiencies of lipid/DNA complexes prepared using *p*CMV-*β*-gal-SPORT reporter gene at lipid/DNA charge ratio of 3:1 were evaluated in the presence of increasing concentrations of added serum in CHO (A), A-549 (B), B16F10 (C), and HepG2 (D) types of cells.

chromatography" using Amberlyst A-26 chloride ion exchange column and 50 mL of chloroform as eluent. ^{1}H NMR (300 MHz, $CDCl_3$) δ/ppm 0.75−0.9 [m, 12H, CH−CH_3 tocopheryl], 1.55 [s, 3H, CH_3-2 tocopheryl], 1.00−1.35 [m,18H, −(CH_2)$_9$ tocopheryl], 1.8−1.9 [m, 2H, CH_2-3 tocopheryl], 2.05 [s, 3H, CH_3-5 tocopheryl], 2.15 [s, 3H, CH_3-8 tocopheryl], 2.20 [s, 3H, CH_3-7 tocopheryl], 2.55−2.6 [t, 2H, CH_2-4 tocopheryl], 3.7−3.8 [s, 6H, N−CH_3], 4.1−4.2 [m, 4H, HO−CH$_2$−CH_2−N−CH$_2$−CH$_2$−O−], 4.35−4.45 [m, 4H, HO−CH_2−CH$_2$−N−CH$_2$−CH_2−O−] . ESIMS (lipid **3**), *m*/*z*: 546 [M$^+$] for $C_{35}H_{64}NO_3^+$. Elemental analysis, calculated: % N, 2.56; % C, 76.87; % H, 11.80. Observed: % N, 2.54; % C, 76.45; % H, 11.35.

Synthesis of AEDHEMT (Lipid 4, Scheme 3). Step a: Synthesis of Intermediate Tertiary Amine: *O*-Aminoethyl-[*N*,*N*′-di-(hydroxyethyl)]-α-tocopherol (III), Scheme 3. The intermediate tertiary amine is synthesized by taking a mixture of triethanolamine (1 g, 6.70 mmol), mesylated tocopherol (3.40 g, 6.70 mmol), and potassium hydroxide (∼4.51 g, 80.39 mmol) 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 and eluting with 1.5−2.5% methanol (v/v) in chloroform afforded 1.3 g (35.27% yield, R_f = 0.8, 10% methanol in chloroform) of the tertiary amine. ^{1}H NMR (300 MHz, $CDCl_3$) δ/ppm 0.8−0.9 [m, 12H, CH−CH_3 tocopheryl], 1.45 [s, 3H, CH_3-2 tocopheryl], 1.00−1.4 [m,18H, −(CH_2)$_9$ tocopheryl], 1.8−1.9[m, 2H, CH_2-3 tocopheryl], 2.05 [s, 3H,CH_3-5 tocopheryl], 2.15 [s, 3H, CH_3-8 tocopheryl], 2.20 [s, 3H, CH_3-7 tocopheryl], 2.45−2.7 [m, 6H, N−CH_2−CH$_2$−O−, (N−CH_2−CH$_2$−OH)$_2$], 2.75−2.859 [t, 2H, CH_2-4 tocopheryl], 3.7−3.85 [m, 6H, N−CH$_2$−CH_2−O−, (N−CH$_2$−CH_2−OH)$_2$]. ESIMS, *m*/*z*: 544 [(M$^+$ − 18) + 1] for $C_{35}H_{63}NO_4^+$.

Step b: Synthesis of *O*-Aminoethyl-[*N*,*N*′-di(hydroxyethyl),*N*′′ methyl]-α-tocopherol, AEDHEMT (Lipid 4, Scheme 3). The intermediate tertiary amine obtained in step a of synthesis of lipid **4** (0.5 g, 0.88 mmol) was quaternized using methyl iodide. The quaternization is carried out following the procedure given in the synthesis of lipid **2** to give the title compound as a quaternary iodide salt,(0.3 g, 58.59% yield, R_f = 0.5, 10% methanol/chloroform). Finally the pure title lipid **4** (0.25 g, 83.33% yield) was obtained by subjecting the quaternized ammonium iodide salt to "repeated chloride ion exchange chromatography" using Amberlyst A-26 chloride ion exchange column and about 90 mL of chloroform as eluent. ^{1}H NMR (300 MHz, $CDCl_3$) δ/ppm 0.8−0.95 [m, 12H, CH−CH_3 tocopheryl], 1.35 [s, 3H, CH_3-2 tocopheryl], 1.00−1.35 [m,18H, −(CH_2)$_9$ tocopheryl], 1.75−1.9 [m, 2H, CH_2-3 tocopheryl], 2.15 [s, 3H, CH_3-5 tocopheryl], 2.18 [s, 3H, CH_3-8 tocopheryl], 2.20 [s, 3H, CH_3-7 tocopheryl], 2.55−2.65 [t, 2H, CH_2-4 tocopheryl], 3.6−3.75 [s, 3H, N−CH$_3$], 3.75−3.9 [m, 4H, N−CH_2-CH$_2$−OH], 4.05−4.15 [m, 2H, N−CH_2−CH$_2$−O−], 4.15−4.25 [m, 4H, N−CH$_2$−CH_2−OH], 4.3−4.4 [m, 2H, N−CH$_2$−CH_2−O−], 4.5−4.6 [brs, 2H,OH]. ESIMS (lipid **4**), *m*/*z*: 558 [M$^+$ − 18] for $C_{36}H_{66}NO_4^+$. Elemental analysis, calculated: % N, 2.43; % C, 74.95; % H, 11.53. Observed: % N, 2.54; % C, 74.45; % H, 11.45.

