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PAPER

Diol glycidyl ether-bridged cyclens: preparation and their applications in gene delivery†

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Polymeric 1,4,7,10-tetraazacyclododecanes (cyclens) using diol glycidyl ether with different chain length as bridges (**5a–e**) were designed and synthesized from various diols, 1,7-diprotected cyclen and epichlorohydrin. The molecular weights of the title polymers were measured by GPC with good polydispersity. Agarose gel retardation and fluorescent titration using ethidium bromide showed good DNA-binding ability of **5**. They could retard plasmid DNA (pDNA) at an N/P ratio of 4–6 and form polyplexes with sizes around 100–250 nm from an N/P ratio of 10 to 60 and relatively low zeta-potential values (5–22 mV). The cytotoxicity of **5** assayed by MTT is much lower than that of 20 kDa PEI. *In vitro* transfection against A549 and 293 cells showed that the transfection efficiency (TE) of **5c**/DNA polyplexes is close to that of 20 kDa PEI at an N/P ratio of 5. Structure–activity relationship (SAR) of **5** was discussed in their DNA-binding, cytotoxicity, and transfection studies. The TE of **5c**/DNA polyplexes could be improved by the introduction of 50 μM of chloroquine, the endosomolytic agents, to pretreated cells. These studies may extend the application areas of macrocyclic polyamines, especially for cyclen.

Introduction

The efficient delivery of nucleic acids into cells is critical for successful gene delivery or gene transfection.[1,2] Among the various viral and non-viral vectors, cationic polymers have been receiving more and more attention and have been considered to be the most substantial competitors to sometimes unsafe viral vectors[3] for gene therapy.[4] These materials can potentially avoid toxicity and immunogenicity,[5] efficiently condense DNA into compact particles, facilitate cellular uptake of nanosized polyplexes,[6] and achieve prolonged gene expression. Since the first performance of polycation-mediated gene delivery in 1987,[7] many polycation materials have been explored for gene transfection applications, with the most intensively studied examples polyethyleneimine (PEI),[8] chitosan,[9] polyamidoamine dendrimers (PAMAM)[10] *etc.*

Among non-viral vectors applied in gene therapy, polyethylenimine (PEI) has successfully been used both under *in vitro* and *in vivo* conditions.[11,12] PEIs are able to form non-covalent complexes with DNA and, recently, have also been reported for the trafficking of siRNA to induce RNAi because of their high cationic charge density at physiological pH.[13] It is assumed that the high gene transfection efficiency (TE) observed with PEI might be attributed its comparatively low pK_a values. The intracellular release of PEI-complexed nucleic acids from endosomes is hypothesized to rely on the protonation of amines in the PEI molecule, which has been explained by the "proton sponge effect".[14] High-molecular weight PEI has higher TE than low-molecular weight PEI, but its cytotoxicity is also higher.[15,16] For further enhancement of TE and biocompatibility, some functional moieties such as poly(ethylene glycol) (PEG),[17] cyclodextrin[18] and chitosan[19] have been introduced into PEI. Although several copolymers displayed lower cytotoxicities along with comparable, or even higher TEs compared with PEI, seeking other materials with novel structures is of great importance.

Our previous studies focused on the synthesis of 1,4,7,10-tetraazacyclododecane (cyclen) derivatives and their interactions with plasmid DNA.[20–22] The cyclen moiety also has repeated ethylenediamine units which is similar to those in PEI. Further, like branched PEI, the four N atoms on the cyclen cycle also show different properties, especially pK_a. Thus, for the easily modification of cyclen derivatives, we had hoped to design and synthesize novel cyclen-based polymeric structures which could be used as efficient non-viral gene vectors. Some bicyclam derivatives have been used as non-viral vectors for specific gene delivery.[23] We also reported cyclen-based cationic lipids[24] or reticular polymers,[25] which could transfer plasmid DNA into cells without the use of extraneous agents. However, the TE was not satisfying. More recently, we prepared a novel linear cyclen based polyamine (LCPA), and it showed comparable TE to that of 25 kDa PEI with relatively low cytotoxicity.[26] It is of great importance to

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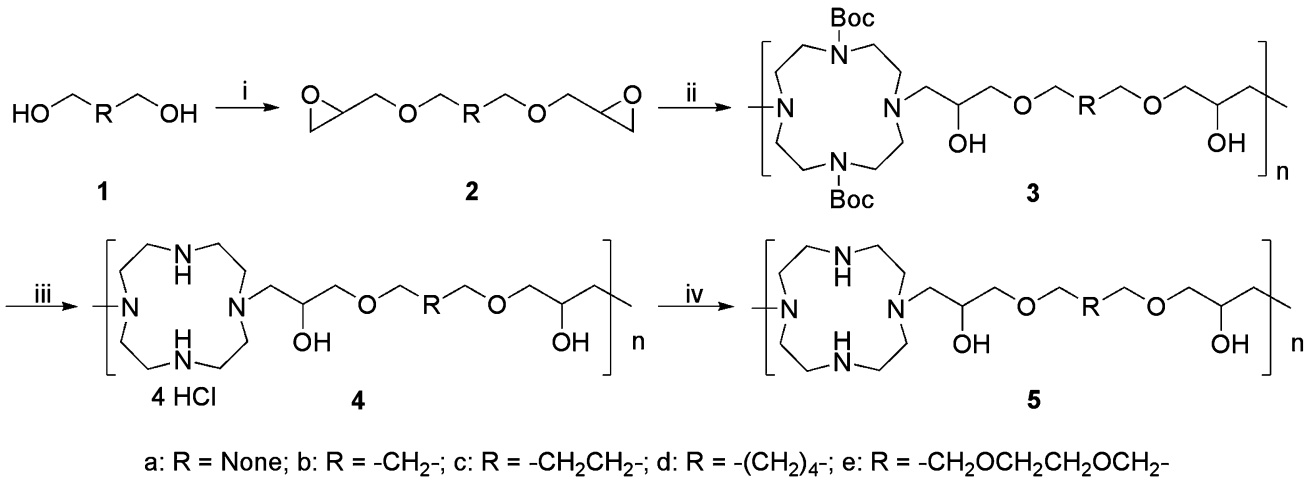

