Organic & Biomolecular Chemistry

Accepted Manuscript

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Cyclen-based cationic lipids containing pH-sensitive moiety as gene delivery vectors

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† Electronic Supplementary Information (ESI) available: Supplemental figures and compounds characterization. See DOI: 10.1039/xxxxxxxx

Abstract

A series of novel cationic lipids based on 1, 4, 7, 10-tetrazacyclododecane (cyclen) with imidazole group as pH-sensitive moiety and various aliphatic long chains were designed and synthesized. Cationic liposomes were prepared by mixing the lipids and helper lipid 1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) under appropriate mole ratio. The liposomes showed good stability and could condense plasmid DNA into nanosized particles (~100 to ~250 nm) with positive zeta-potential (+10~25 mV). CCK-8-based cell viability assays showed relatively lower cytotoxicity of the lipoplexes compared to commercially available lipofectamine 2000. Both enhanced green fluorescent protein and luciferase assays were carried out to investigate the in vitro transfection efficiency (TE) of the lipoplexes. Results showed that the structures of hydrophobic chain and linking bond both significantly affected the TE, and linoleyl-contained lipoplex gave the best TE, which is comparable to lipofectamine 2000. Imidazole group was demonstrated to play important role in the transfection, and the imidazole-absent analog gave dramatically lower TE. Further, it was also found that Ca^{2+} could largely enhance the TE of these lipids, and the optimized TE was about 5 times higher than lipofectamine 2000. Flow cytometry demonstrate that the enhancement of TE by Ca^{2+} was caused by the improvement of cellular uptake. These results suggest that the cyclen-imidazole contained lipids might be promising non-viral gene delivery vectors.

Introduction

Over the past decades, gene therapy has gained significant attention as a potential method for survival against many diseases, whether acquired (such as AIDS or cancer) or inherited (cystic fibrosis *etc*) through a genetic disorder.[1-3] The therapy involves the delivery of a specific gene (usually a DNA plasmid) to the target cells thus combating the disease at the level of its origin.[4] However, because of the unfavorable electrostatic interactions between macromolecular DNA and biological cell surface (both being negatively charged) and the nuclease sensitiveness of non-protected DNA, spontaneous delivery and expression of the supplemented correct copy of the malfunctioning genes into cells is unlikely to be an efficient process.[5] Therefore, to achieve successful gene therapy, development of proper delivery vectors is a significant factor. Generally speaking, gene delivery systems commonly used in gene therapy are mainly classified into two sorts: viral and non-viral (or synthetic) vectors.[6] In terms of viral vectors, despite the significant advantage of high transfection efficiency (TE), a number of observably disadvantages including immunogenicity, toxicity and potential insertional mutagenesis have obstructed the development of these vectors.[7] Conversely, cationic lipids, as the main class of non-viral vectors, are increasingly being demonstrated as the transfection vectors for delivering genes into tissue cells due to their less immunogenic nature, ability to deliver large pieces of DNA, and ease of handling and preparation.[8-10] Since Felgner first reported cationic lipid as synthetic vector in 1987,[11] large amount of cationic lipids have been synthesized as candidates for non-viral gene delivery.[12-16] However, comparing with the viral vector, cationic lipids showed relatively low TE and potential toxicity, which limited their clinical application.

The structure of amphiphilic cationic lipids generally comprises a hydrophilic cationic headgroup, which is attached to hydrophobic tails via a linker. The polar headgroups, which can be quaternary ammoniums,[11] amines[17], guanidiniums[18] and heterocyclics,[19, 20] have one or more positive charges (usually nitrogen atom) to facilitate their interaction with negatively charged nucleic acids by electrostatic attraction, leading to the formation of compact complexes.[21] The hydrophobic tails in cationic lipids are usually composed of one or two long aliphatic chains, which are generally 12-18 carbon atoms in length and can be either saturated or unsaturated. The linking bond usually contains a biodegradable chemical bond (ester, amide

or carbamate) or a non-degradable ether bond. All these domains can significantly affect the TE and toxicity of the cationic lipids.

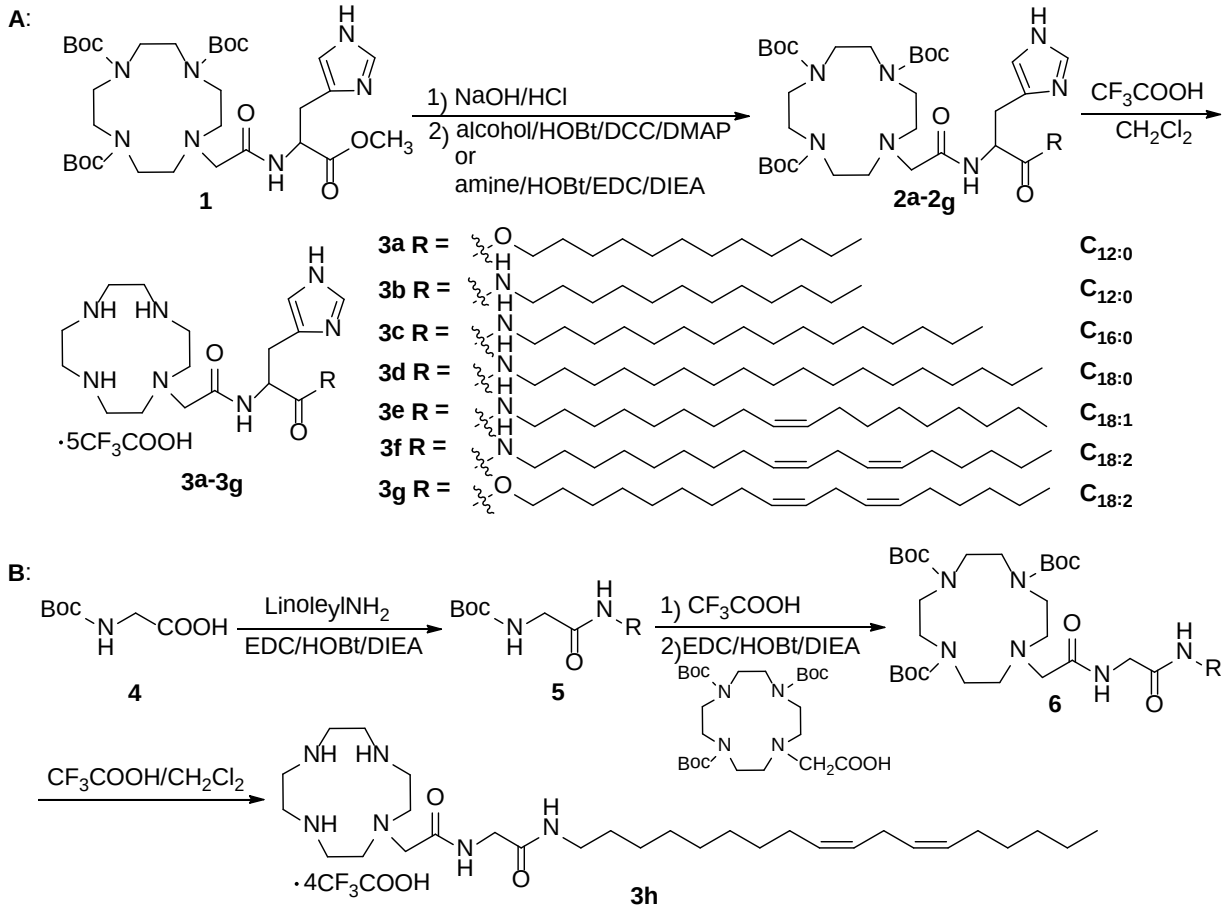
In our efforts to find novel cationic lipids with high TE and good biocompatibility, we first used 1, 4, 7, 10-tetrazacyclododecane (cyclen) as the hydrophilic headgroup for its strong binding ability toward DNA and non-self-folded conformation. Results have demonstrated that cyclen-based lipids might be promising candidates for gene delivery.[22, 23] However, although cyclen has four amine groups with wide range of pK_a values, it lacks the one with the pK_a close to the more acidic endosome environment (pH 5.0-6.5). In other words, cyclen lacks the amino groups having the so-called "proton sponge effect", which might benefit the endosome escape. Thus, introducing pH sensitive group whose pK_a value is in the endosomal pH range into the cyclen-based lipid might promote gene transfection process. In this report, imidazole was chosen for its proper pK_a (~6.0),[24-26] and a series of cyclen-based cationic lipids with histidine in the backbone were synthesized. Their interaction with plasmid DNA was studied, and the structure-activity relationship in the gene delivery mediated by these lipids was discussed.

