Chem Biol Drug Des 2012; **79:** 879–887

Research Article

doi: 10.1111/j.1747-0285.2012.01355.x

Cationic Lipids Containing Cyclen and Ammonium Moieties as Gene Delivery Vectors

Qing-Dong Huang†, Jiang Ren†, Wen-Jing Ou, Yun Fu, Mao-Qiang Cai, Ji Zhang*, Wen Zhu* and Xiao-Qi Yu*

Key Laboratory of Green Chemistry and Technology (Ministry of Education), College of Chemistry, and State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu 610064, China

**Corresponding authors: Xiao-Qi Yu,* xqyu@scu.edu.cn*; Wen Zhu, zhuwen@scu.edu.cn; Ji Zhang, ji_zhang@163.com*

†*These authors contributed equally to this work.*

In this study, two novel cationic lipids containing protonated cyclen and quaternary ammonium moieties were designed and synthesized as non-viral gene delivery vectors. The structures of the two lipids differ in their hydrophobic region (cholesterol or diosgenin). Cationic liposomes were easily prepared from the lipids individually or from the mixtures of each cationic lipid and dioleoylphosphatidylethanolamine. Several studies including DLS, gel retardation assay, and ethidium bromide intercalation assay suggest that these amphiphilic molecules are able to bind and compact DNA into nanometer particles which can be used as non-viral gene delivery agents. Our results from *in vitro* transfection show that in association with dioleoylphosphatidylethanolamine, two cationic lipids can induce effective gene transfection in human embryonic kidney 293 cells, although the gene transfection efficiencies of two cationic lipids were found to be lower than that of lipofectamine 2000™. 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 vectors.

Key words: ammonium, cationic lipids, cyclen, gene delivery, gene transfection

Received 30 April 2011, revised 3 January 2012 and accepted for publication 7 January 2012

The clinical success of gene therapy continues to remain critically dependent on the availability of safe and efficacious gene delivery reagents, popularly known as transfection vectors (1). Generally, existing transfection vectors are classified into two major categories: viral and non-viral. Viral vectors are significantly more efficient in delivering the therapeutic gene as well as inducing gene expression as a result of their highly evolved and specialized components. However, because of their inherent drawbacks such as immunogenicity, restricted targeting of specific cell types, size limitation on DNA, and potential for mutagenesis (2), their clinic applications are limited. Consequently, an increasing number of investigations are being reported on the development of versatile, safe, and efficient non-viral alternatives including cationic lipids (3,4), cationic polymers (5), and dendrimers (6). Because of their less immunogenic nature, robust manufacture ability to deliver large pieces of DNA, and ease of handling and preparation techniques, an upsurge of global interest in developing efficient cationic lipids for gene delivery was witnessed in recent years.

The molecular structure of cationic lipids used for gene delivery generally comprises three segments: a positively charged hydrophilic polar headgroup region (usually consisting of one or more positively charged nitrogen atoms), a non-polar hydrophobic region (usually consisting of one, two, or three hydrocarbon chains or a cholesterol derivative), and a functional linkage such as ether, ester, amide, amidine group, which bridges the headgroup and hydrophobic tail. Variations in any part of the three regions may dramatically influence the gene transfer efficacies of cationic lipids (7,8). Therefore, the systematic modification of each part and understanding of the structure–activity relationship are of great importance. Although several cationic lipid-based transfection kits have been commercially available, those were still far from the requirement of *in vivo* gene therapy because of their potential toxicity and relative low transfection efficiency.

Previous reports have indicated that cationic lipids using polyamine as the headgroups generally have higher gene transfection efficiencies than cationic lipids involving a quaternary amine or a single protonated amine (9,10). However, cationic lipids with long linear polyamine chain as headgroup may also decrease gene delivery efficiency because of their relative low DNA-binding ability, which might be the result of self-folding of the linear lipopolyamine chain (11). On the contrary, cyclic polyamines and branched polyamines are hard to self-fold; therefore, these cationic hydrophilic moieties can be used to increase the DNA-binding ability. Although many cationic amphiphiles involving linear polyamines (12,13) or branched polyamines (14) have been widely studied, it seems that only few examples (15) using macrocyclic polyamines as hydrophilic headgroup on a cationic lipid were investigated. As a typical compound in the macrocyclic polyamine family, 1, 4, 7, 10-tetraazacyclododecane (cyclen) has centralized amine density and non-folded conformations comparing to linear polyamines, and the relatively large pK_a of the protonated amines may ensure its DNA-binding ability. Our previous studies focused on the synthesis of cyclen derivatives,

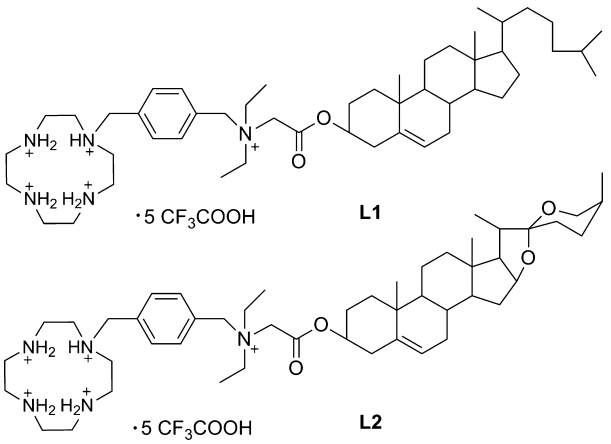


Figure 1: Structures of the cationic lipids (**L1** and **L2**) studied herein.

their interactions with plasmid DNA, and gene delivery abilities (16–19). It was revealed that many cyclen derivatives have great potential to be gene delivery vector. More recently, we designed and synthesized two protonated cyclen and imidazolium salt-based cationic lipids for gene delivery. These lipids could mediate effective gene transfection in association with neutral lipid dioleoylphosphatidylethanolamine (DOPE) and calcium ion (Ca^{2+}). However, with the absence of calcium ion (Ca^{2+}), the two cationic lipids have nearly no gene transfection abilities (20).

On the basis of our above research, we herein designed and synthesized two cationic lipids containing protonated cyclen and quaternary ammonium moieties (**L1** and **L2**, Figure 1). The two lipids have same hydrophilic headgroup (protonated cyclen) and linker group (ammonium salt and ester parts). The only structural difference between them is their hydrophobic region (cholesterol for **L1** and diosgenin for **L2**). Their interactions with plasmid DNA and the properties of formed lipoplexes were examined. The *in vitro* transfection efficiencies toward human embryonic kidney (HEK) 293 cells were investigated. Results indicate that the two cationic lipids have good transfection efficiencies in association with DOPE in HEK 293 cells. Further, the transfection efficiencies of **L2** with diosgenin as hydrophobic group are higher than that of **L1** with cholesterol as hydrophobic group, while **L1** shows lower cytotoxicity than **L2**.

