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Cationic gemini lipids with cyclen headgroups: interaction with DNA and gene delivery abilities†

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A series of novel 1,4,7,10-tetraazacyclododecane (cyclen)-based gemini cationic lipids were synthesized, and L-cystine was used as backbone between the two amphiphilic units. The liposomes formed from the lipids and DOPE could efficiently condense plasmid DNA into nanoparticles with suitable size and zeta-potentials, which might be suitable for gene transfection. These lipids were applied as non-viral gene delivery vectors, and their structure–activity relationship was studied. It was found that both the hydrophobic tails and the linking group could largely influence the transfection efficiency, and the oleylamine derived lipid gave the best transfection results, which were close to the commercially available transfection reagent lipofectamine 2000. The gemini structure would favor the gene transfection, and the transfection efficiency of the gemini lipid was much higher than the mono counterpart. Besides, these lipids have very low cytotoxicity, suggesting their good biocompatibility. Results indicate that such gemini lipids might be promising non-viral gene delivery vectors.

1. Introduction

The key factor toward efficient gene therapy is the development of ideal gene carriers, which may deliver therapeutic genes into target cells.[1] Gene carriers are mainly divided into two types: viral and non-viral. Although viral vectors have relatively high transfection efficiency (TE), their biosafety-related disadvantages such as immunogenicity, oncogenic effects and potential mutagenesis limit their applications.[2] On the other hand, non-viral vectors have been receiving numerous attentions over the past decades[3] for their safety, easy preparation and modification, and reproducibility.[2] Some new strategies to enhance the gene transfection were also developed.[4] As an important class of non-viral vectors, cationic lipids have many advantages such as simple structures, good repeatability, biocompatibility, and potential commercial value. Since the first study of DOTMA as cationic lipid gene vector by Felgner in 1987,[5] various types of lipids and liposomes for gene delivery were reported.[6]

Gemini lipid, which was first raised by Menger and Littau in 1991,[7] refers to the amphiphilic molecules with two identical simple surfactants linked by a spacer. As a result, gemini lipids are always consist of at least two hydrophilic headgroups and two hydrophobic tails.[8] Due to its special structure, gemini lipids need a relatively lower amount to bind DNA compared to their mono counterparts, and the lower dosage would lead to lower toxicity. These lipids may condense DNA into nanoparticles with smaller size, facilitating the endocytosis of the lipid/DNA complex (lipoplex).[9] Up to now, some gemini lipids have been found to exhibit higher TE than commercially available lipofectamine 2000.[10,11] They also showed good serum tolerance in the transfection, suggesting their good biocompatibility.[12]

It has been demonstrated that the gene delivery efficiencies of gemini lipids mainly depend on the molecular architectures of the polar head groups,[13] hydrophobic tails,[14,15] and the nature of backbone[16] as well as spacer.[17,18] In our previous studies, 1,4,7,10-tetraazacyclododecane (cyclen) was first applied as headgroup in the design of efficient non-viral gene delivery vectors.[19–21] It was found that the unique cyclic structure of cyclen may facilitate DNA binding[22] and lower the cytotoxicity of cationic lipids. In this report, we would like to introduce a series of novel cyclen-based cationic gemini lipids with cystine as backbone.[12] Various hydrophobic tails were introduced *via* amide or ester bonds. For comparison, mono counterpart was also prepared. Their interactions with plasmid DNA together with the structure–activity relationship (SAR) in the gene transfection process were studied.

2. Experimental section

2.1. Materials and methods

All chemicals and reagents were obtained commercially and were used as received. Anhydrous acetonitrile, dichloromethane (DCM) and chloroform ($CHCl_3$) were dried and purified under nitrogen by using standard methods and were distilled immediately before use. [4,7,10-Tris(tert-butoxycarbonyl)-1,4,7,10-tetraazacyclododecan-1-yl]acetic acid (tri-boc -cyclen- acetic