Results and discussion

Synthesis of the lipidic compounds

As shown in Scheme 1, a series of novel cationic lipids containing protonated cyclen headgroup and various hydrophobic tails with different chain lengths and saturation degrees were prepared. The linking group between the hydrophilic and hydrophobic domains consists of a histidine moiety, on which an imidazole group exists. Cyclen-histine conjugate **1** was prepared according to our previous report.[22] After hydrolysis with NaOH, compound **1** was directly coupled with long chain fatty amine or alcohol to give the precursors **2a-2g**. Some additives including dicyclohexylcarbodiimide (DCC), 1-hydroxybenzotrizole (HOBt) and 4-dimethylaminopyridine (DMAP) were necessary for the coupling with alcohol, while 1-ethyl-3-(3-dimethyllaminopropyl)carbodiimide (EDC) and N, N-diisopropylethylamine (DIEA) were used in the reaction with amine. Target lipids **3a-3g** were obtained by removing the Boc groups with trifluoroacetic acid in anhydrous CH_2Cl_2. For comparison, analog **3h** without imidazole group was also prepared through similar method (Scheme 1B). These compounds enabled us to studies the structure-activity relationship of such type of cationic

lipids in their gene transfection process. All novel compounds were purified and their structures were confirmed by ^{1}H NMR, ^{13}C NMR and HRMS.



Scheme 1. Synthetic routes of target lipids.

Buffering capability

It is well known that polyethyleneimine (PEI) has been considered as "golden standard" for polymeric gene vectors because of its relatively high TE, which might come from its excellent pH buffering capacity and the consequent "proton-sponge effect".[27] The introducing of imidazole which has pK_a of ~6 may significantly contribute to its endosomal pH buffering capacity. Six lipids (**3b, 3d-3h**) were chosen to examine their buffering capacity by acid-base titration experiments, and 25 KDa PEI was used as control. As shown in Fig. 1, the imidazole-attached molecules (**3b, 3d-3g**) gave relatively slow pH increase with the addition of NaOH as well as PEI, indicating their good buffering capacities. On the contrary, as expected, imidazole-absent **3h** gave a sharper upward trend of pH change, showing its weaker buffering capacity. Further, we calculated their buffering capacities in the pH range of 5.0-6.5 (Table S1),

and the results were consistent with those displayed in the figure. Except **3b**, all imidazole contained lipids have slightly higher buffering capacity than PEI.

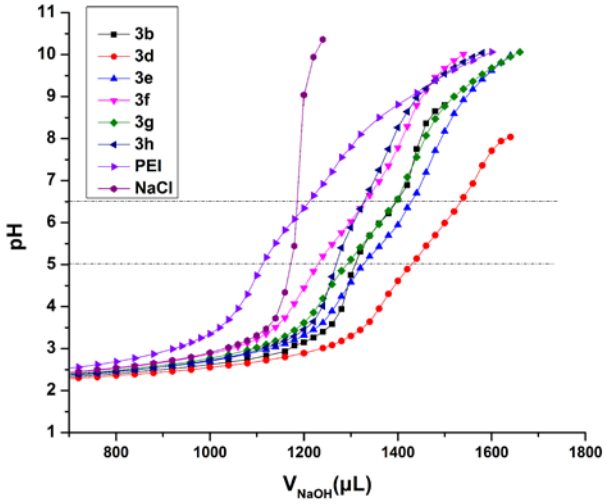


Fig. 1 Acid–base titration profiles of 25 kDa PEI, 150 mM NaCl and lipid solutions. Lipids or PEI (0.050 mmol of amino groups) was first treated with 1 N HCl to adjust pH to 2.0, and then the solution pH was measured after each addition of 20 μL of 0.1 N NaOH.

Formation of liposomes and liposome/DNA complexes (lipoplexes)

As gene carriers, cationic lipids are usually combined with neutral lipids such as 1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), which may promote hexagonal phase formation to enable endosomal escape and release of the nucleic acid.[3] The mole ratio of lipid/DOPE was chosen as 1:3 according to the best behavior in the transfection experiments (Fig. S1 and S2†). The liposomes formed at various N/P ratios with DOPE were subjected to agarose electrophoresis to evaluate the DNA binding ability. As shown in Fig. 2, it was found that all the materials can effectively bind plasmid DNA (pUC 19) and completely retard DNA migration at the N/P ratio of 4. Liposome **3d** was found to completely retard DNA migration at N/P ratio of 2, showing stronger binding ability. This might be contributed by the longer aliphatic tails and the resulted stronger hydrophobic effect. On the other hand, the electron-rich unsaturated bonds in **3e**-**3g** might shield the positive charge of the liposome, resulting in weaker binding than saturated analog **3d**. To further study the effect of the hydrophobic tail of liposome on their DNA affinity, ethidium bromide (EB) exclusion assay was also carried out (Fig. S3†). The results showed that the fluorescent intensities caused by intercalated EB dramatically decreased with the rise of N/P ratio (0-5), indicating the stronger binding between

the liposomes and DNA. The relative fluorescence intensity became approximately stable at N/P ratio of ~5, suggesting a nearly full DNA condensation. It's clearly shown that the fluorescent quenching ability increased along with the increase of chain length (**3a**-**3d**). Meanwhile, like the gel retardation results, **3e**-**3f** with unsaturated chains showed reduced fluorescent quenching and DNA binding ability.

