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Synthesis and gene transfection activity of cyclen-based cationic lipids with asymmetric acyl-cholesteryl hydrophobic tails

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A series of novel 1,4,7,10-tetraazacyclododecane (cyclen)-based cationic lipids with asymmetric double hydrophobic tails (cholesteryl and long aliphatic chains) were designed and synthesized. Lysine was chosen as a linking moiety in the molecular backbone. The liposomes formed from **8** and dioleoylphosphatidylethanolamine (DOPE) could bind and condense plasmid DNA into nanoparticles under a low N/P ratio. These nano-scaled lipoplexes have low cytotoxicity, and might efficiently transfect A549 cells. *In vitro* transfection results revealed that all cationic lipids showed a comparable or better transfection efficiency (TE) than commercially available Lipofectamine 2000. The length and saturation degree of the aliphatic chain would affect their gene transfection performance, and the linoleic acid-containing **8e** could give the best TE.

Introduction

Gene therapy is a promising approach for treating both genetic and acquired diseases. The most challenging issue for the successful application of gene therapy is to develop safe and highly efficient vectors for the delivery of the transgene into host cells.[1] Traditionally, gene delivery systems are broadly based on either viral or non-viral mediated vectors. Despite the high transfection efficiency (TE) of viral vectors, safety concerns have been raised in clinical trials. The inherent drawbacks of viral vectors include immunogenicity, restricted targeting of specific cell types, size limitation on DNA, potential mutagenesis, *etc.*[2] Non-viral vectors, such as cationic liposome/polymer-based systems[2,3] and some naturally existing or artificially synthesized cell-penetrating peptides[4–7] are considered to be less toxic, less immunogenic, and easier to prepare than viral vectors and are potentially more attractive for clinical applications.

Appropriate modification of cationic lipid structures and the study of the structure–activity relationships (SAR) are of great importance for the rational design of efficient lipidic gene vectors.[8] The molecular architectures of cationic lipids include three functional units: a hydrophilic head group, a linkage (including a backbone domain), and a hydrophobic moiety.[9] The cationic hydrophilic domain generally has one or more positively charged groups usually formed from the protonation of amino groups (monocationic and polycationic lipids, respectively).[10] Polyamine-containing lipids are expected to form liposomes with a greater surface charge density than monocationic lipids. In our ongoing studies of novel polycationic lipids for gene transfection, we recently demonstrated the potential of 1,4,7,10-tetraazacyclododecane (cyclen) based gene delivery systems.[11,12] Besides easy modification, the unique cyclic structure and wide-ranging pK_a (ref. 13) of the amino groups on cyclen may facilitate the binding toward negatively charged DNA and other processes in the transfection.

The gene delivery performance of a cationic lipid, including its cytotoxicity and biodegradability in a physiological environment, is largely dependent upon the structure of the linking group between the hydrophobic and the hydrophilic domains.[14,15] The common linkage bonds are ether, ester, amide, carbamate groups, *etc.* Although some commercially available transfection agents such as Lipofectamine 2000 contain ether bridges, other linkages are considered to have lower cytotoxicity and the potential for degradation under special conditions. Carbamate and amide linkers are expected to serve as a reasonable balance between the TE and biocompatibility.[3,16,17] Besides the linkage bonds, the backbone domain also acts as a scaffold on which the cationic lipid is built.[9] The most common backbone is a glycerol-based structure. However, as a natural and biocompatible unit, amino acids were used as building blocks in the design of cationic lipids, and high TEs were achieved in several cases.[18,19]

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The hydrophobic tails of synthesized lipids usually contain a steroid skeleton or a double-chain hydrocarbon with either saturated or unsaturated chains.[9] Among the sterol-containing cationic lipids, cholesterol derivatives are most widely used due to their importance in human metabolic pathways.[20–22] Although many cationic lipids with cholesterol or double-chain hydrocarbon have been extensively studied, lipids with asymmetric hydrophobic tails have received less attention.[23–25] For example, Yingyongnarongkul *et al.* synthesized a series of novel cationic lipids with asymmetric acyl-cholesteryl hydrophobic tails with high TE and low cytotoxicity.[26] In this report, we develop a new series of cyclen-based polycationic lipids with asymmetric acyl-cholesteryl hydrophobic tails, which were connected by the lysine moiety. These materials were proved to have the potential to be efficient gene vectors with relatively low cytotoxicity.

Results and discussion

Synthesis of target cationic lipids

The building blocks of target lipids include a cyclen headgroup, a lysine-containing backbone, a hydrophobic cholesteryl tail and an aliphatic tail with different chain lengths and saturation degrees, which are conducive to the SAR study. The preparation route is shown in Scheme 1. Firstly, commercially available α-*N*-boc-ε-*N*-(2-chlorobenzyloxycarbonyl)-L-lysine (**1**) was coupled with different aliphatic amines (**2a–2e**) in the presence of (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) (EDC·HCl) and *N*-hydroxybenzotriazole (HOBt) to yield **3a–e**. After removal of the boc group by trifluoroacetic acid, the compounds were subsequently reacted with tri-boc-cyclen-acetic acid **4** to give the intermediates **5a–e**. The benzyloxycarbonyl group was then removed by Pd/C under a H_2 atmosphere. The coupling between the product and cholesteryl chloroformate **6** gave the precursors **7a–e**. Finally deprotection led to target lipids **8a–e**. The structures of all new compounds were confirmed by NMR and HRMS.