Materials and Methods

Chemicals and methods

Unless stated otherwise, all chemicals and reagents were obtained commercially and used without further purification. Absolute chloroform ($CHCl_3$) and dichloromethane (CH_2Cl_2) were distilled from calcium hydride (CaH_2) and anhydrous acetonitrile (CH_3CN) from phosphorus pentoxide. The precursor cholesteryl chloroacetate (**2a**) (21) and 1-bromomethyl-4-(4, 7, 10-tris(tertbutyloxycarbonyl)-1′,4′,7′,10′-tetraazacyclododecane-1-yl-methylene)benzene (**4**) (22) were prepared according to the literature. IR spectra were recorded on a Shimadzu FTIR-4200 spectrometer (Shimadzu Inc., Tokyo, Japan) 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 (Varian Inc., Palo Alto, CA, USA), 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 LCQDECA (San José, CA, USA) and a Bruker Daltonics BioTOF mass spectrometer (Bruker Daltonics Inc., CA, USA), respectively. Fluorescence spectra were measured by a Horiba Jobin Yvon Fluoromax-4 spectrofluorometer (Hitachi Inc., Tokyo, Japan). Particle size and zeta potential of liposomes or lipoplexes were measured via dynamic light scattering (DLS) by a Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern, UK) at 25 °C. MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) were purchased from Sigma-Aldrich (St. Louis, MO, USA). MicroBCA 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) coding for luciferase DNA and pEGFP-N1 (Clontech, Palo Alto, CA, USA) coding for EGFP DNA. The Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Invitrogen Corp.

Preparation of diosgenin chloroacetate (2b)

Chloroacetyl chloride (180 mmol) in anhydrous dichloromethane (30 mL) was added dropwise to a stirred solution of diosgenin (36 mmol) and triethylamine (5.8 mL, 41.4 mmol) in anhydrous dichloromethane (100 mL). The resulting reaction mixture was stirred for 6 h at room temperature. Excess chloroacetyl chloride was removed by washing with a 5% solution of sodium hydrogen carbonate (60 mL × 3) and brine(60 mL × 1). The organic phase was dried over anhydrous magnesium sulfate, filtered, and rotary-evaporated. The resulting chloroacetate derivative was obtained as a white solid. IR (per cm): 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, $ClCH_2CO$), 4.42 (m, 1H, diosgenin-H), 4.70 (m, 1H, diosgenin-H), and 5.40 (s, 1H, C=CH-). HR MS ($C_{29}H_{44}ClO_4$ + H): Calcd.491.2928; Found. 491.2934.

General process for the preparation of 3

Chloroacetate derivatives (**2a** and **2b**) (27.1 mmol) were added to a solution of diethyl amine (25.76 g, 325.2 mmol), sodium carbonate(2.9 g, 28 mmol), potassium iodide (0.24 g, 1.4 mmol) in anhydrous chloroform(36 mL), and dimethyl sulfoxide (22 mL). The reaction mixture was heated at 90 °C for 10 h, then chloroform (180 mL) was added, washed the resulting reaction mixture with a 5% solution of sodium hydrogen carbonate (100 mL × 3) and brine (100 mL × 1). The organic phase was dried over anhydrous magnesium sulfate, filtered, and rotary-evaporated to obtain crude residues that were purified by flash chromatography over silica (ethyl acetate) to obtain the corresponding diethyl amine derivatives **3a** and **3b**.

Compound **3a**: IR (cm^{-1}): 3453, 2961, 1736, 1465, 1380, 1276, 1179, 1091, 1009, 954, 842, 804, 726. ^1H NMR ($CDCl_3$, 400 MHz): δ

0.68 (s, 3H, cholesterol-H), 0.85–2.02 (br, 43H, cholesterol-H and $N(CH_2CH_3)_2$), 2.32 (d, 2H, cholesterol-H), 2.66 (m, J = 21.2 Hz, 4H, $N(CH_2CH_3)_2$), 3.30 (s, 2H, NCH_2CO), 4.67 (m, 1H, cholesterol-H), 5.35 (s, 1H, C=CH-). ^{13}C NMR ($CDCl_3$, 100 MHz): 21.02, 22.56, 22.82, 23.83, 24.28, 27.82, 28.01, 28.23, 31.84, 31.89, 35.79, 36.17, 36.58, 36.97, 38.15, 39.51, 39.72, 42.30, 47.73, 50.01, 54.42, 56.12, 56.67, 74.02, 76.81, 77.02, 77.24, 122.70, 139.57, 170.92. ESI MS: 500.45($M+H^+$).

Compound **3b**: IR (per cm): 3423, 2941, 2896, 2848, 1724, 1454, 1377, 1271, 1244, 1141, 1097, 1052, 1010, 982, 959, 899, 867. ^1H NMR ($CDCl_3$, 400 MHz): δ 0.79 (t, J = 6 Hz, 6H, diosgenin-H), 0.96–2.02 (br, 32H, diosgenin-H and $N(CH_2CH_3)_2$), 2.33 (d, 2H, diosgenin-H), 2.65 (q, J = 14.4 Hz, 4H, $N(CH_2CH_3)_2$), 3.30 (s, 2H, NCH_2COO), 3.37 (t, 1H, diosgenin-H), 3.40 (t, 1H, diosgenin-H), 4.40 (m, 1H, diosgenin-H), 4.75 (m, 3H, diosgenin-H), 5.38 (s, 1H, C=CH-). ^{13}C NMR ($CDCl_3$, 100 MHz): 16.27, 17.13, 19.33, 20.79, 27.78, 28.79, 30.28, 31.38, 31.82, 32.02, 36.71, 36.92, 38.11, 39.70, 40.23, 41.59, 47.72, 49.90, 54.37, 56.40, 62.06, 66.81, 73.90, 80.78, 109.24, 122.40, 139.59, 170.89. ESI MS: 528.69 ($M+H^+$).