Synthesis of *O*-Aminoethyl-[*N*,*N*′,*N*′′-tri(hydroxyethyl)]-α-tocopherol, AETHET (Lipid 5, Scheme 3). The intermediate tertiary amine obtained in step a of the synthesis of lipid **4** (0.5 g, 0.88 mmol) was taken into a 25 mL round-bottomed flask, and a huge excess of bromoethanol (6 mL) was added to it. After the reaction mixture was refluxed for 4 days, the solvent was removed on a rotary evaporator. The column chromatographic

purification of the resulting residue using 60−120 mesh size silica and 3−4% (v/v) methanol in chloroform as eluent afforded the title compound as a quaternary bromide salt (0.3 g, 58.59% yield, R_f = 0.5, 10% methanol/chloroform). Finally the pure title lipid **5** (0.25 g, 83.33% yield) as a chloride salt was obtained by subjecting the quaternized ammonium bromide salt to "repeated chloride ion exchange chromatography" using Amberlyst A-26 chloride ion exchange column and 90 mL of chloroform as eluent. ^{1}H NMR (300 MHz, $CDCl_3$) δ/ppm 0.8−0.95 [m, 12H, CH−C$\underline{H}_3$ tocopheryl], 1.35 [s, 3H, C$\underline{H}_3$-2 tocopheryl], 1.00−1.35 [m, 18H, −(C$\underline{H}_2$)$_9$ tocopheryl], 1.75−1.8 [m, 2H, C$\underline{H}_2$-3 tocopheryl], 2.05 [s, 3H, C$\underline{H}_3$-5 tocopheryl], 2.11 [s, 3H, C$\underline{H}_3$-8 tocopheryl], 2.18 [s, 3H, C$\underline{H}_3$-7 tocopheryl], 2.45−2.55 [broad, 2H, C$\underline{H}_2$-4 tocopheryl], 3.55−3.65 [broad, 6H, N−C$\underline{H}_2$−CH$_2$−OH], 3.8−3.95 [m, 2H, N−C$\underline{H}_2$-CH$_2$−O−], 4.1−4.3 [m, 8H, N−CH$_2$−C$\underline{H}_2$−O−, N−CH$_2$−C$\underline{H}_2$−OH]. ESIMS (lipid **5**), m/z: 588 [$M^+ - 18$] for $C_{37}H_{68}NO_5^+$. Elemental analysis, calculated: % N, 2.31; % C, 73.22; % H, 11.29. Observed: % N, 2.34; % C, 73.45; % H, 11.35.

Plasmids. *p*CMV-SPORT-β-gal was amplified in DH5α strain of *Escherichia coli*, isolated by alkaline lysis procedure, and finally purified by PEG-8000 precipitation as described previously.[70] The purity of plasmid was checked by A_{260}/A_{280} ratio (around 1.9) and 1% agarose gel electrophoresis.

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% 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 or DOPE) (concentration of the each lipid and colipid is 0.5 mol, respectively) were dissolved in chloroform 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 4 h. Then 1 mL of sterile deionized water was added to the vacuum-dried lipid film and the mixture was allowed to swell overnight. The vial was then vortexed for 2−3 min at room temperature and occasionally sonicated in a bath sonicator to produce multilamellar vesicles (MLVs). MLVs were then sonicated in an ice bath until clarity using a Branson 450 sonifier at 100% duty cycle and 25 W output power. The resulting clear aqueous liposomes were used in forming lipoplexes.

ζ Potential and Size Measurements. The sizes and the surface charges (ζ potentials) of liposomes and lipoplexes were measured by photon correlation spectroscopy and electrophoretic mobility on a Zetasizer 3000HS$_A$ (Malvern, U.K.). The sizes were measured in deionized water 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, U.S.). The diameters of liposomes and lipoplexes were calculated by using the automatic mode. The ζ potential was measured using the following parameters: viscosity, 0.89 cP; dielectric constant, 79; temperature, 25 °C; $F(K_a)$, 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.