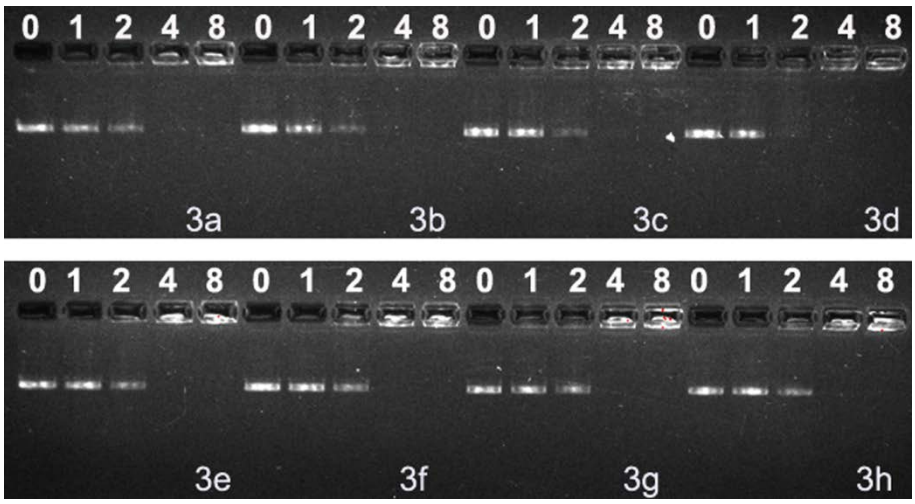


Fig. 2 Electrophoretic gel retardation assays of lipid/DOPE/DNA (pUC 19) complexes at different N/P ratios. The molar ratio of lipid/DOPE was 1:3.

The particle sizes and zeta-potentials of the liposomes and their DNA complexes (lipoplexes) were measured via dynamic light scattering (DLS). The average particle sizes of these liposomes varied from 60 nm to 120 nm (Fig. S4A†). It was confirmed that elongation of the aliphatic chain led to stronger hydrophobic effect, resulting in smaller particles (**3a**-**3d**). The double bonds on the unsaturated chain cause bends, which would hinder the hydrophobic interactions between the amphiphilic molecules, leading to larger liposome size. The zeta potentials of the liposomes were found in the range of +45~57 mV (Fig. S4B†), this cationic property ensures their electrostatic interaction with DNA. Compared to amide-contained analongs **3b** and **3f**, the lipids with ester bond (**3a** and **3g**) gave lower zeta-potentials, indicating that even the oxygen atom near the electro-withdrawing carbonyl group would screen the positive charges. The stability of these liposome solutions was also checked. It was found that even after 10 weeks storage under 4ºC, the particle size and zeta-potentials were seldom changed (Fig. S4C and S4D†).

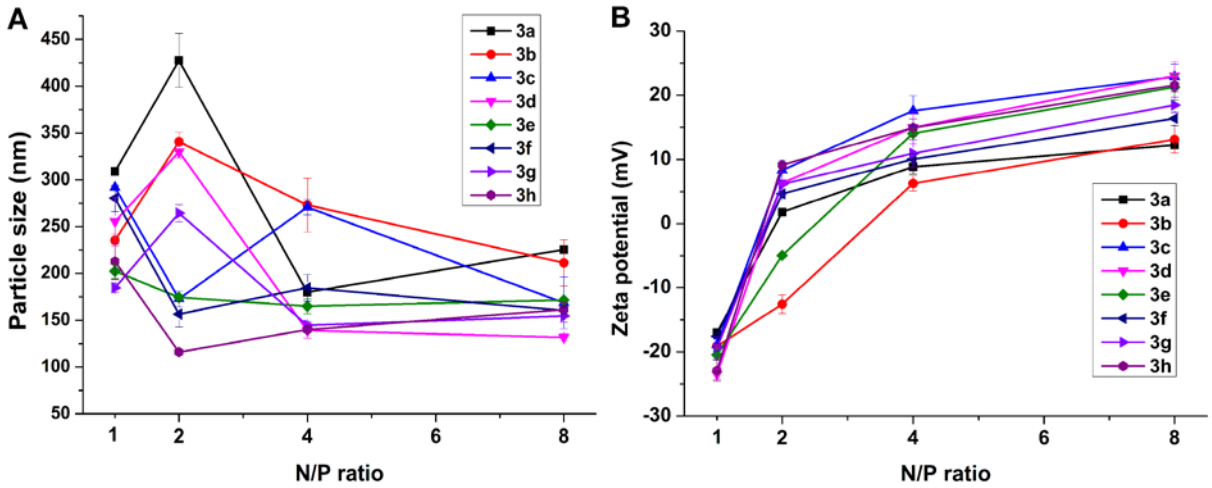


Fig. 3 Mean particle sizes (A) and zeta-potentials (B) of the lipoplexes formed from **3a** to **3h** and pUC 19 DNA under various N/P ratios (DLS at room temperature). Data represent mean ± SD (n = 3).

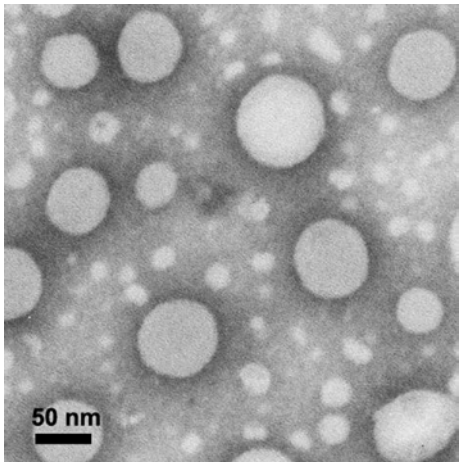


Fig. 4 TEM image of **3f**/DOPE/DNA lipoplex at N/P ratio of 6.

