Linear cyclen-based polyamine as a novel and efficient reagent in gene delivery†

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Linear cyclen-based polyamine (LCPA, $M_w = 7392$, $M_w/M_n = 1.19$) as a novel non-viral gene vector was designed and synthesized from 1,7-diprotected 1,4,7,10-tetraazacyclododecane (cyclen), bis(β-hydroxylethyl)amine and epichlorohydrin. Agarose gel retardation and fluorescent titration using ethidium bromide showed the good DNA-binding ability of LCPA. It could retard pDNA at an N/P ratio of 4 and form polyplexes with sizes around 250–300 nm from an N/P ratio of 10 to 60 and relatively lower zeta-potential values (< +3 mV) even at the N/P ratio of 60. The cytotoxicity of LCPA assayed by MTT is much lower than that of 25 kDa PEI. *In vitro* transfection against A549 and 293 cells showed that the transfection efficiency of LCPA/DNA polyplexes is close to that of 25 kDa PEI at an N/P ratio of 10–15, indicating that the new material could be a promising non-viral polycationic reagent for gene delivery.

Introduction

Safe and efficient gene delivery vectors, including viral and non-viral vectors,[1] are prerequisites for successful gene therapy.[2] As viral approaches often cause notable toxicity and immunogenicity, non-viral systems have received increased attention because of their potential to overcome many inherent challenges of viral vectors.[3] Among non-viral vectors, cationic polymers and cationic lipids are the two major systems. Compared with cationic lipids which have been widely used in gene delivery,[4] cationic polymers possess many advantages such as a good ability to condense DNA and ease of special modifications.[5] Many cationic polymers, such as polyethyleneimine (PEI),[6] polyamidoamine dendrimers,[7] poly(L-lysine) (PLL),[8] poly[α-(4-aminobutyl)-L-glycolic acid],[9] chitosan,[10] poly(β-aminoesters),[11] poly[*N*,*N*-(dimethylamino)ethyl methacrylate][12] and polycationic cyclodextrin,[13] have been used as gene delivery carriers with individual properties.

Among various cationic polymers, PEI is the most studied material for DNA delivery because of its strong buffering capability in the pH region of 7.4–5.1 together with high binding capability towards DNA and a relatively high transfection efficiency. Therefore, PEI has been considered as the gold standard of gene transfection.[3] However, the high cytotoxicity partly caused by its high charge density seriously hampered its therapeutic use. Low-molecular weight (LMW) PEI has much lower cytotoxicity than that of high-molecular weight (HMW) PEI, but its transfection efficiency is also lower.[14] To obtain decreased cytotoxicity and improved transfection efficiency, much attention has been paid to modified PEI. The commonly used methods are to block or graft PEI with biocompatible moieties, such as poly(ethylene glycol) (PEG),[15] chitosan[16] and dextran.[17] Although several copolymers displayed lower cytotoxicities along with comparable, or even higher transfection efficiencies compared with PEI, seeking other materials with novel structures is of great importance.

The positive charge on amino groups of polyamines may facilitate charge interactions with the phosphate backbone of DNA. Increasing knowledge about the crucial role of 1,4,7,10-tetraazacyclododecane (cyclen) in cell biology stimulated wide basic and applied research interests. Macrocyclic complexes with tetraazamacrocyclic ligands, such as cyclen, cyclam and bicyclam, exhibited antitumor or anti-HIV activity.[18] These important and versatile applications stimulate researchers to explore new cyclen-based ligands and complexes with potential chemical, biological and catalytic properties. Thus, many studies have been focused on metal complexes of cyclen and their cleavage ability towards phosphoesters and DNA as artificial enzymes in chemical biology, medicine and gene therapy.[19-21] Some bicyclam derivatives were used as non-viral vectors for specific gene delivery.[22] More recently, we also reported a novel cationic macrocyclic polyamine lipid containing an imidazolium salt group and a cyclen unit, which could transfer plasmid DNA into cells without use of extraneous agent.[23] Moreover, we prepared a reticular cyclen-based polymer from cyclen and epichlorohydrin (EPI),[24] however, the transfection efficiency was not satisfying.