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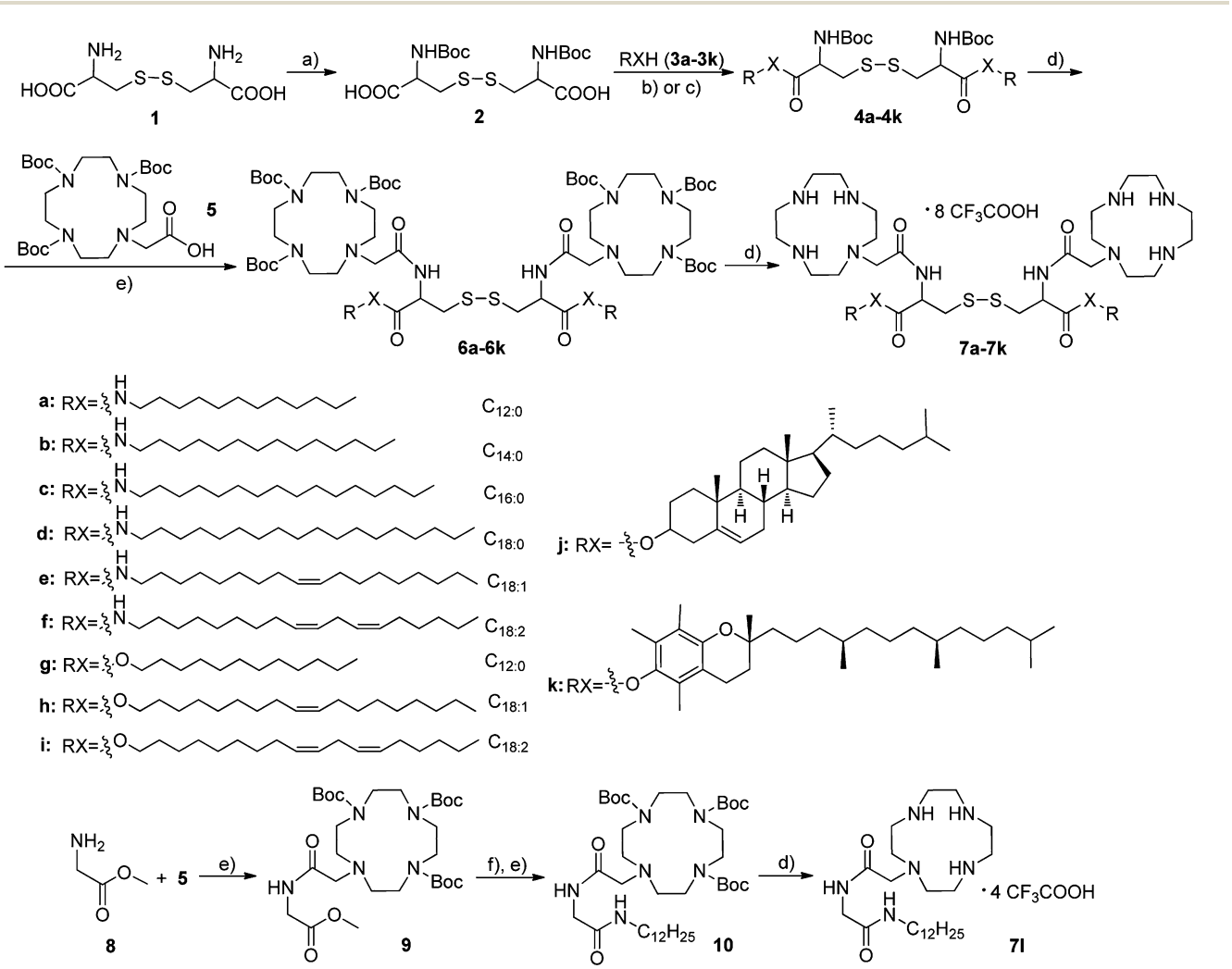
acid, compound **5**, Scheme 1),[23] (9*Z*,12*Z*)-octadeca-9,12-dien-1-amine(compound **3f**)[24] and (9*Z*,12*Z*)-octadeca-9,12-dien-1-ol (compound **3i**)[25] were synthesized according to the literature. The supercoiled plasmid DNA (pUC-19) used in agarose-gel assay was purchased from Takara (Dalian, China). The plasmids used in the study were pEGFP-N1 (Clontech, Palo Alto, CA, USA). A Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). The Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum were purchased from Invitrogen Corp. 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). HEK 293 human embryonic kidney cell lines were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. The NMR spectra were measured on a Varian INOVA-400 spectrometer and the d scale in parts per million was referenced to residual solvent peaks or internal tetramethylsilane (TMS). MS-ESI spectra data were recorded on a Bruker Daltonics BioTOF mass spectrometer.

2.2. Synthesis (detailed analysis data of novel compounds are shown in ESI†)

2.2.1. Preparation of compound 2.[12] To a solution of L-Cystine (compound **1**, 2.4 g, 10 mmol) in 22 mL of 1 N NaOH, BOC anhydride (4.36 g, 20 mmol) was added dropwise. The resulting solution was left stirring at room temperature for overnight. The reaction mixture was washed with hexane and adjusted to pH 2 with a saturated solution of potassium bisulfate, extracted with ethylacetate, dried over anhydrous $MgSO_4$, and filtered. The filtrate was concentrated with a rotary evaporator to yield 2.2 g. Yield: 49.4%.

2.2.2. Preparation of compound 4. 4a–4f: to a mixing solution of compound **2** (880 mg, 2 mmol) and *N*-methylmorpholine (890 mg, 4.4 mmol) in $CHCl_3$, cooled down to 0 °C, isobutylchloroformate (1200 mg, 4.4 mmol) in $CHCl_3$ was added



Scheme 1 Synthesis route of title lipids **7a–7l**. Reagents and conditions: (a) 1 N NaOH, $(Boc)_2O$; (b) *N*-methylmorpholine (NMM), isobutylchloroformate, $CHCl_3$; (c) *N*,*N*-dicyclohexylcarbodiimide (DCC), 4-dimethylamino-pyridine (DMAP), CH_2Cl_2; (d) CF_3COOH, CH_2Cl_2; (e) *N*,*N*-diisopropylethylamine (DIEA), 1-hydroxybenzotriazole (HOBt), (3-dimethylaminopropyl)ethyl-carbodiimidmonohydrochloride (EDCI); (f) NaOH, MeOH.

dropwise for activation of carboxyl.[12] After 1 h, amine (compound **3a–3f**) (4.8 mmol) was added, and the reaction was slowly warmed to room temperature. After 40 h of reaction, the solvent was removed under reduced pressure, then CH_2Cl_2 was added. The mixture was washed with H_2O, saturated aqueous $NaHCO_3$ solution, and brine. The organic layer was dried over anhydrous sodium sulfate and evaporated the solvent to get white solid. The residue was purified by silica gel column chromatography (PE/EA = 5/1, v/v) to obtain compound **4a–4f**. Yield: 33.0–50.3%