Interaction with DNA and characterization of liposome/DNA complexes (lipoplexes)

Cationic liposomes are formed from either individual cationic lipids or more frequently from a combination of cationic lipids and neutral lipids such as dioleoyl phosphatidylethanolamine (DOPE), which might increase the transfection efficiency significantly.[19] Each cationic lipid was mixed in different molar ratios (1 : 0, 1 : 1, 1 : 2, and 1 : 3) with DOPE to determine the optimal combination. According to the best behavior in the transfection experiment (data not shown), the **8**/DOPE ratio of 1 : 2 was used herein. Ethidium bromide (EB) dye replacement and agarose gel electrophoresis assays were first employed to evaluate their binding abilities toward plasmid DNA. Fig. 1 shows the EB replacement assay results of relative fluorescence intensity as a function of the N/P charge ratio in 10 mM of HEPES solution (pH 7.4). It was shown that the fluorescent intensities significantly decreased with the rise of the N/P ratio (0–5). At the N/P ratio of 5, only about 20% of the original fluorescent intensity was found, indicating that these lipids could efficiently displace EB, which previously intercalated into DNA base pairs. The parameters of CE50 which represent the N/P charge ratios for quenching 50% fluorescence intensity of EB were evaluated to be 2.8, 2.5, 2.3, 1.9 and 2.1 for lipids **8a–8e**, respectively. On the other hand, the agarose gel retardation assay was also applied to evaluate the interaction between **8** and pDNA. Results of Fig. 2 show that complete DNA retardation could be achieved at the N/P of 4 for the liposomes formed from all five lipids. These results further demonstrate the strong interaction of **8** with plasmid DNA.

The average particle size, surface charge and morphology of the lipoplexes may largely influence the apparent cytotoxicity, cellular uptake/trafficking, and release of the encapsulated gene.[27] Fig. 3A depicts the N/P ratio dependence of the mean particle sizes of the lipoplexes formed from **8a** to **8e**. The average diameters were observed in the range of 100–380 nm, which strongly depended on the N/P ratio. In a general view, the average diameters gradually dropped with the increase of N/P, and then became stable around 120–150 nm at the N/P ratio of 4, indicating that full DNA condensation was achieved. Meanwhile, surface potentials of the lipoplex nanoparticles were also measured by DLS, and the results are shown in Fig. 3B. Zeta potentials of the five lipoplexes showed a similar trend along with the change of the N/P ratio. The values increased from −12 mV to 38 mV with the rise of the N/P ratio from 2 to 8. The difference of zeta-potentials between these lipoplexes under the N/P of 2 was much larger than those under the N/P of ≥4. This might be attributed to the different binding situation at such a lower N/P ratio (incomplete condensation).

Transmission electron microscopy (TEM) was further performed to get information about the shape and morphology of lipoplexes. A representative electron micrograph of lipoplexes formed from **8e** is shown in Fig. 4. The lipoplexes showed homogeneous spherical particles with sizes in the range of 40–50 nm. From these results we considered that lipoplexes formed from **8** may condense DNA to form nanoparticles with proper sizes for gene delivery. It is worth mentioning that the discrepancy of particle size measured by DLS (158 ± 6 nm) and TEM (40–50 nm) could be attributed to the fact that DLS determines the hydrodynamic diameter of micelles in water, whereas TEM reveals the morphology of the micelles in the dehydrated state.[28]

Cytotoxicity

The cytotoxicity of the prepared lipoplexes was examined in A549 cells by the MTT assay. As shown in Fig. 5, the percentage of viable cells decreased with the rise of the N/P ratio. Under the N/P of 4, the cytotoxicity of these lipids is slightly lower than that of the commercially available transfection agent Lipofectamine 2000. In addition, the results also revealed that the variation of hydrophobic chains would influence the cytotoxicity. Lipoplexes with longer chains led to higher cell viabi-

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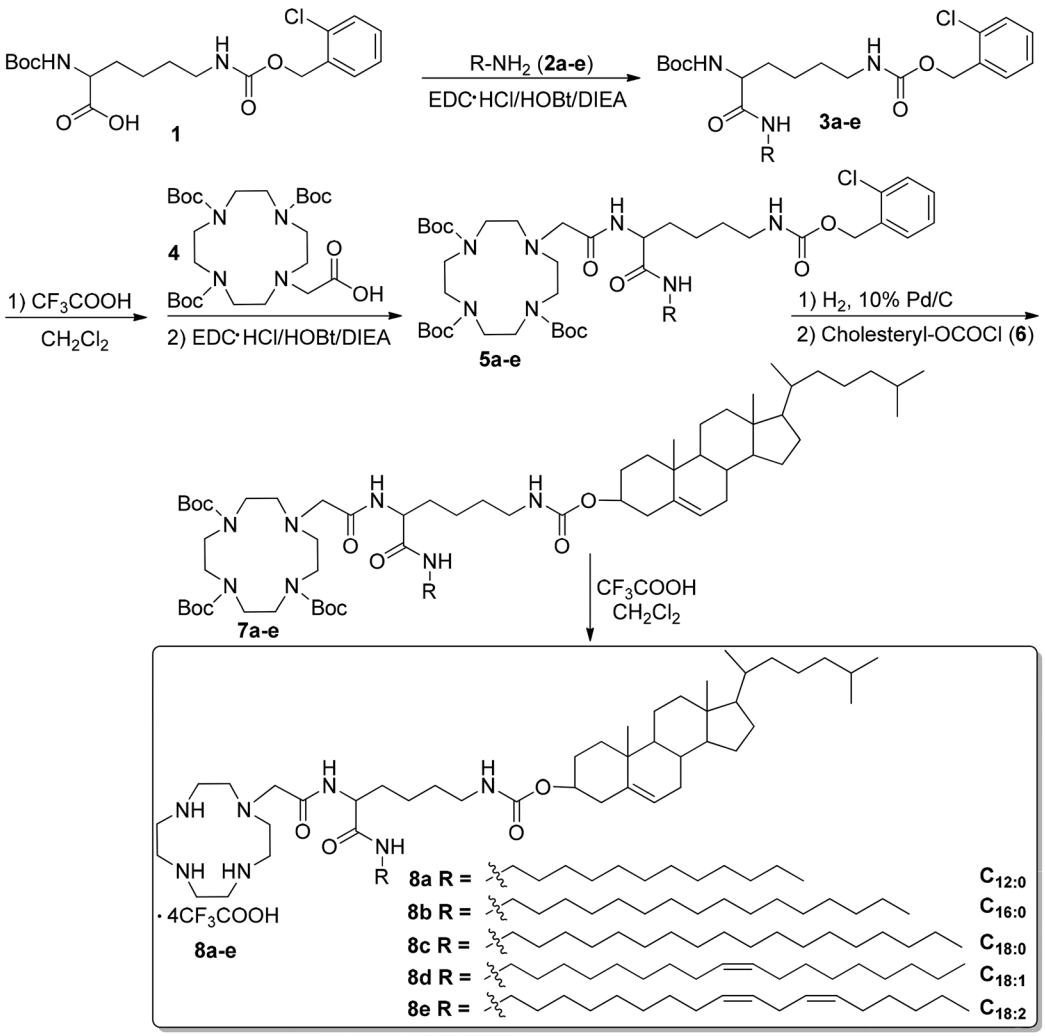