Herein, we wish to report a novel linear cyclen-based polyamine, which is prepared from cyclen, bis(β-hydroxylethyl)amine and epichlorohydrin. The buffer capability of the title compound was detected by acid–base titration. The interaction between the polymer and plasmid DNA was studied by fluorescence titration

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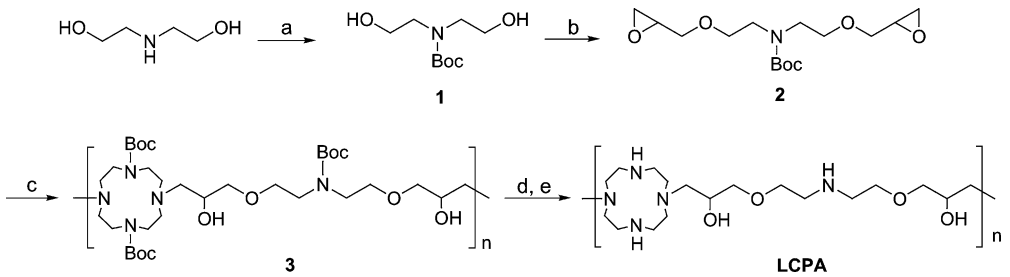

Scheme 1 Synthetic route to LCPA. a) $(Boc)_2O$, Et_3N, $CHCl_3$; b) epichlorohydrin, Bu_4NBr, NaOH, H_2O; c) 1, 7-bis(*tert*-butyloxycarbonyl)-1,4,7,10-tetraazacyclodocane, EtOH; d) HCl (g), CH_2Cl_2; e) NaOH, $CHCl_3$.

and agarose gel electrophoresis. The properties such as particle size and zeta-potential of the formed complexes were also studied. *In vitro* transfection experiments demonstrate that this cyclen-based polymer may act as efficient non-viral vector in gene delivery with low cytotoxicity.

Results and discussion

Synthesis and characterization of LCPA

The preparation of LCPA is shown in Scheme 1. The nitrogen atom of bis(β-hydroxylethyl)amine was first protected by a Boc group to avoid a side reaction caused by the nucleophilic attack of an N atom to epichlorohydrin. Then compound **1** reacted smoothly with epichlorohydrin in the presence of Bu_4NBr–NaOH to give the bridge unit **2**. The protected cyclen-based polymer **3**, in which many new-formed hydroxyls may increase the water-solubility, was subsequently prepared by the reaction between compound **2** and 1,7-bis(*tert*-butyloxycarbonyl)-1,4,7,10-tetraazacyclodocane (diBoc-cyclen) in equal molar ratio. Polymer **3** could be recrystallized from EtOH–hexane. Finally, the water-soluble LCPA could be obtained by deprotection of Boc groups using HCl (g) in dichloromethane. NMR analysis of the final product showed the absence of a singlet at 1.42–1.44 ppm, indicating that the Boc groups were completely cleaved from the polyamine backbone. The final LCPA was analyzed *via* size-exclusion chromatography (SEC) in combination with multiple angle laser light scattering to determine the molecular weight (M_w = 7392, M_w/M_n = 1.19). In addition, we also tried to use diethoxyphosphoryl (DEP) groups for the 1,7-diprotection of cyclen. However, this method was abandoned because of the difficulty of deprotection in the last step.

Buffer capability

Cationic polymers are assumed to have the ability to facilitate the escape of polyamine/DNA complexes from the endosome by the "proton sponge effect" and to promote transfection efficiency.[25] In this study, the buffer capabilities of LCPA and 25 kDa PEI were evaluated by acid–base titration. As shown in Fig. 1, the buffer capability of LCPA is lower than that of PEI. It was reported that the buffer capability of a polycation mainly depends on the presence of primary, secondary and tertiary amines groups. Since LCPA has a lower molecular weight and less density of amino groups in the structure relative to 25 kDa PEI, a lower buffer capacity of LCPA is reasonable.

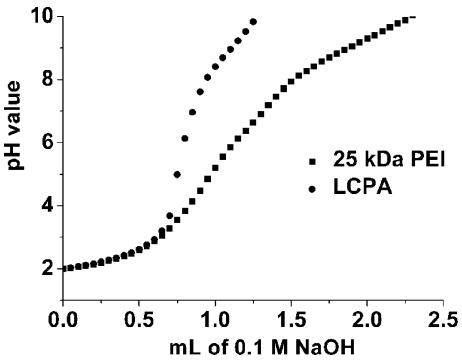

Fig. 1 Acid–base titration profiles of PEI and LCPA. LCPA or PEI (0.25 mmol of amino groups) was first treated with 1 N HCl to adjust pH to 2.0, and then the solution pH was measure after each addition of 50 μL of 0.1 N NaOH. The relatively flat curve of PEI indicates its higher buffer capability than that of LCPA.