4g–4k to the solution of compound **2** (880 mg, 2 mmol) and 4-dimethylamino-pyridine (DMAP) (50 mg) in CH_2Cl_2 at 0 °C, *N*,*N*-dicyclohexyl-carbodiimide (DCC) (0.99 g, 4.8 mmol) in CH_2Cl_2 were added dropwise. After 30 min, the alcohol (**3g–3k**) (4.8 mmol) was added, and the reaction was slowly warmed to room temperature. After 40 h of reaction, the solution was cooled to 0 °C, the precipitate formed was filtered off, evaporated the solvent. The residue was purified by silica gel column chromatography (PE/EA = 5/1, v/v) to obtain **4g–4k**. Yield: 25.4–38.2%

2.2.3. Preparation of compound 6. Step a: Compound **4** (0.5 mmol) was dissolved in anhydrous CH_2Cl_2 (5 mL), then trifluoroacetic acid (CF_3COOH) (5 mL) in anhydrous CH_2Cl_2 (5 mL) was added at 0 °C. After stirring 6 h, the solvent was removed under reduced pressure. The residue was washed with anhydrous ether twice to get pure target compound. Yield: 85%.

Step b: A solution of compound **5** (2.2 mmol) in CH_2Cl_2 (50 mL) was cooled to 0 °C. Sequentially HOBt (367 mg, 2.4 mmol), EDC·HCl (460 mg, 2.4 mmol), *N*,*N*-diisopropylethylamine (DIEA) (517 mg, 4 mmol) were gradually added. After the reaction mixture was stirred for 1 h at 0 °C, add the compound (1 mmol) got from step a. Then the mixed solution was left for stirring at room temperature overnight. The mixture was washed with water, saturated aqueous $NaHCO_3$ solution and saturated brine. The organic layer was dried over anhydrous sodium sulfate and evaporated the solvent to get white solid. The residue was purified by silica gel column chromatography (PE/EA = 1/2, v/v) to obtain compound **6a–6k**. Yield: 37–52%

2.2.4. Preparation of compound 7. Compound **6** (250 mg) was dissolved in anhydrous CH_2Cl_2 (2.5 mL), then trifluoroacetic acid (CF_3COOH) (2.5 mL) in anhydrous CH_2Cl_2 (2.5 mL) was added at 0 °C. After stirring 6 h, the solvent was removed under reduced pressure. The residue was washed with anhydrous ether twice to get pure compound **7a–7k**. Yield: 82–90%

2.2.5. Preparation of compound 9. A solution of compound **5** (1.27 g, 2.4 mmol) in CH_2Cl_2 (50 mL) was cooled to 0 °C. Sequentially HOBt (367 mg, 2.4 mmol), EDC·HCl (460 mg, 2.4 mmol), *N*,*N*-diisopropylethylamine (DIEA) (517 mg, 4 mmol) were gradually added and the reaction mixture was stirred for 1 h at 0 °C. Then compound **8** (280 mg, 2 mmol) was added to the mixture solution, and left it stirring at room temperature overnight. The mixture was washed with water, saturated aqueous $NaHCO_3$ solution and saturated brine. The organic layer was dried over anhydrous sodium sulfate and evaporated the solvent to get white solid. The residue was purified by silica gel column chromatography (PE/EA = 1/4, v/v) to obtain compound **9**. Yield: 51.6%

2.2.6. Preparation of compound 10. Step a: compound **9** (0.7 g, 1.14 mmol) was dissolved in CH_3OH (50 mL), then 2 N NaOH was added to it. The reaction kept stirring at room temperature. After the reaction, the CH_3OH was removed under reduced pressure. Then adding water to it and use 2 N HCl adjust the pH to acidity. Then, CH_2Cl_2 was added, washed with brine twice. The organic layer was dried over anhydrous sodium sulfate and evaporated the solvent to get white solid. Yield: 74%.

Step b: the compound just got (0.48 g, 0.78 mmol) in CH_2Cl_2 (70 mL) was cooled to 0 °C. Sequentially HOBt (172 mg, 1.12 mmol), EDC·HCl (215 mg, 1.12 mmol), *N*,*N*-diisopropylethylamine (DIEA) (210 mg, 1.6 mmol) were gradually added and the reaction mixture was stirred for 1 h at 0 °C. Then the mixed solution was left for stirring at room temperature overnight. The solvent was removed under reduced pressure, then EtOAc was added, the mixture was washed with water, saturated aqueous $NaHCO_3$ solution and saturated brine. The organic layer was dried over anhydrous sodium sulfate and evaporated the solvent to get white solid. The residue was purified by silica gel column chromatography (PE/EA = 1/2, v/v) to obtain compound **10**. Yield: 84.9%.

2.2.7. Preparation of compound 7l. Compound **10** (150 mg) was dissolved in anhydrous CH_2Cl_2 (1.5 mL), then trifluoroacetic acid (CF_3COOH) (1.5 mL) in anhydrous CH_2Cl_2 (1.5 mL) was added at 0 °C. After stirring 6 h, the solvent was removed under reduced pressure. The residue was washed with anhydrous ether twice to get pure compound **7l**. Yield: 80%.

2.3. Preparation of cationic liposome

The cationic lipid and the neutral lipid DOPE in a 1 : 2 mole ratio were dissolved in 2.5 mL anhydrous chloroform in a glass vial. The solvent was removed under reduced pressure with a thin flow of moisture-free nitrogen gas. And the dried lipid film was then kept under high vacuum overnight. The lipid film was hydrated with 2.5 mL of Tris–HCl buffer (10 mM, pH 7.4) 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.[26]

2.4. Amplification and purification of plasmid DNA

pGL-3 and pEGFP-N1 plasmids were used. The former one was seed 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 °C and 220 rpm overnight. The plasmids were purified by an EndoFree Tiangen™ Plasmid Kit. Then, the purified plasmids were dissolved in TE (Tris + EDTA) buffer solution and stored at −80 °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/280 nm (~1.9). The concentrations of plasmids were determined by ultraviolet (UV) absorbance at 260 nm.