Scheme 1 Synthetic routes of title lipids **8a–8e**.

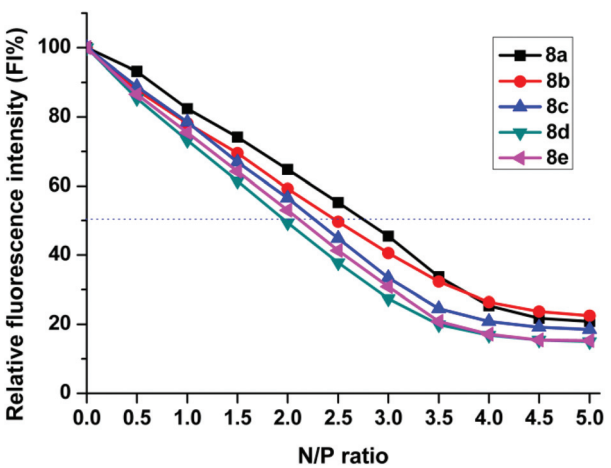

Fig. 1 Fluorescence quenching of EB by **8**/DOPE liposomes under various N/P ratios in 10 mM of HEPES buffer. The molar ratio of **8**/DOPE was 1 : 2.

lity, while unsaturated chains seemed to be harmful to the cells. This might come from the greater membrane fluidity caused by the bended unsaturated long chain.

In vitro gene transfection

Gene transfection experiments were then conducted to assess the suitability of **8** as non-viral vectors for nucleic acid delivery. To directly visualize the infected cells expressing pEGFP-Nl, lipids **8** (prepared at N/P ratios of 4, 6 and 8) mediated eGFP expression in A549 tumor cells was firstly studied and observed using an inverted fluorescence microscope. Fig. 6 shows the eGFP fluorescence microscope images obtained after **8**-promoted transfection. It was found that the transfection mediated by lipoplexes **8a–8d** showed the highest green fluorescence density at the N/P of 4 (Fig. 6A, D, G and J), indicating the largest amount of transfected cells. Meanwhile, lipoplexes **8e** gave the best transfection result at the N/P of 6 (Fig. 6N). Among the five lipids, the linoleic acid-containing **8e** is

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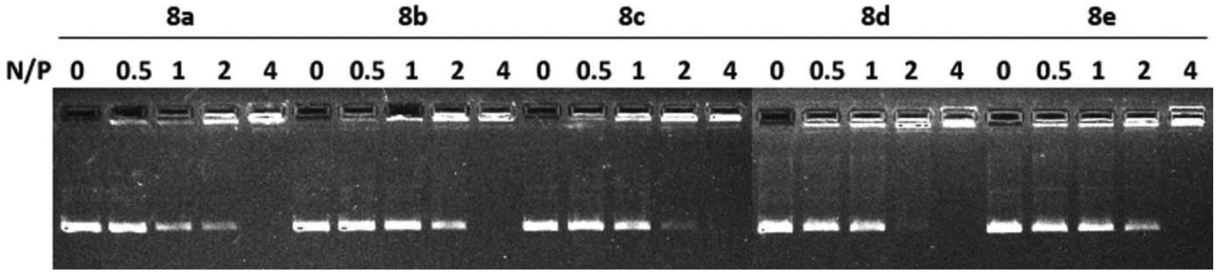

Fig. 2 Electrophoretic gel retardation assays of **8**/DOPE/pDNA complexes at different N/P ratios. The molar ratio of **8**/DOPE was 1 : 2.

