# Common co-lipids, in synergy, impart high gene transfer properties to transfection-incompetent cationic lipids

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**Abstract Efficacious cationic transfection lipids usually need either DOPE or cholesterol as co-lipid to deliver DNA inside the cell cytoplasm in non-viral gene delivery. If both of these co-lipids fail in imparting gene transfer properties, the cationic lipids are usually considered to be transfection inefficient. Herein, using both the reporter gene assay in CHO, COS-1 and HepG2 cells and the whole cell histochemical X-gal staining assay in representative CHO cells, we demonstrate that common co-lipids DOPE, Cholesterol and DOPC, when act in synergy, are capable of imparting improved gene transfer properties to a novel series of cationic lipids (1–5). Contrastingly, lipids 1–5 became essentially transfection-incompetent when used in combination with each of the pure co-lipid components alone.**


*Keywords:* Co-lipids; Non-viral gene therapy; Cationic transfection lipids; Lipofection; Lipid:DNA interactions

## 1. Introduction

Clinical success of gene therapy critically depends upon the bio-safety and efficacies of transfection vectors used in delivering therapeutic genes into the body cells [1–3]. Broadly speaking, the contemporary transfection vectors are classified into two major categories: viral and non-viral. Although, recombinant retroviral vectors in particular, are remarkably efficient in transfecting body cells [4,5] they are potentially capable of: generating replication competent virus through various recombination events with the host genome; inducing inflammatory and adverse immunogenic responses; producing insertional mutagenesis through random integration into the host genome; etc. [6–9]. Recently, it has been reported that retrovirus vector insertion near the promoter of the proto-oncogene LMO2 in two human patients with X-linked severe combined immunodeficiency (SCID-XI) is capable of triggering deregulated premalignant cell proliferation with unexpected frequency [10]. Conversely, cationic lipids, because of their least immunogenic nature, robust manufacture, ability to deliver large pieces of DNA and ease in handling & preparation techniques, are finding increasing uses as the gene transfer reagents of choice in non-viral gene therapy [11–35].

A number of previously reported investigations [11,16–28], including our own [12–15,29–35], have demonstrated that cationic transfection lipids, in general, need to be used in combination with either 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) or Cholesterol as an auxiliary lipid (co-lipid). During screening of libraries of cationic lipids for their transfection properties, if none of these two co-lipids impart gene transfection properties, the lipids are usually considered to be transfection inefficient. Herein, using a novel series of non-glycerol backbone based cationic lipids with polar 2-hydroxyethyl and 2-aminoethyl head-group functionalities (**1**–**5**, Fig. 1), we demonstrate that the common co-lipids DOPE, cholesterol and 1,2-dioleyl-*sn*-glycero-3-phosphocholine (DOPC), when act in synergy, are capable of imparting high gene transfer properties to cationic lipids **1**–**5**. Contrastingly, both the reporter gene expression assay in COS-1, CHO and HepG2 cells and the whole cell histochemical X-gal staining assay in representative CHO cells convincingly demonstrated that the high gene transfection properties of these new lipids were essentially abolished when used in combination with equimolar amounts of individual pure co-lipid components alone. Electrophoresis gel patterns in DNase I sensitivity assay are consistent with the notion that the high transfection properties of the present cationic lipids in association with the equimolar amounts of DOPE, cholesterol and DOPC may partly originate due to reduced DNase I susceptibility of the corresponding lipoplexes. Taken together, the present findings support the notion that use of common co-lipids in synergy may turn out to be rewarding in future design of novel liposomal transfection kits for use in non-viral gene therapy.

## 2. Materials and methods

### 2.1. General procedures and materials

FABMS data were acquired by the liquid secondary ion mass spectrometry (LSIMS) technique using *meta*-nitrobenzyl alcohol as the matrix. LSIMS analysis was performed in the scan range 100–1000 amu at the rate of 3 scans/s. $^1$H NMR spectra were recorded on

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*Abbreviations:* DMEM, Dulbecco's modified Eagle's medium; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; FBS, fetal bovine serum; DOPE, 1,2-dioleyl-sn-glycero-3-phosphoethanolamine; DOPC, 1,2-dioleyl-*sn*-glycero-3-phosphocholine; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; MTT, 3-(4,5,-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; PBS, phosphate-buffered saline

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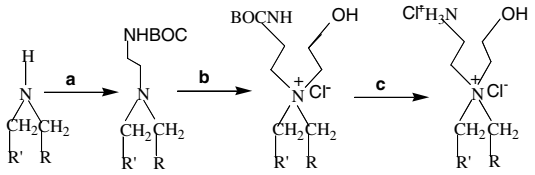


**Where**

**Lipid -1: R = R' = $C_{13}H_{27}$**
**Lipid -2: R = R' = $C_{15}H_{31}$**
**Lipid -3: R = R' = $C_{17}H_{35}$**
**Lipid -4: R = $C_{17}H_{35}$, R' = $C_{17}H_{33}$ (oleyl)**
**Lipid -5: R = R' = $C_{17}H_{33}$ (oleyl)**

**Reagents: (a) Br-$(CH_2)_2$-NHBOC, $K_2CO_3$, ethyl acetate, reflux 48 h**
**(b) 2-chloroethanol (huge excess), $K_2CO_3$, reflux, 32h**
**(c) methanolic HCl, 3h, $Cl^-$ ion exchange resin**

Fig. 1. Synthesis of cationic lipids **1**–**5**.

a Varian FT 200 MHz, AV 300 MHz or Varian Unity 400 MHz. 1-Bromotetradecane, 1-bromohexadecane, 1-bromooctadecane, *n*-tetradecylamine, *n*-hexadecylamine, *n*-octadecylamine were procured from Lancaster (Morecambe, UK). Unless otherwise stated all reagents were purchased from local commercial suppliers and were used without further purification. The progress of the reactions was monitored by thin-layer chromatography on 0.25 mm silica gel plates. Column chromatography was performed with silica gel (Acme Synthetic Chemicals, India, 60–120 mesh). Lipofectamine was purchased from Invitrogen life technologies (USA). Cell culture media, fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), polyethylene glycol 8000, *o*-nitrophenyl-β-D-galactopyranoside (ONPG) and cholesterol were purchased from Sigma, St. Louis, USA. NP-40, antibiotics and agarose were purchased from Hi-media, India. DOPE and DOPC were purchased from Fluka (Switzerland). Unless otherwise stated all the other reagents purchased from local commercial suppliers were of analytical grades and were used without further purification. Purity of all the final lipids (**1**–**5**, Fig. 1) was determined to be more than 95% by analytical HPLC (Shimadzu Model LC10A) using a PARTISIL 5 ODS-3 WCS analytical column (4.6 × 250 mm, Whatman Inc., Clifton, NJ, USA) in two different mobile phases. One solvent system (A) was methanol:acetonitrile:water:trifluoroacetic acid in the ratio 65:10:25:0.05 (v/v) for 15 min with a flow rate of 0.8 mL/min. The other mobile phase (B) was methanol:water: trifluoroacetic acid in the ratio 75:25:0.05 for 15 min with a flow rate of 0.8 mL/min. Peaks were detected by UV absorption at 219 nm. Typical retention times in mobile phase B were: 3.62 min (lipid **1**); 3.61 min (lipid **2**); 3.60 min (lipid **3**); 3.69 min (lipid **4**); 3.59 min (lipid **5**).

### 2.2. Synthesis of N-2-aminoethyl-N,N-di-n-hexadecylamine, N-2-hydroxyethylammonium chloride

*2.2.1. Step (a). Synthesis of N,N-di-n-hexadecyl-N-[2-(N-tert-butyloxycarbonyl)aminoethyl]amine (II, Fig. 1).* A mixture of 2.2 g (4.7 mmol) of *N*,*N*-di-*n*-hexadecylamine (I, Fig. 1, prepared conventionally by refluxing one equivalent each of *n*-hexadecylamine and *n*-hexadecyl bromide in ethyl acetate in presence of 1.1 equivalent of anhydrous potassium carbonate followed by usual work up and column chromatographic purification) and 1.1 g (5.2 mmol) of *N*-*tert*-butoxycarbonyl-2-bromoethylamine (prepared by reacting one equivalent each of 2-bromoethylamine hydrobromide and BOC-anhydride in presence of 2.2 equivalent of triethyl amine in 1:1 dicholoromethane/*N*,*N*-dimethylformamide, v/v, followed by usual work up) was refluxed in 10 mL ethyl acetate in presence of anhydrous potassium carbonate (1.4 g, 10 mmol) for 48 h. The reaction mixture was taken in 100 mL chloroform, washed with water (2 × 100 mL), dried over anhydrous magnesium sulfate and filtered. Chloroform was removed from the filtrate on a rotary evaporator. Silica gel column chromatographic purification of the resulting residue using 60–120 mesh silica gel size and 6% acetone in pet ether (v/v) as the eluent afforded the title compound as light yellow solid (2.0 g, 71% yield, $R_f$ = 0.5, 30:70, v/v, ethyl acetate:pet-ether).