The physical properties of the lipoplexes formed from **3a**-**3h** were subsequently studied. After binding with plasmid DNA, the size of the lipoplex particles increased evidently. As shown in Fig. 3A, all liposomes are able to condense DNA into nanosized particles (~100 to ~300 nm) at N/P ratios of 4 and 8. Under lower N/P ratio, for the incomplete DNA condensation, the particle sizes were irregular. Zeta-potentials of the lipoplexes rose along with the increase of N/P ratio, and the surface charge was observed about +10~25 mV above the N/P of 6 (Fig. 3B). The size and zeta potential for all lipoplexes became stable from N/P of ~4, indicating that full DNA condensation was achieved. Furthermore, transmission electron microscopy (TEM) was used to get direct information about the shape and morphology of lipoplexes. Representive micrograph of lipoplexes formed from **3f** is shown in Fig. 4. Plasmid DNA was compacted into spherical nanoparticles with diameter of 50-80 nm. The particle size

determined by TEM was much smaller than those obtained by DLS (~175 nm). This might be caused by the different experimental conditions: the particle sizes determined by DLS were examined in the hydrated state in solution, while TEM revealed the morphology of the micelles in the dehydrated state.[28]

Cytotoxicity

Low cytotoxicity is prerequisite for a gene vector toward clinic trials. To investigate the cytotoxicity of the cationic liposomes, CCK-8-based cell viability assays was executed in HEK 293 cells, and Lipofectamine 2000 was used as positive control.[29] The results shown in Fig. 5 reveal that cells showed higher tolerance against the synthesized materials than the positive control, especially at N/P ratio used in subsequent transfection assays (≤8). At low N/P ratio of 2 and 4, nearly no cytotoxicity was found. The type of hydrophobic tails seems to have no significant influence on the cytotoxicity. However, lipids with ester bonds (**3a** and **3g**) gave higher cell viability than their amide-contained analogs at higher N/P ratio (~12), which might be due to the biodegradable nature of ester bond.[30]

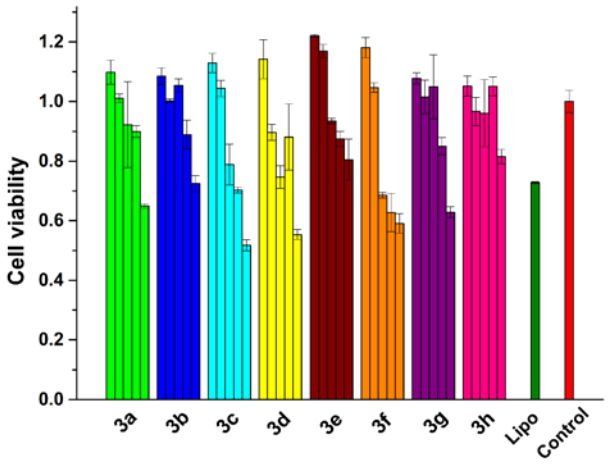


Fig. 5 Cytotoxicity of the lipoplexes prepared at various N/P ratio (2, 4, 8, 12 and 24 in each column group, respectively). Data represent mean ± SD (n = 3).

In vitro gene transfection

The lipid/DOPE ratio used in the transfection experiments was first optimized by using both enhanced green fluorescent protein (eGFP) reporter gene and luciferase reporter gene as delivery cargo. Results show that the eGFP expression was largely dependent on the ratio, and

lipid/DOPE of 1:3 was found to be optimal for the transfection (Fig. S1†). For luciferase reporter gene, 1:3 was also a suitable ratio, while the optimal N/P ratio was lower (Fig. S2†). Therefore, the lipid/DOPE ratio of 1:3 was used in subsequent studies. Fig. 6 shows **3a-3h** mediated eGFP expression in HEK 293 cells observed by an inverted fluorescent microscope, and lipofectamine 2000 was used as control. The transfection mediated by eight lipoplexes showed their respective strongest fluorescent emission at N/P of 4 or 8. Generally, lipids with saturated chains gave poor transfection, while those with unsaturated chains (especially linoleyl-contained **3f**) gave much better results, which was comparable to lipofectamine 2000. For the linoleyl-contained analogs, **3f** with amide linking bond showed much higher TE than **3g**, which contains ester bond in the structure. Moreover, imidazole group evidently play important role in the transfection by such type of lipids, since the imidazole-absent **3h** gave much weaker eGFP expression.

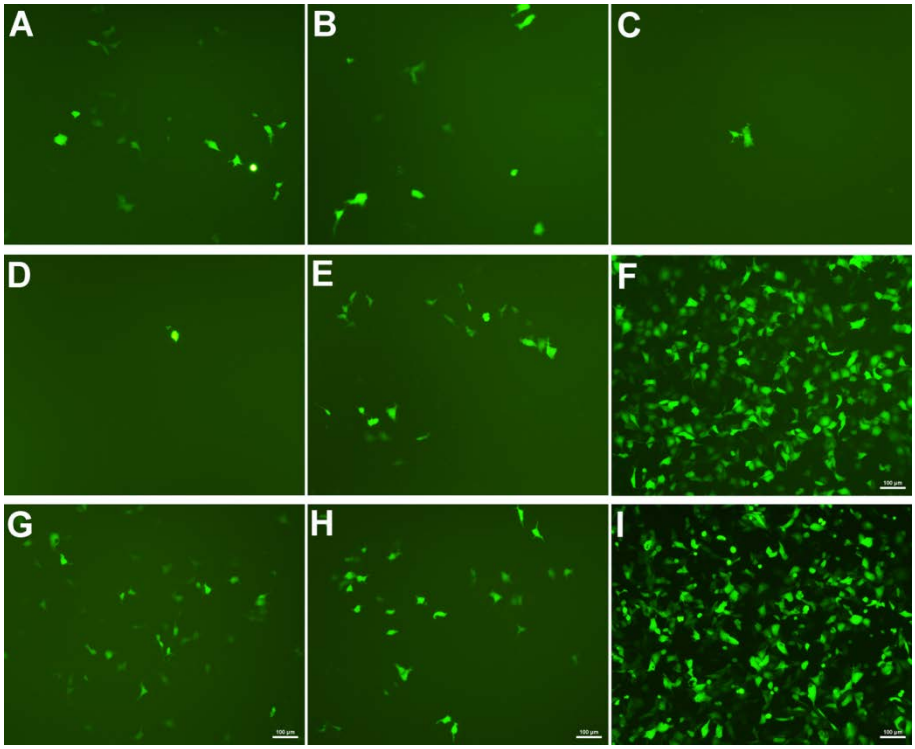


Fig. 6 Fluorescent microscope images of HEK 293 cells transfected by **3a-3h** (A-H, respectively) and lipofectamine 2000 (I). Lipid/DOPE ratio was 1:3, N/P ratios were 4 (C, E, F, G) and 8 (A, B, D, H). The cells were observed by fluorescence microscopy after 24 h transfection.