2.5. 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 309.

2.6. Agarose-gel retardation assay

Lipid/DOPE/pDNA complexes at different N/P ratios (the amino groups of lipids to phosphate groups of DNA) ranging from 0 to 8 were prepared by adding an appropriate volume of lipids to 0.125 μg pUC-19 DNA. The complexes were incubated at 37 °C for 30 min. Then the complexes were electrophoresed on the 1.0% (W/V) agarose gel containing gelred and with Tris-acetate (TAE) running buffer at 110 V for 30 min. DNA was visualized with a UV lamp at wavelength of 312 nm by using BioRad Universal Hood II.

2.7. Ethidium bromide replacement assay

The ability of lipids 7 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^{-1}) 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 was measured. Then CT-DNA (10 μL, 1.0 mg mL^{-1}) was added to the solution and mixed symmetrically, and the measured fluorescence intensity is the result of the interaction between DNA and EB. Subsequently, the solutions of lipid **7a–7k** (0.5 mmol L^{-1}, 4 μL for each addition) and **7l** (1 mmol L^{-1}, 4 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. 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 $\%F = (F - F_{EB})/(F_0 - F_{EB})$, wherein F_{EB} and F_0 denote the fluorescence intensities of pure EB solution and DNA/EB solution, respectively.

2.8. Lipoplex particle sizes and zeta potentials

Sizes and zeta potentials of the lipid/pDNA lipoplexes at various N/P ratios were analyzed at room temperature on a dynamic light scattering system (Zetasizer Nano ZS, Malvern instruments Led) at 25 °C. The lipoplexes particle solutions were first prepared by mixing 7/DOPE lipids and pUC-19 (2 μg mL^{-1}) under diverse N/P ratios in 1 mL deionized water.

2.9. 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.

2.10. Cell culture

Human embryonic kidney transformed 293 (HEK293) cells were incubated in DMEM containing 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin-streptomycin, 10 000 U mL^{-1}) at 37 °C in a humidified atmosphere containing 5% CO_2.

2.11. MTT cytotoxicity assay

Toxicity of lipoplexes toward HEK 293 cells was determined by using a Cell Counting Kit-8 (CCK-8). About 8000 cells per well were seeded into 96-well plates.[27] After 24 h, optimized lipid/DOPE formulations were complexed with 0.2 μg of DNA at various N/P ratios for 30 min; 100 μL of lipoplexes were added to the cells in the absence of serum. After 4 h of incubation, lipoplex solutions were removed, and 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 °C for another 1 h. 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. Blank control and Lipoplex prepared from Lipofectamine 2000 were used as comparisons.

2.12. *In vitro* transfection procedure

Gene transfection of a series of complexes was investigated in HEK293 cells. In order to obtain about 80% confluent cultures at the time of transfection, 24-well plates were seeded with 85 000 cell per well in 500 μL of serum-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 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 100 μL, and the DNA was used at a concentration of 1 μg per 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 100 μL lipoplexes was added to each well. The plates were then incubated for 4 h at 37 °C in a humidified atmosphere containing 5% CO_2. At the end of incubation period, medium was removed, and 500 μL of fresh DMEM medium containing 10% FBS was added to each well. Plates were further incubated for a period of 24 h before checking the reporter gene expression.

For fluorescent microscopy assays, cells were transfected by complexes containing pEGFP-N1. After 24 h incubation, the microscopy images were obtained at the magnification of 100 and recorded using Viewfinder Lite (1.0) software. Control transfection was performed in each case using a commercially

available transfection reagent Lipofectamine 2000™ based on the standard conditions specified by the manufacture.

For luciferase assays, cells were transfected by complexes containing pGL-3. For a typical assay in a 24-well plate, 24 h 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 TEs of the individual lipids was made based on the data for luciferase expressed as relative light units (RLU) mg^{-1} 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.

3. Results and discussion

3.1. Synthesis of target cationic gemini lipids 7

The synthetic route of the cyclen-based gemini lipids **7** is shown in Scheme 1. L-Cystine was chosen as backbone for its symmetric and modifiable structure. The condensation between butyloxycarbonyl (Boc) protected cystine **2** and various amines or alcohols in the presence of *N*-methylmorpholine (NMM) or dicyclohexylcarbodiimide (DCC) gave relative diamides or diesters **4**, respectively. Compound **4** was then deprotected and reacted with tri-Boc-cyclenylacetic acid **5**[23] to give the protected precursors **6**. Target gemini lipids **7** could be smoothly obtained by removing the Boc groups with trifluoroacetic acid, and the resulting trifluoroacetates were used in subsequent studies. In addition, for comparison, the mono counterpart lipid **7l**, which has only one headgroup and one tail, was also prepared *via* similar method from glycine methyl ester **8**. The structures of all new compounds were characterized and confirmed by NMR and HRMS.