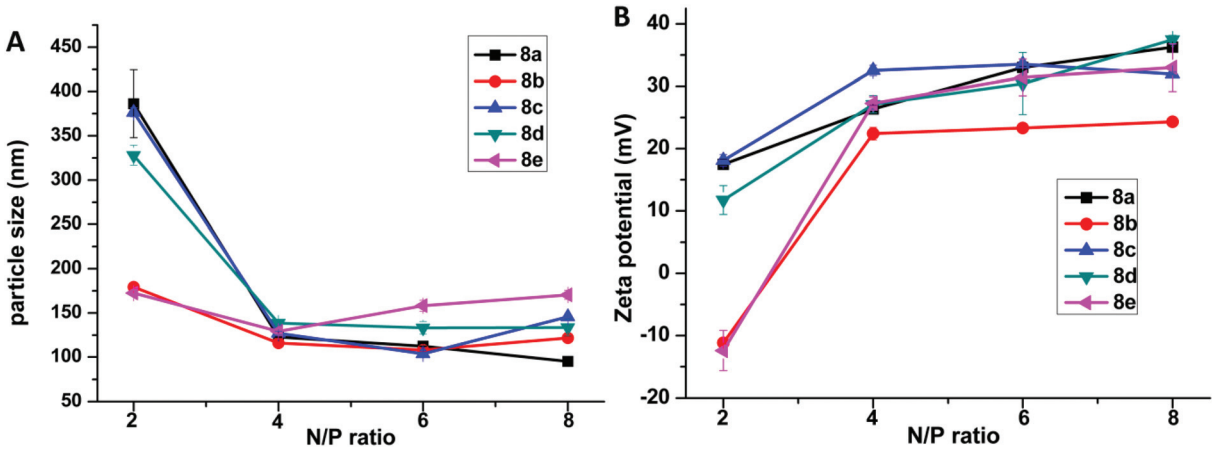

Fig. 3 Mean particle sizes (A) and zeta-potentials (B) of the lipoplexes formed from **8a** to **8e** under various N/P ratios (DLS at room temperature).

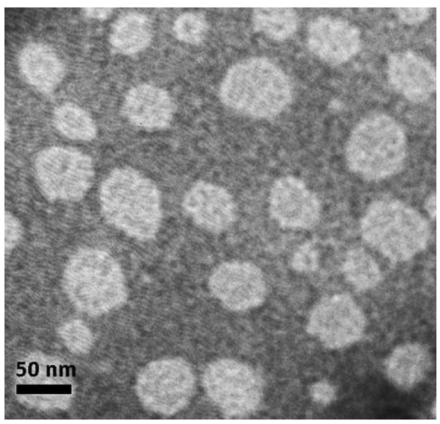

Fig. 4 TEM image of **8e**/DOPE/DNA complexes at the N/P ratio of 6.

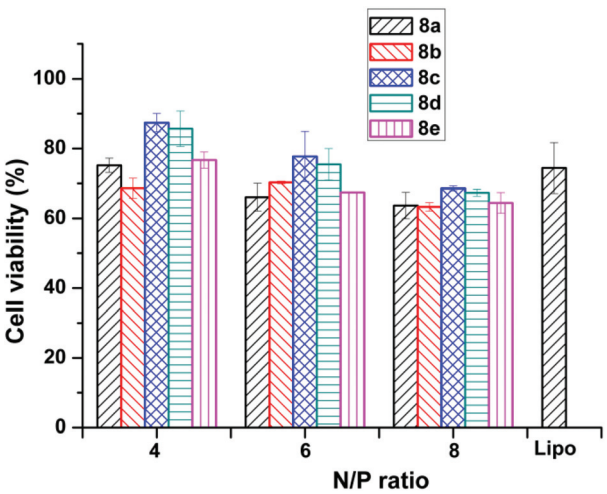

Fig. 5 Cell toxicity of the lipoplexes prepared at various N/P ratios, with Lipofectamine 2000 as the control.

distinctly the best choice for high TE. Subsequently, a luciferase assay was conducted to quantitatively study the TE of **8** in the same cell line. As shown in Fig. 7, the results were quite consistent with those obtained in eGFP experiments. **8e** gave the best TE at the N/P ratio of 6, and the TE was about 1.6 times higher than that of Lipofectamine 2000. Except **8e**, the other four lipids gave their best TE at the N/P of 4. Among the lipids containing saturated long chains, palmitic (hexadecanoic) acid-containing **8b** seemed to be the best transfection reagent, and its TE was close to that of Lipofectamine 2000. It was also reported that linoleic acid modified lipids[29,30] or lipopolymers[28] might show a higher gene delivery efficiency than their analogs. We speculate that the relatively higher DNA-binding ability and better fluidic character may benefit their transfection behavior.

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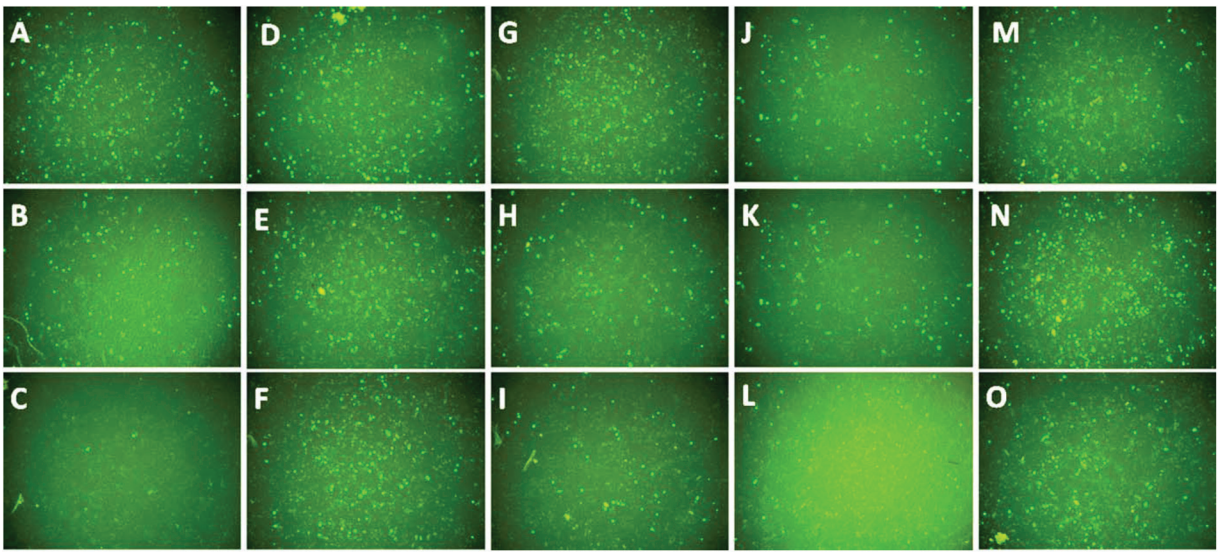