General process for the preparation of 5

A solution of diethyl amine derivatives (**3a** and **3b**) (6 mmol) and 1-bromomethyl4-(4, 7, 10-tris-(tertbutyloxycarbonyl)-1, 4, 7, 10-tetraazacyclododecane-1-yl-methyl)benzene (**4**) (7.9 g, 12 mmol) in anhydrous acetonitrile (30 mL) was refluxed at 90 °C over a period of 60–96 h, after which TLC indicated the disappearance of the starting material diethyl amine derivatives. Then, reaction mixture was cooled and the acetonitril was rotary-evaporated to furnish a yellow residue, and it was purified by chromatography over silica (dichloromethane/methanol, 16:1) to obtain white foamy solid in 45–60% yield.

Compound **5a**: IR (per cm): 3426, 2941, 1743, 1691, 1462, 1414, 1365, 1249, 1218, 1172, 1029, 979, 861, 773. ^1H NMR ($CDCl_3$, 400 MHz): δ 0.68 (s, 3H, cholesterol-H), 0.86–2.05 (br, 72H, cholesterol-H, $N(CH_2CH_3)_2$ and Boc-H), 2.01–2.65 (br, 6H, cholesterol-H and cyclen-H), 3.32–3.69 (br, 12H, cyclen-H), 3.80–3.86 (br, 4H, $N(CH_2CH_3)_2$), 4.44 (s, 2H, Ar-CH_2-cyclen), 4.72 (m, 1H, cholesterol-H), 5.04 (s, 2H, Ar-CH_2-$N(CH_2CH_3)_2$), 5.31 (s, 2H, Et_2NCH_2COO), 7.33–7.36 (m, 2H, benzene-H), 7.50–7.52 (m, 2H, benzene-H). ^{13}C NMR ($CDCl_3$, 100 MHz): 24.21, 27.48, 27.92, 27.99, 28.13, 28.43, 28.62, 31.19, 31.77, 31.84, 35.70, 36.13, 36.51, 36.77, 37.69, 39.45, 39.66, 42.27, 47.72, 49.96, 54.24, 55.34, 55.81, 56.12, 56.63, 63.06, 76.82, 79.48, 123.49, 125.68, 125.74, 127.79, 130.98, 132.59, 133.91, 138.69, 155.36, 155.85, 164.26. ESI MS: 1074.43 (M^+).

Compound **5b**: IR (per cm): 3416, 2951, 3951, 1742, 1691, 1560, 1414, 1367, 1248, 1218, 1172, 1051, 982, 898, 864, 774. ^1H NMR ($CDCl_3$, 400 MHz): δ 0.79 (s, 6H, diosgenin-H), 0.97–2.05 (br, 60H, diosgenin-H, Boc-H and NCH_2CH_3), 2.38 (s, 2H, diosgenin-H), 2.65 (br, 4H, cyclen-H), 3.32–3.57 (br, 14H, cyclen-H and diosgenin-H), 3.70–3.85 (br, 6H, $N(CH_2CH_3)_2$ and benzene-CH_2-cyclen), 4.41–4.42 (br, 3H, diosgenin-H and NCH_2COO), 4.75 (m, 1H, diosgenin-H), 5.03 (m, 2H, benzene-CH_2-$N(CH_2CH_3)$), 5.41 (s, 2H, C=CH-), 7.35 (d, 2H, J = 6.4, benzene-H), 7.49 (d, 2H, J = 7.6, benzene-H). ^{13}C NMR ($CDCl_3$, 100 MHz): 18.39, 19.24, 20.77, 27.44, 28.43, 28.64, 28.74, 30.24, 31.30, 31.78, 31.99, 36.66, 36.72, 37.66, 39.63, 40.21, 41.56, 49.84, 55.25, 55.75, 56.36, 58.16, 62.03, 66.80, 77.31, 79.54, 80.73, 109.26, 123.24, 132.57, 138.74, 164.31. ESI MS: 1103.63 (M^++H).

General process for the preparation of L1 and L2

Precursor (**5a** and **5b**) (1.2 mmol) was suspended in anhydrous dichloromethane (15 mL), then to the resulting solution was added dropwise a solution of trifluoroacetic acid (5 mL) in anhydrous dichloromethane (5 mL) under ice-cold water 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 obtain yellow residue. Then, under stirring, anhydrous ethyl ether was added dropwise into the residue to give the final lipids as a white solid in 98–100% yield.

Cationic lipid **L1**: IR (per cm): 3411, 2947, 2867, 1740, 1687, 1464, 1201, 1174, 1129, 827, 799, 719. ^1H NMR (DMSO-d6, 400 MHz): δ 0.66 (s, 3H, cholesterol-H), 0.84–1.99 (br, 47H, cholesterol-H, $N(CH_2CH_3)_2$), 2.36 (d, 2H, cholesterol-H), 2.75 (s, 4H, cyclen-H), 2.85 (s, 4H, cyclen-H), 3.06 (s, 4H, cyclen-H), 3.14 (m, 4H, cyclen-H), 3.47 (s, 4H, $N(CH_2CH_3)_2$), 4.24 (s, 2H, benzene-CH_2-cyclen), 4.65 (s, 1H, cholesterol-H), 4.70 (s, 2H, benzene-CH_2-$N(CH_2CH_3)_2$), 5.40 (s, 1H, C=CH-), 5.76 (s, 2H, $(CH_3CH_2)_2N$-CH_2-COO), 7.49 (m, 4H, benzene-H). ^{13}C NMR (DMSO-d6, 100 MHz): 22.85, 23.12, 23.66, 24.31, 27.48, 27.86, 28.25, 31.14, 31.81, 35.66, 36.12, 36.53, 36.75, 37.66, 45.11, 47.78, 49.84, 54.94, 55.38, 55.81, 56.04, 56.48, 56.57, 62.04, 76.23, 123.17, 126.81, 131.06, 133.17, 138.62, 139.38, 164.89. HR MS ($C_{49}H_{84}N_5O_2^+$): Calcd. 774.6625; Found. 774.6564.