$^1$H NMR (200 MHz, $CDCl_3$): δ/ppm = 0.9 [t, 6H, $CH_3$–$(CH_2)_{12}$–]; 1.2–1.4 [m, 56H, –$(CH_2)_{14}$–]; 1.5 [s, 9H, –$C(CH_3)_3$]; 2.4 [t, 4H, N($CH_2$–$CH_2$–)$_2$]; 2.5 [t, 2H, N($CH_2$–$CH_2$–NHBOC –)]; 3.1 [m, 2H, N($CH_2$–$CH_2$–NHBOC)]; 4.9 [bm, 1H, N*H*BOC].

*2.2.2. Step (b). Synthesis of N-2-(N-tert-butyloxycarbonyl)aminoethyl-N,N-di-n-hexadecylamine-N-2-hydroxyethylammonium chloride (III, Fig. 1).* 0.18 g (329 mmol) of the intermediate tertiary amine II obtained above in a step was dissolved in huge excess (3 mL) of 2-chloroethanol and anhydrous $K_2CO_3$ (0.22 g, 33 mmol) was added to the solution. The solution was allowed to reflux at 80 °C for 32 h. The reaction mixture was filtered and the unreacted excess 2-choroethanol was removed by repeated chasing with methanol on a rotary evaporator. The residue upon column chromatographic purification using 60–120 mesh size silica gel and 3–4% methanol in dichloromethane (v/v) as eluent afforded the title compound as a white solid (0.08 g, 43% yield, $R_f$ = 0.5, 10% methanol in dichloromethane, v/v).

$^1$H NMR(200 MHz, $CDCl_3$): δ/ppm = 0.9 [t, 6H, $CH_3$–$(CH_2)_{14}$–]; 1.2–1.3 [m, 52H, –$CH_3$ $(CH_2)_{13}$–]; 1.4–1.5 [s, 9H, –CO–O–C($CH_3$)$_3$]; 1.65 [m, 4H, –N$^+$(–$CH_2$–$CH_2$–)$_2$]; 3.4 [m, 4H, –N$^+$(–$CH_2$–$CH_2$–)$_2$]; 3.6 [bm, 6H, –N$^+$–$CH_2$–$CH_2$–NH–Boc; –N$^+$–$CH_2$–$CH_2$–NH–BOC; –N$^+$–$CH_2$–$CH_2$–OH ]; 4.0 [m, 2H, –N$^+$–$CH_2$–$CH_2$–OH]; 5.6 [m, 1H, –N*H*BOC]; 6.5 [m, 1H, –N$^+$–$CH_2$–$CH_2$–O*H* ].

*2.2.3. Step (c). Synthesis of N-2-aminoethyl-N,N-di-n-hexadecylamine-N-2-hydroxyethyl ammonium chloride.HCl (lipid 2).* The intermediate obtained above in step (b) (0.08 g, 0.14 mmol) was dissolved in 1.5 mL of methanol and 0.5 mL of 1 N HCl was added at 0 °C. The resulting solution was left stirred at room temperature for 3 h. 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 8–10% (v/v) methanol-chloroform as eluent followed by chloride ion exchange chromatography using amberlyst A-26 chloride ion exchange resin afforded lipid **2** as a white solid (0.06 g, 88% yield, $R_f$ = 0.2, 10% methanol in chloroform, v/v).

$^1$H NMR(200 MHz, $CD_3OD$): δ/ppm = 0.9 [t, 6H, $CH_3$–$(CH_2)_{13}$–]; 1.2–1.3 [m, 52H, –$CH_3(CH_2)_{13}$–]; 1.65 [m, 4H, –N$^+$(–$CH_2$–$CH_2$–)$_2$]; 3.4 [m, 6H, –N$^+$(–$CH_2$–$CH_2$–)$_2$, –N$^+$–$CH_2$–$CH_2$–OH]; 3.5–3.65 [bm, 4H, –N$^+$–$CH_2$–$CH_2$–$NH_3^+$, –N$^+$–$CH_2$–$CH_2$–$NH_3^+$]; 4.0 [m, 2H, –N$^+$–$CH_2$–$CH_2$–OH].

LSIMS (lipid **2**): *m*/*z*: 554 [M]$^+$ (calcd for $C_{36}H_{78}N_2O$, 83%).

### 2.3. Synthesis of lipids *1* and *3*–*5*

Lipids **1** and **3**–**5** were prepared following the same detail synthetic procedure as described above for the representative lipid **2** except using the appropriate starting secondary amines (I, Fig. 1). All the isolated intermediates gave spectroscopic data in agreement with their structures shown in Fig. 1. The $^1$H NMR and the LSIMS mass spectral data of lipids 1 and 3–5 are provided below.

*2.3.1. N-2-aminoethyl-N,N-di-n-tetradecylamine-N-2- hydroxyethylammonium chloride·HCl (lipid 1).* $^1$H NMR(200 MHz, $CD_3OD$): δ/ppm = 0.9 [t, 6H, $CH_3$–$(CH_2)_{11}$–]; 1.2–1.3 [m, 44H, –$CH_3(CH_2)_{11}$–]; 1.65 [m, 4H, –N$^+$(–$CH_2$–$CH_2$–)$_2$]; 3.4 [m, 6H, –N$^+$(–$CH_2$–$CH_2$–)$_2$, –N$^+$–$CH_2$–$CH_2$–OH]; 3.5–3.65 [bm, 4H, –N$^+$–$CH_2$–$CH_2$–$NH_3^+$, –N$^+$–$CH_2$–$CH_2$–$NH_3^+$]; 4.0 [m, 2H, –N$^+$–$CH_2$–$CH_2$–OH].

LSIMS (lipid **1**): *m*/*z*: 499 [M + 1]$^+$ (calcd for $C_{32}H_{70}N_2O$, 100%).

*2.3.2. N-2-aminoethyl-N,N-di-n-octadecylamine-N-2-hydroxyethylammonium chloride·HCl (lipid 3).* $^1$H NMR(200 MHz, $CD_3OD$): δ/ppm = 0.9 [t, 6H, $CH_3$–$(CH_2)_{15}$–]; 1.2–1.3 [m, 60H, –$CH_3(CH_2)_{15}$–]; 1.65 [m, 4H, –N$^+$(–$CH_2$–$CH_2$–)$_2$]; 3.4 [m, 6H, –N$^+$(–$CH_2$–$CH_2$–)$_2$, –N$^+$–$CH_2$–$CH_2$–OH]; 3.5–3.65 [bm, 4H, –N$^+$–$CH_2$–$CH_2$–$NH_3^+$, –N$^+$–$CH_2$–$CH_2$–$NH_3^+$]; 4.0 [m, 2H, –N$^+$–$CH_2$–$CH_2$–OH].

LSIMS (lipid **3**): *m*/*z*: 611 [M + 1]$^+$ (calcd for $C_{40}H_{82}N_2O$, 100%).

*2.3.3. N-2-aminoethyl-N-oleyl-N-n-octadecylamine-N-2-hydroxyethylammonium chloride·HCl (lipid 4).* $^1$H NMR (200 MHz, $CD_3OD$): δ/ppm = 0.9 [t, 6H, $CH_3$–$(CH_2)_{15}$–]; 1.2–1.3 [m, 52H, –$CH_3(CH_2)_{15}$–, $CH_3$–$(CH_2)_6$–$CH_2$–CH═CH–$CH_2$– $(CH_2)_5$–]; 1.65 [m, 4H, –N$^+$(–$CH_2$–$CH_2$–)$_2$]; 1.90– 2.1 [m, 4H, –$CH_2$–CH═CH–$CH_2$]; 3.4 [m, 6H, –N$^+$(–$CH_2$–$CH_2$–)$_2$, –N$^+$–$CH_2$–$CH_2$–OH]; 3.5–3.65 [bm, 4H, –N$^+$–$CH_2$–$CH_2$–$NH_3^+$, –N$^+$–$CH_2$–$CH_2$–$NH_3^+$]; 4.0 [m, 2H, –N$^+$–$CH_2$–$CH_2$–OH]; 5.3 [m, 2H, –$CH_2$–C*H*═C*H*–$CH_2$].

