European Journal of Pharmaceutics and Biopharmaceutics 78 (2011) 326–335

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Novel cationic lipids possessing protonated cyclen and imidazolium salt for gene delivery

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ARTICLE INFO

Article history:
Received 27 November 2010
Accepted in revised form 15 March 2011
Available online 23 March 2011

Keywords:
Gene therapy
Gene delivery
Cationic liposome
Cyclen
Imidazolium salt
Calcium ion

ABSTRACT

In this study, two novel protonated cyclen and imidazolium salt-containing cationic lipids differing only in their hydrophobic region (cholesterol or diosgenin) have been designed and synthesized for gene delivery. Cationic liposomes were easily prepared from each of these lipids individually or from the mixtures of each cationic lipid and dioleoylphosphatidyl ethanolamine (DOPE). Several studies including DLS, gel retardation assay, ethidium bromide intercalation assay, and TEM demonstrated that these amphiphilic molecules are able to bind and compact DNA into nanometer particles that could be used as non-viral gene delivery agents. Our results from in vitro transfection showed that in association with DOPE, two cationic lipids can induce effective gene transfection in HEK293 cells. Furthermore, the gene transfection efficiencies of two cationic lipids were dramatically increased in the presence of calcium ion (Ca^{2+}). It is notable that the gene transfection abilities of two cationic lipids were maintained in the presence of 10% serum. Besides, different cytotoxicity was found for two lipoplexes. This study demonstrates that the title cationic lipids have large potential to be efficient non-viral gene vector.

1. Introduction

Gene therapy has tremendous potential for both inherited and acquired diseases [1,2]. The clinical success of gene therapy requires a safe and effective gene delivery system, which involves viral or non-viral vectors [3]. The gene delivery efficacies of viral vectors are, in general, superior to those of their non-viral counterparts. However, as viral vectors suffer from numerous biosafety-related disadvantages such as immunogenicity, restricted targeting of specific cell types, size limitation on DNA, and potential for mutagenesis [4], their use in clinic is limited. Compared to viral vectors, non-viral vectors including cationic lipids [2,5], cationic polymers [6,7], dendrimers [8], etc. are highly attractive because of their excellent safety profile despite their low transfection efficiency. Among non-viral vectors, cationic lipids have particularly excellent potential for gene delivery applications because of their lesser immunogenic nature, robust manufacture ability to deliver large pieces of DNA, and ease of handling and preparation techniques [9,10]. Since Felgner et al. first reported the utilization of unnatural diether-linked cationic lipid, N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), as a synthetic carrier to deliver gene into cells in 1987 [11], hundreds of cationic lipids have been synthesized as candidates for non-viral gene delivery [2,5]. However, all those cationic lipids were still far from the requirement of in vivo gene therapy because of their potential toxicity and relative low transfection efficiency. Consequently, the development of novel nontoxic cationic structures and high effective cationic lipids is of great importance.

Previous reports have proved that cationic lipids using polyamine as the headgroups generally have much superior performance in terms of gene delivery than cationic lipids of a quaternary amine or a single protonated amine [12–14]. However, cationic lipids with long linear polyamine chains as headgroups may also decrease gene delivery efficiency because of their relative low binding ability toward DNA, which is the result of self-folding of the linear lipopolyamine chains in cationic lipid structure [15]. Since cyclic polyamines and branched polyamines are hard to self-fold, we postulated that using macrocyclic polyamines or branched polyamines as the hydrophilic headgroups of cationic lipids may solve this problem. Although many cationic amphiphiles of linear polyamines [16,17] or branched polyamines [18] have been widely studied, it seems that nearly no example using

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doi:10.1016/j.ejpb.2011.03.017

macrocyclic polyamines as hydrophilic headgroup on a cationic lipid formulation have been investigated. In addition, because of the dissociation of positive charge on imidazolium salt group, cationic lipids containing imidazolium polar heads [19,20] have been reported to display higher transfection efficiency and reduced cytotoxicity when compared with classical quaternary ammonium cationic lipids. For this reason, we have designed and synthesized a novel 1,4,7,10-tetraazacyclododecane (cyclen)-based and imidazolium-containing cationic lipid **L** (Fig. 1) by linking *N*-dodecylimidazole (NDI) group and cyclen ring via a *p*-xylyl bridge. Unfortunately, results showed that although the liposome prepared from **L** could bind to DNA and transfer it into cell without any other extraneous agent such as cholesterol or dioleoylphosphatidylethanolamine (DOPE), it need high N/P ratio (39:1) to completely bind DNA, and only weak gene express was observed. This might be attributed to its less protonated nitrogen atom and low water solubility [21].

The hydrophobic structures cationic lipids have large effects on both gene transfection efficiency and cytotoxicity. Steroid compounds such as vitamin D [22], bile acids [23], antibiotic steroid [24], cholestane, and litocholic acid [15] were used as hydrophobic region of cationic lipids, which showed promising gene delivery performance and lower cytotoxicity. In this study, two trifluoroacetic acid salts **L1** and **L2**, in which the hydrophilic cyclen headgroup and steroid tails (cholesterol for **L1** and diosgenin for **L2**, Fig. 1) were bridged by imdazolium and an ester group, were designed and synthesized. Stable liposomes could be prepared from the two lipids themselves or in the presence of DOPE. Studies revealed that the liposomes could bind and compact DNA into nanoparticles (lipoplexes). Their cytotoxicities and in vitro transfection efficiencies were systematically investigated. We hope that the study could broaden the applications of macrocyclic polyamines, especially of cyclen.

2. Experimental section

2.1. Materials and methods

Unless otherwise stated, all chemicals and reagents were obtained commercially and used without further purification.

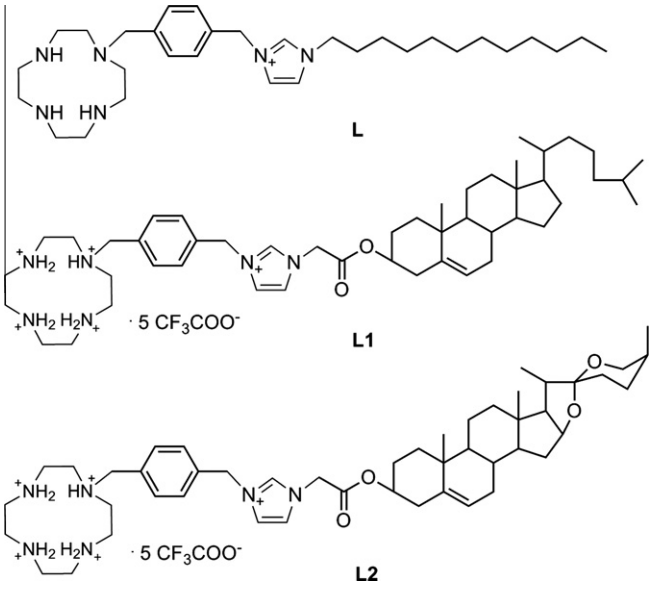


Fig. 1. Molecular structures of the studied cationic lipids.