Fig. 6 Fluorescent microscope images of A549 cells transfected with **8a** (A–C), **8b** (D–F), **8c** (G–I), **8d** (J–L), and **8e** (M–O). The lipid/DOPE ratio was 1 : 2, and N/P ratios were 4 (top row), 6 (middle row) and 8 (bottom row). The cells were observed by fluorescence microscopy after 24 h transfection.

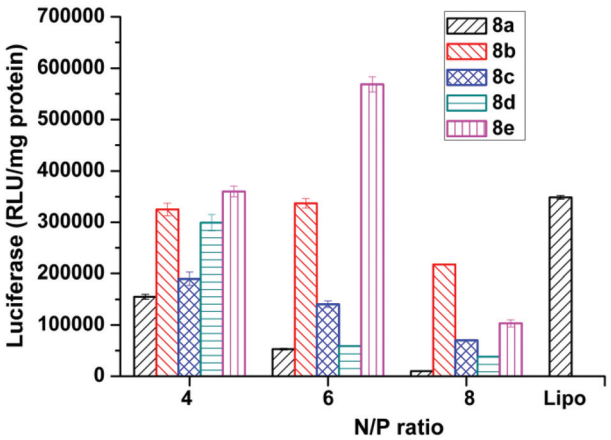

Fig. 7 Transfection efficiencies of lipids **8a–8e** in A549 cells. The molar ratio of lipid/dioleoylphosphatidylethanolamine (DOPE) was 1 : 2.

Conclusion

In summary, a series of cyclen-based cationic lipids with asymmetric double hydrophobic tails were prepared. Lysine was used as a linking group in the molecular backbone. The liposomes formed from the lipids and DOPE could efficiently condense DNA into nanoparticles with proper size and zeta-potential. The length and saturation degree of the aliphatic chain would affect their gene transfection performance. Results showed that the linoleic acid-containing **8e** could give 1.6 times higher TE than the commercially available transfection reagent Lipofectamine 2000. The cytotoxicity of these lipids was similar to that of Lipofectamine 2000. Further modification of such types of lipids and relative mechanism studies are now in progress.

Experimental section

Materials and methods

All chemicals and reagents were obtained commercially and were used as received. Anhydrous chloroform and dichloromethane were dried and purified under nitrogen by using standard methods and were distilled immediately before use. [4,7,10-Tris(*tert*-butoxy-carbonyl)-1,4,7,10-tetraaza-cyclododecan-1-yl] acetic acid (tri-boc-cyclen-acetic acid, compound **4**),[31] and cholesteryl chloroformate (compound **6**)[16] were synthesized according to the literature. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Sigma-Aldrich (St. Louis, MO, USA). A micro-BCA protein assay kit was obtained from Pierce (Rockford, IL, USA). A luciferase assay kit was purchased from Promega (Madison, WI, USA). A endotoxin-free plasmid purification kit was purchased from TIANGEN (Beijing, China). The plasmids used in the gene transfection assay were pGL3 (Promega) coding for luciferase DNA and pEGFP-N1 (Clontech, Palo Alto, CA, USA) coding for enhanced green fluorescent protein (eGFP) DNA. The supercoiled plasmid DNA (pUC-19) used in the agarose-gel assay was purchased from Takara (Dalian, China). Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium, and fetal bovine serum (FBS) were purchased from Invitrogen Corp. A549 (human lung adenocarcinoma epithelial cells) was purchased from the American Type Culture Collection (ATCC). The NMR spectra were measured on a Varian INOVA-400 spectrometer and the *d* scale in parts per

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million was referenced to residual solvent peaks or internal tetramethylsilane (TMS). MS-ESI spectra data were recorded on a Bruker Daltonics BioTOF mass spectrometer.

Preparation of compounds 3a–e

A solution of compound **1** (830 mg, 2 mmol), long chain alkyl amines **2a–e** (2.2 mmol), and HOBt (324 mg, 2.4 mmol) in CH_2Cl_2 (50 mL) was cooled to 0 °C. EDC·HCl (460 mg, 2.4 mmol) and *N*,*N*-diisopropylethylamine (DIEA, 776 mg, 3 mmol) were gradually added, and the reaction mixture was stirred for 1 h at 0 °C and at room temperature overnight. The mixture was washed with saturated aqueous $NaHCO_3$ solution (2 × 20 mL) and saturated brine (20 mL). The organic phase was dried over Na_2SO_4, filtered, and concentrated to afford a white solid, which was further purified by column chromatography on silica gel (EtOAc–PE = 1 : 2, R_f = 0.3) to yield **3** as a white solid.

