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Ring-Opening Polymerization for Hyperbranched Polycationic Gene Delivery Vectors with Excellent Serum Tolerance

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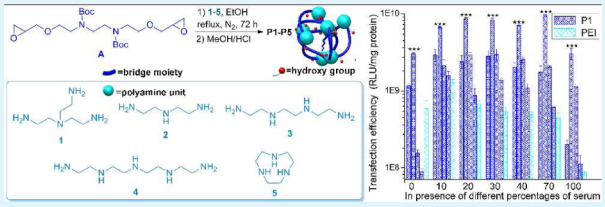
Supporting Information

ABSTRACT: In order to improve the transfection efficiency (TE) and biocompatibility, we synthesized a series of hyperbranched cationic polymers by ring-opening polymerization between diepoxide and several polyamines. These materials can condense plasmid DNA efficiently into nanoparticles that have much lower cytotoxicity than those derived from bPEI. In vitro transfection experiments showed that polymers prepared from branched or cyclic polyamine (**P1** and **P5**) exhibited TE several times higher than 25KDa bPEI. More significantly, serum seemed to have no negative effect on **P1**−**P5** mediated transfection. On the contrary, the TE of **P1** improved, even when the serum concentration reached 70%. Several assays demonstrated the excellent serum tolerance of such polycationic vectors: bovine serum albumin (BSA) adsorption assay revealed considerably lower protein adsorption of **P1**−**P5** than PEI; **P1** showed better DNA protection ability from degradation by DNase I than PEI; flow cytometry results suggested that any concentration of serum may not decrease the cellular uptake of **P1**/DNA polyplex; and confocal laser scanning microscopy also found that serum has little effect on the transfection. By using specific cellular uptake inhibitors, we found that the polyplexes enter the cells mainly via caveolae and microtubule-mediated pathways. We believe that this ring-opening polymerization may be an effective synthetic approach toward gene delivery materials with high biological activity.



KEYWORDS: *nonviral gene vector, serum tolerance, cationic polymer, ring-opening polymerization, cellular uptake*

1. INTRODUCTION

Gene therapy has offered promise for treatment of genetic disorders and cancer of both innate and acquired origin, and therefore, it has attracted great interest in both basic research and clinical trials.[1−5] A main challenge in gene therapy is the efficient in vivo transfer of genetic materials to targeted tissues. Unfortunately, the gene vector with high efficiency and low toxicity, which meet the clinical needs, is currently not available.[6,7] Generally, gene vectors can be divided into viral and nonviral ones. As a main type of nonviral vectors, cationic polymers have gained a great deal of attention because of their obvious advantages, including easy availability (even at large scales), higher gene carrying capacity, enabling repeated administration, and lower host immunogenicity. However, the transfection efficiencies (TE) of nonviral vectors are generally much lower than the viral ones.[8−11] Previous studies found that the TE is closely related to the polycations' features including their molecular weight, topological structure, charge density, nitrogen profile, and protonation range.[12−14] The clarification of the relationship between the structure of vectors and their biological performance remains a challenge for gene therapy.

Besides high TE, low cytotoxicity and good serum tolerance are also necessary for an excellent gene carrier. Among the cationic polymers explored, branched polyethylenimine (bPEI, 25 kDa) is certainly the most studied vectors. Its unique characteristics including high condensation capability toward DNA, strong buffering capacity in the pH range of 7.4−5.1, and relatively high TE made it to be the gold standard for newly designed nonviral vectors, especially polymeric ones. However, the high charge density of bPEI contributes significantly to its evident cytotoxicity due to the interaction with negatively charged cellular membranes, and their TE under serum condition is not satisfying due to their nonspecific interactions with blood components.[15] These defects limited its in vivo application. Previous studies found that the introduction of hydroxyl groups on the cationic polymers can effectively increase TE in the presence of serum and reduce the cytotoxicity.[16,17] On the other hand, it was also confirmed that hyperbranched polycations might have higher TE than linear ones, such as the branched PDMAEMA or brushed PDMAEMA.[18−24] Yamagata et al. compared the dendritic and linear polylysine of similar molecular weight and found that the

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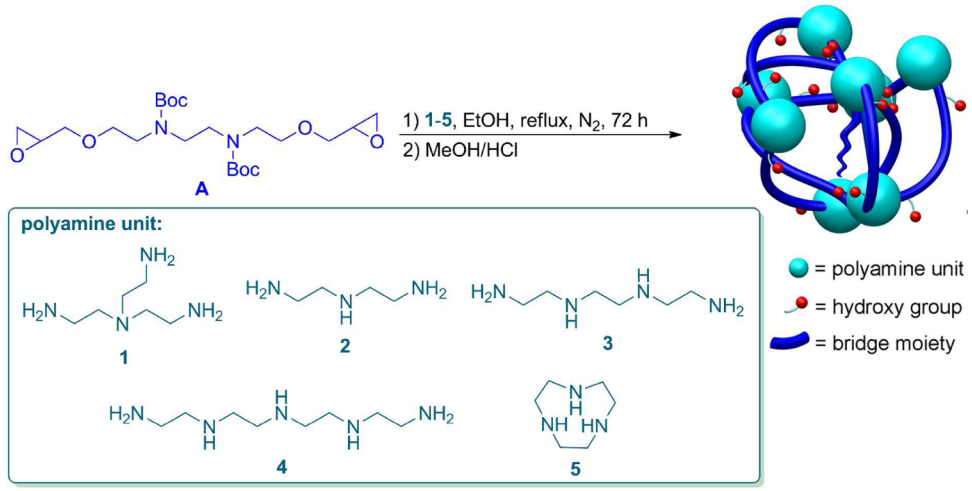
Scheme 1. Schematic Illustration for the Preparation of Hyperbranched Cationic Polymers P1−P5



TE mediated by the dendritic polylysine was 100-fold higher than that of the linear analog.[25] Compared to linear polymeric carriers, the highly branched cationic polymers have compact and globular structures and a great number of various types of amino groups.[26] These structural characteristics may allow them to effectively condense negatively charged DNA into nanosized particles and provide the polymer with appropriate buffering capacities, thereby leading to improved cellular uptake, easier endosome escape, and usually higher TE.