Absolute chloroform ($CHCl_3$) and dichloromethane (CH_2Cl_2) were distilled after being dried with calcium hydride (CaH_2). Anhydrous acetonitrile (CH_3CN) was distilled after being dried with P_2O_5. Chloroacetyl chloride and trifluoroacetic acid were dried and purified under nitrogen by using standard methods. Column chromatography was performed using 200–300 mesh silica gel. All aqueous solutions were prepared from deionized or distilled water. Cholesteryl chloroacetate (**2a**) [25] and the precursor 1-(*p*-bromomethyl benzyl)-4,7,10 tris(tertbutyloxycarbonyl)-1,4,7,10-tetraazacyclododecane **4** [26] were prepared according to reported procedures. IR spectra were recorded on a Shimadzu FTIR-4200 spectrometer as KBr pellets or thin films on KBr plates. The ^1H NMR and ^{13}C NMR spectra were measured on a Varian INOVA-400 spectrometer, and the δ scale in parts per million was referenced to residual solvent peaks or internal tetramethylsilane (TMS). MS-ESI spectra data were recorded on a Finnigan LCQ^{DECA} and a Bruker Daltonics BioTOF mass spectrometer, respectively. Fluorescence spectra were measured by a Horiba Jobin Yvon Fluoromax-4 spectrofluorometer. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Micro-BCA protein assay kit was obtained from Pierce (Rockford, IL, USA). Luciferase assay kit was purchased from Promega (Madison, WI, USA). Endotoxin-free plasmid purification kit was purchased from TIANGEN (Beijing, China). The plasmids used in the study were pGL-3 (Promega, Madison, WI, USA) coding for luciferase DNA and pEGFP-N1 (Clontech, Palo Alto, CA, USA) coding for EGFP DNA. The Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 Medium, and fetal bovine serum were purchased from Invitrogen Corp.

2.2. Preparation of diosgenin chloroacetate (*2b*)

Chloroacetyl chloride (19.8 g, 180 mmol) in anhydrous dichloromethane (30 mL) was added dropwise to a stirred solution of diosgenin (14.9 g, 36 mmol) and triethylamine (5.8 mL, 4.2 g, 41.4 mmol) in anhydrous dichloromethane (120 mL). The resulting reaction mixture was stirred for 4 h at room temperature. Excess chloroacetyl chloride was removed by washing the reaction mixture with a 5% solution of aqueous $NaHCO_3$ (3×50 mL) and brine (50 mL). The organic phase was collected and dried over anhydrous magnesium sulfate and then filtered. The solvent was evaporated under reduced pressure to give the product as a white solid. IR (cm^{-1}): 2953, 2832, 1737, 1452, 1378, 1313, 1164, 1049, 1003, 900. ^1H NMR ($CDCl_3$, 600 MHz): δ 0.80 (t, $J = 6.0$ Hz, 6H, diosgenin-H), 0.98–2.01 (br, 28H, diosgenin-H), 2.37 (d, 2H, diosgenin-H), 3.39 (d, 1H, diosgenin-H), 3.48 (t, $J = 54.6$ Hz, 1H, diosgenin-H), 4.05 (s, 2H, Cl*CH*$_2$CO), 4.42 (m, 1H, diosgenin-H), 4.70 (m, 1H, diosgenin-H), 5.40 (s, 1H, C═C*H*—). HR MS ($C_{29}H_{44}ClO_4 + H$): Calcd. 491.2928; Found. 491.2934.

2.3. General procedure for the preparation of imidazole derivatives 3

Chloroacetate derivative **2** (27.1 mmol) was added to a solution of imidazole (22.15 g, 325.2 mmol), sodium carbonate(2.9 g, 28 mmol), potassium iodine (0.24 g, 1.4 mmol) in anhydrous chloroform (36 mL), and dimethyl sufloxide (22 mL). The reaction mixture was heated at 90 °C for 10 h, chloroform (180 mL) was added, and the resulted solution was washed with a 5% solution of aqueous $NaHCO_3$ (3×100 mL) and brine (100 mL). The organic phase was collected and dried over anhydrous magnesium sulfate and then filtered. The solvent was evaporated under reduced pressure to give crude products, which were purified by flash chromatography over silica (ethyl acetate) to obtain the corresponding imidazole derivatives **3**.

Compound **3a**: IR (cm^{-1}): 3427, 3113, 2930, 2855, 1746, 1509, 1464, 1377, 1291, 1217, 1110, 1078, 1003. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.67 (s, 3H, cholesterol-H), 0.85–1.99 (br, 38H, cholesterol-H), 2.32 (d, 2H, cholesterol-H), 4.67 (s, 3H, NCH_2CO and cholesterol-H), 5.35 (s, 1H, C=C*H*—), 6.96 (s, 1H, imidazole-H), 7.10 (s, 1H, imidazole-H), 7.51 (s, 1H, imidazole-H). ^{13}C NMR ($CDCl_3$, 100 MHz): 19.27, 21.02, 22.57, 22.82, 23.83, 24.27, 27.64, 28.00, 28.21, 31.81, 31.88, 35.78, 36.17, 36.52, 36.83, 37.91, 39.50, 39.68, 42.30, 48.27, 49.95, 56.12, 56.65, 76.08, 119.94, 123.22, 129.66, 137.93, 139.00, 166.80. HR MS ($C_{32}H_{50}N_2O_2$ + H): Calcd.495.3951; Found. 495.3954.

Compound **3b**: IR (cm^{-1}): 3117, 2932, 1751, 1508, 1457, 1379, 1219, 1178, 1052, 984, 901, 816, 752, 663, 617. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 0.80 (m, 6H, diosgenin-H), 0.98–2.00 (br, 32H, diosgenin-H), 2.35 (d, 2H, diosgenin-H), 3.36 (d, 1H, diosgenin-H), 3.40 (d, 1H, diosgenin-H), 4.41 (m, 1H, diosgenin-H), 4.68 (m, 3H, ClCH_2CO and diosgenin-H), 5.39 (s, 1H, C=C*H*—), 6.97 (s, 1H, imidazole-H), 7.11 (s, 1H, imidazole-H), 7.52 (s, 1H, imidazole-H). ^{13}C NMR ($CDCl_3$, 100 MHz): 16.27, 17.13, 19.29, 20.80, 27.60, 28.79, 30.28, 31.35, 31.37, 31.81, 32.01, 36.66, 36.79, 37.88, 39.67, 40.24, 41.60, 48.28, 49.86, 56.39, 62.06, 66.83, 75.99, 80.76, 109.27, 119.93, 122.95, 129.68, 137.92, 139.04, 166.79. HR MS ($C_{32}H_{46}N_2O_4$ + H): Calcd. 523.3536; Found. 523.3532.

2.4. General procedure for the preparation of compounds **5**

A solution of imidazole derivatives **3** (6 mmol) and 1-(*p*-bromomethyl benzyl)-4,7,10 tris(tertbutyloxycarbonyl)-1,4,7,10-tetraazacyclododecane **4** (7.9 g, 12 mmol) in anhydrous acetonitrile (30 mL) was refluxed at 90 °C over a period of 48–96 h until TLC indicated the disappearance of the starting materials. Then, reaction mixture was cooled, and the acetonitrile was rotary-evaporated to give a yellow residue, which could be purified by chromatography over silica (dichloromethane/methanol, 12:1) as white foamy solid in 55–65% yields.