Cationic lipid **L2**: IR (per cm): 3432, 2949, 1743, 1683, 1457, 1203, 1177, 1137, 1055, 1008, 984, 899, 799, 720. ^1H NMR ($CDCl_3$, 400 MHz): δ 0.79 (s, 6H, diosgenin-H), 0.97–2.00 (br, 38H, diosgenin-H and NCH_2CH_3), 2.37 (s, 2H, diosgenin-H), 2.87–3.18 (br, 16H, cyclen-H), 3.35–3.40 (t, J = 21.6, 1H, diosgenin-H), 3.46–3.49 (t, J = 14.0, 1H, diosgenin-H), 3.57 (s, 4H, $N(CH_2CH_3)_2$), 4.03 (s, 2H, benzene-CH_2-Cyclen), 4.40 (m, 1H, diosgenin-H), 4.75 (s, 3H, benzene-CH_2-$N(CH_2CH_3)_2$ and C=CH-), 5.42 (s, 2H, NCH_2COO), 7.44–7.46 (m, 4H, benzene-H). ^{13}C NMR (DMSO-d6, 100 MHz): 30.26, 31.13, 31.35, 31.89, 31.96, 36.68, 36.73, 37.65, 39.54, 39.68, 39.82, 39.96, 40.10, 40.24, 40.38, 41.55, 42.47, 42.59, 45.03, 47.73, 49.79, 54.94, 55.66, 55.79, 56.15, 62.07, 62.24, 66.37, 76.21, 79.68, 80.63, 108.88, 116.23, 118.20, 122.99, 126.82, 131.08, 133.16, 138.56, 139.42, 158.80, 159.02, 164.88. HR MS ($C_{49}H_{80}N_5O_5^+$): Calcd. 802.6210; Found. 802.6207.

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 traces of organic solvent were removed by keeping these films under vacuum above 8 h. The dried films and freshly autoclaved Tris buffer (10 mM, PH 7.4) were preheated to 70 °C, then the Tris buffer (5 mL) was added to the films such that the final concentration of the cationic lipid was 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 which were stored at 4 °C.

Amplification and purification of plasmid DNA

pGL-3 and pEGFP-N1 plasmids DNA 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.

Preparation of liposome/pDNA complexes (lipoplexes)

To prepare the cationic lipid/pDNA complexes (lipoplexes), various amounts of cationic lipid in liposome solutions were mixed with a constant amount of DNA by pipetting thoroughly at various N/P ratios and incubated for 30 min at room temperature. The theoretical N/P ratios of lipid to DNA represent the ratios of charge on cationic lipid (in mol) to nucleotide-based molarity and were calculated by considering the average nucleotide unit mass of 350.

Gel retardation assay

To determine the formation of liposome/pDNA complex (lipoplexes), lipoplexes of various N/P ratios ranging from 0 to 8 were prepared as described earlier. Constant amount of 5 μg DNA was used here. 10 μL of each lipoplexes solution was electrophoresed on the 1% (w/V) agarose gel containing Gold view and Tris–acetate (TAE) running buffer at 110 V for 30 min. DNA was visualized with a UV lamp using a BioRad Universal Hood II (Bio-Rad, Hercules, CA, USA).

Ethidium bromide intercalation assay

Fluorescence emission owing 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 owing 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 EB and 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 $FI\% = (F_x - F_0)/(F_{max} - F_0) \times 100$.

Cell culture

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) at 37 °C in a humidified atmosphere containing 5% CO_2.

Transfection procedures

Twenty four-well plates were seeded with 75 000 cell/well in 500 μL antibiotic-free media 24 h before transfection to obtain about 80% confluent cultures at the time of 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. 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 were 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 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 fluorescent microscopy assays, cells were transfected by lipoplexes 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 (Olympus, Tokyo, Japan). 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 lipoplexes containing pGL-3. For a typical assay in a 24-well plate, 24 h post-transfection as described earlier, the old medium was removed from the wells and the cells were washed twice with 500 μL of pre-chilled PBS. According to the protocol of Luciferase assay kit (Promega), 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) 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). Comparison of the transfection efficiencies of the individual lipids was made based on data for luciferase expressed as relative

light units (RLU)/mg of protein. All the experiments were carried out in triplicates, and results presented are the average of at least two such independent experiments carried out on the same days.

Cytotoxicity assays

Toxicity of lipoplexes toward HEK 293 cells was determined by using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) reduction assay following the literature procedures (23). About 7000 cells/well were seeded into 96-well plates. After 24 h, optimized lipid/DOPE formulations were complexed with 0.5 μg of DNA at various N/P ratios for 30 min. 100 μL of lipoplexes was 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 (Bio-Rad). The cell viability (%) was then calculated as [A_{490}(treated cells)-background]/[A_{490}(Untreated cells)-background] $\times$ 100.

Results and Discussion

Synthesis of cationic lipids

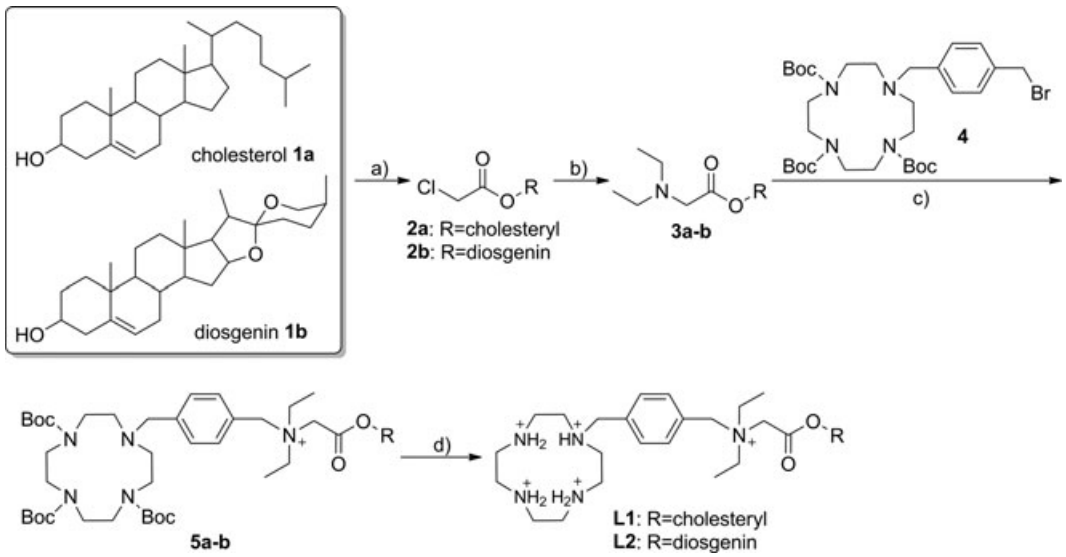
Target cationic lipids **L1** and **L2** were synthesized along the route shown in Scheme 1. Firstly, halogenated derivatives (**2a** and **2b**) were prepared via the acylation of corresponding alcohols **1** (cholesterol or diosgenin) by chloroacetyl chloride. With potassium iodide as catalyst, halogen derivatives **2** then reacted with diethyl amine in the presence of sodium carbonate under 90 °C in chloroform and dimethyl sulfoxide (DMSO) for about 10 h to give diethyl amine derivatives **3**. Subsequent reaction between **3** and compound **4** in acetonitrile led to the Boc-protected cyclen–ammonium compounds **5**. The final cationic lipids **L1** and **L2** were obtained by removing the Boc groups of **5** with trifluoroacetic acid in methylene dichloride. All lipid compounds were characterized by ^{1}H-NMR, ^{13}C-NMR, ESI MS, and IR.