Taking into account both the advantages of hydroxyl groups and the hyperbranched structure, we prepared a series of hyperbranched cationic polymers through the epoxide ring-opening polymerization, in which nonionic hydrophilic hydroxyl groups were introduced.[16,27,28] These compounds were designed to improve cell viability, serum-tolerance, and TE. The interactions between these hyperbranched polymeric materials and DNA were studied in this report, and results revealed that these nonviral vectors have excellent serum-tolerance and much higher TE compared to PEI.

2. EXPERIMENTAL SECTION

2.1. Materials and Methods. All reagents and chemicals were purchased from the commercial providers and used without further purification unless specially noted. Absolute dichloromethane (CH_2Cl_2) and chloroform ($CHCl_3$) were distilled with calcium hydride (CaH_2). Column chromatography was performed using 200−300 mesh silica gel or alkaline Al_2O_3. The ^1H NMR spectra were measured on a Bruker AM400 NMR spectrometer. The molecular weights (M_w and M_n) and distribution (M_w/M_n) of prepared cationic polymers were determined by a gel permeation chromatography (GPC) system (Waters Corp., Milford, MA). The eluent was 0.5 M NaOAc/0.5 M HOAc (pH 4.6) after it was passed through a 0.02 μm film filter. The flow rate was set as 0.5 mL/min.

MicroBCA protein assay kit was purchased from Pierce (Rockford, IL). Cy5 was purchased from Mirus Bio, LLC (Madison, WI). Luciferase assay kit was obtained from Promega (Madison, WI). Endotoxin-free plasmid purification kit was purchased from Tiangen Biotech (Beijing) Co., Ltd. (Beijing, China). The plasmids used in the study were pEGFP-N1 (Clontech, Palo Alto, CA) coding for EGFP DNA and pGL-3 (Promega, Madison, WI) coding forluciferase DNA. FBS was obtained from Life Technologies Corporation (Gibco, Grand Island, NY). DMEM purchased from Invitrogen Corp. Cell Counting Kit-8 was purchased from Dojindo Molecular Technologies, Inc. (Rockville, MD). Branched polyethylenimine (PEI-25 kDa, M_w = 25 000) was supplied by Sigma−Aldrich.

Using cell culture, amplification and purification of plasmid DNA, acid−base titration, ethidium bromide (EB) displacement assay, particle size, and zeta-potential measurement in water, we processed these assays according to our previously reported procedures.[16]

2.2. Synthesis of Cationic Polymers P1−P5. Amino compound (**1**−**5**) (0.8 mmol of amino group) was dissolved in EtOH, and then compound **A**[16] (Scheme 1, 0.8 mmol of epoxy group) was added to the solution. The reaction mixture was further stirred at 80 °C under N_2 atmosphere for 72 h. The solvent was distilled off under reduced pressure. Then, polymers were dissolved in CH_2Cl_2 and stirred by continuously imported HCl gas overnight at room temperature. After remove of solvent, the residue was dissolved in a small amount of water. The product was obtained as pale-yellow solid after dialyzed against deionized water for 3 days (MWCO 3500) and lyophilization.

P1. 55.6% yield. ^1H NMR (400 MHz, D_2O, δ): 2.77−2.88 (m, 6H, $-NHCH_2CH_2N-$), 3.03−3.36 (m, 24H, $-NHCH_2CH_2N-$, $-CH_2CHOHCH_2O-$, $-OCH_2CH_2NHCH_2-$), 3.46−3.70 (m, 12H, $-CH_2CHOHCH_2O-$, $-OCH_2CH_2NHCH_2-$), 4.11−4.17 (m, 3H, $-CH_2CHOHCH_2O-$). GPC: M_w = 51 kDa, PDI = 2.81.

P2. 40.8% yield. ^1H NMR (400 MHz, D_2O, δ): 2.55−2.88 (m, 4H, $-NHCH_2CH_2N-$), 2.89−3.28 (m, 22H, $-NHCH_2CH_2N-$, $-CH_2CHOHCH_2O-$, $-OCH_2CH_2NHCH_2-$), 3.34−3.69 (m, 12H, $-CH_2CHOHCH_2O-$, $-OCH_2CH_2NHCH_2-$), 3.89−4.19 (m, 3H, $-CH_2CHOHCH_2O-$). GPC: M_w = 103 kDa, PDI = 3.88.

P3. 60.5% yield. ^1H NMR (400 MHz, D_2O, δ): 2.55−2.97 (m, $-NHCH_2CH_2N-$ and $-NCH_2CH_2N-$, 8H,); 3.16−3.32 (m, $-NHCH_2CH_2N-$, $-CH_2CHOHCH_2O-$ and $-OCH_2CH_2NHCH_2-$, 28H); 3.49−3.68 (m, $-CH_2CHOHCH_2O-$ and $-OCH_2CH_2NHCH_2-$, 16H); 3.89−4.07 (m, $-CH_2CHOHCH_2O-$, 3H). GPC: M_w = 99 kDa, PDI = 3.55.

P4. 67.2% yield. ^1H NMR (400 MHz, D_2O, δ): 2.75−2.89 (m, $-NHCH_2CH_2N-$ and $-NCH_2CH_2N-$, 12H); 2.94−3.32 (m, $-NHCH_2CH_2N-$, $-CH_2CHOHCH_2O-$ and $-OCH_2CH_2NHCH_2-$, 34H); 3.37−3.69 (m, $-CH_2CHOHCH_2O-$ and $-OCH_2CH_2NHCH_2-$, 20H); 3.89−4.08 (m, $-CH_2CHOHCH_2O-$, 5H). GPC: M_w = 67 kDa, PDI = 5.38.