To quantitatively study the TE of the lipoplexes, luciferase reporter gene was also used as the cargo for the **3**-mediated transfection. Six lipids were chosen for the experiments with N/P ratios of 2, 4 and 8, and the results are shown in Fig. 7, in which similar structure-activity

relationship could be found. Lipid **3f** gave the best TE, which was still some less than that of lipofectamine 2000. The optimal N/P ratio (= 2) was lower than that in eGFP experiments. For the lipids with C18 chain, the saturated one (**3d**) showed much lower TE than unsaturated **3e** and **3f**. The superiority of the unsaturated lipids in transfection might be contributed to their relatively lower DNA binding ability (Fig. 2 and 3), which would benefit the release of DNA to cytoplasm. It is also known that unsaturated chain may increase the membrane fluidity by disrupting membrane package.[6] Some previous studies also found that the linoleyl chain may help to increase membrane-traversing motility, leading to increased uptake or additional intracellular events (e.g. endosomal escape or nuclear leakage) and subsequent better gene expression.[28, 31]

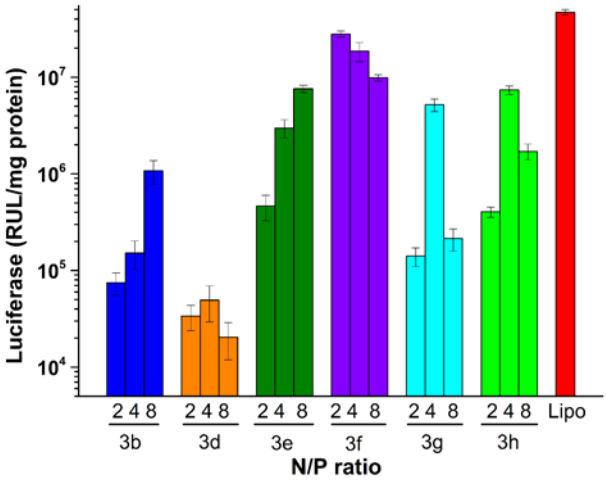


Fig. 7 Luciferase expression in HEK 293 cells transfected by lipid/DOPE/DNA lipoplexes at various N/P ratios under lipid/DOPE ratio of 1:3. Data represent mean ± SD (n = 3).

Many studies have shown that calcium ion could improve the TE of cationic liposomes by facilitating cell uptake through endocytosis and the entrance toward nucleus after endosomal escape.[32-35] To further improve the TE of cyclen-imidazole contained lipids, the effect of calcium ion was subsequently investigated. Transfection mediated by lipid **3f** with lipid/DOPE ratio of 1:3 and N/P ratio of 4 was used as template experiment. EGFP assay was first carried out by adding calcium ion with various concentrations (50 μM - 5 mM, Fig. 8). The fluorescent images show that significant enhancement of TE was observed, and higher concentration of Ca^{2+} led to higher TE. Quantitative analysis of the eGFP fluorescence also confirmed the promotion of TE by Ca^{2+} (Fig. S5†). With the use of 5 mM of Ca^{2+}, the TE of **3f** increased to

nearly 3 times higher than that of lipofectamine 2000. Furthermore, luciferase assays were also processed by using pGL-3 as reporter gene in different cell lines. As shown in Fig. 9, in HEK 293 cells, the TE of **3f** slightly increased with the presence of 1 mM of Ca^{2+} and dramatically increased when the Ca^{2+} concentration rise to 5 mM. Further increase of Ca^{2+} (10 mM) led to decrease of TE, which might be attributed to the increased cytotoxicity. In other words, 10 times improvement of TE was achieved by the addition of Ca^{2+}, and the maximum TE was about 5 times higher than that of lipofectamine 2000. In 7402 cells, similar trend was found, and the TE of **3f** was also largely enhanced with 5 mM of Ca^{2+}. But unfortunately, the maximum TE was still lower than lipofectamine 2000.

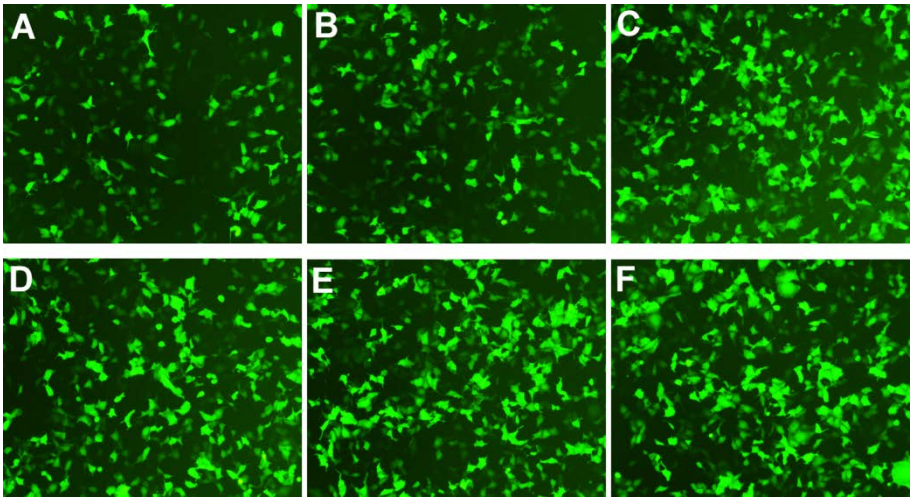


Fig. 8 Fluorescent microscope images of HEK293 cells transfected by **3f** with different Ca^{2+} concentrations: A (without Ca^{2+}), B (with 50 μM of Ca^{2+}), C (100 μM), D (500 μM), E (1 mM) and F (5 mM). The cells were observed by fluorescence microscopy after 24 h transfection.