Formation of LCPA/DNA complexes

DNA condensation capability is a prerequisite for polymeric gene vectors. Gel retardation analysis was performed to confirm the affinity between LCPA and plasmid DNA. As shown in Fig. 2, the electrophoretic mobility of DNA was retarded by the introduction of LCPA, and total DNA retardation was detected at and above N/P ratios of 4. The results suggested that LCPA can bind to DNA through electrostatic interactions between DNA backbone

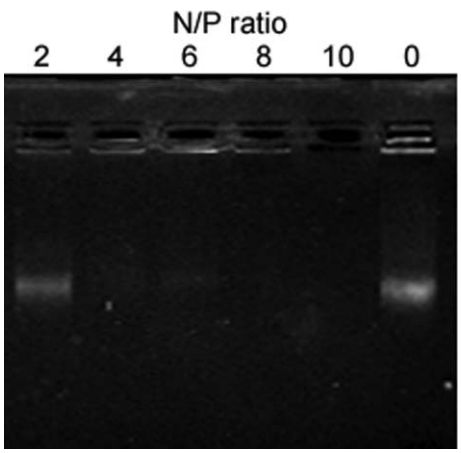

Fig. 2 Electrophoretic mobility of plasmid DNA in the presence of LCPA (ethidium bromide staining). Lanes 1–5 (from left): LCPA/pDNA complex with N/P ratio of 2, 4, 6, 8, and 10, respectively; Lane 6: pDNA control.

and the cationic nitrogen atoms both in the cyclen cycle and on the bridge.

The ethidium bromide (EB) exclusion assay is another useful method in the studies of binding ability of polyamines to DNA. EB has weak fluorescence, but its emission intensity in the presence of DNA could be greatly enhanced because of its strong intercalation between the adjacent DNA base pairs. It was previously reported that this enhanced fluorescence could be quenched, or at least partly quenched by the addition of a second molecule with higher DNA-binding ability, such as polycations. The percent of decreased fluorescence value can be used as a parameter to evaluate the DNA binding affinity of certain materials. Fig. 3 shows that the addition of LCPA to EB pretreated with DNA caused an appreciable decrease in the emission intensity, indicating that EB which bound to DNA was partially replaced by LCPA. The relative fluorescence decreased by approximately 50% at N/P ratio of 5. The results, which are in agreement with the gel retardation analysis results, may confirm the good binding ability of LCPA towards DNA.

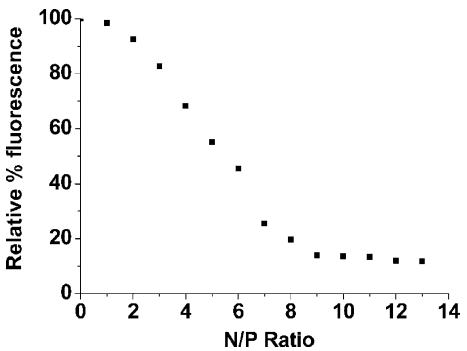

Fig. 3 Change of relative fluorescence of EB bound to DNA by the addition of LCPA to different N/P ratios. All the samples were excited at 497 nm and the emission was measured at 600 nm. DNA concentration was 3.8 μg mL^{-1}.

The appropriate polymer/DNA nanoparticle is of critical important for polyamines used as gene vectors. And the particle size would apparently affect the transfection efficiency of gene vectors. The particle size depends on many parameters, such as DNA concentration, sequence of addition of polyamines or DNA during complex preparation, and ionic strength of the solvent. The particle size of LCPA/pDNA complexes was measured at various N/P ratios, and the results are shown in Fig. 4A. LCPA can efficiently compact pDNA into small nanoparticles. Generally, the hydrodynamic sizes of the complexes decrease with increasing N/P ratios. After reaching the N/P ratio of 10, LCPA can condense DNA into nanoparticles of 250–300 nm in diameter. Gel retardation analysis and ethidium bromide exclusion assay have shown that LCPA has a high bind affinity towards plasmid DNA. Thus under relatively high N/P, more LCPA molecules could bind to DNA without an increase of polyplex size. Indeed, the particles with a size of 100–200 nm are prone to endocytosis. However, a larger size of particles is favorable to cell attachment and subsequent cell uptake by increased sedimentation of complexes.[26] Meanwhile, larger nanoparticles might promote the escape of gene from the polyamine/DNA complexes into the nucleus.[27]

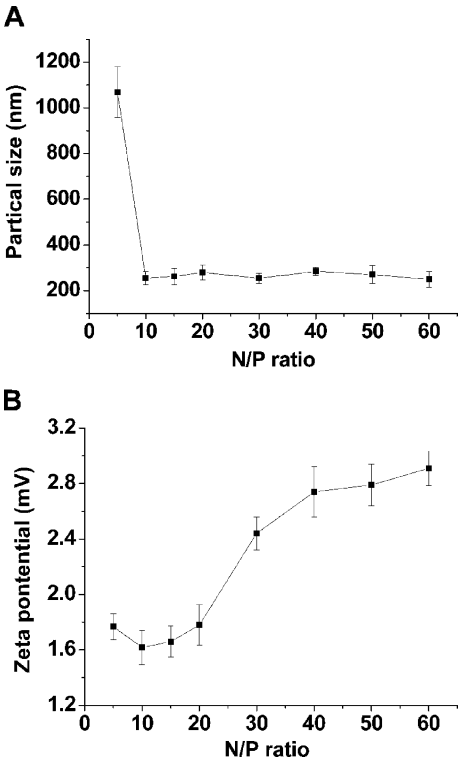

Fig. 4 Average particle size (A) and zeta-potential (B) of LCPA/pDNA at N/P ratios of 5, 10, 15, 20, 30, 40, 50 and 60.

