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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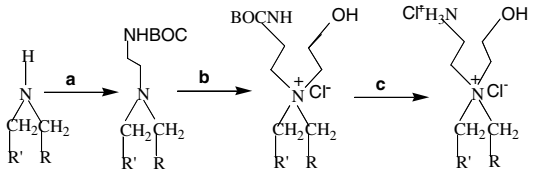

Where

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–