P5. 26.2% yield. ^1H NMR (400 MHz, D_2O, δ): 2.85−2.99 (m, $-NHCH_2CH_2NH-$ and $-NCH_2CH_2N-$, 18H); 3.19−3.35 (m, $-CH_2CHOHCH_2O-$ and $-OCH_2CH_2NHCH_2-$, 12H); 3.43−3.69 (m, $-CH_2CHOHCH_2O-$ and $-OCH_2CH_2NHCH_2-$, 12H); 3.94−4.06 (m, $-CH_2CHOHCH_2O-$, 3H). GPC: M_w = 44 kDa, PDI = 2.70.

2.3. Agarose Gel Retardation Assay. pDNA (0.125 μg/sample) was mixed with the appropriate volume of the polymer solution (in water) to form polyplexes at different w/w ratios (weight ratio of polymer relative to pDNA). Water was added to the complex solution to make the total volume to 15 μL. After incubation at 37 °C for 0.5 h, the polyplexes were loaded into a 1% agarose gel containing GelRed

and electrophoresed at 120 V for 0.5 h. Gels were visualized under an ultraviolet lamp using a Vilber Lourmat imaging system.

2.4. Transmission Electron Microscopy (TEM). TEM (JEM-100CXa) was used to observe the morphologies of the polyplexes. One microgram (1 μg) of pUC-19 was mixed with the appropriate volume of the polymer solution to form complexes at a weight ratio of 6, diluted by water to a total volume of 50 μL, and incubated at 37 °C for 0.5 h. Fifteen minutes before measurement, the polyplexes solution were diluted with water or water containing FBS to 1 mL. The polyplexes suspension was added dropwise to the copper grid. After drying, 0.5% (w/v) phosphotungstic acid was added dropwise to the above grids. The grids were dried at room temperature at atmospheric pressure for several hours before observation.

2.5. Cytotoxicity Assay. The cytotoxicity assay of the copolymers was carried out by using CCK-8 assay with human osteosarcoma (U-2OS) cells. About 7×10^3 cells were seeded in a 96-well plate. The next day, the medium was replaced with 100 μL of fresh medium without FBS, to which 100 μL of complexes at various weight ratio of polymer relative to pDNA was added to achieve a final volume of 200 μL. After 24 h of incubation, polyplexes solutions were removed, and 10 μL of CCK-8 in 90 μL PBS was added to each well for additional 1 h incubation. The absorbance was measured using a microtiter plate reader. The relative cell viability was calculated by the following equation: viability (%) = (optical density of sample/optical density of control) × 100. The cell viability of bPEI 25 kDa was also performed with CCK-8.

2.6. Transfection Procedure. Transfection assays were performed first by using pGL-3 as the reporter gene in U-2OS, HEK293, HeLa, and HepG2 cells according to reported procedure.[16]

Enhanced green fluorescent protein (EGFP) pDNA was also used as the reporter gene to assessed the TE of complexes at their optimal weight ratios in U-2OS cells in the absence or presence of 10% serum using the same procedures. The transfected cells were imaged by using fluorescence microscopy (Nikon TS100) and quantitatively determined by using flow cytometry (FCM; Beckman Coulter, Pasadena, CA). bPEI 25 kDa was used as postive control.

2.7. Protein Adsorption Assay. In brief, 1 mL of polymer solution (1 mg/mL) was mixed with 1 mL of bovine serum albumin solution (2 mg/mL). After shaking for 0.5 h at 37 °C and following full-speed centrifugation, the supernatant was carefully collected. The concentration of BSA in the supernatant was determined by BCA protein assay. The protein adsorbed on the polyplexes was calculated by the following equation:

$$q = (C_i - C_s) \times V/m$$

Where C_i and C_s are the initial BSA concentration (2 mg/mL) and the BSA concentration in the supernatant after adsorption experiments, respectively; V = 2 mL; and m = 1 mg.

2.8. Cellular Uptake of Plasmid DNA (Flow Cytometry). The cellular uptake of the polymer/fluorescein-labeled DNA complexes was analyzed by flow cytometry. The Label IT Cy5 Labeling Kit was used to label pDNA according to the manufacturer's protocol. Briefly, U-2OS cells were seeded in 12-well plates (2.0×10^5 cells/well) and allowed to attach and grow for 24 h. The medium was exchange with serum-free or serum-containing medium. Cells were incubated with complexes containing Cy5-labeled DNA (2 μg of DNA/well, optimal weight ratio of each sample) in media for 4 h at 37 °C. Subsequently, the cells were washed with 1 × PBS and harvested with 0.25% Trypsin/EDTA and resuspended in 1 × PBS. Mean fluorescence intensity was analyzed using flow cytometer (Becton Dickinson and Company). Cy5-Labeled plasmid DNA uptake was measured in the FL4 channel using the red diode laser (633 nm).

2.9. Inhibition Studies. To probe the internalization mechanism of the polyplexes, the cellular uptake study was performed at 4 °C or in the presence of various endocytic inhibitors. Briefly, cells were incubated with polyplexes (at optimal mass ratio or N/P ratio) at 4 °C for 4 h, wherein the energy-dependent endocytosis was blocked. Otherwise, cells were preincubated with various endocytic inhibitors including cytochalasin D (10 mg/mL), genistein (200 μM), and nocodazole (33 μM). Following pretreatment for 30 min, the inhibitor solutions were replaced by the freshly prepared test complexes (Cy5-labeled plasmid DNA) in media containing inhibitors at the same concentrations. After further incubation for 4 h, cells were harvested and analyzed by flow cytometry. In the study, the groups in the presence of test complexes at 37 °C but without inhibitor treatment were used as controls. Results were represented as percentage uptake level of control cells.