3a: (1.06 g, 1.82 mmol, yield 91%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.60–7.39 (m, 2H), 7.33–7.19 (m, 2H), 5.08 (s, 2H), 4.05 (s, 1H), 3.45–3.01 (m, 4H), 2.18–1.11 (m, 35H), 1.00–0.76 (m, 3H). HR-MS (ESI): $C_{31}H_{52}ClN_3NaO_5$ $[M + Na]^+$, 604.3488, found: 604.3495.

3b: (1.15 g, 1.8 mmol, yield 90%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.50–7.34 (m, 2H), 7.30–7.24 (m, 2H), 5.06 (s, 2H), 4.19 (s, 1H), 3.40–3.10 (m, 4H), 1.96–1.75 (m, 2H), 1.71–1.22 (m, 41H), 0.89 (t, J = 6.8 Hz, 3H). HR-MS (ESI): $C_{35}H_{60}ClN_3NaO_5$ $[M + Na]^+$, 660.4114, found: 660.4115.

3c: (1.15 mg, 1.72 mmol, yield 86%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.47–7.30 (m, 2H), 7.26–7.15 (m, 2H), 5.17 (s, 2H), 4.07 (m, 1H), 3.16 (d, J = 2.6 Hz, 4H), 1.77–1.23 (m, 47H), 0.86 (t, J = 6.8 Hz, 3H). HR-MS (ESI): $C_{35}H_{60}ClN_3NaO_5$ $[M + Na]^+$, 688.4427, found: 688.4427.

3d: (1.13 g, 1.7 mmol, yield 85%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.46–7.33 (m, 2H), 7.27–7.21 (m, 2H), 5.40–5.30 (m, 2H), 5.20 (s, 2H), 4.05 (s, 1H), 3.23–3.17 (m, 4H), 2.12–1.91 (m, 4H), 1.78–1.12 (m, 39H), 0.88 (t, J = 6.7 Hz, 3H). HR-MS (ESI): $C_{37}H_{62}ClN_3NaO_5$ $[M + Na]^+$, 686.4270, found: 686.4272.

3e: (1.19 g, 1.8 mmol, yield 90%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.60–7.30 (m, 2H), 7.36–7.00 (m, 2H), 5.51–5.18 (m, 6H), 4.05 (s, 1H), 3.16 (s, 4H), 2.75 − 1.25 (m, 39H), 0.86 (s, 3H). HR-MS (ESI): $C_{37}H_{60}ClN_3NaO_5$ $[M + Na]^+$, 684.4114, found: 684.4115.

Preparation of compounds 5a–e

Compound **3** (1.0 mmol) was dissolved in anhydrous CH_2Cl_2 (4 mL), and then 2 mL CF_3COOH was added at 0 °C. After stirring for 6 h, the solvent was removed under reduced pressure to obtain an oily product which was directly coupled with tri-boc-cyclen-acetic acid **4** (531 mg, 1.0 mmol) in the presence of EDC·HCl (230 mg, 1.2 mmol), HOBt (162 mg, 1.2 mmol), and DIEA (259 mg, 2 mmol) in CH_2Cl_2 by the same synthetic method as that for compound **3**. After column chromatography on silica gel (EtOAc–PE = 2 : 1), **5** was obtained as a white powder.

5a: (706 mg, 0.71 mmol, yield 71%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.52–7.32 (m, 2H), 7.29–7.20 (m, 2H), 5.38–5.06 (m, 2H), 4.31 (s, 1H), 3.73–2.74 (m, 22H), 1.67–1.20 (m, 53H), 0.87 (t, J = 6.8 Hz, 3H). HR-MS (ESI): $C_{51}H_{88}ClN_7NaO_{10}$ $[M + Na]^+$, 1016.6173, found: 1016.6183.

5b: (736 mg, 0.7 mmol, yield 70%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.47–7.29 (m, 2H), 7.26–7.17 (m, 2H), 5.35–5.08 (m, 2H), 4.31 (s, 1H), 3.50–2.62 (m, 22H), 1.59–1.21 (m, 61H), 0.97–0.74 (m, 3H). HR-MS (ESI): $C_{55}H_{96}ClN_7NaO_{10}$ $[M + Na]^+$, 1072.6799, found: 1072.6806.

5c: (788 mg, 0.73 mmol, yield 73%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40–7.20 (m, 4H), 5.12 (s, 2H), 4.28 (s, 1H), 3.6–2.50 (m, 22H), 1.67–1.0 (m, 65H), 0.83–0.80 (m, 3H). HR-MS (ESI): $C_{57}H_{100}ClN_7NaO_{10}$ $[M + Na]^+$, 1100.7112, found: 1100.7118.

5d: (754 mg, 0.7 mmol, yield 70%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.49–7.34 (m, 2H), 7.26–7.24 (m, 2H), 5.50–5.12 (m, 4H), 4.32 (s, 1H), 3.70–2.75 (m, 22H), 2.10–1.94 (m, 4H), 1.82–1.20 (m, 57H), 0.89 (t, J = 6.8 Hz, 3H). HR-MS (ESI): $C_{57}H_{98}ClN_7NaO_{10}$ $[M + Na]^+$, 1098.6956, found: 1098.6962.

5e: (774 mg, 0.72 mmol, yield 72%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.55–7.32 (m, 2H), 7.28–7.25 (m, 2H), 5.57–5.11 (m, 6H), 4.33 (s, 1H), 3.94–2.58 (m, 24H), 2.21–1.93 (m, 4H), 1.76–1.17 (m, 55H), 0.91 (t, J = 6.7 Hz, 3H). HR-MS (ESI): $C_{57}H_{96}ClN_7NaO_{10}$ $[M + Na]^+$, 1096.6799, found: 1096.6797.