3.2. Interactions between the liposomes and plasmid DNA

Cationic liposomes can be formed from either individual cationic lipid or more frequently from a combination of cationic lipid and neutral lipids such as DOPE. Each gemini lipid was mixed in different molar ratios (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 **7**/DOPE ratio of 1 : 2 was used herein. Agarose gel electrophoresis was first performed to investigate their DNA condensation ability, which is known as a primary factor for the cationic lipids serving as gene vectors. As shown in Fig. 1. All of the lipids showed good DNA binding abilities. Full DNA retardation on the gel could be observed from the N/P ratio of 2 except the experiment involving **7f**, which completely retarded DNA from higher N/P ratio. Further, ethidium bromide (EB) dye replacement assay was also carried out to evaluate their DNA binding abilities[21]. Results in Fig. 2 show the relative fluorescence intensities of EB in HEPES solution (pH 7.4, 10 mM) in the presence of various amounts of liposomes. It was observed that the fluorescent intensity dramatically decreased with the increase of N/P ratio, indicating that the liposomes have strong DNA-binding abilities to replace EB. At the N/P ratios of 4, all the liposomes could quench at last 50% of original fluorescence intensity. For the amide contained gemini lipids **7a–7e**, the original fluorescent intensities were quenched to 0–15% at the N/P ratio of 3 (Fig. 2A). Meanwhile, for the ester contained gemini lipids **7g–7k**, the fluorescent quenching abilities were relatively lower (Fig. 2B). Such difference might be attributed to the more negatively charged ester bonds. The chain length might also affect the binding ability, and liposomes with longer hydrophobic chains exhibited better fluorescence quenching ability, indicating tighter DNA binding (**7a–7d**). Higher unsaturation degree of the hydrophobic chain would not benefit the binding, and **7f**-derived liposome showed distinctly weaker fluorescence quenching ability. Moreover, the mono counterpart **7l** showed obviously lower binding ability than the gemini lipids, suggesting that the configuration of gemini liposomes might benefit their interaction toward DNA.

The particle size, surface charge and morphology of the liposome/DNA complexes (lipoplexes) were important factors for their large influences on gene transfection, expecially on the apparent cytotoxicity, cellular uptake/trafficking, and the release of the encapsulated gene. Dynamic light scattering (DLS) assay was applied to measure the sizes and zeta-potentials of the formed lipoplexes, and the results are shown in Fig. 3. The average diameters were observed in a range of 100–650 nm, which strongly depended on the N/P ratios (Fig. 3A). With the rise of N/P ratio, the particle sizes reduced and stabilized at about 100 nm, indicating the full condensation of DNA. As the exceptions, lipoplexes formed from **7f** and **7l** gave larger

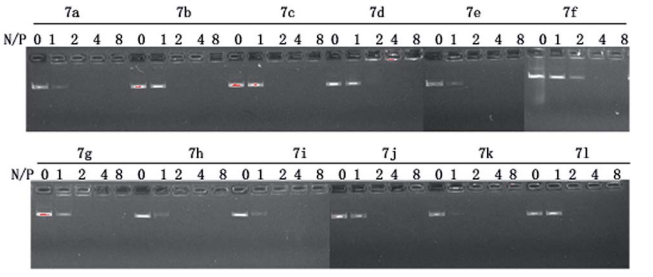


Fig. 1 Electrophoretic gel retardation assays of plasmid DNA with the presence of liposomes at various N/P ratios. The molar ratio of lipid/DOPE was 1 : 2.

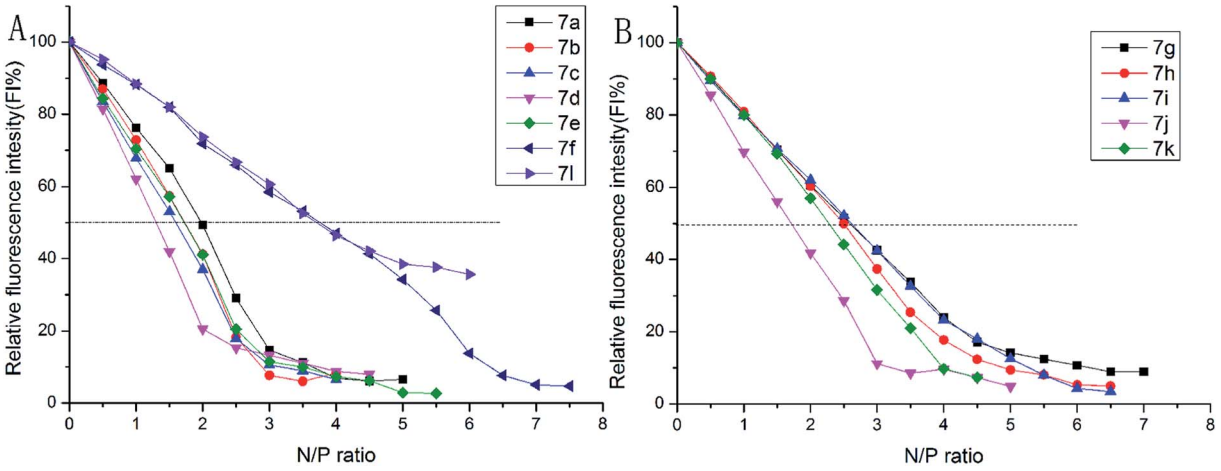


Fig. 2 Ethidium bromide displacement assay by **7a–7f**, **7l** (A) and **7g–7k** (B) under various N/P ratios in 10 mM Hepes buffer solution. The molar ratio of lipid/DOPE was 1 : 2.