LSIMS (lipid **4**): *m*/*z*: 609 [M + 1]$^+$ (calcd for $C_{40}H_{80}N_2O$, 100%).

*2.3.4. N-2-aminoethyl-N,N-di-n-oleyl-N-2-hydroxyethylammonium chloride·HCl (lipid 5).* $^1$H NMR (200 MHz, $CD_3OD$): δ/ppm = 0.9 [t, 6H, $CH_3$–$(CH_2)_{15}$–]; 1.2–1.3 [m, 44H, $CH_3$–$(CH_2)_6$–$CH_2$–

CH=CH–$CH_2$–($CH_2$)$_5$–]; 1.65 [m, 4H, –$N^+$(–$CH_2$–$CH_2$–)$_2$]; 1.90–2.1 [m, 8H, –$CH_2$–CH=CH–$CH_2$]; 3.4 [m, 6H, –$N^+$(–$CH_2$–$CH_2$–)$_2$, –$N^+$–$CH_2$–$CH_2$–OH]; 3.5–3.65 [bm, 4H, –$N^+$–$CH_2$–$CH_2$–$NH_3^+$, –$N^+$–$CH_2$–$CH_2$–$NH_3^+$]; 4.0 [m, 2H, –$N^+$–$CH_2$–$CH_2$–OH]; 5.3 [m, 4H, –$CH_2$–CH=CH–$CH_2$].

LSIMS (lipid **5**): *m/z*: 606 $[M]^+$ (calcd for $C_{40}H_{78}N_2O$, 100%).

### 2.4. Cell culture

COS-1 (SV 40 transformed african green monkey kidney cells), CHO (Chinese hamster ovary) 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% $CO_2$.

### 2.5. Plasmids

pCMV-SPORT-β-gal plasmids were generous gifts from Dr. Leaf Huang (Department of Pharmacogenetics, University of Pittsburgh, School of Medicine, Pittsburgh, PA, USA) and Dr. Nalam Madhusudhana Rao (Centre for Cellular and Molecular Biology, Hyderabad, India). The plasmids were amplified in DH5α strain of *Escherichia coli*, isolated by alkaline lysis procedure and finally purified by PEG-8000 precipitation as described previously [14]. The purity of plasmid was checked by $A_{260}/A_{280}$ ratio (around 1.9) and 1% agarose gel electrophoresis.

### 2.6. Preparation of liposomes and lipid–DNA complexes

Cationic lipids and the co-lipids in chloroform were dried under a stream of $N_2$ gas and vacuum-dessicated for a minimum of 6 h to remove residual organic solvent. The dried lipid film was hydrated in sterile deionized water at cationic lipid concentration of 1 mM for a minimum of 12 h. Liposomes were vortexed to remove any adhering lipid film and probe sonicated until a clear translucent solution formed. The *p*DNA solution was added to the liposomes, mixed properly by pipetting up and down a few times and kept at room temperature for 15–30 min before use.

### 2.7. In vitro transfection studies

Cells were seeded at a density of 15,000 (for COS-1) and 20000 (for CHO and HepG2) per well in a 96-well plate 18–24 h before transfection. 0.30 μg of *p*DNA was complexed with varying amounts of lipids (to give +/− ratios of 0.1:1, 0.3:1, 1:1, 3:1 and 9:1) in plain DMEM (total volume made up to 100 μl) for 20–30 min. The complexes were then added to the cells. After 3 h of incubation, 100 μl of DMEM with 20% FBS was added to the cells. The medium was changed to complete medium containing 10% FBS after 24 h and the reporter gene activity was estimated after 48 h. Cells were washed with phosphate-buffered saline (PBS, 100 μl) and lysed in 50 μl lysis buffer (0.25 M Tris–HCl, pH 8.0, 0.5% NP40). The β-galactosidase activity per well was estimated by adding 50 μl of 2×-substrate solution (1.33 mg/ml of ONPG, 0.2 M sodium phosphate, pH 7.3, 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 calibration curve constructed using pure commercial β-galactosidase enzyme. The transfection values reported are the average values from two replicate experiments performed in the same plate on the same day. Each transfection experiment was performed three times on three different days. The day-to-day variation in transfection efficiency was mostly within 2–3 fold and was dependent on the cell density and condition of the cells.

### 2.8. Toxicity assay

Cytotoxicities of the lipoplexes prepared from the lipids **1**–**5** were assessed by the MTT reduction assay as described earlier [14]. The cytotoxicity assay was performed in 96-well plates by maintaining the same ratio of number of cells to amount of cationic lipid, as used in the transfection experiments. MTT was added 3 h after addition of cationic lipid to the cells. Results were expressed as percent viability = $[A_{540}(\text{treated cells}) - \text{background}/A_{540}(\text{untreated cells}) - \text{background}] \times 100$.

### 2.9. Size measurments

The sizes of liposomes and lipoplexes were measured by photon correlation spectroscopy and electrophoretic mobility on a Zeta sizer 3000 $HS_A$ (Malvern UK). The sizes were measured in deionised water with a sample refractive index of 1.59 and a viscosity of 0.89. The system was calibrated by using the 200 nm + 5 nm polystyrene polymer (Duke Scientific Corp., Palo Alto, CA). The diameters of liposomes and lipoplexes were calculated by using the automatic mode.

### 2.10. DNA binding assay

The DNA binding abilities of the present cationic lipids in combination with varying co-lipids were assessed by the conventional gel retardation assay on a 1% agarose gel (pre-stained with ethidium bromide) across the varying lipid:DNA charge ratios of 1:1 to 9:1. *p*CMV-β-gal (0.30 μg) was complexed with the varying amount of cationic lipids in a total volume of 20 μl in HEPES buffer, pH 7.40, and incubated at room temperature for 20–25 min. 4 μl of 6× loading buffer (0.25% bromophenol blue in 40%, w/v, sucrose in $H_2O$) was added to it and the resulting solution, (24 μl) was loaded on each well. The samples were electrophoresed at 80 V for 45 min and the DNA bands were visualized in the Gel documentation unit.

### 2.11. DNase I sensitivity assay

Briefly, in a typical assay, *p*CMV-SPORT-β-gal (1000 ng) was complexed with the varying amounts of the representative cationic lipid **1** and co-lipids (pure and mixed) using the indicated lipid:DNA charge ratios in Fig. 5 in a total volume of 30 μL in HEPES buffer, pH 7.40, and incubated at room temperature for 30 min on a rotary shaker. Subsequently, the complexes were treated with 10 μL DNase I (at a final concentration of 1 μg/mL) in presence of 20 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 incubated at 60 °C for 10 min in a water bath. The aqueous layer was washed with 50 μL of phenol:chloroform:isoamylalcohol (25:24:1 mixture, v/v) and centrifuged at $10000 \times g$ for 5 min. The aqueous supernatants were seperated, loaded (15 μL) on a 1% agarose gel (pre-stained with ethydium bromide) and electrophoresed at 100 V for 1 h.

### 2.12. The whole cell histochemical 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining assay

Cells expressing β-galactosidase were histochemically stained with the substrate X-gal as described previously [14]. Briefly, 48 h after transfection with lipoplexes in 96-well plates, the cells were washed two times ($2 \times 100$ μL) with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$, pH 7.4) and fixed with 0.5% glutaraldehyde in PBS (225 μL). After 15 min incubation at room temperature, the cells were washed again with PBS three times ($3 \times 250$ μL) and subsequently, were stained with 1.0 mg/mL X-gal in PBS containing 5.0 mM $K_3[Fe(CN)_6]$, and 5.0 mM $K_4[Fe(CN)_6]$ and 1 mM $MgSO_4$ for 2–4 h at 37 °C. Blue colored cells were identified by light microscope (Leica, Germany).

## 3. Results and discussion

### 3.1. Chemistry

The synthetic process adopted for preparing lipids **1**–**5** is shown schematically in Fig. 1. The starting *N*,*N*-dialkylamines (I, Fig. 1) containing appropriate alkyl chain lengths upon refluxing with *tert*-butyloxycarbonyl protected 2-bromoethylamine in anhydrous ethyl acetate in presence of potassium carbonate followed by usual work-up afforded the intermediate tertiary amines (II, Fig. 1). The target lipids were prepared by quaternizing intermediates II with excess of 2-chloroethanol in presence of potassium carbonate followed by acid deprotection of quaternized intermediate (III, Fig. 1) and chloride ion exchange chromatography over Amberlyst A-26. The details of synthetic processes, spectral and analytical characterizations of all the intermediates and final lipids are provided above under Section 2.