Compound **5a**: IR (cm^{-1}): 3424, 2941, 2860, 1749, 1691, 1564, 1461, 1415, 1366, 1249, 1165, 1107, 1027, 978, 859, 773. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.68 (s, 3H, cholesterol-H), 0.85–2.03 (br, 63H, cholesterol-H and Boc-H), 2.36 (d, 2H, cholesterol-H), 2.37–2.38 (br, 4H, cyclen-H), 3.31–3.71 (br, 12H, cyclen-H), 3.73–3.76 (t, J = 11.6, 2H, benzene-CH_2-cyclen), 4.69 (m, 1H, cholesterol-H), 5.37–5.38 (m, 3H, C=*CH*— and benzene-CH_2-imidazolium), 5.49 (s, 2H, imidazolium-CH_2–COO), 7.27 (s, 1H, imidazole-H), 7.32–7.38 (br, 5H, imidazole-H and benzene-H), 10.70 (s, 1H, imidazole-H). ^{13}C NMR ($CDCl_3$, 100 MHz): 22.53, 22.79, 23.80, 24.24, 27.60, 27.97, 28.18, 28.45, 28.66, 30.91, 31.76, 31.86, 35.75, 36.14, 36.49, 36.80, 37.86, 39.47, 39.66, 42.27, 49.94, 50.46, 56.11, 56.65, 79.47, 121.30, 123.28, 123.84, 128.87, 138.93, 165.38, 206.98. HR MS ($C_{63}H_{101}N_6O_8$): Calcd. 1069.7675; Found. 1069.7616.

Compound **5b**: IR (cm^{-1}): 3423, 3145, 3951, 1748, 1690, 1564, 1459, 1415, 1367, 1248, 1163, 1105, 1052, 1006, 982, 920, 900, 862, 774. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.79 (m, 6H, diosgenin-H), 0.96–2.03 (br, 55H, diosgenin-H and Boc-H), 2.36 (d, 2H, diosgenin-H), 2.51–2.70 (br, 4H, cyclen-H), 3.30–3.71 (br, 14H, cyclen-H and diosgenein-H), 3.72 (t, J = 8, 2H, benzene-CH_2-cyclen), 4.42 (m, 1H, diosgenin-H), 4.68 (m, 1H, diosgenin-H), 5.35–5.39 (m, 3H, C=*CH*— and benzene-CH_2-imidazolium), 5.46 (s, 2H, imidazolium-CH_2–COO), 7.20 (s, 1H, imidazolium-H), 7.30–7.39 (br, 4H, benzene-H), 7.44 (s, 1H, imidazolium-H), 10.52 (s, 1H, imidazolium-H). ^{13}C NMR ($CDCl_3$, 100 MHz): 20.77, 27.56, 28.44, 28.65, 28.75, 30.24, 31.30, 31.34, 31.78, 31.99, 36.62, 36.76, 37.83, 39.65, 40.21, 41.56, 49.85, 50.46, 53.23, 56.39, 62.05, 66.79, 76.79, 77.06, 79.47, 80.72, 109.23, 121.38, 122.97, 123.87, 128.86, 137.93, 138.99, 165.45. HR MS ($C_{63}H_{97}N_6O_{10}$): Calcd. 1097.7266; Found. 1097.7002.

2.5. General procedure for the preparation of title lipids ***L1*** and ***L2***

Compound **5** (1.2 mmol) was suspended in anhydrous dichloromethane (15 mL), and then, a solution of trifluoroacetic acid (5 mL) in anhydrous dichloromethane (5 mL) was added dropwise under ice bath and N_2 atmosphere. And then, the obtained mixture was stirred at room temperature for 6 h. The solvent and excess trifluoroacetic acid were removed under reduced pressure to give yellow residue. Then, under stirring, anhydrous ethyl ether was added dropwise into the residue to precipitate the desired lipids as white solid in 98–100% yields.

Lipid **L1**: IR (cm^{-1}): 3436, 3101, 2951, 2867, 1746, 1681, 1565, 1515, 1457, 1414, 1375, 1203, 1174, 1131, 832, 800, 722. ^{1}H NMR (DMSO-d6, 400 MHz): δ 0.60 (s, 3H, cholesterol-H), 0.80–2.00 (br, 41H, cholesterol-H), 2.36 (d, 2H, cholesterol-H), 2.60–2.90 (m, 8H, cyclen-H), 3.00–3.20 (m, 8H, cyclen-H), 3.75 (s, 2H, benzene-CH_2-cyclen), 4.55 (m, 1H, cholesterol-H), 5.20 (m, 2H, benzene-CH_2-imidazolium) 5.35 (s, 1H, C=*CH*—), 5.51 (s, 2H, imidazolium-CH_2–COO), 7.40 (m, 4H, benzene-H), 7.78 (s, 1H, imidazole-H), 7.87 (s, 1H, imidazole-H), 9.36 (s, 1H, imidazole-H). ^{13}C NMR (DMSO-d6, 100 MHz): 27.86, 28.24, 31.80, 35.67, 36.13, 36.52, 36.81, 37.97, 39.35, 39.42, 39.56, 39.77, 39.97, 42.32, 42.53, 42.64, 45.17, 47.61, 49.85, 50.22, 52.17, 55.48, 56.05, 56.58, 75.96, 79.71, 116.14, 119.12, 122.71, 122.96, 124.70, 128.82, 131.05, 134.28, 136.49, 137.93, 139.55, 158.78, 159.09, 159.40, 166.73. HR MS ($C_{48}H_{77}N_6O_2$): Calcd. 769.6108; Found. 769.6102.

Lipid **L2**: IR (cm^{-1}): 3430, 3101, 2949, 1747, 1682, 1566, 1455, 1203, 1133, 1055, 984, 834, 800, 722. ^{1}H NMR (DMSO-d6, 400 MHz): δ 0.72 (m, 6H, diosgenin-H), 0.89–1.92 (br, 31H, diosgenin-H), 2.31 (d, 2H, diosgenin-H), 2.71–2.83 (br, 8H, cyclen-H), 3.04–3.19 (br, 9H, cyclen-H and diosgenein-H), 3.38 (d, 1H, diosgenein-H), 3.74 (s, 2H, benzene-CH_2-cyclen), 4.26–4.28 (m, 1H, diosgenin-H), 4.52 (m, 1H, diosgenin-H), 5.26 (s, 2H, benzene-CH_2-imidazolium), 5.35 (s, 1H, C=*CH*—), 5.51 (s, 2H, imidazolium-CH_2–COO), 7.38–7.42 (br, 4H, benzene-H), 7.79 (s, 1H, imidazolium-H), 7.87 (s, 1H, imidazolium-H), 9.36 (s, 1H, imidazolium-H). ^{13}C NMR (DMSO-d6, 100 MHz): 28.92, 30.25, 31.37, 31.89, 31.94, 36.67, 36.77, 37.94, 39.28, 39.49, 39.70, 39.91, 41.54, 42.49, 42.61, 45.15, 47.60, 49.79, 50.22, 52.19, 55.50, 56.16, 62.26, 66.38, 75.97, 80.63, 108.89, 122.69, 122.79, 124.71, 128.82, 131.06, 134.26, 136.46, 137.90, 139.59, 166.72. HR MS ($C_{48}H_{73}N_6O_4$): Calcd. 797.5693; Found. 797.5691.

2.6. Preparation of cationic liposome

Individual cationic lipid (0.005 mmol) or its mixture with DOPE in the desired mole ratio was dissolved in anhydrous chloroform (5 mL) in autoclaved glass vials. Thin films were made by slowly rotary-evaporating the solvent at room temperature. Last trace of organic solvent was removed by keeping these films under vacuum above 8 h. The dried films and Tris–HCl buffer (10 mM, pH 7.4) were preheated to 70 °C, and then the buffer was added to the films to the final lipid concentration of 1 mM. The mixtures were vortexed vigorously until the films were completely resuspended. Sonication of these suspensions for 20 min in a bath sonicator at 60 °C afforded the corresponding cationic liposomes that were stored at 4 °C.