Zeta-potential is an indicator of surface charges on the polymer/pDNA nanoparticles. A positively charged surface allows electrostatic interaction with anionic cell surfaces and facilitates cellular uptake.[14a,27] The zeta-potential values of LCPA/DNA complexes were also measured at N/P ratios ranging from 5 to 60 (Fig. 4B). The net surface charge of the LCPA/DNA complexes stabilized at the N/P range of 5–20, and increased at the N/P ratio of 20 and above. This zeta-potential range of 1.6–3.0 mV is much lower than that of most reported polycation/DNA complexes (about 20 mV) and is comparable to the results of PEG-PEI copolymers which act as effective gene carriers.[28] This relatively low zeta-potential might be attributed to the repeated hydroxyl groups and ether bonds which can screen the positive charge in the structure of LCPA. Moreover, the compartmentation of cationic cyclen moieties by the bis(β-hydroxylethyl)amine-epichlorohydrin bridges also decreased the density of amino groups. On the other hand, the increase of zeta potential under higher N/P might be owing to the larger number of LCPA molecules binding to DNA. It is known that polyamines with high positive surface charge may cause high cytotoxicity and decreased transfection efficiency. Thus, we hope that the low positive charge of LCPA may decrease the cytotoxicity and benefit gene transfection.

Cytotoxicity

The cytotoxicity of cationic polymers is thought to be caused by damage from the interaction with plasma membrane or other cellular compartments, and researches have found a rough correlation between toxicity and transfection efficiency.[29] The cytotoxicity of LCPA was evaluated in A549 and 293 cells by MTT assay, and the 25 kDa PEI was used as the control. As

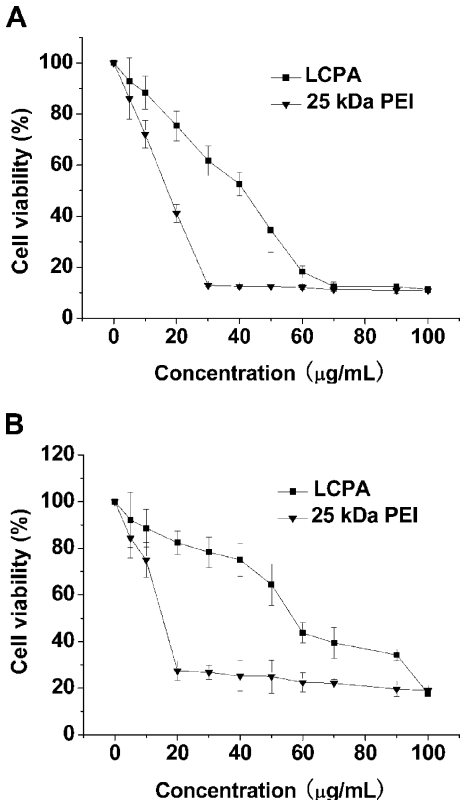

Fig. 5 Relative cell viabilities of LCPA and 25 kDa PEI in (A) A549 cells and (B) 293 cells.

shown in Fig. 5, 25 kDa PEI displayed serious cytotoxicity in two cell lines and the relative cell viability of PEI were less than 20% when its concentration was over 30 μg mL^{-1}. In comparison with 25 kDa PEI, LCPA indicated relatively high cell viabilities at 30 μg mL^{-1} concentration (65% and 80% relative cell viability for A549 and 293 cells, respectively). The much lower cytotoxicity of LCPA may be ascribed to the low positive charge, which is caused by the relatively lower density of amino groups, on the surface of the complexes. Additionally, cell-dependent cytotoxicities were found for LCPA, and A549 cells showed a relatively severe weakness against the cytotoxicity of both PEI and LCPA (Fig. 5A).

In vitro transfection

The gene transfection efficiency of LCPA/DNA complexes was assessed by *in vitro* delivery experiments of luciferase reporter gene (plasmid pGL-3) into A549 and 293 cells. 25 kDa PEI was used for comparison because of its high transfection efficiency and easiness to get. PEI/DNA polyplexes were prepared at an N/P ratio of 10, and LCPA/DNA complexes were prepared at various N/P ratios ranging from 5 to 40. Fig. 6 showed the effect of different N/P ratios on transfection efficiency. The transfection efficiency of LCPA/DNA complexes increased with the increase of N/P ratio, and the maximal transfection efficiency was observed at N/P ratio of 15 in both of the two cell lines, while the optimal N/P ratio for PEI is 10 (only the transfection data of PEI under N/P of 10 are shown). A further increase of N/P ratio might lead to higher cytotoxicity and decreased transfection efficiency. The results showed that the transfection efficiency of the PEI/DNA