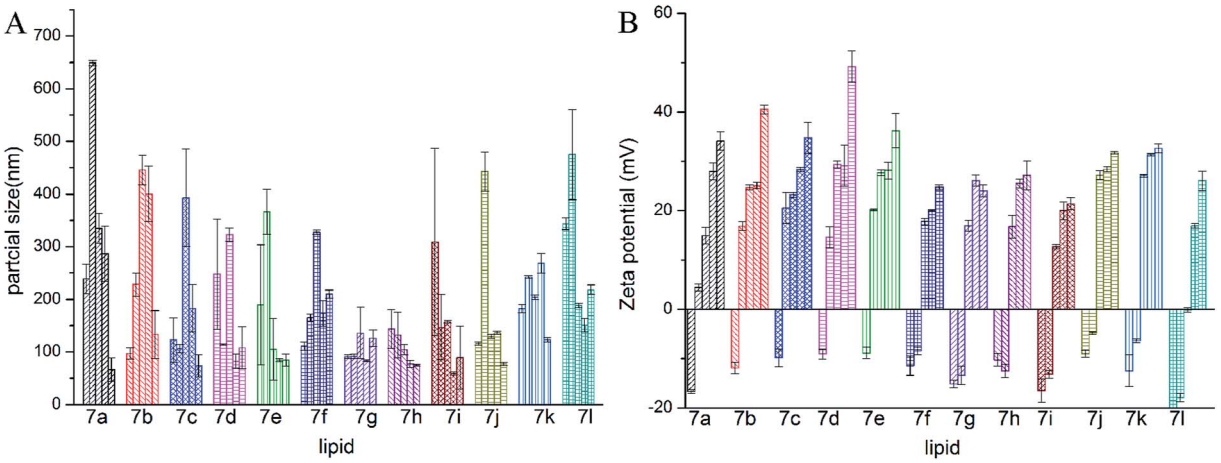


Fig. 3 Mean particle sizes (A) and zeta-potentials (B) of the lipoplexes formed from **7** at N/P ratio of 1, 2, 4, 6, 8.

particles under N/P of 8, and this might be due to their relatively lower DNA binding ability, which is in accordance with the fluorescence quenching results. In addition, the stability of the lipoplexes was also studied, and it was shown that the sizes of the particles involving lipids **7d**, **7e** and **7l** seldom changed during 24 h incubation, while the lipofectamine 2000-contained particles exhibited obvious size increase (Fig. S1†). Such results suggest the good stability of the lipoplexes. Zeta potentials of all the lipoplexes showed similar trends. With the increase of N/P ratio, the zeta potentials reversed from negative to positive at N/P of 2 or 4, and finally reached around +35 mV. At N/P ratio of 2, the surface charges of lipoplexes derived from **7a–7e** turned to positive, while other lipoplexes still remained negatively charged. Such results also suggest that the amide-contained gemini lipids **7a–7e** have stronger DNA binding ability, which was similar to the EB displacement results (Fig. 2A). Additionally, the mono counterpart **7l** was again proved to have lower DNA binding ability. Among the lipoplexes studied, the zeta-potential of the one derived from **7l** turned to positive at the largest N/P ratio, indicating that more liposome was needed for the condensation of DNA.

Transmission electron microscopy (TEM) was further performed to get direct information about the shape and morphology of lipoplexes. Representative electron micrograph of lipoplexes formed from **7e** and lipofectamine 2000 are shown in Fig. 4 (A and C respectively), which reveal that the lipoplex show almost spherical particles with the diameters in the range of 50–100 nm. Further, the **7e**/DNA lipoplex in serum environment was also investigated by the same assay. Result in Fig. 4B shows that the complex is stable in the presence of serum, and no obvious change of particle size or morphology was observed.

3.3. Cytotoxicity

Low cytotoxicity is necessary for efficient gene carriers. The cell viability of the lipoplexes formed from cationic gemini lipids as a function of N/P ratio was evaluated in HEK 293 cells by CCK-8, and commercially available lipofectamine 2000 was used for comparison. As shown in Fig. 5, even at high N/P ratios, the

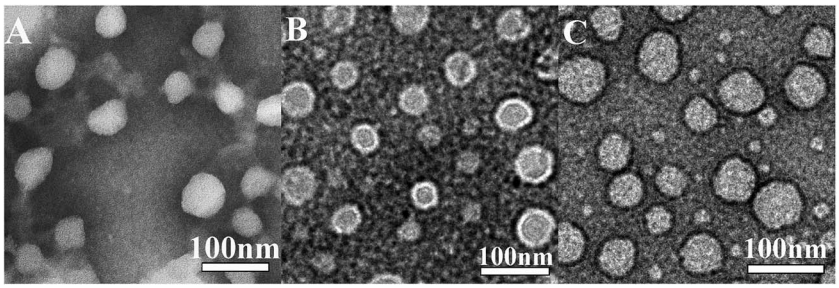


Fig. 4 TEM image of lipoplexes. (A & B) **7e**/DOPE/DNA lipoplex at N/P ratio of 12 in deionized water (A) and 50% of FBS (B); (C) lipofectamine 2000/DNA lipoplex in deionized water.