Scheme 1 Synthetic route of linear cyclen-based cationic polymers. (i) Epichlorohydrin, Bu_4NBr, NaOH, H_2O. (ii) 1,7-diBoc cyclen, EtOH, reflux, 40 h. (iii) Anhydrous HCl, CH_2Cl_2. (iv) aqueous NaOH.

further investigate the structure–activity relationship (SAR) of this type of cationic polymer. In this report, we prepared a series of cyclen-based linear polymers with different diol glycidyl ethers as a bridge. Their interactions with plasmid DNA and the properties of formed polyplexes were examined. The *in vitro* TEs towards two cell-lines were investigated to reveal the SAR of this type of cationic polymers in gene delivery.

Results and discussion

Synthesis and characterization of compounds 5

The preparation route of polymers **5** is shown in Scheme 1. Diols of various chain length reacted with epichlorohydrin in the presence of Bu_4NBr–NaOH–H_2O to give the diglycidyl ethers **2**. The protected cyclen-based polymers **3**, in which the new-formed hydroxyls and ether bonds might increase their solubility and biocompatibility, were prepared by the ring-opening polymerization between equal molar amounts of 1,7-diprotected cyclen and **2** in refluxing ethanol under nitrogen. The bulky and electron-withdrawing Boc groups on cyclen might hamper the reaction, and it needs 40 h to achieve a satisfying polymerization degree. Before deprotection, crude products of **3** were recrystallized by chloroform/cyclohexane to remove the relatively lower molecular weight portion. Anhydrous HCl gas was then used to remove Boc groups on the cyclen ring. During the deprotection process, the clear CH_2Cl_2 solution of **3** turned turbid, and the hydrochloride salt of **4** was precipitated. ^{1}H NMR (ESI†) showed the absence of a singlet at 1.42–1.44 ppm, indicating that the Boc groups were completely cleaved from the polyamine backbone. Polymers **4** were dialyzed against pure water for 2 d to ensure their polydispersity, and the molecular weights (M_w) of **5** measured by GPC are listed in Table 1. Higher M_w was obtained for **5c**, however, the M_ns of the polymers did not have large difference, suggesting that similar polymerization degrees were achieved for the five polymers. The relatively low degree of polymerization might be attributed to the two bulky and electron-withdrawing Boc groups. The ring-opening reactions using some 1,7-dialkyl substituted cyclens always lead to high polymerization degrees (unpublished results).

Table 1 Molecular weights of polymers **5** measured by GPC

Polymer	M_w	M_n	PDI	Calculated *n* value
5a	4349	3084	1.41	9
5b	3519	2607	1.35	7
5c	8416	3932	2.14	10
5d	3491	2586	1.35	7
5e	3666	2657	1.38	6

All of the target polymers could dissolve well in water under studied concentrations.

Buffer capability

Efficient gene transfer mediated by PEI is in part due to its high buffering capacity, which is responsible for the timely release of polyplexes from the endosome and the lysosome by the "proton sponge effect".[27] Acid–base titration experiments were carried out for PEI and **5** to evaluate the buffering capacity of the new polymers. As shown in Fig. 1, the buffer capabilities of **5** were slight lower than that of 20 kDa PEI. This lower capability could be ascribed to the lower molecular weight and lower density of amino groups relative to PEI. On the other hand, we speculated that the four amino groups with different pK_a values on the macrocycle would contribute to the buffer capability.[28]

Formation of 5/DNA complexes

One prerequisite for a polymeric gene carrier is the ability to condense DNA into compact structures. The formation of a polyplex can reduce the electrostatic repulsion between DNA and the cell surface and can protect DNA against enzymatic degradation by nucleases in cytoplasm or serum.[29] 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 **5**, and total DNA retardation was detected at and above N/P ratios of 4 (for **5b** and **5d**) and 6 (for **5a**, **5c** and **5e**). The results suggested that **5** can bind to DNA through electrostatic interactions between the DNA backbone and the cationic nitrogen atoms in the cyclen moiety.

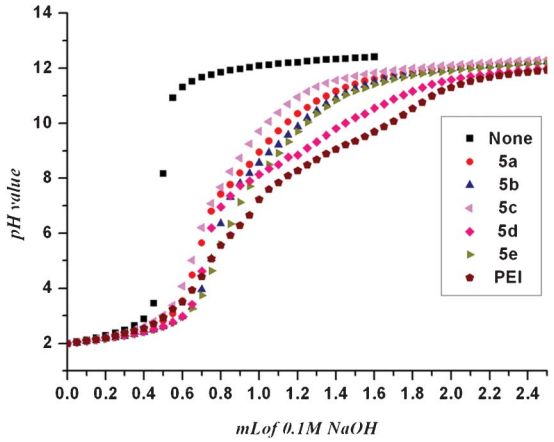

Fig. 1 Determination of the buffering capacity of PEI, water and **5** by acid–base titration. After polymer solution (0.25 mmol of amino groups) was prepared, the pH of the solution was raised to 2.0 with 1 M HCl, and then the solution pH was measured after each addition of 50 μL of 0.1 M NaOH. The pH of all solutions was measured at room temperature. The relatively flat curve of PEI indicates its higher buffer capability than those of **5**.