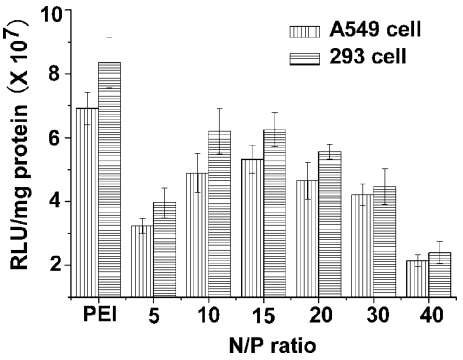

Fig. 6 Luciferase expression in A549 and 293 cells transfected by 25 kDa PEI/DNA (N/P = 10) and LCPA/DNA complexes at different N/P ratios. For comparison with Fig. 5, the concentrations of LCPA in the transfection experiments were 7.5, 15, 22.5, 30, 45, 60 μg mL^{-1} for the N/P ratio of 5, 10, 15, 20, 30, 40, respectively.

complexes is about 1.3 times better than that of LCPA/DNA complexes. In addition, comparing the two types of cells, the transfection efficiency in 293 cells is higher than those in A549 cells within the range of N/P ratios tested. This difference may be attributed to the lower cytotoxicity of LCPA in 293 cells than that in A549 cells.

Results showed that LCPA can act as an effective non-viral gene vector with relatively low cytotoxicity and comparable transfection efficiency to that of 25 kDa PEI. In to the chemical structure of LCPA, the unique macrocyclic system affords four amino groups with different pK_a values (>9 for two amino groups and <5 for other two) for each cyclen moiety.[30] These values give a relative measure of the basicity of the amino groups. The ones with higher pK_a would help DNA binding and the formation of polyplex, while the other amino groups with lower pK_a are expected to remain unprotonated in the polyplex, presumably due to the lower protonation power and the steric restriction, directing to the enhanced buffering capacity in the endosomal compartment.[31] To directly visualize the infected cells expressing pEGFP-Nl, enhanced green fluorescent protein expression in A549 and 293 cells was observed by an inverted fluorescent microscope. According to the results of the luciferase assay, LCPA/DNA and PEI/DNA (control) complexes were used at the optimal N/P ratios of 15 and 10, respectively. The results were in accordance with the luciferase expression in the same two cell lines. As shown in Fig. 7, the images also indicated that the density of transfected cells by LCPA/DNA is close to that caused by 25 kDa PEI/DNA complexes in both cell lines. For the N/P ratios of LCPA/DNA complexes in the range of 10 to 40, good transfection with low cytotoxicity were observed in both 293 and A549 cell lines. Further increase (>40) or decrease (<10) of the N/P ratio led to much lower transfection efficiency, only a few cells that expressed GFP were observed by microscopy (data not shown). Similar results were obtained in luciferase expression experiments. This low transfection efficiency was attributed to the liability to degradation under low N/P and the difficulty to release DNA under high N/P. The transfection efficiency of LCPA/DNA complexes towards 293 cells is higher than that towards A549 cells, which was consistent with luciferase experiments.

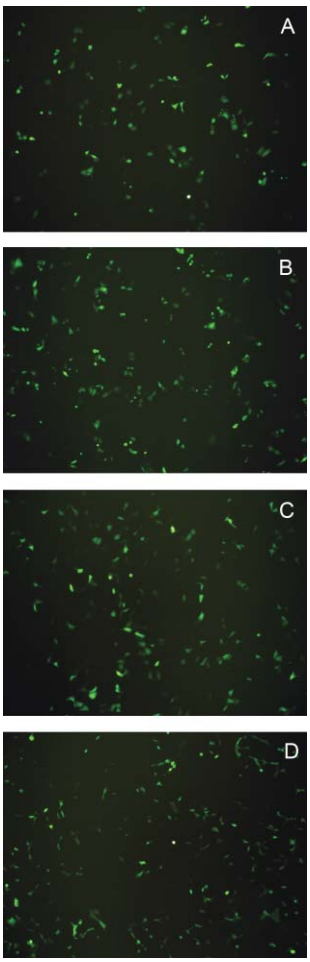

Fig. 7 Fluorescent microscope images of pEGFP-transfected cells: (A) LCPA in A549 at N/P = 15, (B) 25 kDa PEI in A549 at N/P = 10, (C) LCPA in 293 at N/P = 15, (D) 25 kDa PEI in 293 at N/P = 10. These 4 cm-wide images were obtained after 68-fold reduction from original pictures, which were recorded at the magnification of 100×.