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. 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 the 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. 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%. The day to day variation in transfection efficiency values for identically treated transfection plates was mostly within 2- to 3-fold and was dependent on the cell density and condition of the cells.

DNA Binding Assay. DNA−lipid complexes were formed by mixing 5 μL of plasmid DNA (0.1 μg/μL in 10 mM Hepes buffer, pH 7.4) with varying amounts of cationic lipids so that the final lipid/DNA charge ratios were maintained at 0.3:1 to 9:1 in a total volume of 50 μL. Complexes were incubated for 30 min at room temperature after which 15 μL of each lipoplex was loaded on a 1% agarose gel and electrophoresed (100 V, 2 h). The bands were visualized with ethidium bromide staining.

DNase I Sensitivity Assay. Briefly, in a typical assay, 1.5 nmol of DNA (500 ng) was complexed with lipid using the indicated lipid/DNA charge ratios in DMEM in a volume of 40 μL, and the mixture was incubated at room temperature for 30 min on a rotary shaker. Subsequently, the complexes were treated with DNase I (at a final concentration of 1 ng/1.5 nmol of DNA) in the presence of 10 mM $MgCl_2$ and incubated for 20 min at 37 °C. The reactions were then halted by adding EDTA (to a final concentration of 50 mM), and the mixture was 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 10000g for 5 min. The aqueous supernatants were separated, loaded (15 μL) on a 1% agarose gel, and electrophoresed at 100 V for 2 h. The bands were visualized with ethidium bromide staining.

Toxicity Assay. Cytotoxicities of the lipids **1**−**5** were assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay as earlier reported.[71] The cytotoxicity assay was performed in 96-well plates by maintaining the same ratio of number of cells to amount of cationic lipid/DNA complexes, as 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 of MTT (5 mg/mL in PBS). 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.

Cellular Uptake Assay. CHO cells were plated in a 96-well plate at a density of 20 000 cells per well. After 16−24 h, cells were treated with lipoplexes comprising 0.3 μg of *p*CMV-SPORT-β-gal DNA and rhodamine-PE labeled liposomes at molar ratio (lipid/DNA) 3:1 according to the usual transfection procedure described above. After that, cells were lysed with 100 μL of 1× lysis buffer (0.1% NP 40) for 30 min and the fluorescence was measured by a microplate fluorescent reader (FLX 800, Bio-Tek Instruments Inc., U.S.) using filter sets for red channels. The fluorescence of lipoplexes with the same amount of cell-lysates (in a total volume of 100 μL) was also measured and considered as total or 100% fluorescence. The

percentage uptake was calculated using the formula

$$\% \text{ uptake} = 100 \times (\text{fluorescence intensity of the fluorescence lipoplex treated cell lysate} - \text{background}) / (\text{fluorescence intensity of lipoplex added to the cells} - \text{background})$$

Cellular Uptake Observed under Inverted Microscope. Cells were seeded at a density of 10 000 cells/well in a 24-well plate usually 18−24 h prior to the treatment. *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) using 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 completely from the wells and the cells were observed under inverted microscope.

Cellular α5GFP Expression Study. For cellular α5GFP expression experiments in CHO, 30 000 cells were seeded in each well of 24-well plate 18−24 h before the transfection. Then 0.9 μg of α5GFP plasmid DNA encoding green fluorescent protein was complexed with liposomes of lipids **1**−**5** and control lipid **6** at molar ratio (lipid/DNA) 3:1 in plain DMEM medium (total volume made up to 100 μL) for 30 min. Just prior to transfection, cells plated in the 24-well plate were washed with PBS (2 × 100 μL) followed by addition of lipoplexes. After 4 h of incubation 400 μL of complete media was added to the cells. After 24 h, the medium was removed from each well, and cells were washed with PBS (2 × 200 μL). Finally 200 μL of PBS was added to the cells and visualized under the inverted fluorescent microscope to observe the cells expressing the green fluorescent protein.

Transfection Biology in 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**−**5**) 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, at which all 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.

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Supporting Information Available: ^{1}H NMR spectra of lipids **1**−**6** and intermediates; mass spectra of lipids **1**−**6** and intermediates; elemental analysis data for lipids **1**−**6**; synthesis of control lipid **6**; gel retardation assay and sensitivity of the lipoplexes upon treatment with DNase I using gel electrophoresis of cationic liposome/DNA complexes of lipids **4** and **5** in the presence of anionic lipids. This material is available free of charge via the Internet at http://pubs.acs.org.

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