2.10. Confocal Laser Scanning Microscopy (CLSM) Analysis. U-2OS cells were seeded in a 35 mm confocal dish (Φ = 15 mm) at a density of 2×10^4 cells per well. After 24 h under standard incubation conditions, the medium was exchanged with serum-free or serum-containing medium. Cells were incubated with Cy5-labeled DNA complexes (0.3 μg of DNA/well, optimal weight ratio of each sample) in media for 4 h at 37 °C. Subsequently, cells were rinsed twice with PBS (pH 7.4) and fixed with 4% paraformaldehyde for 10 min, and nuclear staining was done with DAPI. The CLSM observation was performed using Leica TCS SP5 at excitation wavelengths of 405 nm for DAPI (blue), 633 nm for Cy5 (red), respectively.

3. RESULTS AND DISCUSSION

3.1. Synthesis and Characterization. A variety of cationic hyperbranched polymers had been synthesized and studied as nonviral gene vectors.[29,30] The purpose of this study was to rationally design and evaluate a novel class of potentially safe nonviral gene delivery hyperbranched polymers. The coexistence of various types of amino groups in these polymeric vectors (such as branched PEI) might benefit their transfection activities.[31,32] As shown in Scheme 1, the hyperbranched target polymers **P1**−**P5** were prepared by ring-opening polymerization between different polyamines (**1**−**5**) and diol glycidyl ether **A**, which has been proven to be an excellent bridge group in the design of novel nonviral gene vectors.[16] Compared to the linear polymers prepared from **A**,[16] the hyperbranched ones are easier to prepare because no protection on amino groups is needed. However, a common problem in the preparation of reticular or hyperbranched polymers is the formation of insoluble gel-like materials. Fortunately, we found that compound **A** and the polyamines reacted well in the refluxed ethanol under nitrogen atmosphere, and no insoluble material was formed, even after a long reaction time. The target polymers are highly soluble in many solvents such as water, dimethyl sulfoxide, and methanol. GPC and ^{1}H NMR were used to characterize their structures. The M_w values of **P1**−**P5** were found to be in the range of 44−103 kDa, which are larger than the commonly used bPEI (25 kDa). Polyamine compounds with linear structure (**2**−**4**) seem to have slightly higher reaction activity with **A** than that of **1** and **5**, and the cationic polymers **P2**−**P4** give relatively larger molecular weights.

3.2. Buffering Capability. Acid−base buffering capacity has been reported as an important factor to determine the performance of the polymer−DNA complex (polyplex) in several aspects including the stability in cell culture medium, cell internalization, and lysosomal release of loaded genes.[33] The buffering capacities of prepared polymers in aqueous NaCl solution was assessed by acid−base titration and is shown in Table 1. It was found that 33.3−40.8% of amine groups could be protonated in the endosomal−lysosomal pH range (7.4−5.1). These values are definitely higher than that of bPEI 25

Table 1. Buffering Capacity of P1−P5 and bPEI (25 kDa)

polymers	P1	P2	P3	P4	P5	bPEI
buffering capacity (%)	40.8	36.6	33.3	34.7	36.7	15.6

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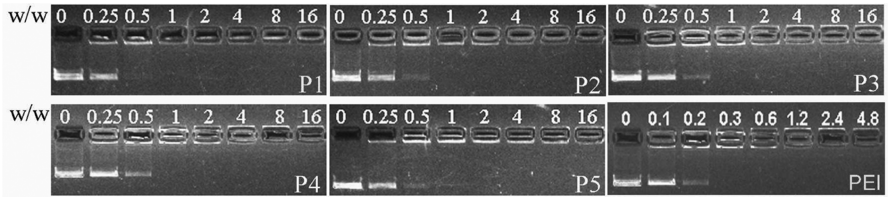

Figure 1. DNA condensation by cationic polymers at various weight ratios evaluated by the gel retardation assay.

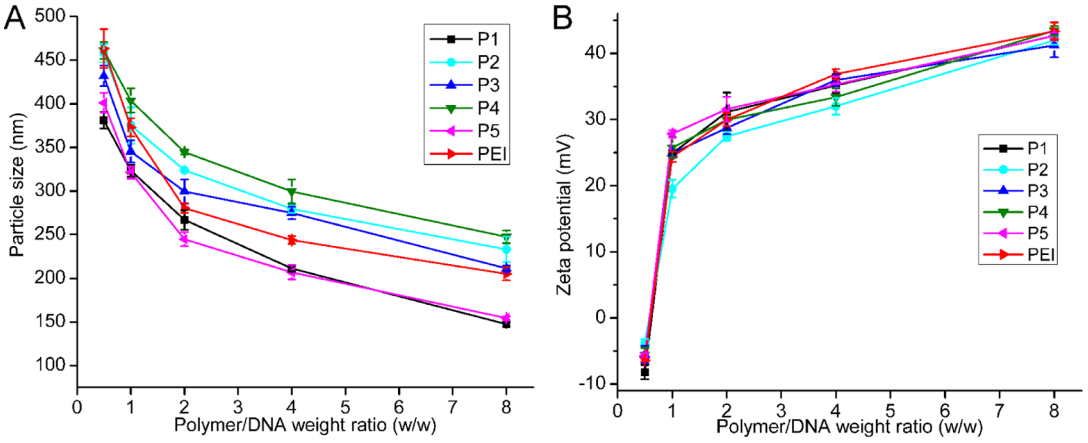

Figure 2. (A) Particle size and (B) zeta potential of polyplexes obtained at various weight ratios. Data represent mean ± SD (n = 3).