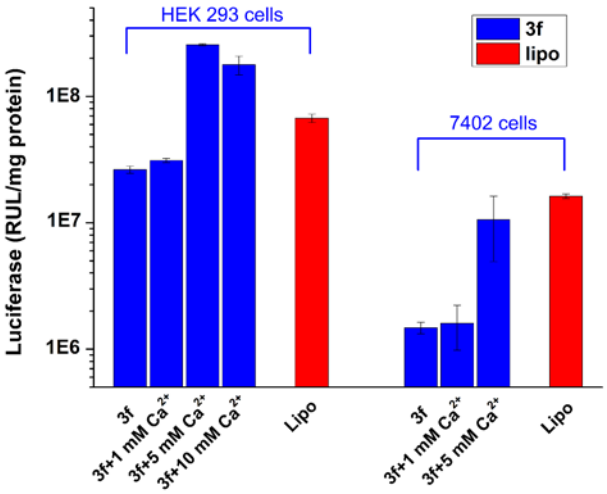


Fig. 9 Luciferase expression in HEK 293 cells and 7402 cells transfected by Lipid **3f**/DOPE/DNA complexes (lipoplexes) at N/P ratio of 4 in the absence and in the presence of 1, 5 and 10 of mM Ca^{2+}. Data represent mean ± SD (n = 3).

It was considered that Ca^{2+} could enhance transfection of lipoplexes by increasing their cellular uptake.[33] The fluorescence-activated cell sorting (FACS) technique was applied after incubation of HEK 293 cells with the lipoplexes containing Cy5-labelled DNA in the presence of various concentrations of calcium ions. The incubation was processed at the optimized N/P ratio of 4 for 4 h. Fig. 10 shows that the percentage of Cy5-positive cells largely increased by the addition of 5 mM of Ca^{2+}, suggesting that the improvement of TE by Ca^{2+} could be attributed to the enhancement of cellular uptake. It's worth mentioning that although the TE of **3f**/Ca^{2+} was much higher than lipofectamine 2000 (Fig. 9), the percentage of Cy5-positive cells by **3f**/Ca^{2+} was still less. Such results indicate that the **3f**-derived lipoplex may exhibit much better intracellular delivery behavior, leading to higher gene expression. Confocal laser scanning microscopy (CLSM) was also carried out to directly observe the distribution of Cy5-labelled DNA. As shown in Fig. 11, after 4 h transfection by **3f**, many of the red pGL-3 plasmids were located within the cells, and the use of Ca^{2+} led to higher density of red fluorescence in the nuclei. For comparison, in the transfection by analog **3h** without imidazole moiety in the structure, the red fluorescence was mainly located in the cytoplasm but not nuclei. This also suggests that imidazole plays important role in intracellular delivery, and its enhanced buffering capacity might benefit the endosome escape and subsequently lead to higher TE.

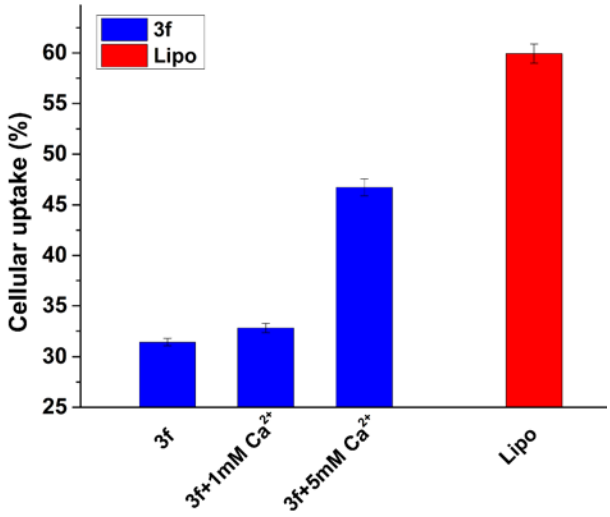


Fig. 10 Cellular uptake (percentage of Cy5-positive cells) of lipoplexes (N/P=4) in HEK 293 cells quantified by flow cytometry analysis. lipofectamine 2000 was used as control. Data represent mean ± SD (n = 3).

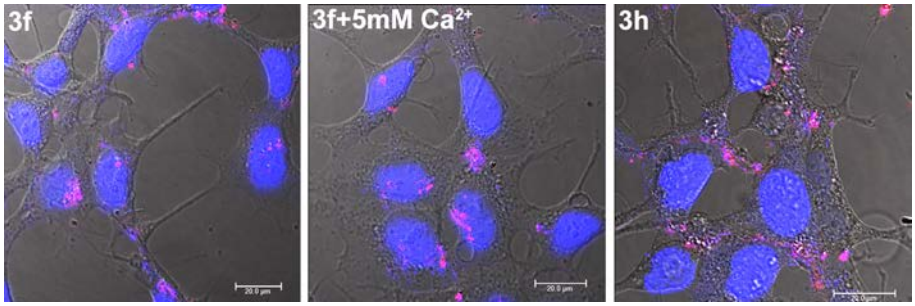


Fig. 11 CLSM images of HEK 293 cells transfected with Cy5-labeled DNA by the lipoplexes at optimal N/P ratio. pGL-3 plasmids were stained red by Cy5 and the nuclei of cells were stained blue by DAPI.

Conclusions

In summary, a series of novel cyclen-based cationic lipids containing various aliphatic chains were designed and synthesized. An imidazole group with pH buffering ability was introduced by using histidine as building block in the preparation. For structure-activity relationship studies, lipids with different linking bonds (e.g. **3f** and **3g**) and the analog without imidazole group (**3h**) were also prepared. Acid-base titration confirmed the good buffering capacity of these lipids. The liposomes formed from the lipid and DOPE showed good stability and could efficiently condense plasmid DNA into nanoparticles with proper sizes and zeta-potentials. Besides their low cytotoxicity, these lipoplexes also showed good in vitro gene transfection efficiency. Linoleyl-contained lipid **3f** gave comparable TE to commercially available lipofectamine 2000. The structures of hydrophobic chain and linking bond significantly affected the TE, and imidazole group was demonstrated to play important role in the transfection by such type of lipids. Furthermore, Ca^{2+} could largely enhance the TE of these lipids, and the optimized TE was about 5 times higher than lipofectamine 2000. The results not only suggest that such lipids with cyclen-imidazole structure might be promising non-viral gene vectors, but also guide us in further modification and optimization of lipidic gene delivery materials.

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. 1, 4, 7, 10-tetraazacyclododecanes (cyclen) was purchased from Quzhou Synpartner Pharmaceutical Technology Co., Ltd. The ^{1}H NMR and ^{13}C NMR spectra were measured on a Bruker AM400 NMR spectrometer. Proton Chemical shifts of NMR spectra were given in ppm relative to internals reference TMS (1H, 0.00 ppm). HRMS spectral data was recorded on a Bruker Daltonics Bio TOF mass spectrometer. 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). pUC19 DNA was purchased from TIANTAI (Chengdu, China). The plasmids used in the study were pGL-3 (Promega, Madison, WI, USA) coding for luciferase and pEGFP-N1 (Clontech, Palo Alto, CA, USA) coding for EGFP. The Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum were purchased from Invitrogen Corp.