Formation of liposomes

The cationic liposomes were prepared from each cationic lipid with or without the participation of neutral lipids (helping lipids) such as DOPE. The influence of DOPE on transfection was ascribed to their tendency to adopt the inverted hexagonal HII phase that seems to be necessary for the DNA release from the endosomal compartment, leading to dramatic enhancement of the transfection efficiencies (24). For this reason, according to the modified procedures reported in the literature (25), each of cationic lipids was mixed in different molar ratios (1:1, 1:2, 1:3, 1:4, 1:5) with DOPE to optimize the lipid/DOPE formulation. Under the studied mole ratios of cationic lipids against neutral lipids, the particle size of all liposome vesicles ranged from 50 to 100 nm with zeta potentials in the range of 25–50 mV. The mean particle sizes of the liposome vesicles were 96.4 and 60.1 nm for **L1**/DOPE (1:3) and **L2**/DOPE (1:3), respectively. With the same DOPE usage, the mean zeta potentials for these two nanoparticles were 44.1 (**L1**/DOPE) and 35.5 mV (**L2**/DOPE). It was evident that **L1**-based liposome has bigger size and slightly higher zeta potential than **L2**-based liposomes.

Formation and characterization of liposome/DNA complexes (lipoplexes)

Electrostatic interactions between plasmid DNA and liposomes under different N/P ratios were firstly studied by conventional electrophoretic gel retardation assays. As shown in Figure 2, **L1**/DOPE (1:3) could partly retard plasmid DNA at N/P ratio of 2 and completely retard plasmid DNA at N/P ratio of 4, while **L2**/DOPE (1:3)



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

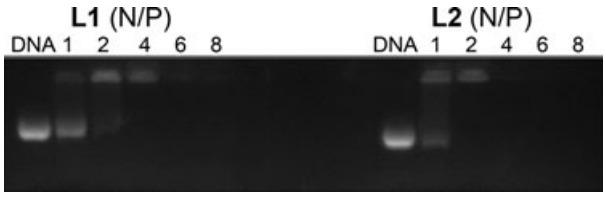


Figure 2: Electrophoretic gel retardation assays of **L1**(**L2**)-based lipoplexes at various N/P ratios (1, 2, 4, 6, 8) by keeping the lipid/DOPE ratio at 1:3.

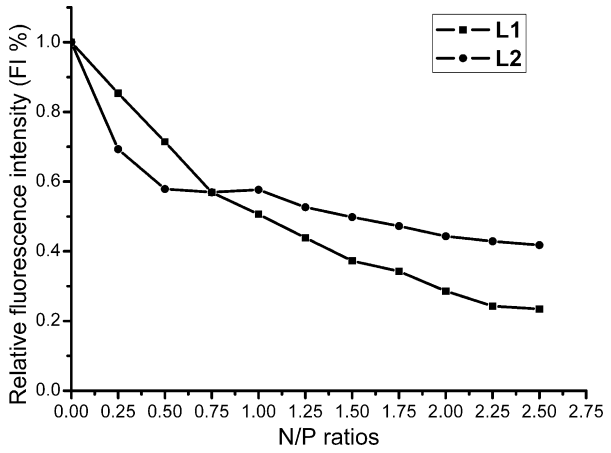


Figure 3: Change in relative fluorescence of EB bound to CT-DNA by the addition of **L1**(**L2**)-based liposomes to different N/P ratios. All the lipid/DOPE ratio is 1:3.

completely inhibit the electrophoretic mobility of plasmid DNA at N/P ratio of 2. The results indicate that the liposomes formed from **L1** and **L2** could bind to plasmid DNA through electrostatic interactions, and **L2** might have stronger DNA-binding ability than **L1**. The ethidium bromide (EB)-participated fluorescence spectroscopy also demonstrates their binding ability. EB has weak fluorescence, but its emission intensity in the presence of CT-DNA can be greatly enhanced because of its strong intercalation between the adjacent DNA base pairs. This enhanced fluorescence could be quenched, or at least partly quenched by the addition of a second molecule with higher DNA-binding ability. Figure 3 shows that the addition of liposomes to EB pretreated with DNA caused appreciable decrease in the emission intensity, indicating that the EB was partially replaced by the liposomes.

For lipoplexes used as gene vectors, appropriate size and zeta potential are of great importance. Figure 4A shows the mean particle sizes and zeta potentials of lipoplexes studied herein at various N/P ratios. Similar to the gel retardation assays, results here also show that **L2**/DOPE has higher DNA condensation ability than **L1**/DOPE. Under relatively higher N/P ratios (>2), **L2**-based liposome could efficiently condense DNA into nanoparticles with the sizes around 130 nm, which was smaller than those formed by **L1**/DOPE/DNA. Additionally, the sizes of **L1**/DOPE/DNA at N/P ratio of 2 suddenly changed to above 700 nm compared with those at other N/P ratios, indicating a partial condensation as shown in the gel retardation assays (Figure 2). The zeta potentials shown in Figure 4B gave an increased trend along with the increase in N/P ratio, and the values reached a plateau of 35 mV for **L1**/DOPE/DNA and 30 mV for **L2**/DOPE/DNA. The N/P ratios at which the zeta potential reached a plateau for **L1** and **L2** were 4 and 2, respectively, which were consistent with those at which DNA was completely retarded in gel retardation experiments.

In vitro *transfection*

Fluorescent microscopy assays and luciferase assays were performed to evaluate the *in vitro* transfection abilities of title lipids (**L1** and **L2**) in HEK 293 cells. To find out the optimized lipid/DOPE ratio, we firstly carried out a luciferase transfection experiments by varying the lipid/DOPE ratios (1:1, 1:2, 1:3, 1:4, 1:5) at the N/P ratio of 10 using pGL-3 plasmid DNA toward HEK 293 cells. As shown in Figure 5, lipoplexes formed from **L1** and **L2** displayed maximum transfection efficiency at lipid/DOPE mole ratio of 1:3 and 1:5, respectively. For the transfection involving **L2**, very low transfection efficiencies were obtained when the lipid/DOPE ratio was below 1:5. More usage of DOPE (lipid/DOPE = 1:6 and 1:7) did not induce higher transfection efficiencies for **L2** (data not shown).