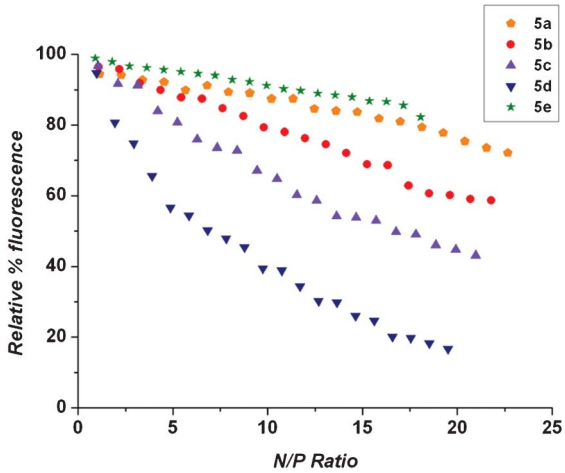

Fig. 3 Change of relative fluorescence of EB bound to DNA by the addition of **5** to different N/P ratios. All the samples were excited at 497 nm and the emission was measured at 600 nm. DNA concentration was 10 μg mL^{-1} in NaCl solution.

Ethidium bromide (EB) is a cationic dye that is widely used to probe DNA–cationic agent interactions. The ethidium ion shows a dramatic increase in fluorescence efficiency when it intercalates into DNA. The displacement of EB from DNA upon complex formation with polycations has been used widely to test the ability of polymers to bind with DNA. Fluorescence intensity may be quenched with the addition of cationic agents which can bind to DNA with a higher binding affinity to premixed DNA–EB solutions. Fig. 3 shows that the addition of **5** to EB pretreated with DNA caused appreciable decrease in the emission intensity, indicating that EB that was bound to DNA was partially replaced by **5**. The bridge length might affect the extent of fluorescence quenching, and the polymer with the longer bridge showed the better fluorescent quenching ability. For instance, although polymer **5d** has the lowest density of amino groups, it could quench the fluorescence of EB to the greatest extent. We supposed that the distance between two cationic cyclen moieties in polymer **5d** might fit that between two anionic phosphate groups in DNA, leading to more compact condensation. For polymer **5e**, the multi-ether bridge might indeed increase its biocompatibility, but at the same time, the oxygen-rich chain would harshly decrease its cationic charge density. As a result, **5e** displayed the lowest fluorescence quenching ability.

The appropriate size of the polymer/DNA nanoparticle is of critical important for polyamines as gene vectors into the target cells.[30] The desirable *in vivo* vectors should efficiently compact pDNA into small (below 200 nm) nanoparticles, which is very useful for particle stabilization, efficient endocytosis and gene transfer.[31] The particle sizes of **5**/pDNA complexes were measured at various N/P ratios, and the results are shown in Fig. 4A. As the polyamines were added, the particle sizes of the polymer/DNA complexes decreased sharply as a result of the electrostatic interaction and then hardly changed with further increase of N/P ratio. All five polymers could efficiently compact pDNA into small nanoparticles with diameters of 100–200 nm, which are prone to endocytosis. The polyplex formed from multi-ether-bridged polymer **5e** and pDNA gave the biggest size of almost all studied **5**/DNA complexes, especially under N/P ratios from 10 to 40. This could be also explained by its lower cationic charge density which led to weaker electrostatic interaction with DNA and the hydration effect due to the presence of ethylene glycol units on the bridge.

The zeta-potential could indicate the surface charges on the polymer/DNA nanoparticles. The zeta-potential of carrier/DNA complexes is closely connected with cellular uptake, the cytoplasmatic transport, and the migration through the nucleopore.[32] The zeta-potential values of **5**/DNA complexes were also measured at N/P ratios ranging from 5 to 60 (Fig. 4B). At low N/P ratio of 5–10, the zeta-potentials of the complexes increased sharply with the increase of N/P ratio. On the other hand, at higher N/P ratios of 15–60, these values appeared to reach a maximum plateau of

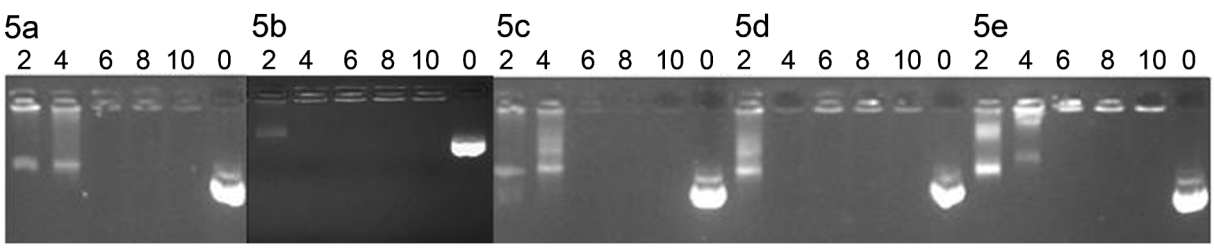

Fig. 2 Agarose gel electrophoresis retardation assays of **5**/DNA complexes. Plasmid DNA was mixed with **5** at different N/P ratios: 2, 4, 6, 8, and 10, respectively. Mark "0": pDNA control.