*3.1.1. Transfection properties of lipids **1–5** with coventional co-lipids.* The findings in a number of previously reported investigations [11,16–28] including our own [12–15,29–35], demonstrated that cationic lipids, in general, need either DOPE or cholesterol to be used as an auxiliary lipid for transfecting cells. Based on these earlier observations, particularly given the high gene transfer efficacies of the dihydroxy analogs of the present lipids [32,33,35], our initial expectation was that the presently designed lipids **1–5** would exhibit reasonably good gene transfer properties in combination with either pure DOPE or pure cholesterol. However, in stark contrast to this expectation, the results in reporter gene expression assay (Figs. 2A–F) using *p*-CMV-SPORT-β-gal plasmid DNA revealed a surprising transfection profile. Except showing some weak to moderate efficacies in transfecting CHO cells at high lipid:DNA charge ratios in presence of DOPE, lipids **1–5** were found to be either essentially transfection-incompetent or poorly transfecting in COS-1, HepG2 and CHO cells when used with equimolar amounts of either pure DOPE or pure cholesterol as co-lipid (Figs. 2A–F). Similarly poor transfection profiles of lipids **1–5** were also observed when used in combination with DOPC (data not shown).

*3.1.2. Transfection properties of lipids **1–5** used in combination with equimolar amounts of DOPE, cholesterol and DOPC.* After observing the above-mentioned disappointing transfection profiles of lipids **1–5** used in combination with pure conventional co-lipids, we decided to probe the influence of mixture of commonly used co-lipids such as DOPE, cholesterol and DOPC, if any, in modulating the transfection profile of lipids **1–5**. To our surprise, we observed remarkably improved transfection efficacies of lipids **1** and **2** when used in combination with equimolar amounts of DOPE, cholesterol and DOPC. Such improved transfection profiles of lipids **1** and **2** were observed in all three cells (COS-1, HepG2 and CHO) when lipids **1–5** were

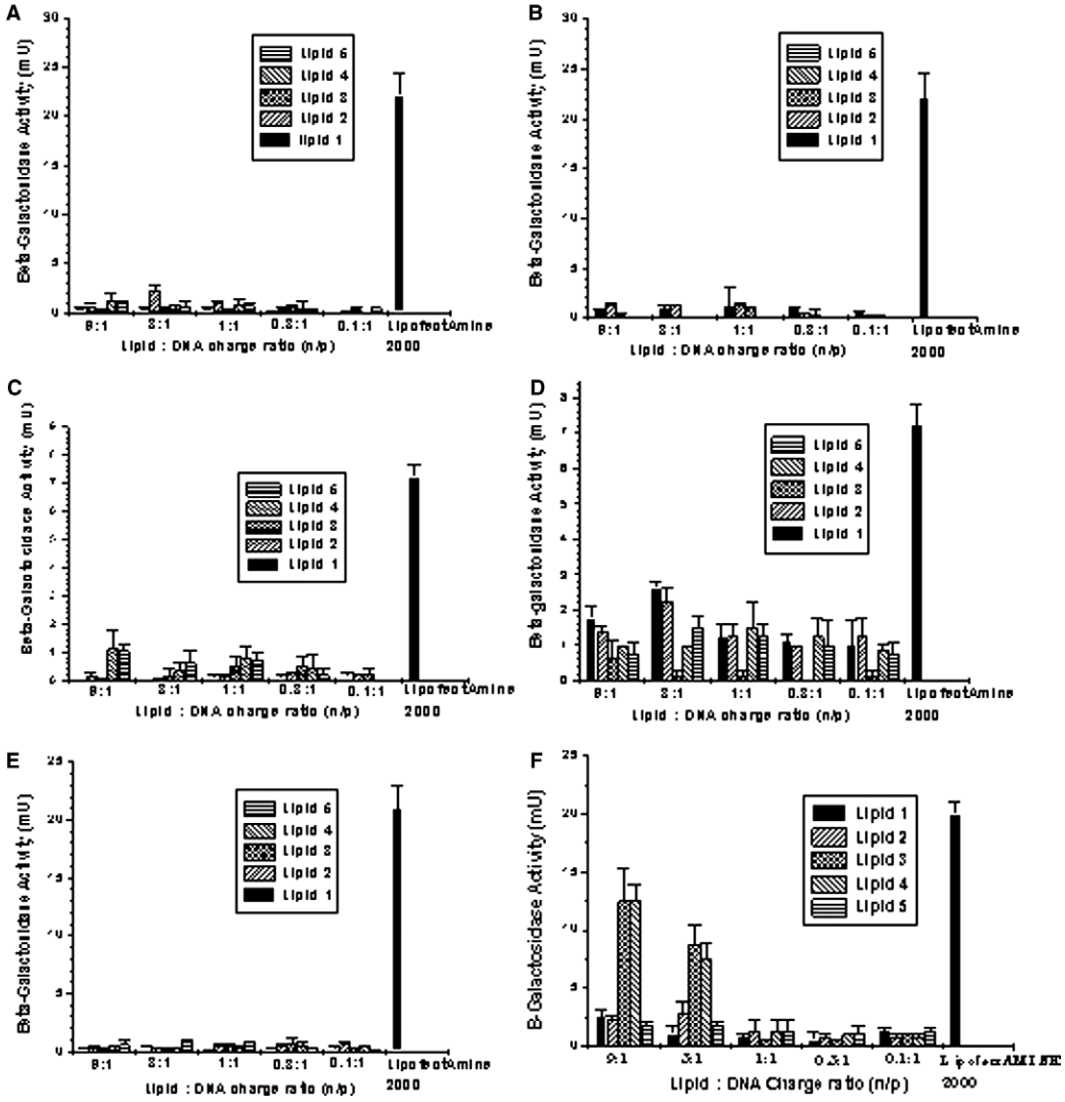


Fig. 2. In vitro transfection efficiencies of lipids **1–5** in presence of pure common co-lipids. The efficiencies of the lipids in transfecting COS-1 (A, B), HepG2 (C, D) and CHO (E, F) cells in combination with usual co-lipids cholesterol (A, C & E) and DOPE (B, D & F) were compared to that of the commercially available reagent lipofectamine 2000. The β-galactosidase activities in each well was converted to an absolute β-galactosidase milliunits using standard curve constructed with pure (commercial) β-galactosidase. All the lipids were tested on the same day and the data presented is the average value of two replicate experiments performed on the same day.

used at 1:1:1:1 mole ratio of lipid:DOPE:Chol:DOPC (Fig. 3A–C). Notably, the transfection efficacies, of lipid **1**, when used with 1:1:1:1 mole ratio of lipid:DOPE:Chol:DOPC, were found to be comparable to or better than that of commercially available lipofectamine in all the three cells (Fig. 3A–C). The maximum transfection efficacies of lipid **1** in combination with the mixed co-lipids were observed at lipid:DNA charge ratios of 1:1 or 3:1 in HepG2 and CHO cells (Fig. 3B and C) and at 9:1 lipid:DNA charge ratio in COS-1 cells (Fig. 3A). Towards evaluating the transfection profile at intermediate lipid:DNA charge ratios of 2:1 and 3:2, the efficacies of lipid **1** in transfecting the representative CHO cells were also separately measured at lipid:DNA charge ratios of 9:1, 3:1, 2:1, 3:2 and 1:1. Lipid **1** in combination with the mixed co-lipids at intermediate lipid:DNA charge ratios of 2:1 and 3:2 were found to be about 2-fold less than those at lipid:DNA charge ratio of 3:1 (Fig. 3D). However, lipid **1** used with pure DOPE or pure cholesterol remained transfection-incompetent at these intermediate lipid:DNA charge ratios in CHO cells (Fig. 3D). In addition, towards gaining insights into whether or not the enhanced transfection efficacies of the present lipids in presence of co-lipids are due to the nature or amount of co-lipids or both, transfection profiles of the most efficacious lipid **1** were evaluated in combination with each individual co-lipid using lipid:co-lipid mole ratios 1:3 in representative CHO cells. The transfection results shown in Fig. 4 revealed an interesting feature. Lipid **1** was found to be essentially transfection-incompetent when used in combination with the co-lipids cholesterol and DOPC at 1:3 mole ratios of lipid to co-lipid (Fig. 4). Thus, the enhanced transfection efficacies of lipids **1** and **2** in presence of equimolar amounts of common co-lipids are more likely due to the nature of the mixed co-lipids than their amounts. However, lipid **1** at 1:3 mole ratio of lipid:DOPE (particularly at 3:1 and 1:1 lipid:DNA charge ratios) was moderately efficient in transfecting CHO cells (being about two fold less efficient than lipid **1** used in combination with equimolar amounts of the mixed co-lipids, Figs. 3D and 4). All these transfection results, taken together, are consistent with the existence of an exquisite transfection enhancing properties of the equimolar mixture of the commonly used co-lipids DOPE, cholesterol and DOPC. Clearly, further cell biology experiments need to