2.7. Amplification and purification of plasmid DNA

pGL-3 and pEGFP-N1 plasmids were used. The former one was used as the luciferase reporter gene, which was transformed in JM109 *Escherichia coli*, and the latter one was used as the enhanced green fluorescent protein reporter gene, which was transformed in *E. coli* DH5α. Both plasmids were amplified in *E. coli* grown in LB media at 37 °C and 220 rpm overnight. The plasmids were purified

by an EndoFree TiangenTM Plasmid Kit. Then, the purified plasmids were dissolved in TE buffer solution and stored at −20 °C. The integrity of plasmids was confirmed by agarose gel electrophoresis. The purity and concentration of plasmids were determined by the ratio of ultraviolet (UV) absorbances at 260 nm to 280 nm.

2.8. Preparation of lipid/DOPE/DNA complexes (lipoplexes)

To prepare the liposome/pDNA complexes (lipoplexes), various amounts of cationic lipids were mixed with a constant amount of DNA by pipetting thoroughly at various N/P ratio, and the mixture was incubated for 30 min at room temperature. The theoretical N/P ratio represents the charge ratio of cationic lipid to nucleotide base (in mole) and was calculated by considering the average nucleotide mass of 350.

2.9. Gel retardation assay

To determine the formation of liposome/pDNA complex (lipoplexes), lipoplexes of various N/P ratios ranging from 1 to 12 were prepared as described above. Constant amount of 5 μg DNA was used here; 10 μL of each lipoplexes solution was electrophoresed on the 1% (W V^{-1}) agarose gel containing Gold view and Tris–acetate (TAE) running buffer at 110 V for 20 min. DNA was visualized with a UV lamp using a BioRad Universal Hood II.

2.10. Ethidium bromide intercalation assay

Fluorescence emission due to ethidium bromide (EB) at 605 nm was monitored in a fluoromax-4 spectrofluorimeter (excitation wavelength was 497 nm with 3 nm slit widths for both excitation and emission). Typically fluorescence emission was measured for EB (5 μM) in 10 mM Tris, pH 7.4 buffer. To this solution, CT-DNA (11 μM) was added, and the fluorescence emission due to EB upon intercalative complexation with DNA was measured again at 25 °C. Then, aliquots of a given cationic liposome (0.68 μM) were added into the EB/CT-DNA solution for further measurement. If F_0 is the fluorescence intensity (FI) of unintercalated, F_{max} is the FI of fully intercalated EB, and F_x is the FI for a given concentration of liposome, then %FI was calculated as $\%FI = (F_x - F_0)/(F_{max} - F_0)$.

2.11. Dynamic light scattering (DLS)

Particle size and zeta potential of liposomes or lipoplexes at various N/P ratio were measured by a dynamic light scattering system (Zetasizer Nano ZS, Malvern Instruments Led) at 25 °C. Then, 100 μL of liposomes or lipoplexes prepared as aforementioned was diluted to 1 mL with deionized water, RPMI 1640 (or containing 10% serum), or DMEM (or containing 10% serum) prior to measurement.

2.12. Cell culture

HEK (human embryonic kidney) 293 cells were incubated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin–streptomycin, 10,000 U mL^{-1}) at 37 °C in a humidified atmosphere containing 5% CO_2.

2.13. Transfection procedure

In order to obtain about 80% confluent cultures at the time of transfection, 24-well plates were seeded with 75,000 cell/well in 500 μL antibiotic-free media 24 h before transfection. For the preparation of lipoplexes applied to cells, various amounts of liposomes and DNA were serially diluted separately in both serum- and antibiotic-free DMEM culture medium; then, the DNA solutions were added into liposome solutions and mixed briefly by pipetting up and down several times, after which the mixtures were incubated at room temperature for about 30 min to obtain lipoplexes of desired N/P ratios, the final lipoplexes volume was 200 μL, and the DNA was used at a concentration of 2 μg/well unless otherwise noted. After 30 min of complexation, old cell culture medium was removed from the wells, cells were washed once with serum-free DMEM, and the above 200 μL lipoplexes was added to each well. The plates were then incubated for 4 h at 37 °C in a humidified atmosphere containing 5% CO_2. At the end of incubation period, medium was removed, and 500 μL of fresh DMEM medium containing 10% FBS was added to each well. Plates were further incubated for a period of 24 h before checking the reporter gene expression.

For calcium ion-promoted transfection studies, lipoplexes applied to cells were prepared as follows: appropriate amounts of liposomes, DNA, and $CaCl_2$ were serially diluted, respectively, in both serum- and antibiotic-free DMEM or serum-free RPMI 1640 (or RPMI 1640 containing 10% FBS); then, the DNA solutions were added into liposome solutions and mixed briefly by pipetting up and down several times, after which the mixtures were immediately added to $CaCl_2$ solution and incubated at room temperature for about 30 min to obtain lipoplexes that were applied to cells. The final lipoplex volume was 200 μL, the DNA was used at a concentration of 2 μg/well unless otherwise noted, and the $CaCl_2$ (100 mM) stock solution was prepared in dH_2O and sterilized by filtering.

For fluorescent microscopy assays, cells were transfected by complexes containing pEGFP-N1. After 24 h incubation, the microscopy images were obtained at the magnification of 100 × and recorded using Viewfinder Lite (1.0) software. Control transfection was performed in each case using a commercially available transfection reagent Lipofectamine 2000™ based on the standard conditions specified by the manufacture.

For luciferase assays, cells were transfected by complexes containing pGL-3. For a typical assay in a 24-well plate, 24 h post-transfection as described above, the old medium was removed from the wells, and the cells were washed twice with 500 μL of pre-chilled PBS. According to Luciferase assay kit (promega) manufacture, 100 μL of 1 × cell lysis buffer diluted with PBS was then added to each well, and the cells were lysed for 30 min in a horizontal rocker at room temperature. The cell lysate was transferred completely to Eppendorf tubes and centrifuged (4000 rpm, RT) for 2 min; the supernatant was transferred to Eppendorf tubes and stored in ice. For the assay, 20 μL of this supernatant and 100 μL of luciferase assay substrate (Promega) were used. The lysate and the substrate were both thawed to RT before performing the assay. The substrate was added to the lysate, and the luciferase activity was measured in a luminometer (Turner designs, 20/20, Promega, USA) in standard single-luminescence mode. The integration time of measurement was 10,000 ms. A delay of 2 s was given before each measurement. The protein concentration in the cell lysate supernatant was estimated in each case with Lowry protein assay kit (PIERCE, Rockford, IL, USA). Comparison of the transfection efficiencies of the individual lipids was made based on the data for luciferase expressed as relative light units (RLU)/mg of protein. All the experiments were done in triplicates, and results presented are the average of at least two such independent experiments done on the same days.

2.14. Cytotoxicity assays

Toxicity of lipoplexes toward HEK293 cells was determined by using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay following literature procedures [25].

About 7000 cells/well were seeded into 96-well plates. After 24 h, optimized lipid/DOPE formulations were complexed with 0.2 μg of DNA at various N/P ratios for 30 min; 100 μL of lipoplexes were added to the cells in the absence of serum. After 4 h of incubation, lipoplex solutions were removed, and 200 μL of media with 10% FBS was added. After 24 h, 20 μL of MTT solution was added, and the cells were incubated further for 4 h. Blue formazan crystals were seen at well when checked under microscope. Media were removed, 150 μL of DMSO was added per well, and then, plates were incubated on a shaker for 10 min at room temperature to dissolve blue crystal. The absorbance was measured using a microtiter plate reader. The % viability was then calculated as [[A490(treated cells)-background]/[A490(Untreated cells)-background]] × 100. Lipoplexes prepared from Lipofectamine 2000 were used as control.