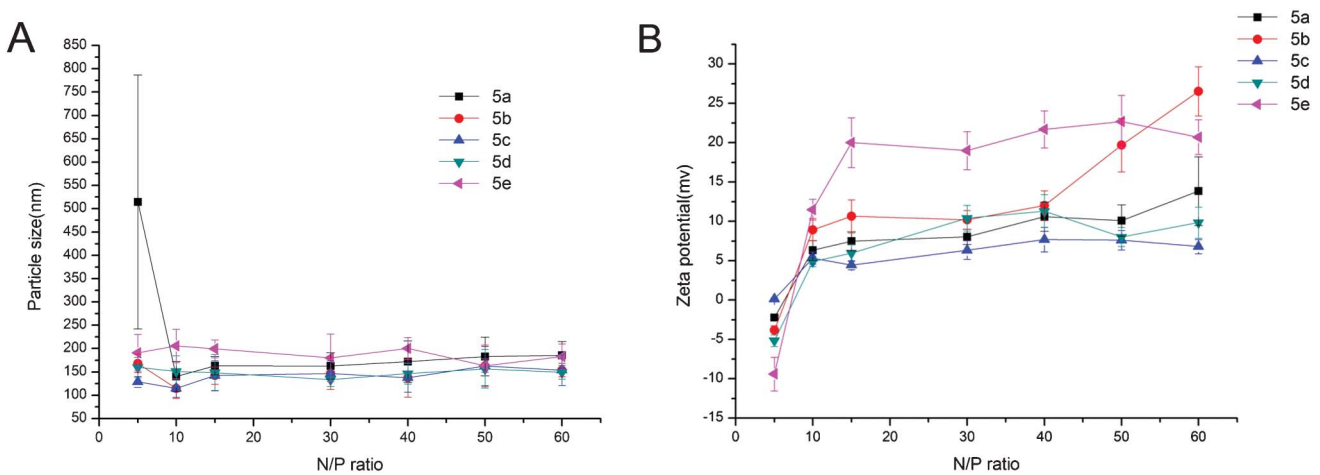

Fig. 4 Average particle size (A) and zeta-potential (B) of **5**/pDNA at N/P ratios of 5, 10, 15, 30, 40, 50 and 60.

about 5–20 mV, which was in the range of most reported bio-active polycation/DNA complexes. The bridge length seemed to have no regular influence on zeta-potentials, and **5a–d**/DNA complexes gave the values of 5–10 mV at the N/P ratios of 10–40. These values were higher than that of previously studied LCPA, which has a secondary N atom in the bridge (1.6–3.0 mV).[26] To our surprise, the zeta-potential values increased with the decrease of the amino density in the order of LCPA, **5a–d**, and **5e**. The polyplex formed from multi-ether-bridged polymer **5e** and pDNA gave the lowest zeta-potential at low N/P ratio, but the value increased to about 20 mV when the N/P increased to 15, and the value was much higher than those of the polyplexes formed from **5a–d**.

Cytotoxicity

The cytotoxicity of cationic polymers is thought to be caused by damage from the interaction with plasma membrane or other cellular compartments, and researchers have found a rough correlation between toxicity and TE. Fig. 5 shows the viability of A549 and 293 cells after incubation for 24 h with various concentrations of **5** assessed by the MTT assay, and 20 kDa PEI was used as the control. Polyamines **5** exhibited improved compatibility as compared to 20 kDa PEI, which displayed serious cytotoxicity in both of the two cell lines (less than 20% of cells survived when its concentration was up to 30 μg mL^{-1}). In comparison, **5** displayed relatively high cell viabilities at 30 μg mL^{-1} concentration (>70% cell viability for both cell lines). The reduced cytotoxicity of **5** may be ascribed to the more biocompatible hydroxyl- and ether-containing bridge. The introduction of the bridge moiety could also reduce the density of amino groups, and therefore decrease charge density. There was no distinct relationship between the bridge length (**5a–d**) and cytotoxicity, and **5b** seemed to be the most biocompatible one of the four polymers. For **5e**, the PEG-like multi-ether bridge might benefit its biocompatibility and lead to lower cytotoxicity. Additionally, cell-dependent cytotoxicities were found for **5**, and A549 cells showed a relative weakness against the cytotoxicity of the prepared materials.

In vitro transfection

The gene transfection efficiencies of **5**/DNA complexes were assessed by *in vitro* delivery experiments of luciferase reporter gene (plasmid pGL-3) into A549 and 293 cells. PEI (20 kDa) was used as the control in this work, and the PEI/DNA complex was prepared