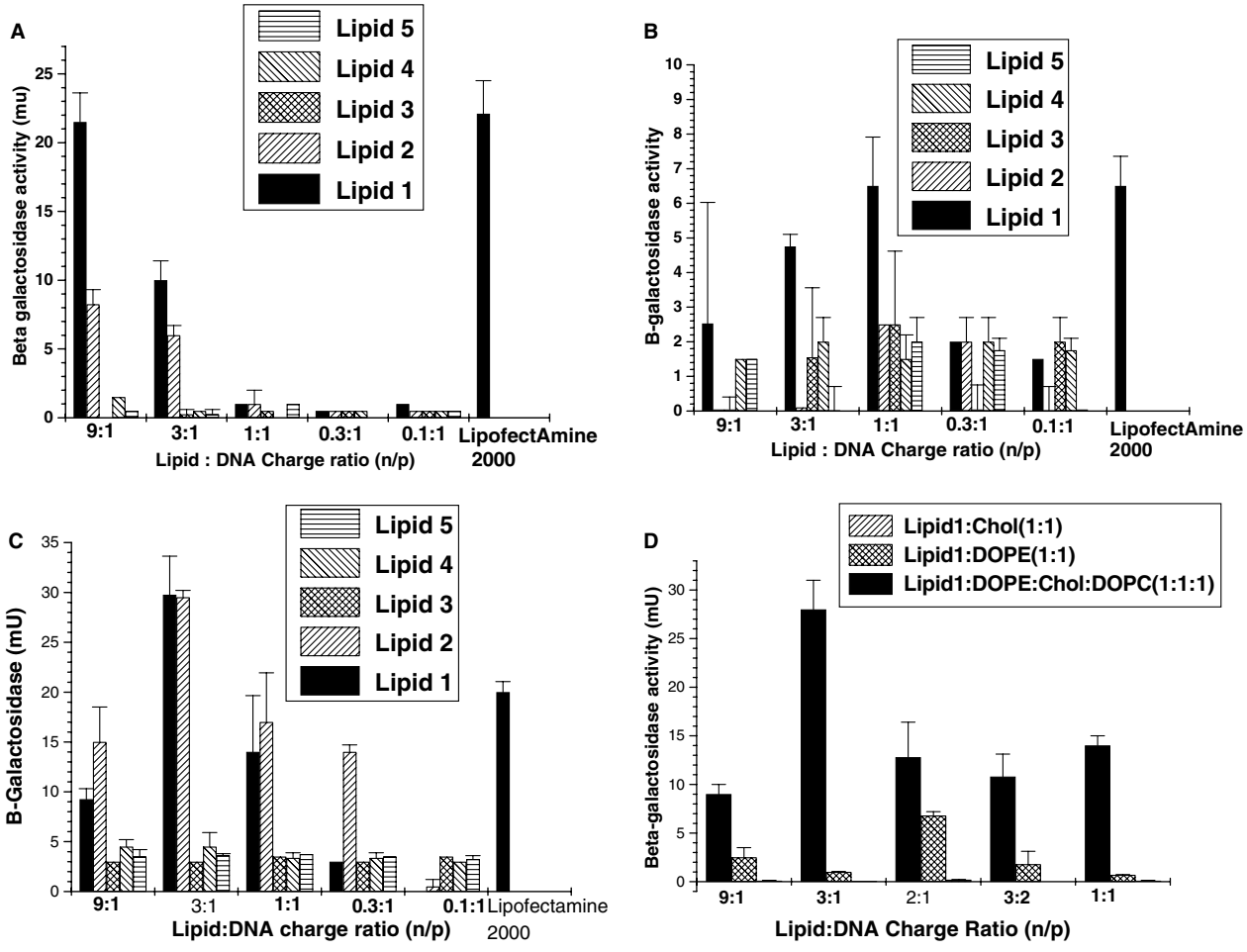


Fig. 3. In vitro transfection efficiencies of lipids **1**–**5** used in combination with equimolar amounts of mixture of common co-lipids. The efficiencies of the lipids in transfecting COS-1 (A), HepG2 (B) and CHO (C) cells in presence of DOPE/cholesterol/DOPC (at 1:1:1:1 mole ratio of lipid:DOPE:Chol:DOPC) were compared to that of the commercially available reagent lipofectamine 2000. (D) The efficacies of lipid **1** (used in combination with equimolar amounts of DOPE, cholesterol and DOPC as well as those in combination with pure DOPE and pure cholesterol) in transfecting representative CHO cells across the entire lipid:DNA charge ratios including the missing lipid:DNA charge ratios 2:1 and 3:2 in (A)–(C). The β-galactosidase activities in each well was converted to an absolute β-galactosidase milli-units using standard curve constructed with pure (commercial) β-galactosidase. All the lipids were tested on the same day and the data presented is the average value of two replicate experiments performed on the same day.

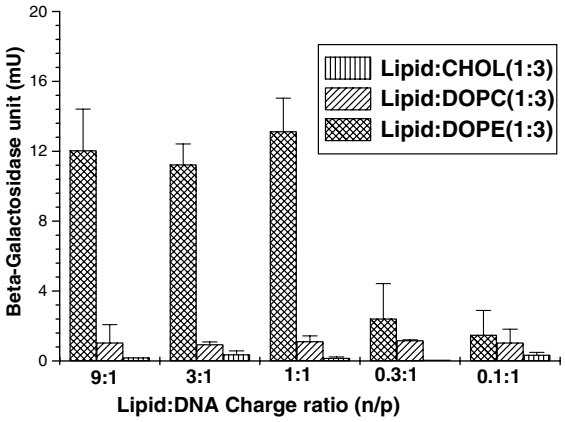


Fig. 4. In vitro transfection efficiencies of lipid **1** used in combination with pure co-lipids DOPE, cholesterol and DOPC at lipid:co-lipid mole ratios of 1:3 in representative CHO cells. The β-galactosidase activities in each well was converted to an absolute β-galactosidase milli-units using standard curve constructed with pure (commercial) β-galactosidase. All the lipids were tested on the same day and the data presented is the average value of two replicate experiments performed on the same day.

be carried out in future towards gaining mechanistic insights into the origin of the observed enhanced transfection properties of the present lipids in presence of equimolar mixtures of these common co-lipids.

The phenomenal influence of mixture of common co-lipids DOPE/Chol/DOPC towards imparting impressive transfection properties to otherwise essentially transfection incompetent cationic lipids **1**–**5** (Fig. 3A–C) was further confirmed by whole cell histochemical X-gal staining in representative CHO cells. Fully consistent with the findings in the reporter gene expression assay (Fig. 3C), the number of X-gal stained CHO cells transfected with lipid **1** used in combination with equimolar amounts of DOPE, cholesterol and DOPC were observed to be remarkably higher than that transfected with lipid **1** in combination with pure co-lipids DOPE and cholesterol (Fig. 5A). The percent of transfected and X-gal stained CHO cells for all the lipids **1**–**5** in combination with equimolar amounts of the mixed co-lipids as well as those for lipid **1** used with the co-lipids DOPE and cholesterol alone at representative lipid:DNA charge ratios of 3:1 are shown in Fig. 5B. The results summarized in Figs. 3–5, taken together, demonstrate that lipid **1** with the myristyl tail is the most efficacious cationic lipid among lipids **1**–**5** at lipid:DNA charge ratio of 3:1 and 1:1 when used in combination with equimolar amounts of common co-lipids DOPE:Chol:DOPC.