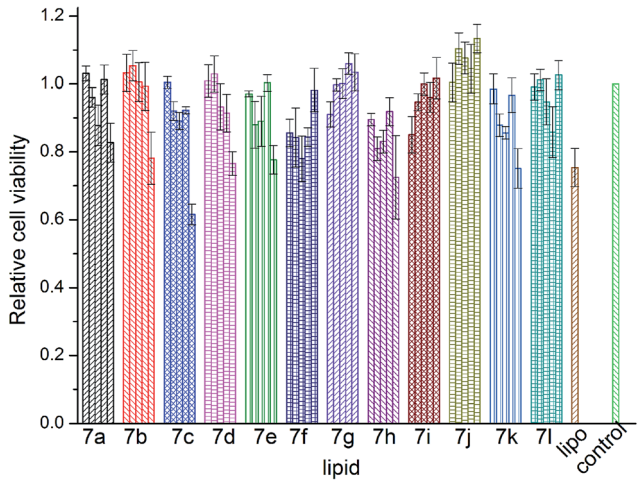


Fig. 5 Cytotoxicity of the lipoplexes prepared at various N/P ratios under lipid/DOPE ratio of 1 : 2. Data represent mean ± SD ($n = 3$). Each lipoplex were at N/P ratio of 2, 4, 8, 12, 24, respectively.

relative cell viabilities treated with the lipoplexes were distinctly higher than those involving lipofectamine 2000. Nearly no cytotoxicity was observed for lipoplexes at N/P ratios lower than 12, which was the highest N/P ratio used in the transfection experiments. The results suggest that the cyclen-based gemini lipids are biocompatible and suitable for gene delivery.

3.4. *In vitro* gene transfection

Studies on the structure–activity relationship of cationic liposome are quite important and helpful for further design of ideal gene carriers. Among the twelve lipids, six of them (**7a**, **7e**, **7g**, **7h**, **7k**, **7i**) were first selected for the transfection of luciferase reporter gene. Fig. 6A gives the quantitatively transfection results in HEK 293 cells at N/P ratios of 4, 8 and 12 with lipofectamine 2000 and naked DNA as controls. These six lipids have large structural difference on the hydrophobic moiety, and this led to distinct diversity of their TE. Lipid **7e** with oleylamine as the hydrophobic group gave the best TE, which was only slightly less than lipofectamine 2000. For the lipids with same aliphatic chains, the amide-contained lipids (**7a** and **7e**) gave several times higher TE than their ester-contained analogs (**7g** and **7h**). We also found similar amide preference in our previous studies on the lipidic gene vectors.[28] Tocopherol has been found to be a potential candidate for the hydrophobic group of lipidic gene vectors.[12,19] However, the tocopherol-contained lipid **7k** was much less effective compared to **7e** with aliphatic long chain. Besides, by comparison with **7a**, the TE of mono counterpart **7l** decreased for an order of magnitude, indicating the advantage of gemini structure. Further, the effect of unsaturation degree of the hydrophobic chains on the transfection was then investigated. As shown in Fig. 6B, N/P ratios of 8 and 12, at which better TEs were obtained in previous studies, were applied in the transfection experiments

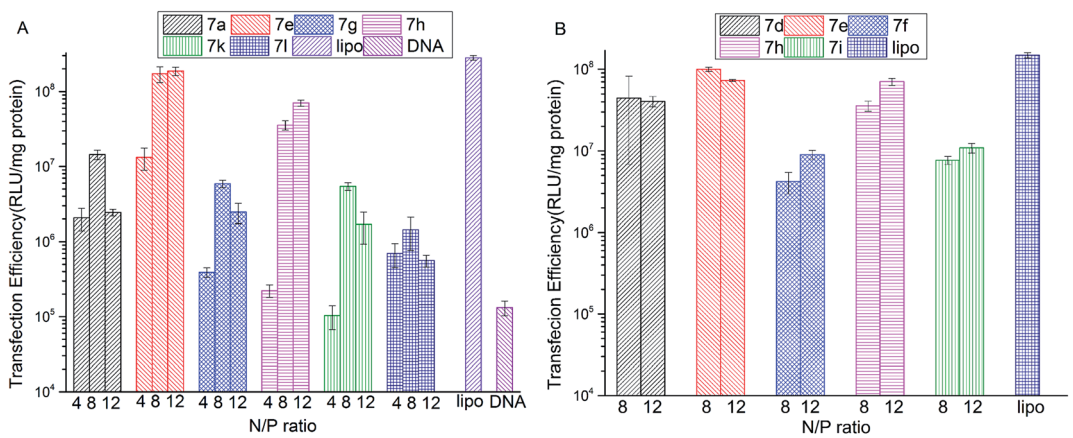


Fig. 6 Transfection efficiencies of lipoplexes in HEK 293 cells at various N/P ratios under lipid/DOPE ratio of 1 : 2.

using **7d**, **7e**, **7f** and **7i**. The oleyl group was found to be the best choice for the transfection, and oleyl-contained lipids **7e** and **7h** gave the best TEs. The linoleyl-contained **7f** and **7i** gave much decreased TEs, which were even lower than the lipid with saturated chains (**7d**). As many reported and some commercially available transfection reagents also have oleyl moiety in their structures, such results might be reasonable. The introducing of monounsaturated hydrophobic chain would facilitate membrane disruption and DNA escape from endosome by increasing the membrane fluidity.[15] Further increase of unsaturation degree may lead to difficulty of liposome forming and subsequent DNA binding, which would hamper the transfection process. The above structure–activity relationships may afford us clues for further optimization of gemini lipid gene delivery materials.

4. Conclusion

In summary, a series of cyclen-based cationic gemini lipids with two symmetrical head groups and two hydrophobic tails were designed and synthesized. Cystine was used as backbone between the two amphiphilic units. Liposomes could be smoothly formed by these lipids and helper lipid DOPE. The interaction between the liposome and plasmid DNA was studied. Results reveal that these liposomes have strong DNA binding ability, and full DNA condensation could be achieved at the N/P ratio of 2–4. The lipoplexes have proper sizes and zeta-potentials, which might be suitable for gene transfection. These materials were applied as non-viral gene delivery vectors, and their structure–activity relationship was investigated. Subtle changes in the structure of the lipids would lead to large effect on the TE. The oleyl amine derived lipid **7e** gave the best TE, which was close to the commercially available transfection reagent lipofectamine 2000. Besides, these lipids have very low cytotoxicity, suggesting their good biocompatibility. Further modifications and extended application of such type of gemini lipids are currently underway.