2.15. Transmission electron microscopy

Transmission electron microscopy was carried out to determine the morphology and particle size distribution of the optimized lipoplexes by negative staining using 1% uranyl acetate. A 10 μL sample of freshly prepared lipoplexes was loaded onto Formvar-coated, 400 mesh copper grids and allowed to remain for 1 min. Excess fluid was wicked off the grids by touching their edges to filter paper, and 10 μL of 1% uranyl acetate was applied on the same grid after which the excess stain was similarly wicked off. The grid was air dried for 20 min, and the specimens were observed under TEM (JEM 100CX II) operating at an acceleration voltage of 80 keV.

3. Results and discussion

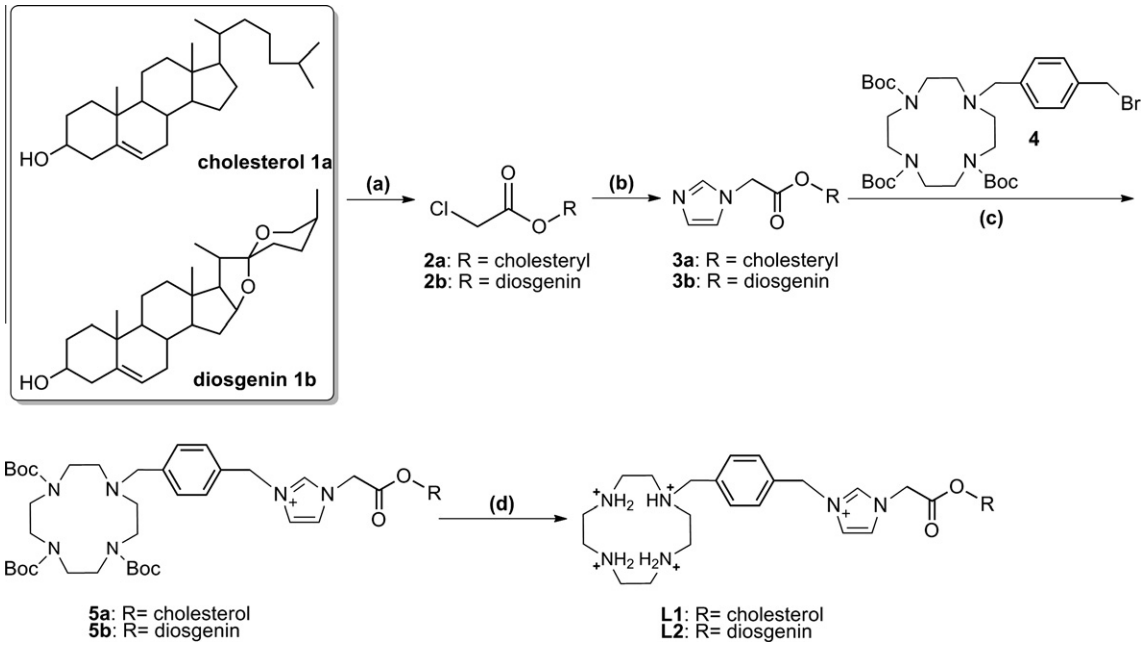
3.1. Synthesis of cationic lipids

The title lipids, whose protonated cyclen headgroup and a steroid tails were bridged by imdazolium and an ester group, were designed and synthesized along the route shown in Scheme 1. Considering the influence of hydrophobic moiety of cationic lipids on their gene transfection efficiency [27] and the unique structure of diosgenin that has an acetal group, we selected diosgenin for the first time as the hydrophobic region of cationic lipid to formulate the diosgenin-based cationic lipid **L2**. As shown in the scheme, chloroacetate derivatives **2** were first prepared through esterification between corresponding steroids (cholesterol **1a** or diosgenin **1b**) and chloroacetyl chloride. With potassium iodide as catalyst and sodium carbonate as base, chloroacetate derivatives **2** react with imidazole in chloroform and dimethyl sulfoxide at 90 °C for about 10 h to give the imidazole derivatives **3**, which could further react with precursor **4** in acetonitrile to form tri-Boc-protected lipids **5**. The final products **L1** and **L2** were obtained by removing the Boc groups by trifluoroacetic acid in CH_2Cl_2. To improve the water solubility, the trifluoroacetic acid salt was directly used for further studies. All new compounds were characterized by IR, ^{1}H NMR, ^{1}C NMR, and HRMS.

3.2. Formation of liposomes and lipid/DOPE/DNA complexes (lipoplexes)

Cationic liposomes are formed from either individual cationic lipid or more frequently from a combination of cationic lipid and neutral lipids, such as DOPE, which usually dramatically enhance the transfection efficiency. Each of cationic lipids was mixed in different molar ratios (1:0, 1:1, 1:2, 1:3, and 1:4) with DOPE to determine the most auspicious combination. Under these mole ratios, the particle size of formed liposomes ranged from 40 nm to 180 nm with zeta potentials in the range of 20–75 mV. Under the optimized mole ratio of 1:3 obtained in transfection experiments (see Fig. 7), the mean particle sizes of the liposome vesicles were 47.6 and 68.3 nm for **L1**/DOPE and **L2**/DOPE, respectively. Meanwhile, the zeta potentials for these two nanoparticles were 50.9 (**L1**/DOPE) and 24.9 mV (**L2**/DOPE). **L1**-based liposome has distinctly smaller size and higher zeta potential, and these differences were undoubtedly originated from the different hydrophobic moieties of cationic lipids.

Liposome/DNA complexes (lipoplexes) were prepared by combining constant amount of DNA with varying amounts of liposomes. Conventional electrophoretic gel retardation assay was first used to study the electrostatic interactions between plasmid



Scheme 1. Synthesis of target lipids **L1** and **L2**. Reaction conditions: (a) chloroacetyl chloride/Et_3N/CH_2Cl_2, 4 h, rt; (b) imidazole/sodium carbonate/potassium iodine/$CHCl_3$/DMSO, 10 h, 90 °C; (c) CH_3CN, reflux; (d) trifluoroacetic acid/CH_2Cl_2, 6 h, rt.

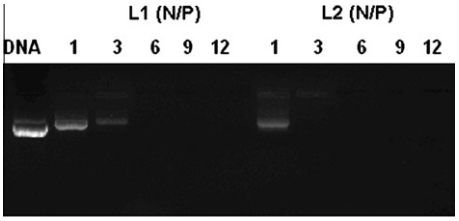


Fig. 2. Electrophoretic gel retardation assays of liposomes/DNA complexes (lipoplexes) at different N/P ratios. The molar ratio of lipid/DOPE was 1:3.

DNA and cationic lipids under different N/P ratios. As shown in Fig. 2, the liposomes formed from **L1** and **L2** could partly retard plasmid DNA at N/P ratio of 3. Further, both liposomes completely inhibit the electrophoretic mobility of plasmid DNA at N/P $\geqslant$ 6, indicating that they have similar DNA-binding abilities. Compared with lipid **L** (Fig. 1) that need high N/P ratio (39/1) to completely retard DNA [21], **L1** and **L2** showed dramatically increased DNA-binding abilities.