kDa (15.6%), indicating their good buffering capacity, which might be attributed to the various types of amino groups in the structures of the hyperbranched polymers. Although the polymers formed from branched or cyclic polyamine (**P1** and **P5**) have smaller molecular weights, their buffering capacities are higher than those of polymers prepared from linear polyamines (**P2**−**P4**). The most critical reason might lie in the structural and environmental differences of the internal amines, which may affect the tendency of the polymers to undergo pH-dependent conformational changes and self-assembling in solution.[34]

3.3. Formation of Polymer/DNA Complexes (Polyplexes). A prerequisite for cationic polymers to be used as gene vectors is their DNA binding capability. Gel retardation assay was used to confirm the interaction between the polymers and plasmid DNA, and the binding ability was indicated by the retarding weight ratio (polymer/DNA, w/w) where the mobility of pUC-19 DNA could be entirely retarded. As shown in Figure 1, all polymers showed good DNA binding ability, and they could completely retard the migration of DNA in agarose gel from weight ratio of ∼1. Because the polymers have lower charge density than PEI, their slightly weaker binding ability compared to PEI is reasonable. The molecular weight seemed to have little effect on their DNA retardation ability. It was known that polymers with higher molecular weight always have lower necessary amount for DNA condensation.[35−38] We consider that polymers formed from branched or cyclic polyamine (**1** and **5**) may have more typical hyperbranched structures, which may benefit their interaction toward DNA. Further, the stability of the polyplex against DNase I (which exists in serum) was also studied by gel electrophoresis. The results (Figure S1, Supporting Information) show that polymer **P1** (w/w > 0.5) could protect DNA from degradation, and the amount of **P1** needed for protection is lower than PEI. On the other hand, the DNA affinities of the polymers were also characterized by the fluorescence quenching experiment using ethidium bromide (EB). The results obtained by EB exclusion assays (Figure S2, Supporting Information) showed that upon the addition of polymers (**P1**−**P5** or PEI), the fluorescence intensity of EB decreased rapidly. The weight ratio at which the fluorescence signal reaches 50% of the original value (FI_{50}), suggesting an effective DNA condensation, was about 0.5 for **P1**−**P5** and 0.2 for PEI. These results were in good agreement with those obtained by the gel retardation experiments.

The positive surface charge and proper particle size of polyplex were necessary for efficient gene delivery.[39−41] Complexes with the sizes between 50 and several hundred nm were suitable for cell uptake.[42] The sizes and zeta-potentials of polyplexes derived from **P1**−**P5** were determined by dynamic light scattering (DLS), and the results are shown in Figure 2. In general, the particle size decreased, whereas the zeta potential increased with the increase of weight ratio. As stated above, polymers formed from branched or cyclic polyamine (**P1** and **P5**) exhibited tighter DNA affinity, and the polyplexes derived from these two polymers gave smaller mean diameters compared to other polyplexes (Figure 2A). Figure 2B showed that the zeta-potential of the polyplexes turned to be positive at a relatively low w/w ratio (0.5). Moreover, we also examined the possible changes of particle sizes in the presence of different serum levels (10−100%; Table S1, Supporting Information). With the increase of serum concentration, the particle sizes of polyplexes only slightly increased, indicating that no apparent aggregation occurred. The high serum stability might be induced by the hydroxyl groups newly formed from ring-opening reaction of epoxides.[16,17]

The morphology of polyplex was further investigated by TEM. As shown in Figure 3A and 3B, the polyplexes at weight ratio of 6 were observed as particles with regular spherical shape in the sizes ranging from 50 to 100 nm. Compared with **P5**/DNA polyplex, **P1**/DNA polyplex had a smaller size and a

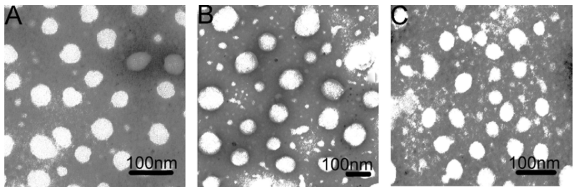

Figure 3. TEM images of (A) **P1**/DNA and (B) **P5**/DNA polyplexes at w/w of 6 in deionized water. (C) **P1**/DNA polyplex at w/w of 6 in water with 10% serum.

more regular shape. It is worth mentioning that the particle of polyplex formed from **P1** did not give any morphological change, even in the presence of 10% FBS (Figure 3C), also indicating its good serum tolerance.

3.4. Cytotoxicity. Excellent biocompatibility is significant for synthetic gene vectors. The high cytotoxicity of 25 kDa bPEI and some cationic lipids has severely limited their in vivo and clinical applications. The amino groups are usually positively charged under physiological conditions, and this may induce the cytotoxicity of gene vectors.[43] The cell viability after 4 h of incubation with the polyplexes studied herein as a function of weight ratio was evaluated in human osteosarcoma (U-2OS) cells by using CCK-8 assay. It should be noted that the weight ratio for CCK-8 assay was same as that used in the transfection experiments. As shown in Figure 4, the cytotoxicity

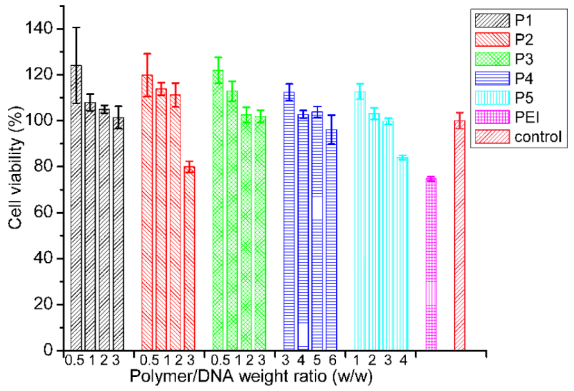

Figure 4. Cytotoxicity of polyplexes at different weight ratios toward U-2OS cells; 25 kDa bPEI was used as control (w/w = 1.4). Data represent mean ± SD (n = 3).