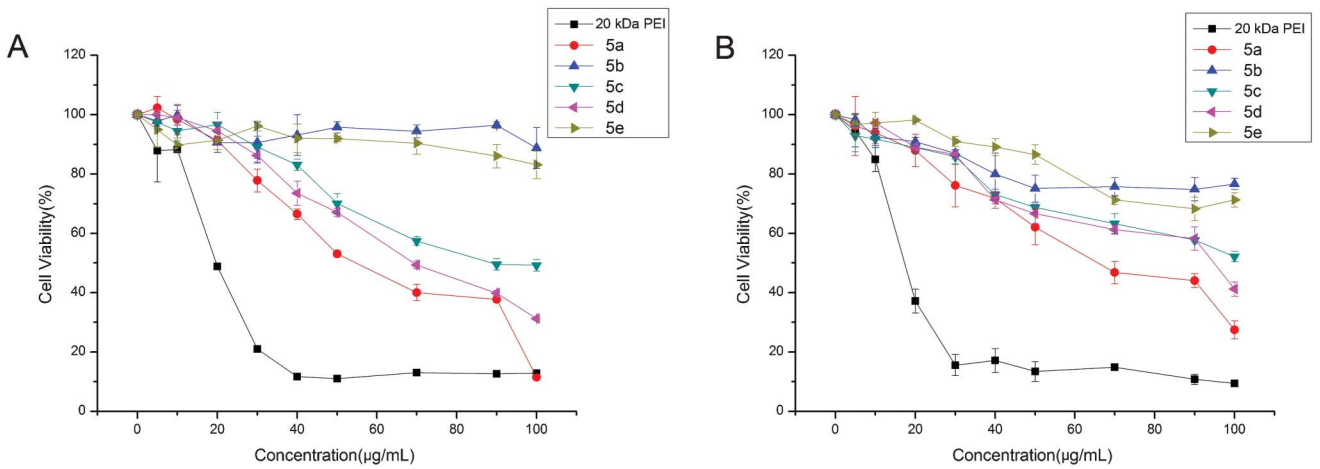

Fig. 5 Relative cell viabilities of **5** and 20 kDa PEI in (A) A549 cells and (B) 293 cell.

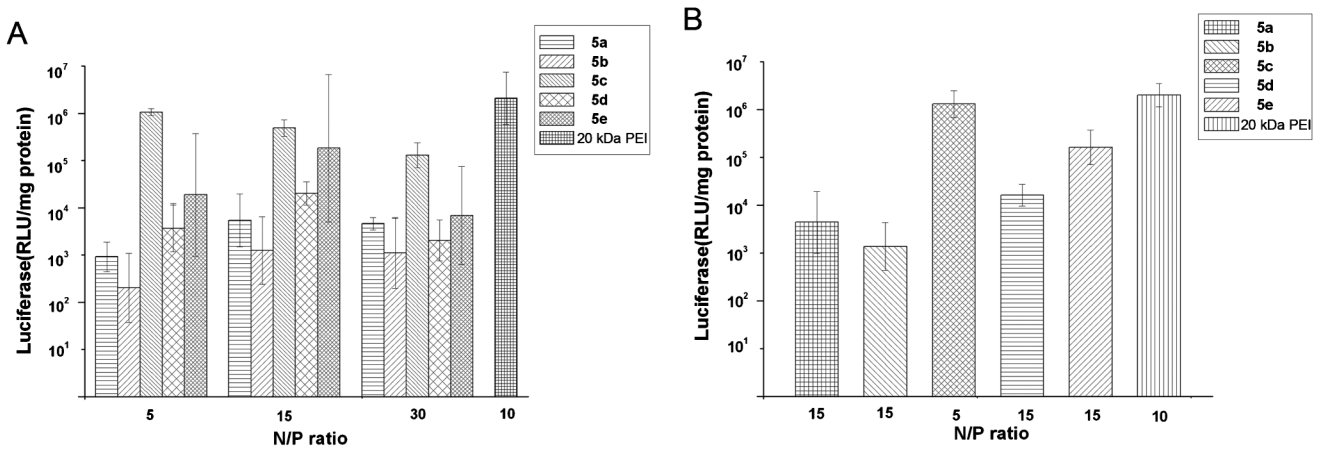

Fig. 6 (A) Luciferase expression in A549 cells transfected by 20 kDa PEI/DNA (N/P = 10) and **5** at N/P ratios 5, 15 and 30. (B) Lucifecrase expression in 293 cells transfected by 20 kDa PEI/DNA (N/P = 10) and **5** at optimal N/P ratios obtained in (A). For comparison with Fig. 5, the concentrations of **5a–e** in the transfection experiments at N/P of 5 were 4.20, 4.37, 4.55, 4.89, 5.26 μg mL^{-1}, respectively.

at an N/P ratio of 10 (optimal ratio for PEI). Fig. 6A shows the TEs of **5**/DNA complexes in A549 cells at various N/P ratios of 5, 15, and 30. Results showed that, except for **5c**, whose complex with DNA gave the best TE at the N/P of 5, all other polymer/DNA complexes gave the best TEs at N/P of 15. Further increase of the N/P ratio might lead to higher cytotoxicities and decreased TEs (data not shown). The binding affinity of the polymer towards DNA and the stability of the formed polyplexes are already well-recognized as the key factors in determining the TE.[33] Both the weak condensation ability of the cationic polymers and the excessively stable polyplex are harmful to the gene transfection. Usually, complexes which decompose too early always lead to little or no gene expression, while highly tight combination may result in slow release of DNA and therefore low TE. As different bridge lengths would affect the DNA binding ability of polymer **5**, we hope to find a balance between DNA binding ability and TE. As shown in Fig. 6A, the TE was significantly influenced by the bridge length. The highest binding ability (**5d**, Fig. 3) did not result in highest TE, however, the **5c**/DNA complex showed the best TE at the N/P of 5, which was comparable to that of PEI. As the weakest material in DNA binding among the five polymers, **5e** showed higher TE than those of **5a**, **5b** and **5d**. This might be due to the higher biocompatibility of its bridge structure and lower cytotoxicity. Similar results were obtained in the experiments involving 293 cells (Fig. 6B), in which the N/P ratios were used according to the best value in Fig. 6A.