The binding ability of the liposomes (lipid/DOPE = 1:3) to CT (calf thymus) DNA was further studied by fluorescence spectroscopy with the use of ethidium bromide (EB). As shown in Fig. 3A, the addition of liposomes to EB pretreated with DNA caused appreciable decrease in the emission intensity, indicating that EB that bound to DNA was partially replaced by the lipids. The fluorescence quench results were also quite consistent with the following classical Stern–Volmer equation [28]:

$$F_{max}/F_x = 1 + K_{sv}(Q)$$

where F_{max} and F_x are as aforementioned in experimental section, Q is the mole concentration (mol/L) of lipid, and K_{sv} is the quenching constant (namely binding constant), which reflect the binding ability of lipid to DNA. After linear fitting (Fig. 3B), the K_{sv} values (namely binding constant) of the **L1**/DOPE and **L2**/DOPE were obtained as $1.02 \times 10^5\ M^{-1}$ ($R^2 = 0.99788$) and $1.69 \times 10^5\ M^{-1}$ ($R^2 = 0.99288$), respectively, showing that **L2** has better DNA-binding ability than **L1**.

The appropriate size and zeta potential of lipoplexes are of critical important for cationic lipids used as gene vectors. For easier deposition on cell membrane and subsequent uptake, the lipoplexes with moderate diameters (from 0.4 to 1.4 μm) were most effective for in vitro gene transfection [29]. The mean particle size and zeta potential of lipoplexes at various N/P ratio (as in gel retardation assays) in water were shown in Fig. 4. The two liposomes could bind and compact DNA into nanoparticles at various N/P ratios (1–12, Fig. 4A). To our surprise, the sizes of both lipoplexes at N/P ratio of 3 suddenly changed to above 700 nm compared with about 100 nm at other N/P ratios. The zeta potentials shown in Fig. 4B give an increased trend along with the increase in N/P ratios. Generally, the lipoplex formed from **L1** showed relatively higher zeta potential (about −4 to 40 mV) than that formed from

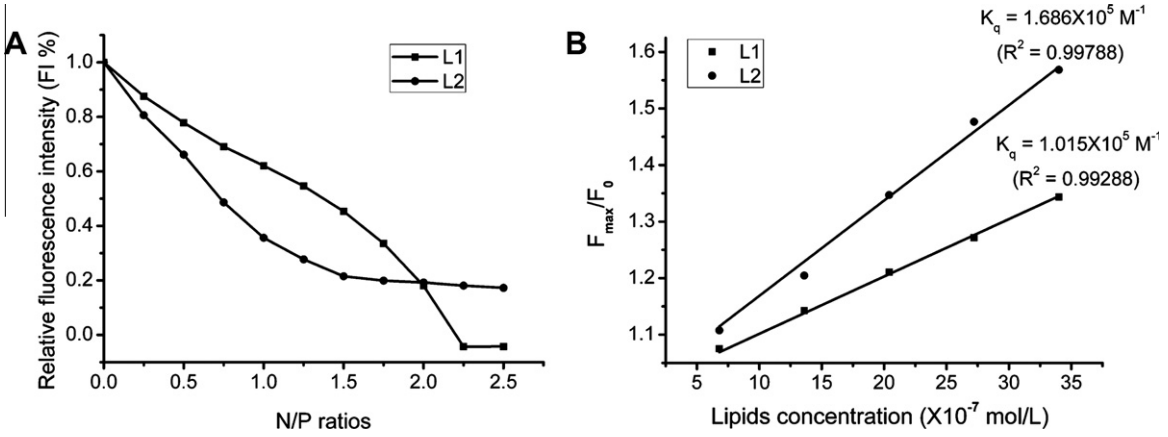


Fig. 3. (A) Change of relative fluorescence of EB bound to DNA by the addition of **L1**/DOPE or **L2**/DOPE to different N/P ratios. The lipid/DOPE ratio was 1:3. (B) Calculation of DNA-binding constant for the two liposomes according to the Stern–Volmer equation.

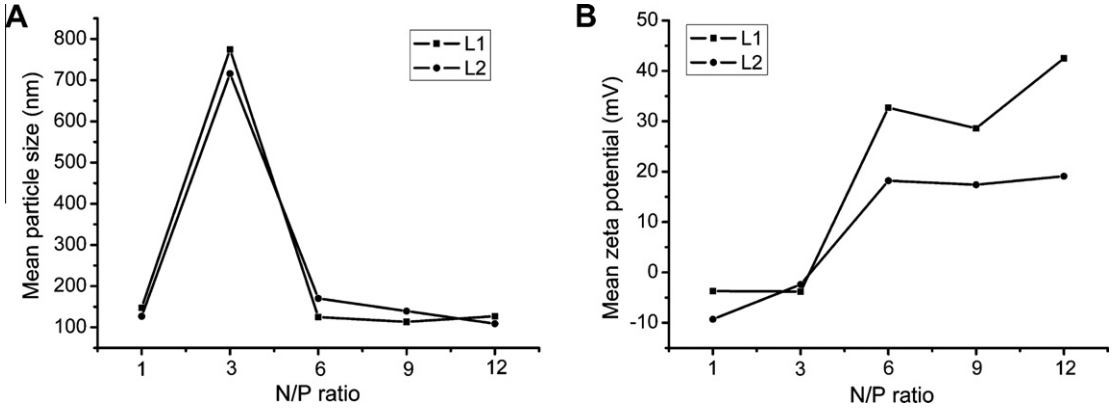


Fig. 4. Particle sizes (A) and zeta potentials (B) of lipid/DOPE/DNA (mole ratio of lipid/DOPE: 1:3) complexes at different N/P ratios by DLS.

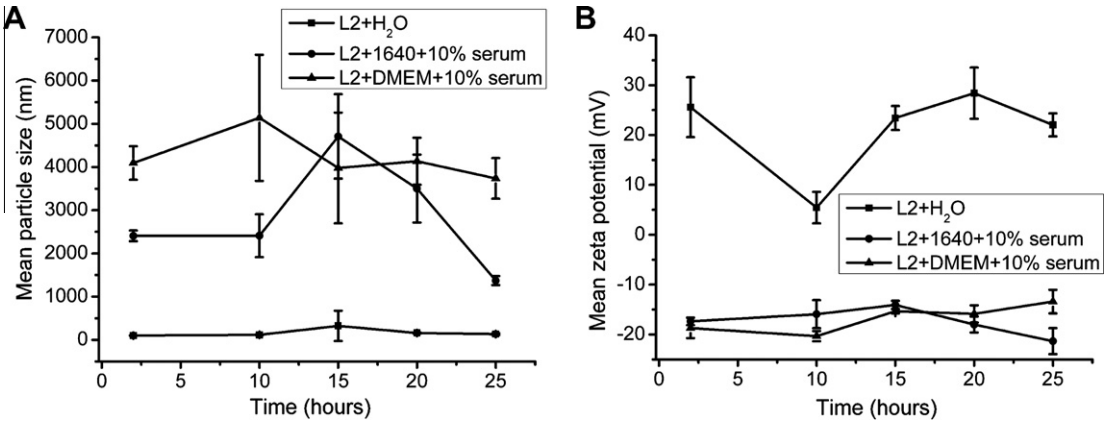


Fig. 5. Particle sizes (A) and zeta potentials (B) of **L2**/DOPE/DNA lipoplexes (N/P = 6) at different times by DLS.

L2 (about −10 to 20 mV). Both lipoplexes have low particle sizes (100–200 nm) and moderate positive zeta potentials (20–30 mV) at the N/P ratio of 6, indicating that DNA could be tightly compacted into nanoparticles with proper size and electric charge under such N/P ratio. These results were consistent with those obtained from gel retardation assays.