of polyplexes gradually increased with an increase of the weight ratios due to the presence of a high amount of cationic charge density, which may lead to the damage of cell membranes. At a lower weight ratio ($\leq$2), almost no cytotoxicity was observed, and the cell viabilities in the presence of studied polyplexes were much higher than that involving 25 kDa bPEI, even at relatively higher weight ratios. Similar assays with 24 h incubation with those polyplexes were also carried out, and the data (Figure S3, Supporting Information) are in accordance with the results above. Comparing to the structure of bPEI, we believe that the lower cytotoxicity may be attributed to the newly formed hydroxyl groups, which can benefit the biocompatibility of the polymeric materials used for gene delivery.[16,17] It is also proved that the ring-opening polymerization from bis-epoxides is an effective synthetic approach toward materials with low cytotoxicity.

3.5. In Vitro Gene Transfection. The TE of the polyplexes derived from **P1**−**P5** was first evaluated in U-2OS cells by luciferase transfection assay. Figure 5A shows the relative TE of **P1**−**P5** in the absence of serum at various w/w ratios in comparison with 25 kDa bPEI, which was used at its optimal N/P ratio of 10 (w/w = 1.4).[44] To our delight, it was found that at the optimal w/w ratios, **P1** and **P5** gave 5.0 and 2.7 times the TE as compared with bPEI, respectively. Meanwhile, **P2** and **P3** only gave moderate TE, and **P4** exhibited poor transfection ability. We believe that the increase of hyperbranched degree (using branched or cyclic polyamine as starting material) would increase the TE of cationic polymers, while the vectors prepared from linear polyamine exhibit much weaker transfection ability. As stated above, polymers with higher branched structures (**P1** and **P5**) may bind DNA more tightly and form smaller nanoparticles, which would benefit the endocytosis of polyplexes.

It was found that the cationic vector-mediated transfection would be inhibited in the presence of serum.[45−47] Previous results in DLS and TEM experiments have shown good serum tolerance of the hyperbranched polymers. The transfection ability of these polymers in the presence of serum was also estimated by the same method. As shown in Figure 5B, the relative TE is maintained for all synthesized polymers with the presence of 10% of serum. Similarly, **P1** and **P5** also showed higher TE among the polymers. Furthermore, the TE of **P1**/DNA polyplex was subsequently studied in the presence of different concentrations of serum (0, 10, 20, 30, 40, 70, and 100%). The results shown in Figure 5C further confirm that the TE of **P1** would not be reduced by the addition of serum. On the contrary, the TE improved even the serum concentration reached 70%, and the TE obtained in the presence of 100% of serum was comparable with that obtained without serum. As comparison, for PEI, when the serum concentration was higher than 10%, the TE began to decrease (Figure 5C), and only about 15% of TE was obtained in the presence of 100% of serum (compared to the TE without serum). In other words, the TE of **P1** was 5.0, 5.2, 12.9, 14.9, 14.3, 21.2, and 35.2 times higher than that of 25 kDa bPEI with the serum concentrations of 0, 10, 20, 30, 40, 70, and 100%, respectively, indicating the excellent serum tolerance of **P1**. Such an advantage might be attributed to its special structure, which contained both hyperbranched polyamine (like bPEI, for DNA binding and buffering capacity) and oxygen-rich backbone (like PEG, for biocompatibility). Besides, the TE of the **P1** was also evaluated in other cell lines including HeLa, HepG2, and HEK 293 in the absence or presence of 10% serum. As shown in Figure 5D, **P1** exhibits higher TE than bPEI. More importantly, the relative TE (compared to bPEI) with serum is evidently higher than that obtained in the serum-free experiment, further indicating the good biocompatibility of **P1**.

To visually compare the gene transfer ability of the studied cationic polymers, we also performed transfection experiments on U-2OS cells using pGFP as reporter gene. The weight ratios were used according to the optimal results from luciferase assays. Unlike luciferase expression results (Figure 5A), almost all polymers induced good to excellent GFP expression, and the fluorescent densities were similar to that of PEI (Figure S4, Supporting Information), whether in the presence of serum or not. To quantitatively evaluate the TE of the polymers toward GFP reporter gene, we applied flow cytometry to determine the amount of the EGFP-positive U-2OS cells and relative fluorescence intensity (RFI). As shown in Figure 6A, the percentages of positive cells induced by these polymers were varied. **P1** and **P5** gave more positive cells than PEI, while the