Preparation of compound 2a-2g

Compound **1** (341 mg, 0.500 mmol) was dissolved in methanol (20 mL). 2 N NaOH (10 mL) was added. After stirring for 2 h at room temperature, the solvent was removed under reduced pressure. The solution was acidified to pH 3 by 2 N HCl, and then the solvent was removed under reduced pressure to obtain a white product (the acid intermediate) which was directly coupled with amine (laurylamine, hexadecylamine, stearylamine, oleylamine, linoleylamine, 0.500 mmol) in the presence of 1-hydroxybenzotrizole (HOBt, 81.0 mg, 0.529 mmol), 1-ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride (EDC·HCl, 115 mg, 0.600 mmol) and N, N-diisopropylethylamine (DIEA, 260 mg, 2.01 mmol) in 50 mL CH_2Cl_2 at 0 ºC for 1 h and at room temperature overnight. The EDC condensation product was then washed with saturated aqueous $NaHCO_3$ solution (2×50 mL) and saturated brine (50 mL). The organic phase was dried over Na_2SO_4, filtered, and concentrated to afford an oil, which was further purified by column chromatography on silica gel (CH_2Cl_2/MeOH = 20:1, v/v) to yield the synthetic precursor (**2b**-**2f**). On the other hand, the above-mentioned acid intermediate might also directly coupled with alcohol (lauryl alcohol, linoleyl alcohol, 0.500 mmol) in the presence of HOBt (81.0 mg, 0.529 mmol), dicyclohexylcarbodiimide (DCC, 124 mg, 0.602 mmol), N, N-dimethylaminopyridine (DMAP, 6.10 mg, 0.050 mmol) in 50 mL CH_2Cl_2 at 0 ºC

for 1 h and at room temperature overnight. The DCC condensation mixture was then filtered, and the filtrate was evaporated under reduced pressure, and a little amount of ethyl acetate was added. The mixture was maintained at 0 ºC for half an hour and filtered. The filtrate was evaporated under reduced pressure to give the crude products which were purified by chromatography over silica gel (CH_2Cl_2/MeOH = 20:1, v/v) to yield the synthetic precursor (**2a** and **2g**). The analysis data of these compounds were shown in supporting information.

Preparation of title lipids 3a-3g

Compound **2** (3.00 mmol) was suspended in anhydrous dichloromethane (5 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. After the solvent and trifluoroacetic acid were removed, lipid **3a-3g** were directly obtained as light yellow solid by treating the residues with anhydrous ethyl ether twice. The analysis data of these compounds were shown in supporting information.

Preparation of lipid 3h

linoleylamine (828 mg, 3.12 mmol) was added to the anhydrous dichloromethane solution (60 mL) of Boc-glycine (compound **4**, 526 mg, 3.00 mmol), EDC·HCl (690 mg, 3.60 mmol), HOBt (550 mg, 3.59 mmol) and DIEA (778 mg, 6.01 mmol) in ice-salt-bath at 0 ºC for 1h and then at room temperature overnight. The mixture was then washed with saturated aqueous $NaHCO_3$ solution (2×50 mL) and saturated brine (50 mL).The organic phase was dried over Na_2SO_4, filtered, and concentrated to afford an oil, which was further purified by column chromatography on silica gel (CH_2Cl_2/MeOH = 20:1, v/v) to yield a light yellow oil **5**.

Compound **5** (423 mg, 1.00 mmol) was suspended in anhydrous dichloromethane (5 mL), and then, a solution of trifluoroacetic acid (2 mL) in anhydrous dichloromethane (5 mL) was added dropwise under ice bath and N_2 atmosphere, the obtained mixture was stirred at room temperature for 6 h. And then, the solvent was removed under reduced pressure to obtain a brown product which was directly coupled with triBoc-cyclen-acetic acid (531 mg, 1.00 mmol) in the presence of EDC·HCl (230 mg, 1.20 mmol), HOBt (184 mg, 1.21 mmol) and DIEA (778 mg, 6.01 mmol) in 50 mL CH_2Cl_2 at 0 ºC for 1 h and at room temperature overnight. The

mixture was then washed with saturated aqueous $NaHCO_3$ solution (2×50 mL) and saturated brine (50 mL). The organic phase was dried over Na_2SO_4, filtered, and concentrated to afford a viscous liquid, which was further purified by column chromatography on silica gel (CH_2Cl_2/MeOH = 20:1, v/v) to yield the synthetic precursor **6**.

Compound **6** (601 mg, 0.719 mmol) was suspended in anhydrous dichloromethane (5 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. After the solvent and trifluoroacetic acid were removed, lipid **3h** was directly obtained as brown oil by treating the residues with anhydrous ethyl ether twice.

The analysis data of these compounds were shown in supporting information.

Acid-base titration

Briefly, lipids (0.050 mmol of amino groups) were dissolved in 15 mL of 150 mM NaCl aqueous solution, and 1N HCl was added to adjust pH to 2.0. Aliquots (40 μL or 20 μL for each) of 0.1 M NaOH were added, and the solution pH was measured with a pH meter (pHS-25) after each addition. For comparison, NaCl (150 mM) and PEI (25 kDa) were used under the same experimental conditions. The buffering capacity, defined as the percentage of amine groups becoming deprotonated from pH 5.0 to 6.5, was calculated from equation:

$$\text{Buffer capacity (\%)} = 100[(\Delta V_1\ \text{NaOH} - \Delta V_2\ \text{NaOH}) \times 0.1\ \text{M}]/\ \text{N mol}$$

wherein ΔV_1 NaOH is the volume of NaOH solution (0.1 M) required to bring the pH value of the cationic lipid solution from 5.0 to 6.5, ΔV_2 NaOH is the volume of NaOH solution (0.1 M) required to bring the pH value of the NaCl solution from 5.0 to 6.5, and N mol is the total moles of protonatable amine groups in the cationic lipid.

Preparation of cationic liposomes and liposome/DNA complexes (lipoplexes)

Individual cationic lipid (0.0025 mmol) or its mixture with DOPE in the desired mole ratio was dissolved in anhydrous chloroform (2.5 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 ºC, and then the buffer was added to the films resulting

in the final lipid concentration of 1.0 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 ºC afforded the corresponding cationic liposomes that were stored at 4 ºC.

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.

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 M109 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 medium at 37 ºC 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 -80 ºC. 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.