The unique structure of cyclen affords four nitrogen atoms with different pK_a values.[34] We had hoped that the two with higher pK_a would help DNA binding and formation of polyplexes, while the other two with lower pK_a would remain unprotonated in the polyplex, directing the enhanced buffering capacity in the endosomal compartment. However, the basicity of the latter two N atoms is too weak (pK_a < 2) to be protonated in the endosomal environment (pH 5.0–6.2), thus will not benefit the buffer capability and subsequent transfection. The well-known lysosomotropic reagent chloroquine (CQ) was tested for the purpose of promoting the TE of **5c**. CQ is a weak base that is known to buffer the endosome and is often used to enhance the transfection efficiency of some cationic polycation/DNA complexes.[35,36] In quite a few literatures using CQ to improve the transfection, 100 μM was a generally used concentration, indicating that a certain concentration (could not be too low) of CQ was needed for the improvement. Indeed, as shown in Fig. 7, lower concentrations of CQ (12.5 and 25 μM) significantly decreased the TE. We speculated that under lower concentrations, CQ could not exert its buffer capacity smoothly, while on the contrary, its toxicity negatively affected the transfection. It was found that 50 μM of CQ seemed to be the optimal concentration, and almost double TE was obtained in the transfection using this dosage of CQ. Further increase of the CQ concentration led to a harsh decrease of TE and mass cell death due to its high cytotoxicity. In summary, as the buffer reagent CQ could notably promote the TE of these polymers, we believed that the TE of the linear cyclen-based polymer could be enhanced by proper modifications towards their buffer capability.

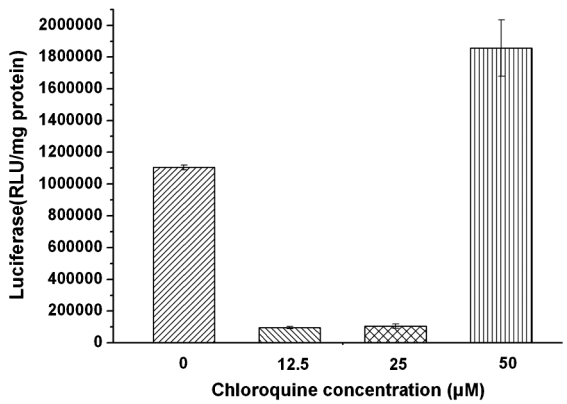

Fig. 7 Luciferase expression in A549 cells transfected by **5c**/DNA complex at N/P ratios 5 in the presence of 0, 12.5, 25, 50 μM of chloroquine.

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. The N/P ratios were used according to the optimal results of the luciferase assay, and the pictures are shown in Fig. 8. These 4 cm wide images