### *3.2. Lipid:DNA binding interactions and lipoplex sensitivities to DNase I*

Towards gaining insights into possible existence of any electrostatic binding interactions difference between the plasmid DNA and the present cationic lipids in combination with varying co-lipids, we performed both the conventional electrophoretic gel retardation assay and DNase I sensitivity assays across the lipid:DNA charge ratios 9:1, 3:1 and 1:1. Results of the simple gel retardation assay (Fig. 6) for representative lipid **1** revealed an interesting feature. Lipid **1** in combination with mixed co-lipids was capable of completely inhibiting the electrophoretic mobility of plasmid DNA across the entire lipid:DNA charge ratios of 1:1–9:1 (Fig. 6). However, lipid **1** when used in combination with either DOPE or Cholesterol co-lipids, was able to inhibit the mobility of DNA only at the highest lipid:DNA charge ratios of 9:1 (Fig. 6). Such gel patterns are consistent with the notion that sub-optimal li-

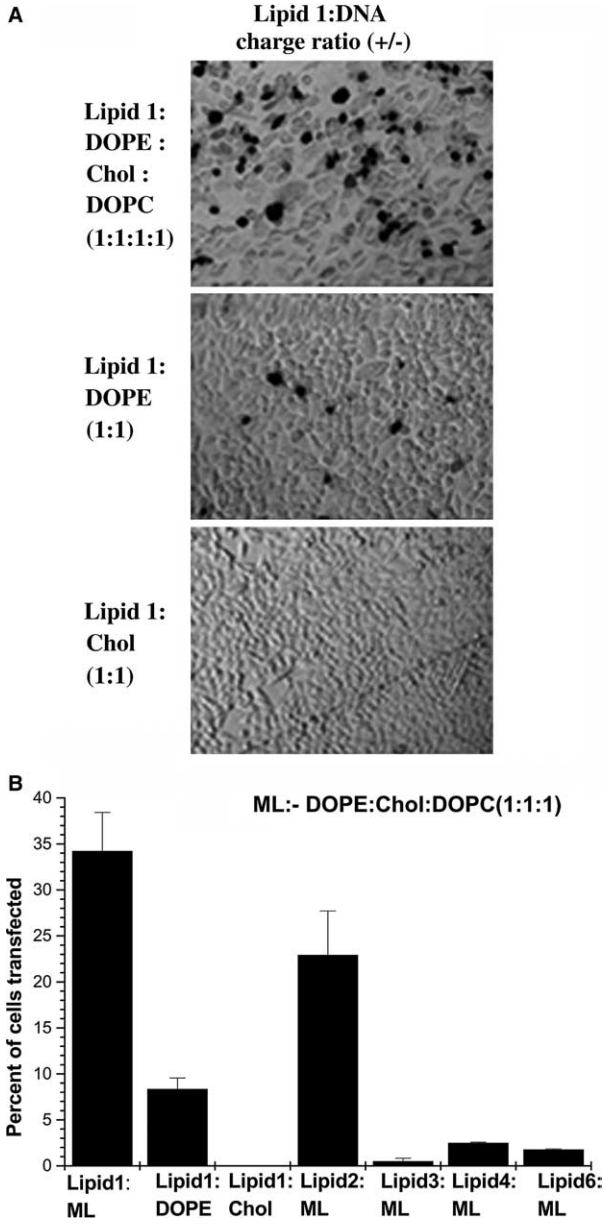


Fig. 5. Whole cell histochemical X-gal staining assay. (A) Histochemical whole cell X-gal staining of transfected CHO cells. Lipoplexes were prepared with mixed co-lipids, conventional co-lipids DOPE and cholesterol and lipid **1** (at lipid:DNA charge ratios of 3:1 and lipid:DOPE:Chol:DOPC mole ratio of 1:1:1:1). Cells expressing β-galactosidase were stained with X-gal as described in the text. (B) Percents of representative X-gal stained CHO cells transfected with lipid **1** used in combination with pure common co- lipids DOPE, Chol, and mixed co-lipids (ML, DOPE:Chol:DOPC) and percents of representative X-gal stained CHO cells transfected with lipids **2**–**5** and co-lipids (ML, DOPE:Chol:DOPC) at lipid:DNA charge ratio of 3:1. Cells expressing β-galactosidase were stained with X-gal as described in the text. A minimum of 100 cells were counted to estimate the percent of cells transfected. The values shown are average of three independent measurements.

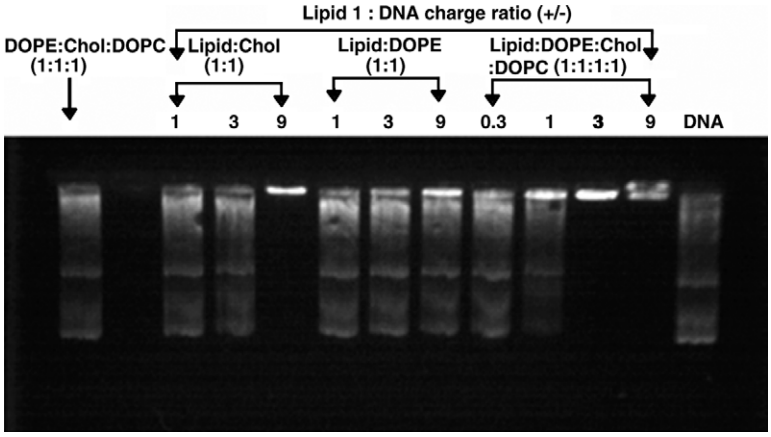


Fig. 6. Electrophoretic gel retardation assay. DNA binding gel patterns for lipoplexes prepared using cholesterol (lanes 3–5 from left), DOPE (lanes 6–8 from left) and fusogenic co-lipids (lanes 9–12 from left). The gel patterns for DNA associated with liposomes prepared using mixed co-lipids (1:1:1 DOPE/Chol/DOPC) in absence of lipid **1** are shown in the extreme left lane. The lipid:DNA charge ratios are indicated at the top of each lane. The details of treatment are as described in the text.

pid:DNA binding interactions could play some role in abolishing the in vitro gene transfer efficacies of the present lipids when used in combination with a single conventional co-lipid. An important point needs to be emphasized at this point of discussion. The overall transfection efficacy of a given cationic lipid depends on the efficiencies of each of the multiple mechanistic steps involved in the lipofection pathway such as endocytotic cellular uptake, release of plasmid DNA from the endosomal compartments to cell cytoplasm, nuclear trafficking of the endosomally released DNA and the final transgene expression [36–38]. Thus, a strongly DNA-binding lipid (such as lipid **1** in combination with equimolar amounts of common co-lipids, Fig. 6) can also be highly transfection-competent in spite of the expected poor DNA dissociation from the lipoplex.

Next we measured the accessibilities of lipoplexes prepared with varying co-lipids to DNase I by DNase I protection experiments using the representative lipoplexes of lipid **1** across the lipid:DNA charge ratios 9:1, 3:1 and 1:1. 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. Electrophoretic gel patterns in such DNase I sensitivity assay (Fig. 7A) revealed a rather surprising trend. Both at 3:1 and 1:1 lipid:DNA charge ratios, band intensities of inaccessible (and therefore undigested) DNA associated with lipoplexes of conventional co-lipids (DOPE or cholesterol) and lipid **1** were found to be significantly reduced compared to those associated with the transfection efficient lipoplexes made from lipid **1** and the mixed co-lipids across the range of lipid:DNA charge ratios of 9:1 to 1:1 (Fig. 7A). This result is consistent with the plasmid DNA associated with lipoplexes of mixed co-lipids being more protected against attack by DNase I than that complexed with lipoplexes of conventional co-lipids (DOPE or cholesterol).

Towards probing any hitherto unsuspected inherent DNA-protection properties of the co-lipid components (1:1:1 mole ratio of DOPE:Cholesterol:DOPC), next we carried out the DNase I protection experiment using plasmid DNA complexed with only the three lipid components namely DOPE:Chol:DOPC (at a mole ratio of 1:1:1 without the presence of lipid **1**). Reasonably intense undigested DNA bands were detected in the gel in such control experiment (the extreme left lane, Fig. 7A), a result in agreement with the existence of high inherent DNA binding properties of equimolar mixtures of common co-lipids. With a view to measure the DNase I sensitivities of lipoplexes prepared with only cationic lipid and DNA (in absence of any co-lipid), we performed the DNase protection experiment using representative cationic lipid **1** across the entire lipid:DNA charge ratios of 9:1–0.1:1. The observed electrophoretic gel pattern is shown in Fig. 7B. The results summarized in Figs. 7A and B demonstrate that at lipid:DNA charge ratios 9:1 and 3:1, DNA associated with the lipoplexes prepared in presence of mixed co-lipids and in absence of any co-lipid are least DNase I sensitive. However, at lipid:DNA charge ratio of 1:1, lipoplexes prepared with mixed co-lipid appeared to be less DNase I sensitive than those prepared with pure cationic lipid (Fig. 7A and B). Thus, broadly speaking, the high gene transfer properties of the present lipids may, in part, originate from such strong DNA binding interactions of mixed co-lipids. However, the origin of the strikingly reduced DNase I sensitivities for lipoplexes **1** prepared with mixed co-lipids is not clear at this stage of investigation.