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References

1 (*a*) G. M. Rubanyi, *Mol. Aspects Med.*, 2001, **22**, 113–142; (*b*) A. El-Aneed, *Eur. J. Pharmacol.*, 2004, **498**, 1–8.
2 A. El-Aneed, *J. Controlled Release*, 2004, **94**, 1–14.
3 X. Guo and L. Huang, *Acc. Chem. Res.*, 2011, **45**, 971–979.
4 (*a*) Y. Su, R. Mani and M. Hong, *J. Am. Chem. Soc.*, 2008, **130**, 8856–8864; (*b*) Y. Su, S. Li and M. Hong, *Amino Acids*, 2013, **44**, 821–833.
5 T. R. G. P. L. Felgner, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Northrop, G. M. Ringold and M. Danielsen, *Proc. Natl. Acad. Sci. U. S. A.*, 1987, **84**, 7413–7417.
6 S. Bhattacharya and A. Bajaj, *Chem. Commun.*, 2009, 4632–4656.
7 F. M. Menger and C. A. Littau, *J. Am. Chem. Soc.*, 1993, **115**, 10083–10090.
8 F. M. Menger, J. S. Keiper and V. Azov, *Langmuir*, 1999, **16**, 2062–2067.
9 M. Kumar, K. Jinturkar, M. R. Yadav and A. Misra, *Crit. Rev. Ther. Drug Carrier Syst.*, 2010, **27**, 237–278.
10 V. Floch, N. Legros, S. Loisel, C. Guillaume, J. Guilbot, T. Benvegnu, V. Ferrieres, D. Plusquellec and C. Ferec, *Biochem. Biophys. Res. Commun.*, 1998, **251**, 360–365.
11 T. Zhou, A. Llizo, P. Li, C.-x. Wang, Y. Guo, M. Ao, L. Bai, C. Wang, Y. Yang and G. Xu, *J. Phys. Chem. C*, 2013, **117**, 26573–26581.
12 B. Kedika and S. V. Patri, *Mol. Pharm.*, 2012, **9**, 1146–1162.
13 D. Zhi, S. Zhang, S. Cui, Y. Zhao, Y. Wang and D. Zhao, *Bioconjugate Chem.*, 2013, **24**, 487–519.
14 R. Koynova, B. Tenchov, L. Wang and R. C. MacDonald, *Mol. Pharm.*, 2009, **6**, 951–958.
15 D. Zhi, S. Zhang, B. Wang, Y. Zhao, B. Yang and S. Yu, *Bioconjugate Chem.*, 2010, **21**, 563–577.
16 B. Kedika and S. V. Patri, *Bioconjugate Chem.*, 2011, **22**, 2581–2592.
17 M. Rajesh, J. Sen, M. Srujan, K. Mukherjee, B. Sreedhar and A. Chaudhuri, *J. Am. Chem. Soc.*, 2007, **129**, 11408–11420.
18 R. Mukthavaram, S. Marepally, M. Y. Venkata, G. N. Vegi, R. Sistla and A. Chaudhuri, *Biomaterials*, 2009, **30**, 2369–2384.
19 Q. Liu, Q.-Q. Jiang, W.-J. Yi, J. Zhang, X.-C. Zhang, M.-B. Wu, Y.-M. Zhang, W. Zhu and X.-Q. Yu, *Bioorg. Med. Chem.*, 2013, **21**, 3105–3113.
20 B.-Q. Liu, W.-J. Yi, J. Zhang, Q. Liu, Y.-H. Liu, S.-D. Fan and X.-Q. Yu, *Org. Biomol. Chem.*, 2014, **12**, 3484–3492.
21 Q.-D. Huang, G.-X. Zhong, Y. Zhang, J. Ren, Y. Fu, J. Zhang, W. Zhu and X.-Q. Yu, *PLoS One*, 2011, **6**, e23134.
22 R. M. Izatt, K. Pawlak, J. S. Bradshaw and R. L. Bruening, *Chem. Rev.*, 1991, **91**, 1721–2085.
23 J. W. Jeon, S. J. Son, C. E. Yoo, I. S. Hong, J. B. Song and J. Suh, *Org. Lett.*, 2002, **4**, 4155–4158.
24 C. Fong, D. Wells, I. Krodkiewska, P. G. Hartley and C. J. Drummond, *Chem. Mater.*, 2006, **18**, 594–597.
25 M. Sodeoka, R. Sampe, S. Kojima, Y. Baba, T. Usui, K. Ueda and H. Osada, *J. Med. Chem.*, 2001, **44**, 3216–3222.
26 Q. Liu, W.-J. Yi, Y.-M. Zhang, J. Zhang, L. Guo and X.-Q. Yu, *Chem. Biol. Drug Des.*, 2013, **82**, 376–383.
27 K. Luo, C. Li, L. Li, W. She, G. Wang and Z. Gu, *Biomaterials*, 2012, **33**, 4917–4927.
28 B. Wang, W.-J. Yi, J. Zhang, Q.-F. Zhang, M.-M. Xun and X.-Q. Yu, *Bioorg. Med. Chem. Lett.*, 2014, **24**, 1771–1775.