The effect of N/P ratio on the gene transfection was then firstly evaluated by fluorescent microscopy assay, which was conducted in

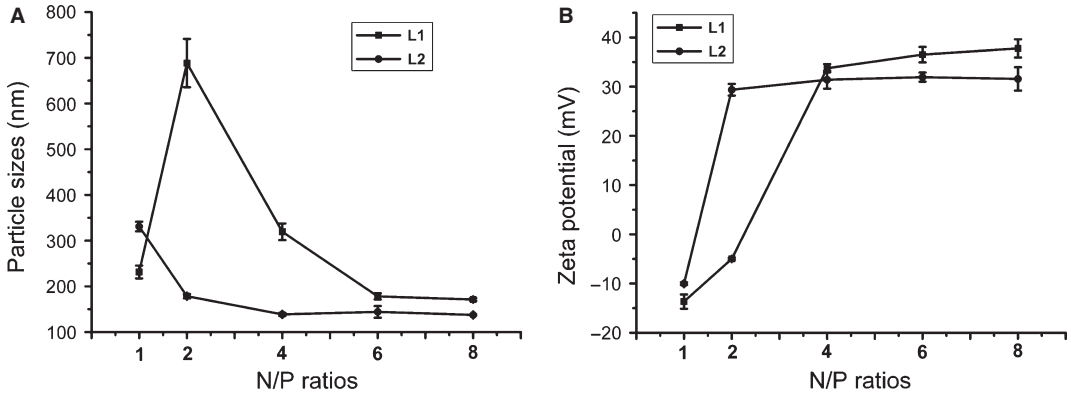


Figure 4: Particle sizes (A, average of three measurements) and zeta potentials (B, average of 10 measurements) of lipid/DOPE/DNA complexes at different N/P ratios by DLS. All the lipid/DOPE ratio is 1:3.

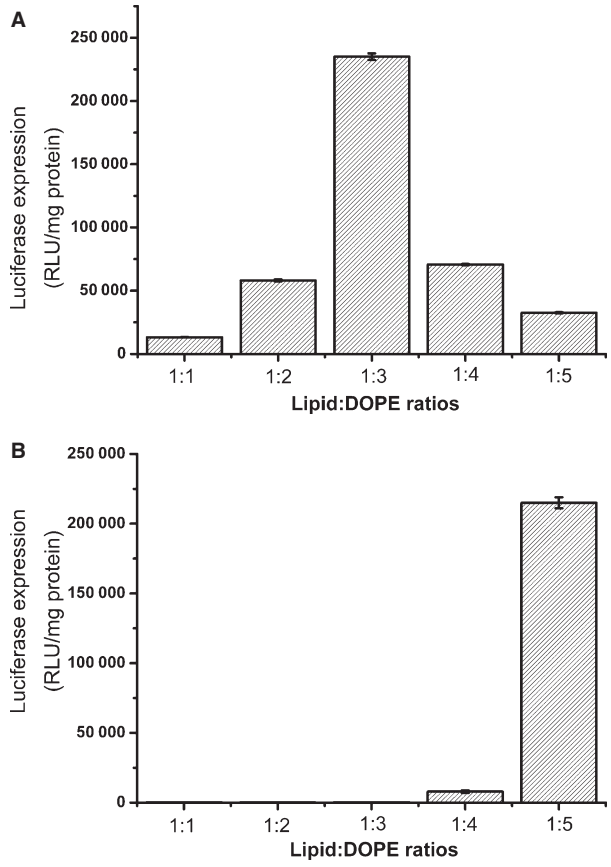


Figure 5: Transfection efficiencies of **L1** (A) and **L2** (B) at various lipid/DOPE ratio by keeping N/P ratio at 10 in human embryonic kidney 293 cells. Concentration of the pGL-3 plasmid DNA = 2 μg/well and data are expressed as relative light units (RLU)/mg of protein.

HEK 293 cells by keeping the lipid/DOPE ratio at 1:3 (**L1**) and 1:5 (**L2**). The N/P ratios in the experiments were varied from 4 to 10. As shown in Figure 6, obvious EGFP expression was observed especially at N/P ratio of 6–8 for both **L1** and **L2**. Subsequent luciferase assays (Figure 7) demonstrate that **L1** and **L2** give their best transfection efficiency at N/P ratio of 10–8, respectively, the optimized gene transfection efficiency of **L2** was about 60% of that obtained by using commercially available Lipofectamine 2000^{TM} as transfection vector, and diosgenin-containing **L2** have superior gene transfection performance than cholesterol-containing **L1**, indicating that the slight difference in the hydrophobic region of these lipids not only affects the physicochemical characteristics of formed lipoplexes, but also affects the optimal transfection conditions and the final transfection efficiencies. These results were consistent with those found by other researchers who demonstrated that variations in any part of the three regions may dramatically influence the gene transfer efficacies of cationic lipids (7,8). 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 showed excellent gene transfection (26–28). However, results herein suggest 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. In addition, different from the aforementioned protonated cyclen- and imidazolium salt-based cationic lipids, which could mediate effective gene transfection only with the presence of calcium ion (Ca^{2+}) (20), the cationic lipids **L1** and **L2** here could induce effective gene transfection without the calcium ion (Ca^{2+}). In most case, the addition of calcium ion (Ca^{2+}) could promote the uptake of lipoplexes by cells through ion channel mechanism and then increase transfection efficiencies; however, the strategy could not be applied in *in vivo* gene transfection because of high cytotoxicity induced by Ca^{2+} (29). In a word, our results here suggest that