Conclusions

A new type of polycation, linear cyclen-based polyamine (LCPA) was designed and synthesized from 1,7-diprotected 1,4,7,10-tetraazacyclododecane (cyclen), bis(β-hydroxylethyl)amine and epichlorohydrin. This new polymeric material showed enough ability to condense DNA into nanoparticles with lower cytotoxicity compared with 25 kDa PEI for its relatively low charge density. *In vitro* transfections of reporter genes of luciferase and enhanced green fluorescent protein against A549 and 293 cell lines suggested that the transfection efficiency of LCPA/DNA complexes is close to that of 25 kDa PEI/DNA complexes, indicating that the new type of polyamine could be a promising non-viral polycationic reagent for gene delivery. As a new type of polyamine which is easily modified, some shorter or N-rich bridge groups can be introduced to increase the charge density of LCPA. Considering the possibility of increased cytotoxicity induced by the increase of charge density, some bio-degradable groups such as ester and disulfide bridges can be constructed. Relative studies that focus on the elucidation of the structure–efficiency relationship of such kinds of polycations are now in progress.

Experimental procedures

Chemicals and instruments

All chemicals and reagents were obtained commercially and were used as received. Anhydrous ethanol (CH_3CH_2OH), anhydrous dichloromethane, triethylamine (NEt_3) and epichlorohydrin were dried and purified under nitrogen by using standard methods and were distilled immediately before use. 1,7-Bis(*tert*-butyloxycarbonyl)-1,4,7,10-tetraazacyclodocane was prepared according to the literature.[32] High molecular weight PEI (branched, average molecular weight 25 kDa: 25 kDa PEI) and MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The plasmids used in the study were pGL-3 (Promega, Madison, WI, USA) coding for luciferase DNA and pEGFP-N1 (Clontech, Palo Alto, CA, USA) coding for EGFP DNA. The Dulbecco's Modified Eagle's Medium (DMEM), 1640 Medium 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).

MS-ESI spectra data were recorded on a Finnigan LCQ^{DECA} and a Bruker Daltonics BioTOF mass spectrometer respectively. The ^{1}H NMR spectra were obtained on a Varian INOVA-400 spectrometer. $CDCl_3$ was used as solvent and TMS as the internal reference. IR spectra were measured with a Shimadzu FTIR-4200 spectrometer. Fluorescence spectra were measured by a Horiba Jobin Yvon Fluoromax-4 spectrofluorometer. The molecular weight of polyamine was determined by size-exclusion chromatography (SEC) in combination with multiple angle laser light scattering (Wyatt Technology Corporation), incorporating Shodex columns (OHPAK KB-803).

Preparation of title polyamine

***N*-*tert*-Butyloxycarbonyl-bis(β-hydroxylethyl)amine (1).** Bis-(β-hydroxylethyl)amine (1.05 g, 0.01 mol) was dissolved in $CHCl_3$, triethylamine was added to adjust the pH to 9. At room temperature, a solution of $(Boc)_2O$ (2.38 g, 0.011 mol) in $CHCl_3$ was added dropwise to the above solution. After stirring overnight at room temperature, the reaction mixture was condensed under reduced pressure. The residue was purified by alumina gel column chromatography (v/v 9 : 1, CH_2Cl_2–MeOH) to give compound **1** as colorless oil. Yield: 82%. IR (CH_2Cl_2, cm^{-1}): 3389, 2971, 2931, 2877, 1669, 1475, 1412, 1367, 1234, 1170, 1077, 880, 775. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.25 (br, 1H, OH), 4.11 (br, 1H, OH), 3.78 (t, J = 4.4 Hz, 4H, CH_2O), 3.42 (t, J = 4.8 Hz, 4H, NCH_2), 1.46 (s, 9H, Boc). ^{13}C NMR (400 MHz, $CDCl_3$): δ 156.42, 80.31, 61.80, 52.24, 28.40. MS (ESI): m/z = 228.1 $[M + Na]^+$.

***N*-*tert*-Butyloxycarbonyl-bis(2-(oxiran-2-ylmethoxy)ethyl)amine (2).** A mixture of epichlorohydrin (2.1 mL, 26.3 mmol), sodium hydroxide pellets (1 g, 26.3 mmol), water (0.12 mL, 6.6 mmol), tetrabutylammonium bromide (71 mg, 0.22 mmol) and compound **1** (0.9 g, 4.4 mmol) was stirred for 4 h at 40 °C. The reaction mixture was filtered off and the solid was washed with dichloromethane. The combined organic layer was