### *3.3. Particle sizes, zeta potentials and toxicity profiles*

Measurements of particle sizes using dynamic laser light scattering technique revealed that the sizes of all three representative lipoplexes prepared using lipid **1** in combination with DOPE, Cholesterol and DOPE/Chol/DOPC increase gradually from around 350 nm to about 700 nm in presence of DMEM as the lipid:DNA charge ratios of lipoplexes increases from 1:1 to 9:1 (Table 1). Thus, the lipoplex nano-sizes are not likely to play any major role behind the observed contrasting transfection profiles. In addition, the global surface potentials of the lipoplexes prepared from lipid **1** in combination with equimolar amounts of pure DOPE, pure cholesterol and DOPE/Chol/DOPC were measured in presence of DMEM using dynamic laser light scattering instrument equipped with zeta-sizing capacity. Interestingly, across the entire lipid:DNA charge ratios 9:1 to 1:1, the surface charges of the lipoplexes prepared with both pure co-lipids and mixed co-lipids were found to be overall negative in presence of DMEM varying

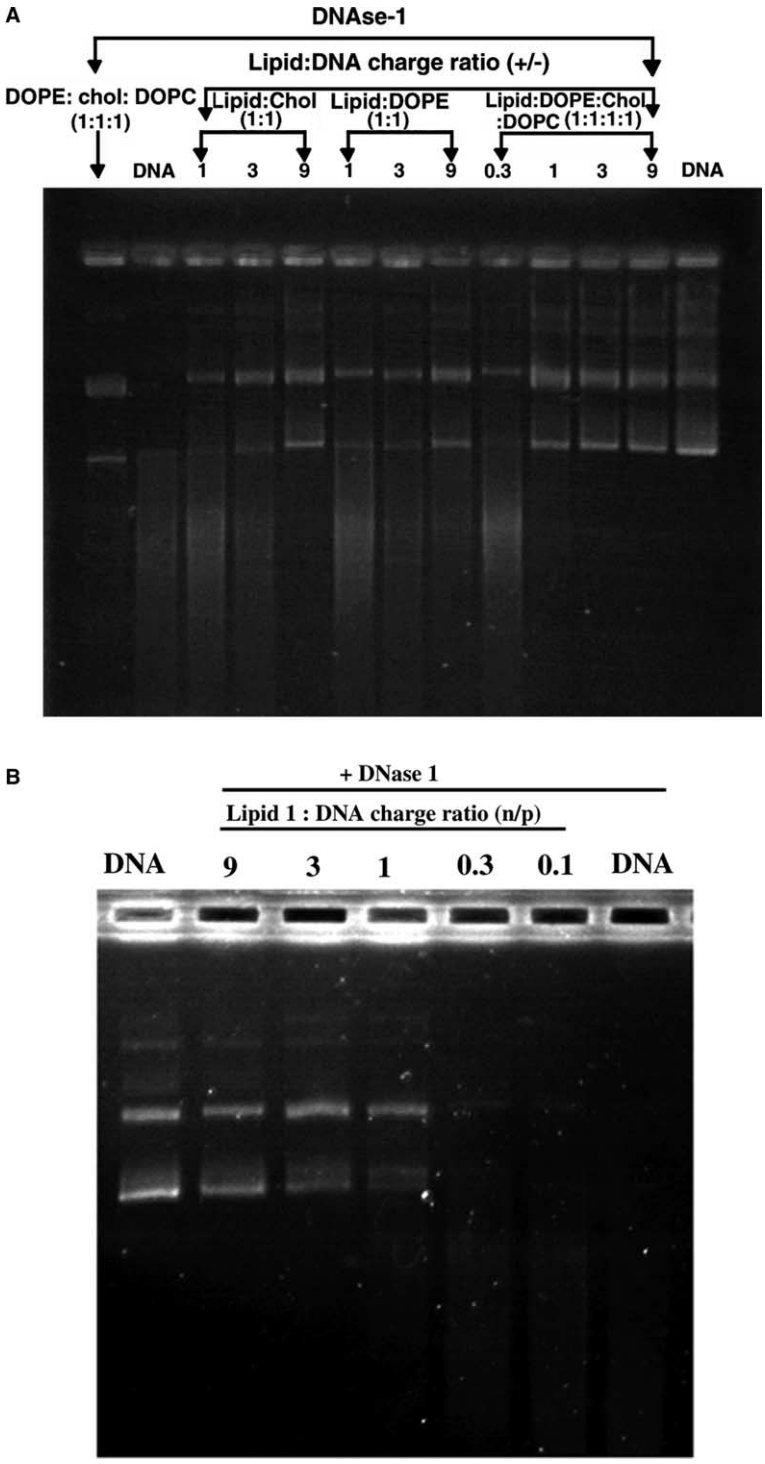


Fig. 7. DNase I protection experiment. (A) Lipoplexes were prepared with representative lipid **1** and cholesterol (lanes 3–5 from left), DOPE (lanes 6–8 from left) and co-lipids (lanes 9–12 from left). The gel patterns for DNA associated with liposomes prepared using mixed co-lipids (1:1:1 DOPE/Chol/DOPC) in absence of lipid **1** are shown in the extreme left lane. (B) Lipoplexes were prepared with representative pure lipid **1** without the use of any co-lipid. The lipid:DNA charge ratios are indicated at the top of each lane. The details of treatment are as described in the text.

within the range −11 to −36 mV (Table 1). Thus, the results summarized in Tables 1 and 2 support the notion that both lipoplex size and surface potentials are unlikely to have any dominant influence in modulating the transfection efficacies of the present lipids. Towards gaining some insights into the cell viability profiles of the present transfection efficient lipoplexes, MTT-based cell viability assays were performed in representative CHO cells across the entire range of lipid:DNA charge ratios used in the actual transfection experiments. Percent cell viabilities upon treatment with lipoplexes prepared from **1** to **5** were found to be high (70–100%) in CHO cells across the lipid:DNA charge ratios (Fig. 8).

Table 1
Sizes (nm) of lipoplexes in plain DMEM

| No. | Sample code | Lipid:DNA charge ratio (n/p) | | | | |
|---|---|---|---|---|---|---|
| | | 9:1 | 3:1 | 2:1 | 3:2 | 1:1 |
| 1. | Lipid1:Chol(1:1) | 710 ± 9.1 | 639.3 ± 9.4 | 512.0 ± 5.8 | 457.7 ± 1.8 | 494.3 ± 1.2 |
| 2. | Lipid1:DOPE(1:1) | 527.6 ± 5.6 | 522.9 ± 4.1 | 452.7 ± 6.9 | 450.3 ± 8.7 | 350.1 ± 3.68 |
| 3. | Lipid1:DOPE:CHOL:DOPC(1:1:1:1) | 698.5 ± 8.5 | 596.1 ± 5.9 | 552.6 ± 4.4 | 433.8 ± 5.7 | 352.2 ± 9.2 |

Table 2
Zeta potential (mV) of lipoplexes in plain DMEM

| No. | Sample code | Lipid:DNA charge ratio (n/p) | | | | |
|---|---|---|---|---|---|---|
| | | 9:1 | 3:1 | 2:1 | 3:2 | 1:1 |
| 1. | Lipid1:DOPE(1:1) | −11.1 ± 0.9 | −16.1 ± 1.9 | −32.0 ± 1.4 | −28.4 ± 1.9 | −34.0 ± 1.1 |
| 2. | Lipid1:CHOL(1:1) | −13.9 ± 2.1 | −18.6 ± 3.3 | −33.8 ± 1.0 | −35.0 ± 1.1 | −36.1 ± 6.4 |
| 3. | Lipid1:DOPE:CHOL:DOPC(1:1:1:1) | −14.5 ± 2.7 | −20.7 ± 4.9 | −32.3 ± 2.9 | −31.5 ± 1.8 | −25.5 ± 7.6 |

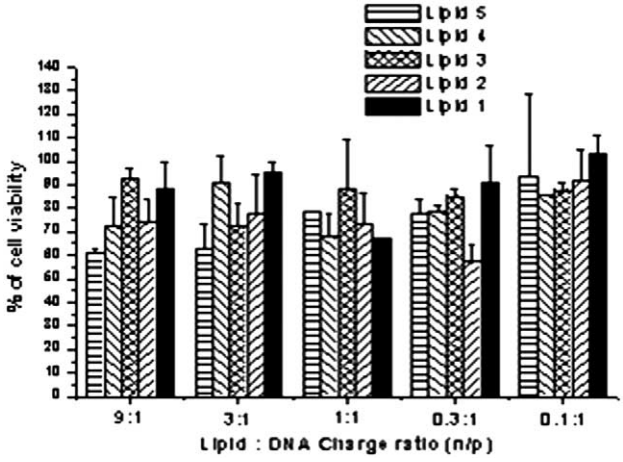


Fig. 8. Percent cell viability upon treatment with lipoplexes. MTT-assay based percent cell viabilities of lipoplexes prepared with lipids **1**–**5** and mixed co-lipids (DOPE/Chol/DOPC) in representative CHO cells. The percent cell viability values shown are average of triplicate experiments performed on the same day.