Preparation of compounds 7a–e

Compound **5** (1.0 mmol) was dissolved in methanol (20 mL), and then 10% palladium on carbon (0.5 g, containing 50% water) was added. The 2-chlorobenzyl group was removed from **5** using a hydrogen atmosphere (balloon pressure) at room temperature for 24 h. The catalyst was removed by filtration, and the filtrate was concentrated. The residue was dissolved in CH_2Cl_2 (30 mL). Triethylamine (101 mg, 2.0 mmol) and cholesteryl chloroformate **6** (460 mg, 1.0 mmol) were gradually added, and the reaction mixture was stirred at room temperature overnight. The mixture was washed with saturated aqueous $NaHCO_3$ solution (20 mL) and saturated brine (20 mL). The organic phase was dried over Na_2SO_4, filtered, and concentrated to afford a white solid, which was further purified by column chromatography on silica gel (EtOAc–PE = 2 : 1, R_f = 0.3) to yield **7** as a white solid.

7a: (1.10 g, 0.89 mmol, yield 89%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 5.33 (s, 1H), 4.29 (s, 1H), 3.53–2.61 (m, 22H), 2.33–0.57 (m, 100H). HR-MS (ESI): $C_{71}H_{127}ClN_7NaO_{10}$ $[M + Na]^+$, 1260.9537, found: 1260.9542.

7b: (1.04 g, 0.8 mmol, yield 80%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 5.33 (s, 1H), 4.21 (s, 1H), 3.66–2.60 (m, 22H), 2.37–0.46 (m, 108H). HR-MS (ESI): $C_{75}H_{135}ClN_7NaO_{10}$ $[M + Na]^+$, 1317.0163, found: 1317.0172.

7c: (953 mg, 0.72 mmol, yield 72%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 5.33 (s, 1H), 4.19 (s, 1H), 3.32–2.67 (m, 22H), 2.38–0.65 (m, 112H). HR-MS (ESI): $C_{77}H_{139}ClN_7NaO_{10}$ $[M + Na]^+$, 1345.0476, found: 1345.0479.

7d: (990 mg, 0.75 mmol, yield 75%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 5.76 (s, 2H), 5.33 (s, 1H), 4.21 (s, 1H), 3.32–2.67 (m, 22H), 2.38–0.53 (m, 110H). HR-MS (ESI): $C_{77}H_{137}ClN_7NaO_{10}$ $[M + Na]^+$, 1343.0319, found: 1343.0324.

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7e: (1.12 g, 0.85 mmol, yield 85%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 5.70 (s, 4H), 5.33 (s, 1H), 4.19 (s, 1H), 3.30–2.65 (m, 22H), 2.38–0.53 (m, 108H). HR-MS (ESI): $C_{77}H_{135}ClN_7NaO_{10}$ $[M + Na]^+$, 1341.0163, found: 1341.0162.

Preparation of title lipids 8a–8e

Compound **7** (0.5 mmol) was suspended in anhydrous CH_2Cl_2 (4 mL), and then a solution of CF_3COOH (2 mL) in anhydrous dichloromethane (2 mL) was added dropwise under an ice bath and a N_2 atmosphere. The obtained mixture was stirred at room temperature for 6 h. After the solvent and CF_3COOH were removed, the residue was washed with anhydrous ether twice to afford the title lipids **8** as an oily solid.

8a: Yield 80%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 5.36 (s, 1H), 4.22 (s, 1H), 3.73–2.73 (m, 22H), 2.49–0.61 (m, 74H). HR-MS (ESI): $C_{56}H_{104}ClN_7NaO_4$ $[M + H]^+$, 938.8144, found: 938.8145.

8b: Yield 82%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 5.36 (s, 1H), 4.20 (s, 1H), 3.80–2.71 (m, 22H), 2.49–0.61 (m, 82H). HR-MS (ESI): $C_{60}H_{112}ClN_7NaO_4$ $[M + H]^+$, 994.8770, found: 994.8770.

8c: Yield 80%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 5.37 (s, 1H), 4.21 (s, 1H), 3.74–2.75 (m, 22H), 2.45–0.51 (m, 86H). HR-MS (ESI): $C_{62}H_{116}ClN_7NaO_4$ $[M + H]^+$, 1022.9083, found: 1022.9087.

8d: Yield 74%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 5.44–5.32 (m, 3H), 4.20 (s, 1H), 3.47–2.63 (m, 22H), 2.38–0.51 (m, 81H). HR-MS (ESI): $C_{62}H_{114}ClN_7NaO_4$ $[M + H]^+$, 1020.8927, found: 1020.8929.

8e: Yield 70%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 5.45–5.32 (m, 5H), 4.20 (s, 1H), 3.48–2.61 (m, 22H), 2.31–0.56 (m, 77H). HR-MS (ESI): $C_{62}H_{112}ClN_7NaO_4$ $[M + H]^+$, 1018.8770, found: 1018.8774.

Preparation of cationic liposomes

The neutral lipid dioleoylphosphatidylethanolamine (DOPE; 0.0025 mmol) was combined with **8** (0.0025 mmol) in chloroform and dried with nitrogen gas under reduced pressure to remove the chloroform solvent. The lipid film was hydrated with 2.5 mL of MilliQ water to the final lipid concentration of 1 mM. The samples were sonicated in a bath sonicator to generate small unilamellar vesicles according to previously described procedures.[17] After being cooled to room temperature, the liposome solution was filtered through a 0.22 μm filter to generate homogeneous size of lipid vesicles.