dried with anhydrous magnesium sulfate. The solvent and excess epichlorohydrins were distilled off under reduced pressure and the residue was purified by silica gel column chromatography (v/v 25 : 1, CH_2Cl_2–MeOH) to give compound **2** as colorless oil. Yield: 61%. IR (CH_2Cl_2, cm^{-1}): 2968, 2926, 2863, 1690, 1462, 1410, 1366, 1250, 1151, 1111, 851, 737. ^1H NMR (400 MHz, $CDCl_3$): δ 3.75 (dd, $J = 11.2$, 2.2 Hz, 2H, CH_2O), 3.64–3.58 (m, 4H, CH_2O), 3.49–3.44 (m, 4H, CH_2O + NCH_2), 3.41–3.36 (m, 2H, NCH_2), 3.16–3.12 (m, 2H, CH), 2.79 (dd, $J = 4.8$, 4.8 Hz, 2H, ring CH_2), 2.61 (dd, $J = 4.8$, 2.8 Hz, 2H, ring CH_2), 1.45 (s, 9H, Boc). ^{13}C NMR (400 MHz, $CDCl_3$): δ 155.44, 79.69, 71.85, 69.87, 50.80, 48.01, 44.23, 28.42. MS (ESI): $m/z = 340.2$ $[M + Na]^+$.

Polymer 3. 1,7-Bis(*tert*-butyloxycarbonyl)-1,4,7,10-tetraazacyclododecane (1.29 g, 3.47 mmol) was dissolved in C_2H_5OH, then compound **2** (1.1 g, 3.47 mmol) was added to the solution. Under the protection of N_2, the reaction mixture was stirring at 80 °C for 24 h. The solvent was removed under reduced pressure. The residue was dissolved in $CHCl_3$, cyclohexane was added to precipitate polymer **3** (1.15 g). IR (CH_2Cl_2, cm^{-1}): 3422, 2974, 2929, 2867, 1691, 1461, 1412, 1366, 1248, 1159, 979, 774, 735. ^1H NMR (400 MHz, $CDCl_3$): δ 3.85 (br, 2H, CH), 3.52–2.95 (m, 24H, other Hs), 2.60–2.25 (m, 8H, cyclen CH_2 adjacent to alkyl N), 1.44–1.42 (m, 27H, Boc).

LCPA. Polymer **3** (1.1 g) was dissolved in CH_2Cl_2, and HCl gas was imported. The reaction mixture was stirring overnight at room temperature. The solvent was removed under reduced pressure, and the residue was dissolved in a small amount of water. Then 5 N NaOH aqueous solution was added to adjust pH to 12. The alkaline solution was extracted with hot $CHCl_3$. The organic layer was dried over anhydrous Na_2SO_4. After removing the solvent, LCPA was obtained as a white solid (510 mg). The molecular weight of LCPA ($M_w = 7392$, $M_w/M_n = 1.19$) was measured by size-exclusion chromatography (SEC) in combination with multiple angle laser light scattering. IR (CH_2Cl_2, cm^{-1}): 3355, 2924, 2856, 1495, 1359, 1116, 948, 732. ^1H NMR (400 MHz, $CDCl_3$): δ 3.88 (m, 2H, CH), 3.58–3.44 (m, 12H, $CH_2OCH_2CH_2NCH_2CH_2OCH_2$), 2.76–2.50 (m, 20H, other Hs).

Acid–base titration

Briefly, LCPA (0.25 mmol of amino groups) was dissolved in 5 mL of 150 mM NaCl aqueous solution, and 1 N HCl was added to adjust pH to 2.0. Aliquots (50 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, PEI (25 kDa) was used under same experimental conditions.

Cell culture

HEK (human embryonic kidney) 293 cells and human non-small-cell lung carcinoma A549 cells were incubated respectively in Dulbecco's Modified Eagle's Medium (DMEM) and 1640 Medium containing 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin-streptomycin, 10000 U mL^{-1}) at 37 °C in a humidified atmosphere containing 5% CO_2.

Amplification and purification of plasmid DNA

pGL-3 and pEGFP plasmids were used. The former one as the luciferase reporter gene was transformed in *E. coli* JM109 and the latter one as the green fluorescent protein gene was transformed in *E. coli* DH5α. Both plasmids were amplified in terrific broth media at 37 °C overnight. The plasmids were purified by an EndoFree Tiangen™ Plasmid Kit. Then the purified plasmids were dissolved in TE buffer solution and stored at –20 °C. The integrity of plasmids was confirmed by agarose gel electrophoresis. The purity and concentration of plasmids were determined by ultraviolet (UV) absorbance at 260 and 280 nm.

Agarose gel retardation assay

LCPA/DNA complexes at different N/P ratios (the amino groups of LCPA to phosphate groups of DNA) ranging from 2 to 10 were prepared by adding an appropriate volume of LCPA (in 150 mM NaCl solution) to 0.8 μL of pEGFP-N1 DNA (120 ng μL^{-1} in 40 mM Tris-HCl buffer solution). The complexes were diluted by 150 mM NaCl solution to a total volume of 6 μL, and then the complexes were incubated at 37 °C for 30 min. After that the complexes were electrophoresed on the 0.7% (W V^{-1}) agarose gel containing EB and with Tris-acetate (TAE) running buffer at 110 V for 30 min. DNA was visualized with a UV lamp using a BioRad Universal Hood II.