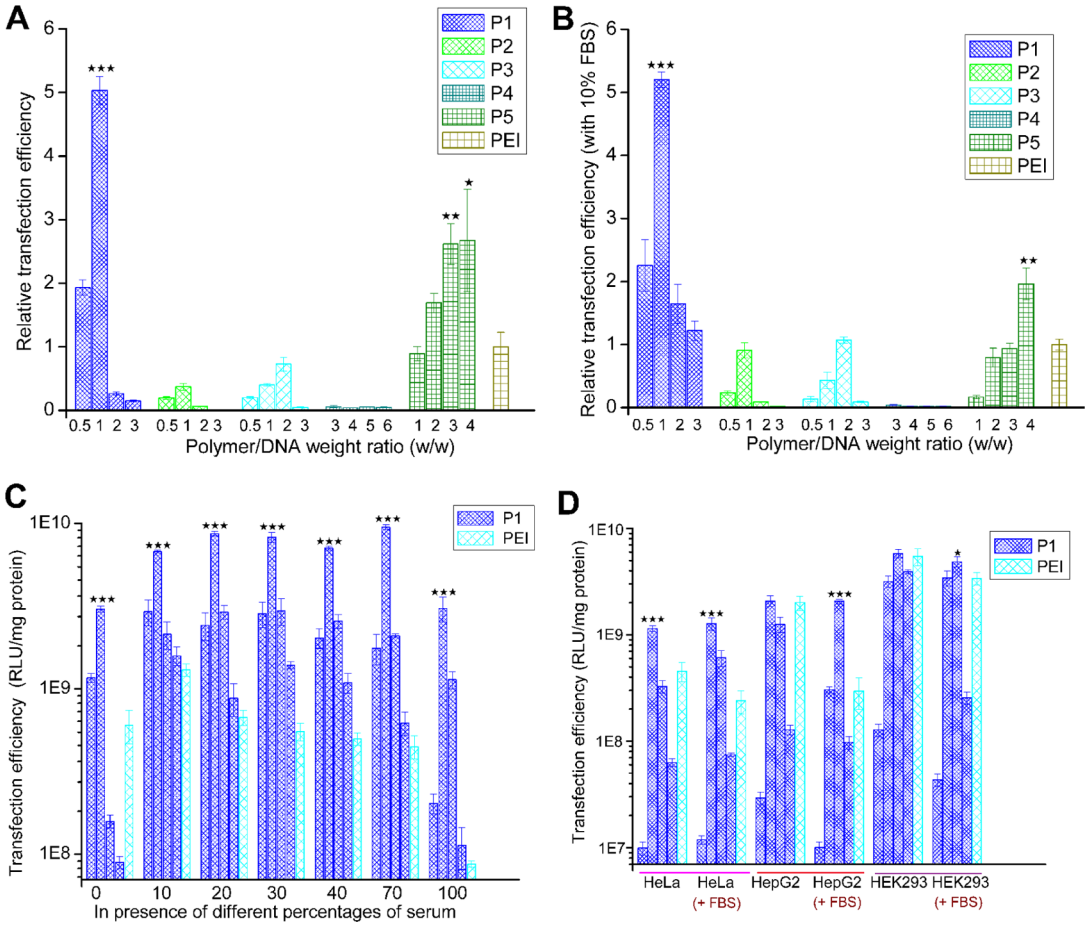

Figure 5. Luciferase gene expression transfected by polyplexes at different weight ratios in comparison with 25 kDa bPEI (N/*P* = 10) in U-2OS cells (unless otherwise noted). Data represent mean ± SD (n = 3). (A) Without serum (B) With serum (10%) (C) TE of **P1** in the presence of serum (different concentration), the weight ratio in each group were 0.5, 1, 2, 3. (D) TE of **P1** in the absence or presence of 10% serum in different cells, the weight ratios in each group were 0.5, 1, 2, and 3. (★, $p < 0.05$; ★★, $p < 0.01$; ★★★, $p < 0.001$ vs PEI complexes).

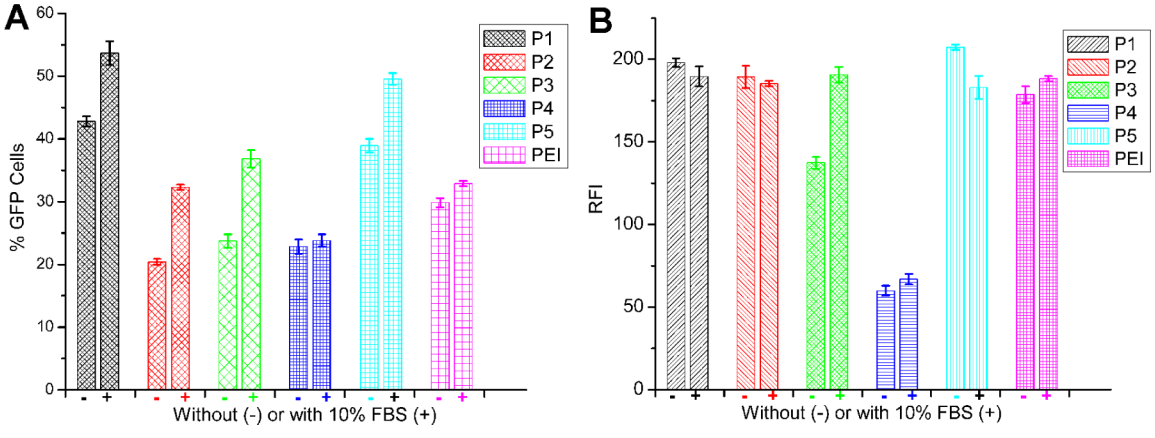

Figure 6. Transfection efficiencies of **P1**−**P5** at the optimal weight ratio without or with 10% of serum in comparison with bPEI. Data were expressed as the percentage of cells expressing (A) eGFP and (B) RFI determined by flow cytometry analysis.

percentages from **P2**−**P4** were much lower. However, all polymers gave almost equal RFI, except **P4**, which also has lowest TE in luciferase assay. Such results indicate that there is no direct relationship between the amount of GFP-positive cells and the fluorescent intensity of GFP. Similarly, 10% of serum seemed to have a positive effect on the amount of transfected cells, but for RFI, the effect of serum is unclear.