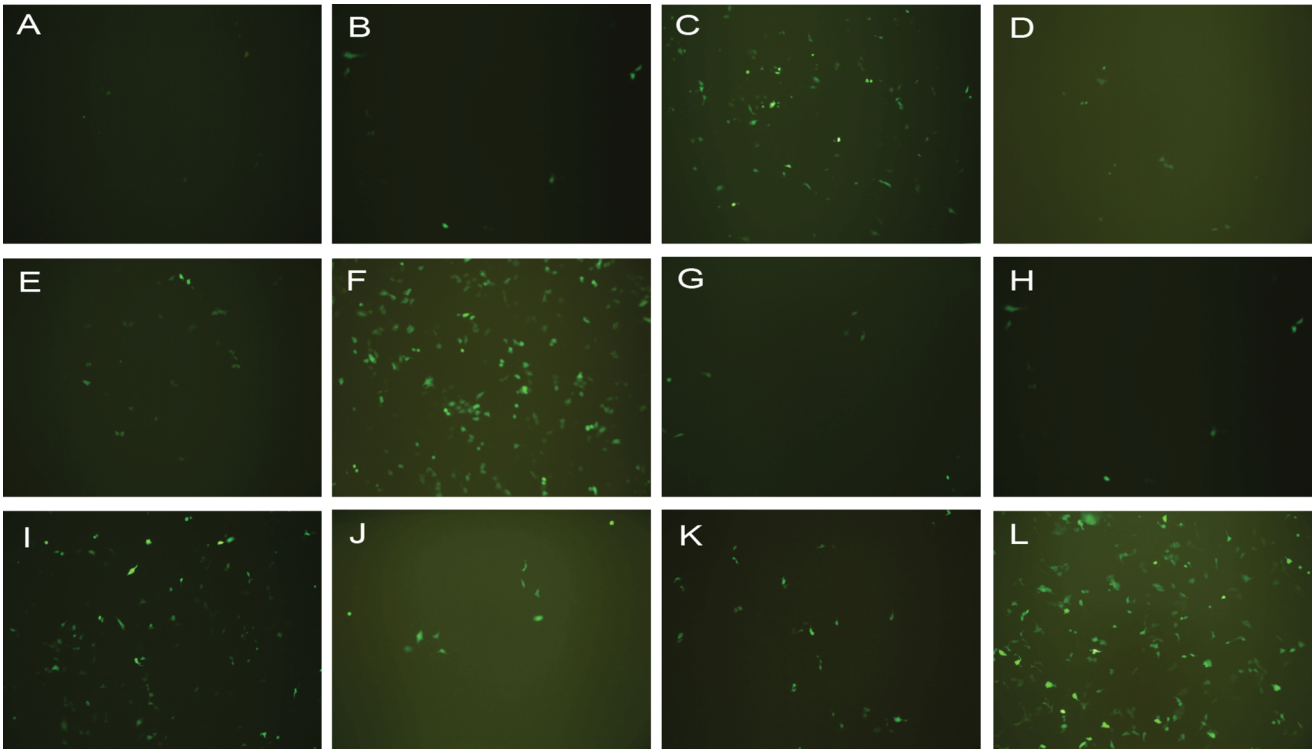

Fig. 8 Fluorescent microscope images of pEGFP-transfected cells: A–F: A549 cells; G–L: 293 cells. A and G: **5a** at N/P of 15; B and H: **5b** at N/P of 15; C and I: **5c** at N/P of 5; D and J: **5d** at N/P of 15; E and K: **5e** at N/P of 15, F and L: 20 kDa PEI at N/P of 10.

were obtained after 68-fold reduction from original pictures, which were recorded at the magnification of 100×. Polymer **5c** gave the strongest fluorescence among the five polymers (Fig. 8C, 8I), nevertheless the density of transfected cells was less than that observed in the experiment using PEI/DNA complex (Fig. 8F, 8L). The TEs according to the density of transfected cells gave the order of **5c** > **5e** > **5d** > **5a–b**, which was in accordance with the results in luciferase assays processed in the same two cell lines.

Conclusions

A new type of cationic polymer, linear cyclen-based polyamines bridged by diol glycidyl ethers with different chain lengths were designed and synthesized from 1,7-diprotected 1,4,7,10-tetraazacyclododecane (cyclen), diols and epichlorohydrin. These new polymeric materials could efficiently bind to plasmid DNA at low N/P ratios and condense DNA to nanoparticles with proper sizes and zeta-potentials. The cytotoxicity of these polymers assayed by MTT is much lower than that of 20 kDa PEI. *In vitro* transfections of reporter genes of luciferase and enhanced green fluorenscent protein against A549 and 293 cell lines suggested that higher binding ability might not result in higher TE, however, the **5c**/DNA complex showed the best TE at the N/P of 5. 50 μM of chloroquine was found to benefit the transfection, and almost double TE was obtained compared to the experiment without the use of CQ. According to these results combined with our previous studies, we considered that after proper structure modifications, this new type of marcrocyclic polyamine-based polymeric material would become a promising non-viral polycationic reagent for gene delivery. These studies may extend the application areas of macrocyclic polyamines, especially for cyclen, and related studies that focus on the purposeful modification of such a kind of polycations are now in progress.

Experimental section

General methods and materials

All chemicals and reagents were obtained commercially and were used as received. Anhydrous ethanol (EtOH), anhydrous dichloromethane, triethylamine (Et_3N) 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-tetraazacyclododecane was prepared according to the literature.[37] High molecular weight PEI (branched, average molecular weight 20 kDa: 20 kDa PEI), chloroquine diphosphate ($C_{18}H_{26}ClN_3 \cdot 2H_3PO_4$, M_w = 515.87) 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, pEGFP-N1 (Clontech, Palo Alto, CA, USA) coding for EGFP DNA and calf thymus DNA (CT DNA) (Sigma Aldrich). 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). The luciferase assay kit was purchased from Promega (Madison, WI, USA). An endotoxin-free plasmid purification kit was purchased from TIANGEN (Beijing, China).

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. Fluorescence spectra were measured by a Horiba Jobin Yvon Fluoromax-4 spectrofluorometer. The molecular weight of polyamine was determined by gel permeation chromatography (GPC) (Waters 515 pump, Waters 2410 Refractive Index Detector (25 °C), incorporating Shodex columns OHPAK KB-803). A filtered mixture of 0.2 mol L^{-1} HAc/NaAc buffer which contained 20% CH_3CN (volume ratio) was used as mobile phase with a flow rate of 0.5 mL min^{-1}. Molecular weight analyses were calculated against poly(ethylene glycol) standards of number average molecular weights ranging from 200 to 80 000.

Preparation of diglycidyl ether intermediates 2

Compounds **2** was prepared according to the literature.[38] In a typical procedure, a mixture of epichlorohydrin (9.34 mL, 0.12 mol), glycol (0.02 mol) sodium hydroxide pellets (4.8 g, 0.12 mol), water (0.5 mL, 0.028 mol), and tetrabutylammonium chloride (0.3224 g, 0.001 mol) was vigorously stirred for 2 h at 40 °C. The solid produced in the reaction was filtered off and washed with dichloromethane. The combined organic layer was dried with anhydrous magnesium sulfate. The solvent and excess epichlorohydrin were distilled off to give a yellow oil. The residue was purified by silica gel column chromatography to give the product **2**. The analytical data of compounds **2a–2c** were identical with those in our previous reports.[38] New compounds **2d** and **2e** are described as follows:

1,6-Hexanediol diglycidyl ether **2d**. Colorless liquid, yield: 68.2%. Eluent for silica gel column chromatography: n-hexane/ethyl acetate = 10 : 1 (v/v). IR (neat): ν (cm^{-1}) 3055, 2995, 2933, 2756, 1635, 1459, 1391, 1340, 1253, 1107, 909, 851, 761. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 3.72-3.68 (m, 2H, CHCH_2O), 3.53–3.47 (m, 4H, CH_2CH_2O), 3.45–3.43 (m, 2H, CHCH_2O), 3.72–3.43 (m, 2H, CH), 2.80–2.78 (m, 2H, ring CH_2), 2.61–2.59 (m, 2H, ring CH_2), 1.59–1.57 (d, 4H, $OCH_2CH_2CH_2$), 1.38–1.35 (m, 4H, $OCH_2CH_2CH_2CH_2$). ^{13}C NMR (400 MHz, $CDCl_3$): δ (ppm) 71.30, 71.22, 50.61, 43.89, 29.45, 25.74. MS (ESI): m/z = 253.12 ([M + Na]$^+$, 100).