Ethidium bromide displacement assay

The ability of LCPA 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 mg mL^{-1}) was put into quartz cuvette containing 2.5 mL of 150 mM NaCl solution. After shaking, the fluorescence intensity of EB was measured. Then DNA (9.5 μL, 1 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 LCPA (0.55 mg mL^{-1}, 4 μL for each addition) were added to the above solution for further measurement. All the samples were excited at 497 nm and the emission was measured at 600 nm.

Particle size and zeta-potential measurements

Particle size and zeta-potential were measured by a Zeta Nano Series (Malvern Instruments Led) at 25 °C. The LCPA/DNA complexes at various N/P ratios ranging from 10 to 60 were prepared by adding an appropriate volume of LCPA solution (in 150 mM NaCl solution) to 100 μL of pEGFP-N1 DNA solution (50 μg mL^{-1} in 40 mM Tris-HCl buffer solution) with the final volume of 200 μL Then the complexes were incubated at room temperature for 30 min. After that the complexes were diluted by 150 mM NaCl solution to 1 mL prior to measurement.

Cell viability assay

Toxicity of LCPA toward 293 cells and A549 cells was determined by using MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay following literature procedures. The 293 cells (6000 cells/well) and A549 cells

(14000 cells/well) were seeded into 96-well plates. The cells were then incubated in a culture medium containing LCPA with a particular concentration for 24 h. After that, the medium was replaced with 200 μL of fresh medium, and 20 μL of sterile filtered MTT (5 mg mL^{-1}) stock solution in PBS was added to each well. After 4 h, unreacted dye was removed by aspiration. The formazan crystals were dissolved in 150 μL DMSO per well and measured spectrophotometrically in an ELISA plate reader (model 550, Bio-Rad) at a wavelength of 570 nm. The cell survival was expressed as follows: Cell viability = $(OD_{treated}/OD_{control}) \times 100\%$.

In vitro transfection

Luciferase assay. The 25 kDa PEI was used as the positive control due to its high transfection efficiency *in vitro* and *in vivo*. The plasmid pGL-3 was used as a reporter gene. Transfections of pGL-3 plasmid mediated by LCPA in 293 cells and A549 cells were studied as compared with 25 kDa PEI. 293 cells or A549 cells were seeded at a density of 6×10^4 cells/well in the 24-well plate with 0.5 mL of medium containing 10% FBS and incubated at 37 °C for 24 h. Then the complexes were prepared at N/P ratios ranging from 10 to 40 by adding 1.5 μg plasmid DNA to an appropriate volume of LCPA solution.

Before transfection, the cells were washed by serum-free medium, and then the LCPA/DNA complexes were added with serum-free medium for 4 h at 37 °C. Then the serum-free medium was replaced by flash medium containing 10% FBS, and the cells were further incubated for 24 h. After that, the medium was removed. The luciferase assay was performed according to manufacture's protocols (Promega). Relative light units (RLUs) were measured with chemiluminometer (FLOROSKAN ASCENT FL). The total protein was measured according to a BCA protein assay kit (Pierce). Luciferase activity was expressed as RLU/mg Protein. Data are shown as mean±standard deviation (SD) based on 3 independent measurements. The statistical significance between two sets of data was calculated using Student's t-test. A P value < 0.05 was considered statistically significant.

Green fluorescent protein assay. Transfections of pEGFP-N1 plasmid mediated by LCPA in 293 cells and A549 cells were also evaluated. The best N/P ratio of 15 in 293 cells and A549 cells determined from the luciferase assay were used. 293 cells and A549 cells were inoculated at a density of 2.4×10^5 and 3×10^5 cells/well in 24-well plates respectively, 24 h prior to transfection. LCPA/DNA complexes were prepared by adding an appropriate volume of LCPA solution (in 150 mM NaCl solution) to 50 μL pEGFP-N1 DNA solution (30 μg mL^{-1} in 40 mM Tris-HCl buffer solution) with the final volume of 100 μL. Then the complexes were incubated at room temperature for 30 min. The plates were washed by PBS twice, and 100 μL LCPA/DNA complexes were then added to a well with an additional 150 μL of medium without FBS. The final concentration of DNA in the complexes was calculated to be 6 μg mL^{-1}. After 4 h of incubation, the LCPA/DNA complex containing medium was replaced with 0.5 mL of fresh medium containing 10% FBS, and the cells were further incubated for 24 h at 37 °C. The cells were directly observed by an inverted microscope (Olympus IX 71). The microscopy images were obtained at the magnification of 100× and recorded using Viewfinder Lite (1.0) software.

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