To determine the stability of the formed liposomes and lipoplexes in different media, the particle size and zeta potential of **L2**-based liposomes (**L2**/DOPE = 1:3) and lipoplexes at different times have been measured in water, RPMI 1640 medium, and DMEM medium with or without 10% serum. The **L2**/DOPE liposome was stable in water, and no evident change of particle size was observed even after 4 days (data not shown). For the lipoplex formed from **L2**/DOPE/DNA, the results in Fig. 5 showed that in the absence of serum, the lipoplex is stable in water with relatively small particle size (100–200 nm) and positive zeta potential. However, after addition of 10% serum, the particle size increased dramatically to 1–5 μm, while zeta potential was reversed to negative. These results indicated that the lipoplex is prone to bind with the serum proteins, promoting aggregation and possibly phagocytosis. On the other hand, the positive surface charge was neutralized by the negatively charged proteins, leading to the charge reverse.

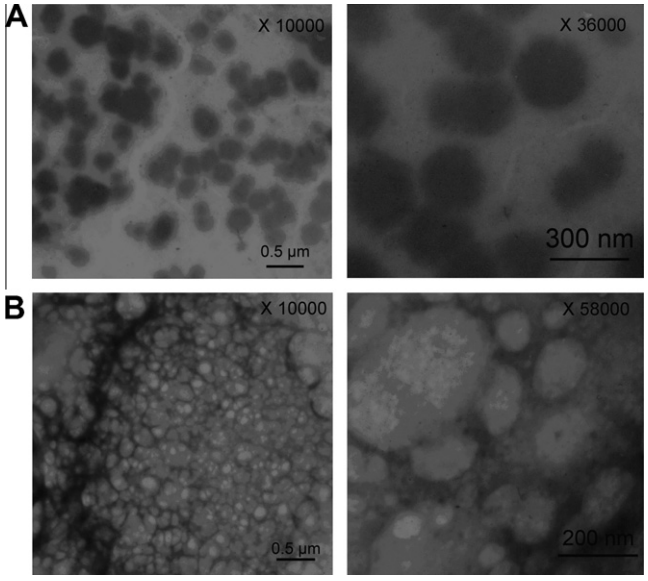


Fig. 6. Representative transmission electron micrographs of the lipoplexes prepared from **L1**/DOPE/DNA (A) and **L2**/DOPE/DNA (B).

Transfection efficiency of cationic lipids has been linked to not only the physicochemical characteristics but also the morphology of lipoplexes. To get further insights into the lipid–DNA complexation, we examined the lipoplexes formed at the lipid/DOPE ratio of 1:3 and N/P ratio of 1:6 by transmission electron microscopy. Representative electron micrographs of the lipoplexes are shown in Fig. 6. The images showed that **L1**/DOPE/DNA lipoplex gave homogeneous spherical particles, while **L2**/DOPE/DNA lipoplex gave non-homogeneous oval-shaped particles. The sizes of these nanoparticles were found to be in the range of 100–250 nm, which was in accord with the results described in DLS analysis.

3.3. In vitro transfection

The gene delivery efficiencies of many of the cationic lipid-based formulations depend on the lipid/DOPE ratio in liposomes and on the molecular structure of the cationic lipids. To find out the optimized lipid/DOPE ratio, we carried out the fluorescent microscopy assay by using pEGFP-N1 plasmid DNA as reporter gene in HEK293 cells. The mole ratio of cationic lipids against DOPE was varied (lipid/DOPE ratio of 1:0, 1:1, 1:2, 1:3, and 1:4) under the N/P ratio of 6. The pictures listed in Fig. 7 showed that two neat lipids were found to have no transfection ability (Fig. 7A and F). Distinct results could be obtained as that **L1**/DOPE/DNA lipoplexes have less transfection efficiency (Fig. 7B–E) than **L2**/DOPE/DNA (Fig. 7G–J). For both lipids, the lipid/DOPE ratio of 1:3 (Fig. 7D and I) was found to be the best combination for efficient transfection. Therefore, this ratio was used in our subsequent investigations.

Calcium ion was used to improve transfection efficiency of cationic liposomes by facilitating the cell uptake of nucleic acids through endocytosis and the entrance toward nucleus after endosomal escape [30,31]. To investigate the effect of calcium ions on the gene transfection performance of the title lipids, keeping the lipid/DOPE ratio at 1:3 and N/P ratio at 6, we carried out fluorescent microscopy assay by varying the concentration (0–20 μM) of calcium ions in transfection medium and using pEGFP-N1 plasmid DNA as reporter gene in HEK293 cells. The results shown that significant transfection enhancement was achieved in the presence of 4–6 μM calcium ions (data not shown). To further verify the observations, luciferase assays were also processed by using pGL-3 as reporter gene in the same cell type. As shown in Fig. 8A, the transfection efficiency of **L1**/DOPE/DNA lipoplex increased with the addition of Ca^{2+} to 4 mM, while further addition of Ca^{2+} led to less transfection efficiency. To our delight, dramatic increase in transfection efficiency was found in the experiments involving **L2** as cationic lipid. In the presence of 6 mM of Ca^{2+}, the luciferase expression in the transfection was found as about seven times as

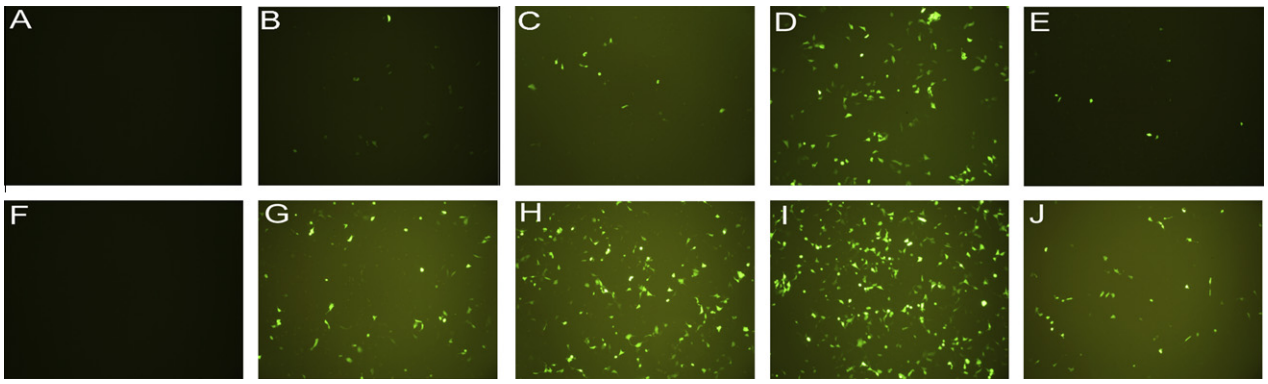


Fig. 7. Fluorescent microscope images of HEK293 cells transfected by **L1**-based (A–E) and **L2**-based (F–J) lipoplexes at the lipid/DOPE ratio of 1:0 (A, F), 1:1 (B, G), 1:2 (C, H), 1:3 (D, I), and 1:4 (E, J). N/P ratios in all experiments were 6. The cells were observed by fluorescence microscopy after 24 h transfection. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