Triethylene glycol diglycidyl ether **2e**. Colorless liquid, yield: 41.4%. Eluent for silica gel column chromatography: n-hexane/ethyl acetate = 4 : 1 (v/v). IR (neat): ν (cm^{-1}) 3055, 2997, 2872, 1637, 1461, 1349, 1253, 1105, 912, 854, 759. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 3.78 (dd, J = 11.6, 2.8 Hz, 2H, CHCH_2O), 3.71–3.65 (m, 12H, OCH_2CH_2O), 3.42 (dd, J = 11.6, 6 Hz, 2H, CHCH_2O), 3.17–3.13 (m, 2H, CH), 2.78 (dd, J = 4.8, 4.8 Hz, 2H, ring CH_2), 2.60 (dd, J = 2.4, 4.8 Hz, 2H, ring CH_2). ^{13}C NMR (400 MHz, $CDCl_3$): δ (ppm) 71.74, 70.43, 70.32, 70.30, 50.48, 43.78. MS (ESI): m/z = 285.11 ([M + Na]$^+$, 100).

Preparation of linear cyclen-based polymer 5

1,7-Bis(*tert*-butyloxycarbonyl)-1,4,7,10-tetraazacyclodocane (0.75 g, 2 mmol) was dissolved in 1 mL of C_2H_5OH, then compound **2** (2 mmol) was added to the solution. Under the protection of N_2, the reaction mixture was stirred at 80 °C for 40 h. The solvent was removed under reduced pressure to give polymer **3**, which was then recrystallized by chloroform/cyclohexane. Polymer **3** was dissolved in CH_2Cl_2, and HCl gas was imported to the solution. The reaction mixture was stirred overnight at room temperature, and white solid precipitated. The solvent was then removed under reduced pressure to produce the hydrochloride product **4**. After dialysis (MWCO 3.5 kDa) against pure water for 2 d, the water solution of **4** was treated with 2 M NaOH aqueous solution to adjust the pH to 12. The alkaline solution was extracted with hot $CHCl_3$. The organic layer was dried over anhydrous Na_2SO_4. The final products **5** were obtained after removing the solvent under reduced pressure. These polymers were characterized by IR, ^{1}H NMR and GPC (see the ESI for the spectra†).

Acid–base titration

Polymer **5** (0.25 mmol of amino groups) was dissolved in 5 mL of 150 mM NaCl aqueous solution, and 1 M HCl was added to adjust the 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 (20 kDa) and a blank were used under same experimental conditions.

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

5/DNA complexes at different N/P ratios (the amino groups of **5** to phosphate groups of DNA) ranging from 2 to 10 were prepared by adding an appropriate volume of **5** (in 150 mM NaCl solution) to 0.8 mL of pEGFP-N1 DNA (120 ng mL^{-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) 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 **5** 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 CT DNA (4.08 μL, 6.24 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 **5** (1 mg mL^{-1}, 2.5 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 **5**/DNA complexes at various N/P ratios ranging from 10 to 60 were prepared by adding an appropriate volume of **5** solution (in 150 mM NaCl solution) to 100 mL 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 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.

Cell viability assay

Toxicity of **5** toward 293 cells and A549 cells was determined by using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide) reduction assay following literature procedures. The 293 cells (6000 cells/well) and A549 cells (14000 cells/well) were seeded into 96-well plates. After being incubated for 24 h, the culture medium was replaced with the medium containing **5** with a particular concentration, and the cells were incubated for another 24 h. After that, the medium was replaced again 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, BioRad) 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 20 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 **5** in 293 cells and A549 cells were studied as compared with 20 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 5 to 30 by adding 1.5 μg plasmid DNA to an appropriate volume of **5** solution. Before transfection, the cells were washed by serum-free medium, and then the **5**/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 the manufacturer's protocols (Promega). Relative light units (RLUs) were measured with a chemiluminometer (FLOROSKANASCENT 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 the Student's t-test. A P value < 0.05 was considered statistically significant.

In the transfection of plasmid pGL-3 involving chloroquine, A549 cells were seeded at 7.5×10^4 cells/well in the 24-well plate with 0.5 mL of medium containing 10% FBS and incubated at 37 °C for 24 h. **5c**/DNA (N/P ratio = 5) complexes with different concentration of chloroquine were prepared by adding appropriate amount chloroquine to the **5c**/DNA complex solution.

Green fluorescent protein assay. Transfections of pEGFP-N1 plasmid mediated by **5** in 293 cells and A549 cells were also evaluated. The best N/P ratio of each polymer in 293 cells and A549 cells determined from the luciferase assay was 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. **5**/DNA complexes were prepared by adding an appropriate volume of **5** 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 of **5**/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 **5**/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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