Gel retardation assay

To determine the formation of liposome/pDNA complex (lipolexes), lipoplexes of various N/P ratios ranging from 0 to 8 were prepared as described above. Constant amount of 0.125 μg DNA was used here; 15 μL of each lipoplexes solution was electrophoresed on the 1% (WV^{-1}) agarose gel containing Gold view and Tris–aceate (TAE) running buffer at 110 V for 30 min. DNA was visualized with a UV lamp using a BioRad Universal Hood II.

Ethidium bromide replacement assay

The ability of lipids **3** to condense DNA was studied using ethidium bromide (EB) exclusion assays. Fluorescence spectra were measured at room temperature in air by a Horiba JobinYvon Flu-oromax-4 spectrofluorometer and corrected for the system response. EB (5 μL, 1.0 mg/mL)

was put into quartz cuvette containing 2.5 mL of 10 mM 4-(2-hydroxyethyl)-1-piperazinee-thanesulfonic acid (HEPES) solution (pH7.4). After shaking, the fluorescence intensity of EB (F_{EB}) was measured. Then CT-DNA (10 μL, 1.0 mg/mL) was added to the solution and mixed symmetrically, and the measured fluorescence intensity (F_0) is the result of the interaction between DNA and EB. Subsequently, the solutions of lipid **3a-g** (1.0 mmol/L, 3.2 μL for each addition) and lipid **3h** (1.0 mmol/L, 4.0 μL for each addition) were added to the above solution for further measurement (F_X). All the samples were excited at 520 nm and the emission was measured at 600 nm. The pure EB solution and DNA/EB solution without cationic liposome were used as negative and positive controls, respectively. The percent relative fluorescence (%F) was determined using the equation $\%F = (F_X-F_{EB})/(F_0-F_{EB})$, wherein F_{EB} and F_0 denote the fluorescence intensities of pure EB solution and DNA/EB solution, respectively.

Dynamic light scattering (DLS)

Particle size and zeta potential of liposomes or lipoplexes at various N/P ratios were measured by a dynamic light scattering (DLS) system (Zetasizer Nano ZS, Malvern Instruments Led) at 25 ºC. Lipoplexes were prepared by following the instructions used for transfection and measured with 1 μg DNA/mL sample.

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 nanoparticles solution (10 μL). A few minutes after the deposition, the 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

HEK 293 cells (human embryonic kidney), Hela cells, HEPG2 cells were incubated in Dulbecco's modified Eagle's medium (DMEM) and BEL-7402 cells (human hepatocellular

carcinoma cell) was incubated in 1640 containing 10% fetal bovine serum (FBS) and 1‰ antibiotics (penicillin–streptomycin, 10,000 U mL^{-1}) at 37 ºC in a humidified atmosphere containing 5% CO_2.

Cytotoxicity assays

Toxicity of lipoplexes toward HEK 293 cells was determined by using a Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). About 1.2×10^4 cells/well were seeded into 96-well plates. After 24 h, optimized lipid/DOPE formulations were completed with 0.2 μg of pEGFP-N1 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 100 μL of media with 10% FBS was added. After 24 h, 10 μL CCK-8 was added to each well and the plates were incubated at 37 ºC for another 1h. Then, the absorbance of each sample was measured using an ELISA plate reader (model 680, BioRad) at a wavelength of 450 nm. The cell viability (%) was obtained according to the manufacturer's instruction. Lipoplex prepared from Lipofectamine 2000 was used as control.

In vitro transfection procedure

In order to obtain about 80% confluent cultures at the time of transfection, 24-well plates were seeded with 85,000 cell/well in 500 μL of 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 anti-biotic-free 1640 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 100 μL, and the DNA was used at a concentration of 1 μg/well. After 30 min of complexation, old cell culture medium was removed from the wells, cells were washed once with serum-free 1640, and the above 100 μL lipoplexes was added to each well. The plates were then incubated for 4 h at 37 ºC in a humidified atmosphere containing 5% CO_2. At the end of incubation period, medium was removed, and 500 μL of fresh 1640 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 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^{TM} based on the standard conditions specified by the manufacture. After 24 h transfection of pEGFP plasmid, lysed cells and take 100 μL lysate to measure the fluorescence intensity. Excitation wavelength was 485 nm and the emission wavelength was 538 nm. Lipofectamine 2000^{TM} was chosen as control.

For luciferase assays, cells were transfected by complexes containing pGL-3. For a typical assay in a 24-well plate, 24 h posttransfection as described above, the old medium was removed from the wells, and the cells were washed twice with 500 μL of prechilled 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.

Flow cytometry assay

The cellular uptake of the liposome/fluorescein labeled-DNA complexes was analyzed by flow cytometry. The Label IT Cy5 Labeling Kit was used to label pDNA with Cy5 according to the

manufacturer's protocol. Briefly, HEK 293 cells were seeded onto 12-well plates (1.8×10^5 cells/well) and allow to attach and grown for 24h. Before transfection, the medium was replaced with serum-free culture medium. Cells were incubated with Cy5 labeled DNA nanoparticles (2 μg DNA/well, optimal N/P ratio of each sample) in media for 4 h at 37°C. Subsequently, the cells were washed with 1× PBS and harvested with 0.25%Trypsin/EDTA and resuspended in 1× PBS. Cy5-labeled plasmid DNA uptake was measured in the FL4 channel using the red diode laser (633 nm). Data from 20,000 events were gated using forward and side scatter parameters to exclude cell debris. The flow cytometer was calibrated for each run to obtain a background level of ~ 1% for control samples (i.e., untreated cells).

Confocal laser scanning microscopy (CLSM)

HEK 293 cells were seeded at a density of 2.5×10^5 cells per well in 35 mm confocal dish (Φ = 15 mm), 24 h prior to transfection. For transfection in the absence of serum, the medium was exchange with serum-free medium (for transfection with serum, the medium was exchanged with serum-containing medium). Complexes of liposomes and Cy5-labeled pGL-3 at a given concentration were added to each well. After incubation at 37 °C for 4 h, cells were rinsed for 3 times with PBS (pH 7.4), fixed with 4% paraformaldehyde (dissolved with PBS buffer) for 10 min, nuclear staining was done with DAPI. The CLSM observation was performed using confocal laser scanning microscope (CLSM, Leica TCS SP8) at excitation wavelengths of 405 nm for DAPI (blue), 633 nm for Cy5 (red), respectively.

Acknowledgements

This work was financially supported by the National Program on Key Basic Research Project of China (973 Program, 2012CB720603), the National Science Foundation of China (No. 21232005), and the Specialized Research Fund for the Doctoral Program of Higher Education (20120181130006) in China. J. Z. thanks the Program for New Century Excellent Talents in University (NCET-11-0354). We also thank Analytical & Testing Center of Sichuan University for the TEM analysis.

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