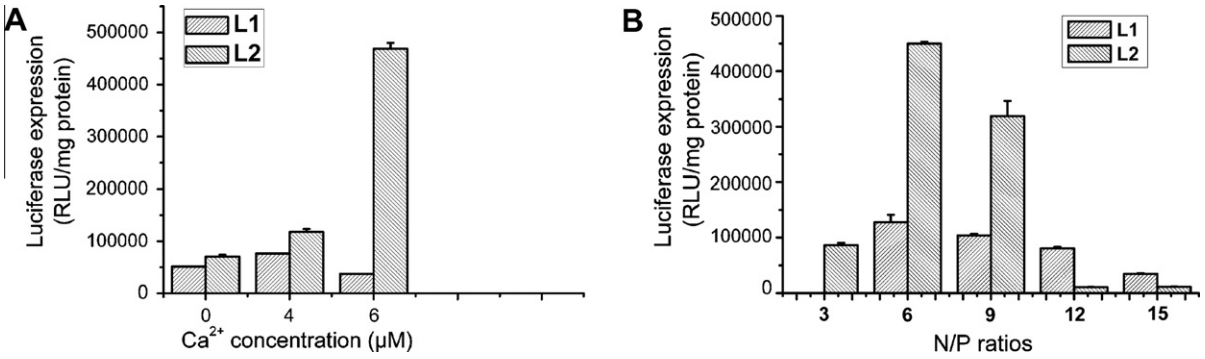


Fig. 8. (A) Luciferase expression in HEK293 cells transfected by Lipid/DOPE/DNA complexes (lipoplexes) at N/P ratio of 6 in the absence (control) and in the presence of 4 and 6 μM of Ca^{2+}. (B) Luciferase expression in HEK293 cells transfected by Lipid/DOPE/DNA complexes (lipoplexes) at various N/P ratios (3–15) in the presence of 6 μM of Ca^{2+}. pGL-3 was used as reporter gene in each case, and the concentration of Ca^{2+} was calculated with respect to the final volume (200 μL) of the transfection solution applied to cells.

more than that without the using of Ca^{2+}. According to the results, Ca^{2+} was employed at the concentration of 6 mM in subsequent experiments.

The lipoplexes were then subjected to luciferase assays at different N/P ratios (3–15) using optimized conditions studied above. As shown in Fig. 8B, the lipoplexes formed from **L1** and **L2** showed maximum transfection efficiencies at N/P ratio of 6. Furthermore, at optimized N/P ratios studied in our experiments, **L2** obviously yielded much higher transfection than **L1**, indicating that minor structure variation in the hydrophobic moiety might lead to large difference in transfection efficiency. Cholesterol, as a major component of cell membrane, was most commonly used as hydrophobic moiety of cationic amphiphiles because of its easy fusion with cell membranes. Cholesterol-based cationic lipids have been widely studied for gene delivery and shown excellent gene transfection [32,33]. However, results herein suggested that in the studied type of cationic lipids, diosgenin is much suitable to be the hydrophobic group for high in vitro gene transfection than cholesterol.

Serum has been reported to be the major hurdle in liposome-mediated gene delivery, and many studies showed that the transfection efficiency of cationic lipoplexes would decrease in the presence of serum. The negatively charged serum proteins might cause aggregation of cationic lipoplex by electrostatic interaction, leading to increased toxicity. Luciferase expression assays were carried out to estimate the effect of serum in the transfection system studied herein. As shown in Fig. 9, the transfection efficiencies dramatically decreased in the experiments using 10% serum in DMEM medium. However, by using RPMI 1640 as culture medium, different changes of transfection efficiency were found for the two lipoplexes. For **L2**-based lipoplex, transfection efficiency also decreased, and the amount of expressed luciferase was found as half of those obtained in the experiment without the use of serum. Meanwhile, for **L1**-participated transfection, increased luciferase expression was found in the experiments using RPMI 1640 as culture in the presence of 10% serum, but the transfection efficiency was still lower than that obtained in **L2**-participated transfection.

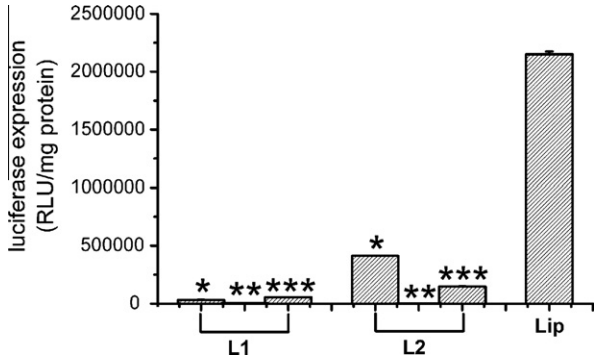


Fig. 9. Luciferase expression in HEK293 cells transfected by Lipid/DOPE/DNA complexes (lipoplexes) under optimized conditions. Cells were transfected in DMEM culture medium without (∗) or with (∗∗) 10% serum or in RPMI 1640 culture medium with 10% serum (∗∗∗). Lipofectamine 2000™ (Lip) was used as control.

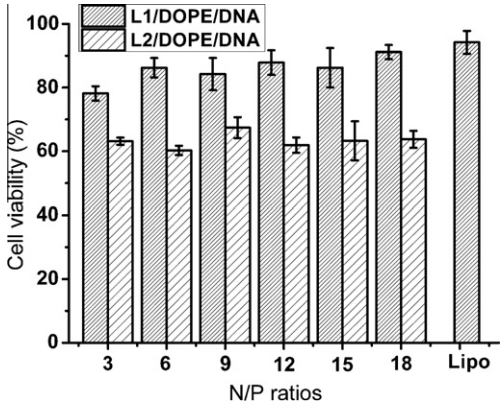


Fig. 10. Cytotoxicity of **L1**/DOPE/DNA and **L2**/DOPE/DNA exhibited toward HEK293 cells at different N/P ratios using lipid/DOPE ratio of 1:3. 0.5 μg of DNA was used, and the toxicity result of lipoplex prepared from lipofectamine 2000 was used as control.

3.4. Cytotoxicity

MTT-based cell viability assays were performed in HEK293 cells across the entire range of N/P used in the transfection experiments, and the results were shown in Fig. 10. Although both lipoplexes led to slight lower cell viability than lipofectamine 2000, they showed good biocompatibility. Under the routine transfection conditions, the cell viabilities were stable even under high N/P ratios. Lipoplex formed from **L1** displayed lower toxicity than that prepared from **L2**, indicating that as one of the factors that may influence the transfection efficiency, toxicity itself could not be a standard for the estimation of transfection efficiency.

4. Conclusion

In this study, we designed and synthesized two novel protonated cyclen and imidazolium salt-based cationic lipids, which differ only in their hydrophobic region (cholesterol or diosgenin). In association with DOPE, two cationic lipids can easily form liposomes and effectively bind and compact DNA into nanoparticles, which were characterized by size and zeta potential measurements and TEM. The gene transfection efficiencies of two cationic lipids could be dramatically increased in the presence of calcium ion (Ca^{2+}). Further, although DLS showed that aggregation might occur in the presence of 10% serum, the gene transfection abilities of two cationic lipids were maintained with serum. Different cytotoxicities were found for two lipoplexes. Although the transfection efficiencies of title lipids were lower than that of commercially available lipofectamine 2000, we believe that this type of cationic lipids has large potential to be efficient non-viral gene vectors. Further studies aimed to elucidate the structure activity relationship and to improve the transfection efficiency of such type of cationic lipids are now in progress.

Acknowledgments

This work was financially supported by the National Science Foundation of China (Nos. 20972104, 20902062, and 20725206), the Program for Changjiang Scholars and Innovative Research Team in University, the Key Project of Chinese Ministry of Education in China, and the Scientific Fund of Sichuan Province for Outstanding Young Scientist. We also thank Analytical & Testing Centre of Sichuan University for NMR analysis.

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