Amplification and purification of plasmid DNA

pGL3 and pEGFP-N1 plasmids were used in the gene transfection assay *in vitro*. The former one as the luciferase reporter gene was transformed in *Escherichia coli* JM109, and the latter one as the green fluorescent protein reporter gene was transformed in *E. coli* DH5a. Both plasmids were amplified in terrific broth media at 37 °C overnight. The plasmids were purified by an EndoFree Tiangen TM Plasmid Kit (Tiangen Biotech, Beijing, China). 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 of plasmid was checked by the A260/A280 ratio (~1.9). The concentrations of plasmids were determined by ultraviolet (UV) absorbance at 260 nm.

Preparation of lipid/DOPE/DNA complexes (lipoplexes)

To prepare the lipid/DOPE/pDNA complexes (lipoplexes), various amounts of cationic lipids were mixed with a constant amount of DNA by pipetting thoroughly at various N/P ratios, 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 (mole ratios) and was calculated by considering the average nucleotide mass of 350.

Ethidium bromide replacement assay

The ability of lipid **8** to condense DNA was studied using ethidium bromide (EB) exclusion assays. Fluorescence spectra were measured at room temperature in air by a Horiba Jobin Yvon Fluoromax-4 spectrofluorometer and corrected for the system response. EB (5 μL, 1.0 mg mL^{-1}) was put into a quartz cuvette containing 2.5 mL of 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) solution (pH 7.4). After shaking, the fluorescence intensity of EB was measured. Then, CT DNA (10 μL, 1.0 mg mL^{-1}) was added to the solution with mixing. The measured fluorescence intensity is the result of the interaction between DNA and EB. Subsequently, the solutions of lipid **8** (1.0 mM, 2 μL for each addition) were added to the above solution for further measurement. All the samples were excited at 520 nm, and the emission was measured at 600 nm.

Agarose-gel retardation assay

Lipid **8**/DOPE/pDNA complexes at different N/P ratios (the amino groups of lipids to phosphate groups of DNA) ranging from 0.5 to 4 were prepared by adding an appropriate volume of lipids (in PBS solution) to 0.125 μg pUC-19 DNA. The complexes were incubated at 37 °C for 30 min. Thereafter, the complexes were electrophoresed on the 1.0% (W/V) agarose-gel-containing gelred and with Tris-acetate running buffer at 110 V for 30 min. DNA was visualized with a UV lamp at a wavelength of 312 nm using a Bio-Rad Universal Hood II (Bio-Rad, Hercules, CA, USA).

Lipoplex particle sizes and zeta potentials

Sizes and zeta potentials of the lipid/pDNA lipoplex at various N/P ratios were analyzed at room temperature on a dynamic light scattering (DLS) system (Zetasizer Nano ZS; Malvern Instruments Led, Malvern, UK) at 25 °C. The lipoplex particle solutions were first prepared by mixing **8**/DOPE lipids and pDNA (1 μg mL^{-1}) under diverse N/P charge ratios in 1 mL deionized water.

Transmission electron microscopy (TEM)

TEM images were obtained on a JEM-100CX (JEOL) transmission electron microscope at an acceleration voltage of 100 kV. The TEM samples were prepared by dipping a copper grid with Formvar film into the freshly prepared nanoparticle solution (10 μL). A few minutes after the deposition, the

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aqueous solution was blotted away with a strip of filter paper and then the samples were dried for 2 min at room temperature. The samples were stained with phosphotungstic acid (ATP) aqueous solution and dried in air.

Cell culture

Human non-small-cell lung carcinoma A549 cells were incubated in DMEM containing 10% FBS and 1% antibiotics (penicillin 10 000 U mL^{-1} and streptomycin 10 000 μg mL^{-1}) at 37 °C under a humidified atmosphere containing 5% CO_2.

MTT cytotoxicity assay

The toxicity of lipoplexes toward A549 cells was determined by the MTT assay following literature procedures.[7] About 7000 cells per 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. Lipoplexes prepared from Lipofectamine 2000 were used as the control.

In vitro transfection assays

A549 cells were seeded into 24-well plates at a density of 80 000 cells per well in 0.5 mL of complete medium. Twenty-four hours prior to transfection experiments, the medium was replaced with 1 mL fresh culture medium with or without FBS.[13] Lipid **8**/DNA complexes equivalent to 1 μg of plasmid DNA at the desired N/P ratios were added to each well and incubated with cells for 4 h. The medium was then replaced with 0.5 mL of fresh complete medium and incubated for a further 24 h at 37 °C. For fluorescence microscopy assays, cells were transfected with complexes containing pEGFP-N1. After 24 h of incubation, the microscopy images were obtained at the magnification of 100× and recorded using the VIEWFINDER LITE (1.0) software (Olympus, Tokyo, Japan). For luciferase assays, cells were transfected with lipoplexes containing pGL3 plasmid DNA. The luciferase assay was performed using the Picagene luciferase assay kit (Toyo Ink, Tokyo, Japan). The transfected cells were washed three times with PBS and lysed in a cell lysis buffer. The lysate was centrifuged at 10 000*g* at 4 °C for 2 min, and the supernatant was subjected to the luciferase assay. The relative light unit (RLU) of chemiluminescence was measured using a luminometer (Turner designs, 20/20; Promega), and the luminescent RLU values were normalized to the protein content as determined by a BCA assay. Transfection with Lipofectamine 2000/DNA complexes was performed as a positive control. All transfection experiments were performed in triplicate.

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