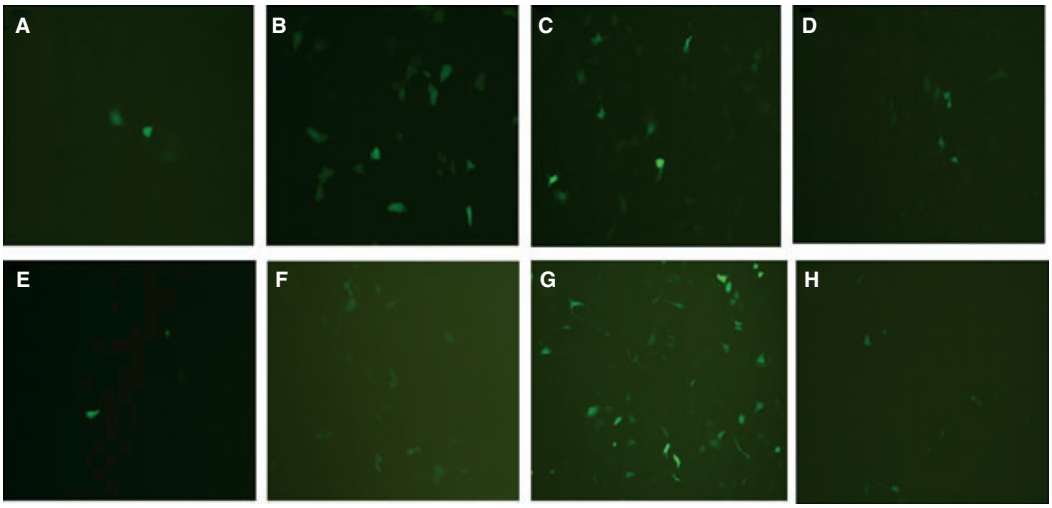


Figure 6: Fluorescent microscope images (100×) of human embryonic kidney 293 cells transfected by **L1**-based lipoplexes (A-D) and **L2**-based lipoplexes (E-H) at the N/P ratio of 4 (A, E), 6 (B, F), 8 (C, G), 10 (D, H). All lipid/DOPE ratios were at 1:3 (for **L1**) and 1:5 (for **L2**), and the pEGFP-N1 plasmids DNA was used as report gene.

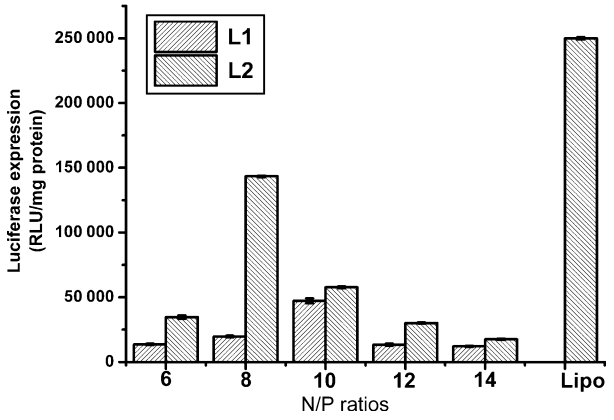


Figure 7: Transfection efficiencies of **L1** and **L2** at various N/P ratios keeping lipid/DOPE mole ratio at 1:3 (for **L1**) and 1:5 (for **L2**) in human embryonic kidney 293 cells. Lipofectamine 2000™ (Lipo) was used as control. Concentration of the pGL-3 plasmid DNA = 2 μg/well and data are expressed as relative light units (RLU)/mg of protein.

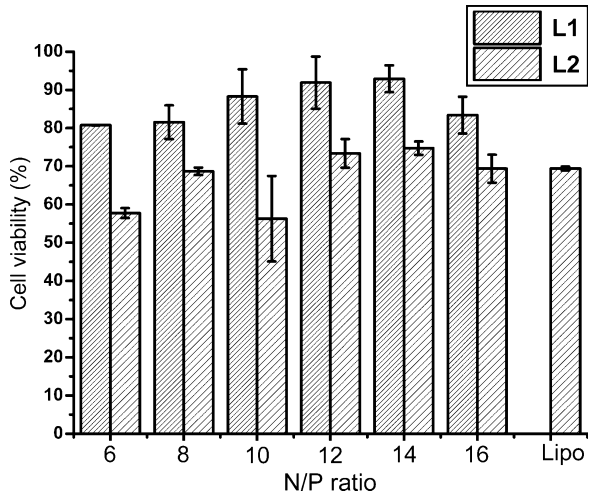


Figure 8: Cell viabilities (%) of **L1**(**L2**)-based lipoplexes at various N/P ratios in human embryonic kidney 293 cells. The lipid/-DOPE ratios were kept at 1:3 (for **L1**) and 1:5 (for **L2**) and concentration of the pGL-3 plasmid DNA was 0.5 μg/well. Lipofectamine 2000™ (Lipo) was used as control.

the two cationic lipids **L1** and **L2**, especially **L2,** have the potential to be effective gene transfection vectors.

Cytotoxicity

The cytotoxicity of lipoplexes was measured by MTT-based cell viability assays, which were performed in HEK 293 cells across the entire range of N/P ratios used in the luciferase expression assays, and the results are shown in Figure 8. Overall, the formed lipoplexes showed good biocompatibility. Under the routine transfection conditions, the cell viabilities were stable even under high N/P ratios. In most cases, the cell viabilities of studied lipoplexes were higher than that of Lipofectamine 2000™. In addition, the cell viability of lipoplexes formed from **L1** was found to be higher than that of lipoplexes formed from **L2**, while the optimal transfection efficiencies of **L1** were lower than that of **L2**. That is to say, for the two lipids studied herein, the one with higher cytotoxicity has higher *in vitro* transfection efficiency.

Conclusion and Future Directions

In this study, based on our previous researches, we designed and synthesized two novel cyclen and ammonium-based cationic lipids (**L1** and **L2**) with different hydrophobic regions. Two cationic lipids can easily form liposomes and effectively bind and compact DNA into nanoparticles. Although their transfection efficiencies were lower than that of the commercially available Lipofectamine 2000™, it was found that in association with DOPE, the two cationic lipids could induce effective gene transfection in HEK 293 cells. Furthermore, diosgenin-containing **L2** have superior gene transfection performance than cholesterol-containing **L1** in spite of its slightly higher cytotoxicity, indicating that diosgenin might be a superior hydrophobic group in the design of cationic lipids of high gene transfection efficiencies.

Acknowledgments

This work was financially supported by the National Science Foundation of China (Nos. 20972104, 20902062), 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 Center of Sichuan University for NMR analysis.