## 4. Conclusion

Using a novel series of non-glycerol backbone based cationic lipids with polar 2-hydroxyethyl and 2-aminoethyl head-group functionalities (**1**–**5**, Fig. 1), we have demonstrated that commonly used co-lipids DOPE, cholesterol and DOPC, when act in synergy, are capable of imparting high gene transfer properties to cationic lipids which are otherwise either essentially transfection incompetent or poorly transfecting when used in combination with either DOPE or Cholesterol. Both reporter gene expression assays in CHO, COS-1 and HepG2 cells and the whole cell histochemical X-gal staining assays in representative CHO cells revealed that the improved gene transfer properties of the present cationic lipids get severely compromised when the cationic lipids are used in combinations with pure individual co-lipid components. Electrophoresis gel patterns in DNase I sensitivity assay are consistent with the notion that the high transfection properties of the present cationic lipids in association with equimolar amounts of DOPE, cholesterol and DOPC may partly originate due to the significantly reduced DNase I susceptibility of the corresponding lipoplexes. Taken together, the present findings support the notion that use of common co-lipids in synergy may turn out to be rewarding in future design of novel liposomal transfection kits for use in non-viral gene therapy.

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## References

[1] Verma, I.M. and Somnia, M. (1997) Nature 389, 239–242.
[2] Anderson, W.F. (1998) Nature 392, 25–30.
[3] Yla-Herttuala, S. and Martin, J.F. (2000) Lancet 355, 213–222.
[4] Mountain, A. (2000) Trends Biotechnol. 18, 119–128.
[5] Daly, G. and Chernajovsky, Y. (2000) Mol. Ther. 2, 423–434.
[6] Yang, Y., Nunes, F.A., Berevsci, K., Furth, E.E., Gonczol, E. and Wilson, J.M. (1994) Proc. Natl. Acad. Sci. USA 91, 4407–4411.
[7] Knowles, M.R., Hohneker, K.W., Zhou, Z., Olsen, J.C., Noah, T.L., Hu, P.C., Leigh, M.W., Engelhardt, J.F., Edwards, L.J., Jones, K.R. and Boucher, R.A.A (1995) New Engl. J. Med. 333, 823–831.
[8] Crystal, R.G., McElvaney, N.G., Rosenfeld, M.A., Chu, C.S., Mastrangeli, A., Hay, J.G., Brody, S.L., Jaffe, H.A., Eissa, N.T. and Danel, C. (1994) Nat. Genet. 8, 42–51.
[9] Lehman, S. (1999) Nature 302, 517–518.
[10] Hacein-Bey-Abina, S., Von Kalle, C., Schmidt, M., McCormack, M.P., Wulffraat, N., Leboulch, P., Lim, A., Osborne, C.S., Pawliuk, R., Morillon, E., Sorensen, R., Forster, A., Fraser, P., Cohen, J.I., de Saint Basile, G., Alexander, I., Wintergerst, U., Frebourg, T., Aurias, A., Stoppa-Lyonnet, D., Romanana, S., Radford-Weiss, I., Gross, F., Valensi, F., Delabesse, E., Macintyre, E., Sigaux, F., Soulier, J., Leiva, L.E., Wissler, M., Prinz, C., Rabbitts, T.H., Le Deist, F., Fischer, A. and Cavazzana-Calvo, M. (2003) Science 401, 415–419.
[11] Gruneich, J.A., Price, A., Zhu, J. and Diamond, S.L. (2004) Gene Ther. 11, 668–674.
[12] Karmali, P.P., Kumar, V.V. and Chaudhuri, A. (2004) J. Med. Chem. 47, 2123–2132.
[13] Singh, R.S. and Chaudhuri, A. (2004) FEBS Letters 556, 86–90.
[14] Mahidhar, Y.V., Rajesh, M. and Chaudhuri, A. (2004) J. Med. Chem. 47, 3938–3948.
[15] Valluripalli, V. and Chaudhuri, A. (2004) FEBS Lett. 571, 205–211.
[16] Madry, I. and Trippel, S.B. (2000) Gene. Ther. 7, 286–291.

[17] Li, S. and Huang, L. (1997) Gene Ther. 4, 891–900.
[18] Hood, J.D., Bednarski, M., Frausto, R., Guccione, S., Reisfeld, R.A., Xiang, R. and Cheresh, D.A. (2000) Science 296, 2404–2407.
[19] Bell, P.C., Bergsma, M., Dolbnya, I.P., Bras, W., Stuart, M.C.A., Rowan, A.E., Feiters, M.C and Engberts, J.B.F.N. (2003) J. Am. Chem. Soc. 125, 1551–1558.
[20] Shi, N.Y., Zhang, Y., Zhu, C., Boado, R.J. and Pardridge, W.M. (2001) Proc. Natl. Acad. Sci. USA 98, 12754–12759.
[21] van der Woude, I., Wagenaar, A., Meekel, A.A., ter Beest, M.B., Ruiters, M.H., Engberts, J.B. and Hoekstra, D. (1997) Proc. Natl. Acad. Sci. USA 94, 1160–1165.
[22] Chen, Q.-R., Zhang, L., Stass, S.A. and Mixson, A.J. (2000) Gene Ther. 7, 1698–1705.
[23] Shi, N. and Pardridge, W.M. (2000) Proc. Natl. Acad. Sci. USA 97, 7567–7572.
[24] Kono, K., Torikoshi, Y., Mitsutomi, M., Itoh, T., Emi, N., Yanagie, H. and Takagishi, T.N. (2001) Gene Ther. 8, 5–12.
[25] Templeton, N.S., Lasic, D.D., Frederik, P.M., Strey, H.H., Roberts, D.D. and Pavalkis, G.N. (1997) Nat. Biotech. 15, 647–652.
[26] Zhu, N., Liggitt, D., Liu, Y. and Debs, R. (1993) Science 261, 209–211.
[27] Zhu, J., Munn, R.J. and Nantz, M.H. (2000) J. Am. Chem. Soc. 122, 2645–2646.
[28] Vigneron, J.P., Oudrihi, N., Fauquet, M., Vergely, L., Bradley, J.C., Basseville, M., Lehn, P. and Lehn, J.M. (1996) Proc. Natl. Acad. Sci. USA 93, 9682–9686.
[29] Majeti, B.K., Singh, R.S., Yadav, S.K., Reddy, B.S., Ramakrishna, S., Diwan, P.V., Madhavendra, S.S. and Chaudhuri, A. (2004) Chem. Biol. 11, 427–437.
[30] Singh, R.S., Gonçalves, C., Sandrin, P., Pichon, C., Midoux, P. and Chaudhuri, A. (2004) Chem. Biol. 11, 713–723.
[31] Kumar, V.V., Pichon, C., Refregiers, M., Guerin, B., Midoux, P. and Chaudhuri, A. (2003) Gene Ther. 10, 1206–1215.
[32] Singh, R.S., Mukherjee, K., Banerjee, R., Chaudhuri, A., Hait, S.K., Moulik, S., Ramadas, Y., Vijayalakshmi, A. and Rao, N.M. (2002) Chem. Eur. J. 8, 900–909.
[33] Srilakshmi, G.V., Sen, J., Chaudhuri, A., Ramdas, Y. and Rao, N.M. (2002) Biochim. Biophys. Acta (Biomembranes) 1559, 87–95.
[34] Banerjee, R., Mahidhar, Y.V., Chaudhuri, A., Gopal, V. and Rao, N.M. (2001) J. Med. Chem. 44, 4176–4185.
[35] Banerjee, R., Das, P.K., Srilakshmi, G.V., Chaudhuri, A. and Rao, N.M.N. (1999) J. Med. Chem. 42, 4292–4299.
[36] Zabner, J., Fasbender, A.J., Moninger, T., Poellinger, K.A. and Welsh, M.J. (1995) J. Biol. Chem 270, 18997–19007.
[37] Xu, Y. and Szoka Jr., F.C. (1996) Biochemistry 35, 5616–5623.
[38] Bally, M.B., Harvie, P., Wong, M.P.F., Kong, S., Wasan, E.K. and Reimer, D.L. (1999) Adv. Drug Del. Rev. 38, 291–315.