References

1. Anderson W.F. (1998) Human gene therapy. Nature;392:25–30.
2. Check E. (2003) Harmful potential of viral vectors fuels doubts over gene therapy. Nature;423:573–574.
3. Bhattacharya S., Bajaj A. (2009) Advances in gene delivery through molecular design of cationic lipids. Chemical Commun; 463:2–4656.
4. Mintzer M.A., Simanek E.E. (2009) Nonviral vectors for gene delivery. Chem Rev;109:259–302.
5. Park J.H., Lee S., Kim J.H., Park K., Kim K., Kwon I.C. (2008) Polymeric nanomedicine for cancer therapy. Prog Polym Sci;33:113–137.
6. Dutta T., Jain N.K., McMillan N.A.J., Parekh H.S. (2010) Dendrimer nanocarriers as versatile vectors in gene delivery. Nanomed-Nanotechnol Biol Med;6:25–34.
7. Bajaj A., Kondaiah P., Bhattacharya S. (2008) Effect of the nature of the spacer on gene transfer efficacies of novel thiocholesterol derived gemini lipids in different cell lines: a structure-activity investigation. J Med Chem;51:2533–2540.

8. Bajaj A., Kondaiah P., Bhattacharya S. (2008) Gene transfection efficacies of novel cationic gemini lipids possessing aromatic backbone and oxyethylene spacers. Biomacromolecules;9:991–999.
9. Liberska A., Unciti-Broceta A., Bradley M. (2009) Very long-chain fatty tails for enhanced transfection. Org Biomol Chem;7:61–68.
10. Ewert K.K., Evans H.M., Zidovska A., Bouxsein N.F., Ahmad A., Safinya C.R. (2006) A columnar phase of dendritic lipid-based cationic liposome-DNA complexes for gene delivery: hexagonally ordered cylindrical micelles embedded in a DNA honeycomb lattice. J Am Chem Soc;128:3998–4006.
11. Fujiwara T., Hasegawa S., Hirashima N., Nakanishi M., Ohwada T. (2000) Gene transfection activities of amphiphilic steroid-polyamine conjugates. BBA-Biomembranes;1468:396–402.
12. Aissaoui A., Martin B., Kan E., Oudrhiri N., Hauchecorne M., Vigneron J.P., Lehn J.M., Lehn P. (2004) Novel cationic lipids incorporating an acid-sensitive acylhydrazone linker: synthesis and transfection properties. J Med Chem;47:5210–5223.
13. Morozova N.G., Maslov M.A., Petukhova O.A., Andronova S.V., Grishaeva A.O., Serebrennikova G.A. (2010) Synthesis of lipid mediators based on 1,2-dialkylglycerol and cholesterol for targeted delivery of oligo- and polynucleotides into hepatocytes. Russ Chem Bullet;59:251–259.
14. Ewert K., Ahmad A., Evans H.M., Schmidt H.W., Safinya C.R. (2002) Efficient synthesis and cell-transfection properties of a new multivalent cationic lipid for nonviral gene delivery. J Med Chem;45:5023–5029.
15. Islam R.U., Hean J., van Otterlo W.A.L., de Koning C.B., Arbuthnot P. (2009) Efficient nucleic acid transduction with lipoplexes containing novel piperazine- and polyamine-conjugated cholesterol derivatives. Bioorgan Med Chem Lett;19:100–103.
16. Huang Q.D., Chen H., Zhou L.H., Huang J., Wu J., Yu X.Q. (2008) A novel macrocyclic polyamine cationic lipid containing an imidazolium salt group: synthesis, characterization and its transfection activity as a gene delivery vector. Chem Biol Drug Des;71:224–229.
17. Zhou L.H., Yang M., Zhou H., Zhang J., Li K., Xiang Y.Z. *et al.* (2009) Novel reticular cyclen-based polymer as gene vector in DNA transfection. Chem Biol Drug Des;73:216–224.
18. Xiang Y.Z., Feng Z.H., Zhang J., Liao Y.L., Yu C.J., Yi W.J. *et al.* (2010) Linear cyclen-based polyamine as a novel and efficient reagent in gene delivery. Organ Biomol Chem;8:640–647.
19. Yi W.J., Feng Z.H., Zhang Q.F., Zhang J., Li L.D., Zhu W. *et al.* (2011) Diol glycidyl ether-bridged cyclens: preparation and their applications in gene delivery. Organ Biomol Chem;9:2413–2421.
20. Huang Q.D., Ou W.J., Chen H., Wang J.Y., Zhang J., Zhu W. *et al.* (2011) Novel cationic lipids possessing protonated cyclen and imidazolium salt for gene delivery. Eur J Pharm Biopharma;78:326–335.
21. Deck L.M., Baca M.L., Salas S.L., Hunsaker L.A., Vander Jagt D.L. (1999) 3-alkyl-6-chloro-2-pyrones: selective inhibitors of pancreatic cholesterol esterase. J Med Chem;42:4250–4256.
22. Kimura E., Kikuchi M., Kitamura H., Koike T. (1999) Selective and efficient recognition of thymidylylthymidine (TpT) by Bis(Zn-II-cyclen) and thymidylylthymidylylthymidine (TpTpT) by tris(Zn-II-cyclen) at neutral pH in aqueous solution. Chem-Eur J;5:3113–3123.
23. Hansena M.B., Nielsena S.E., Berg K. (1989) Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J Immunol Methods;119:203–210.
24. Koiwai K., Tokuhisa K., Karinaga R., Kudo Y., Kusuki S., Takeda Y. *et al.* (2005) Transition from a normal to inverted cylinder for an amidine-bearing lipid/pDNA complex and its excellent transfection. Bioconjugate Chem;16:1349–1351.
25. Bajaj A., Paul B., Kondaiah P., Bhattacharya S. (2008) Structure-activity investigation on the gene transfection properties of cardiolipin mimicking gemini lipid analogues. Bioconjugate Chem;19:1283–1300.
26. Mevel M., Kamaly N., Carmona S., Oliver M.H., Jorgensen M.R., Crowther C. *et al.* (2010) DODAG; a versatile new cationic lipid that mediates efficient delivery of pDNA and siRNA. J Control Release;143:222–232.
27. Medvedeva D.A., Maslov M.A., Serikov R.N., Morozova N.G., Serebrenikova G.A., Sheglov D.V. *et al.* (2009) Novel cholesterol-based cationic lipids for gene delivery. J Med Chem;52:6558–6568.
28. Zidovska A., Evans H.M., Ahmad A., Ewert K.K., Safinya C.R. (2009) The role of cholesterol and structurally related molecules in enhancing transfection of cationic liposome-DNA complexes. J Phys Chem B;113:5208–5216.
29. Lam A.M.I., Cullis P.R. (2000) Calcium enhances the transfection potency of plasmid DNA-cationic liposome complexes. BBA-Biomembranes;1463:279–290.