3.6. Bovine Serum Albumin (BSA) Adsorption.

The cationic polycation/DNA complexes may interact with negatively charged serum proteins via nonspecific electrostatic interaction, which might screen the positive charge of polyplexes and lead to aggregation.[48] The ability to resist protein adsorption would help to stabilize the polyplexes in the presence of serum and facilitate effective gene delivery.[49] The serum-conditioned TE was largely influenced by the structural

and physicochemical changes of polyplexes upon exposure to serum. To further study the highly serum-tolerant ability of these polymers, such as **P1**, we then carried out a protein BSA adsorption experiment. Results revealed that the BSA adsorption of all studied polymers was much lower than bPEI (Figure 7). Because the zeta-potentials of all polyplexes are

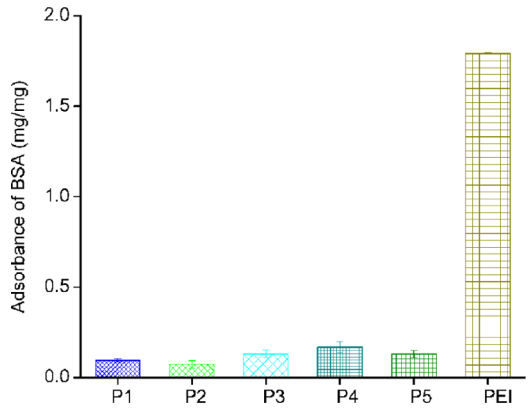

Figure 7. Protein adsorption of synthesized polymers and bPEI.

evidently positive (> +25 mV, Figure 2B), we believe that the special structures of the polymers may resist the electrostatic protein adsorption. Such resistance most likely come from the "hydroxyl effect"[16,17] of the electron-rich backbone: the ether and hydroxyl groups formed from the polymerization play a role similar to PEG, which has been proven to be able to enhance the biocompatibility of the cationic vectors.[50,43]

3.7. Mechanism Studies. To further study the transfection mechanism mediated by these polymers, several types of assays were performed in U-2OS cells to elucidate the fate of the target gene. First, cellular uptake of the polyplexes formed from **P1**−**P5** and bPEI was investigated by flow cytometry. After a 4 h incubation of polyplexes with U-2OS cells, the percentage of cells positive for Cy5-labeled pDNA was calculated. Results showed that all tested polyplexes exhibited excellent cellular uptake, and the percentage of transfected cells were found to be close to 100% (Figure 8A). Because the TE of the tested polymers varied considerably (as shown in Figure 5A), it could be drawn that good cellular uptake is a necessary but not sufficient condition for good TE. Subsequently, a similar assay was also carried out in the presence of different percentages of serum in the same cell line. As shown in Figure 8B, serum severely hampered the cellular uptake of the polyplexes derived from bPEI, and the percentage of positive cells rapidly decreased with the rise of serum concentration. On the contrary, to our delight, the cellular uptake of **P1**/DNA polyplex maintained at any tested serum concentrations. These results further demonstrate the excellent serum tolerance of such polymeric gene vectors.

The uptake mechanism of **P1**/DNA polyplex was further studied. As shown in Figure 9, the cellular uptake after

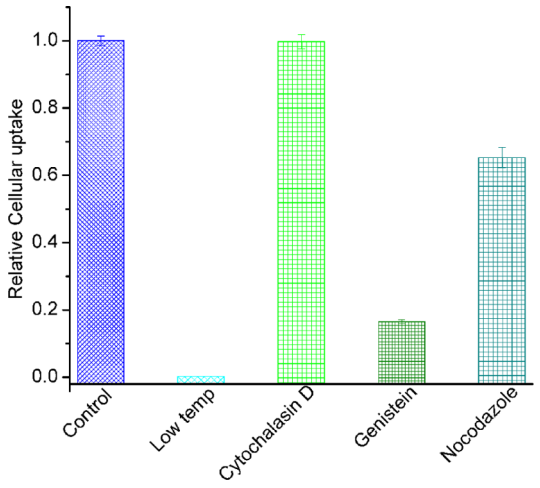

Figure 9. Relative cellular uptake of **P1**/DNA polyplexes at optimal transfection w/w ratio in U-2OS cells at 4 °C or in the presence of various endocytic inhibitors quantified by flow cytomety analysis.

incubation at 4 °C[51] almost ceased, while the uptake of PEI/DNA polyplex at the same temperature was seldom affected (data not shown). Such results indicated that the bPEI polyplexes enter the cells mainly via an energy-independent and nonendocytosis pathway. By comparison, **P1**/DNA appeared to enter the cell by an energy-dependent process. To elucidate the possible cellular uptake pathway of **P1**/DNA, the cells were treated with different chemical inhibitors. **P1**-mediated transfections were performed in the presence of cytochalasin D, genistein, or nocodazole, which may inhibit the pathway of macropinocytosis, caveolae, and microtubule-mediated endocytosis, respectively. Results revealed that cytochalasin D did not inhibit the uptake at all, suggesting that macropinocytosis is not possible for **P1**-mediated trans-

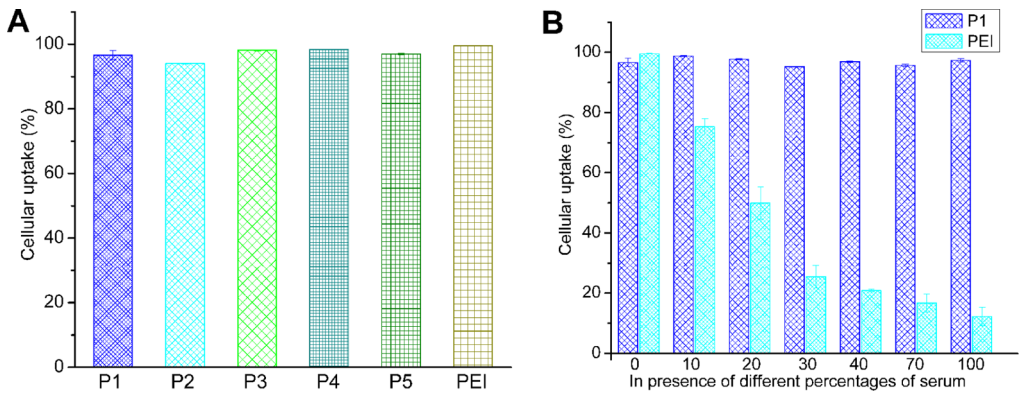

Figure 8. Cellular uptake of polyplexes at optimal transfection w/w ratio in U-2OS cells quantified by flow cytometry analysis. (A) Without serum and (B) with different concentrations of serum.

fection (Figure 9.) On the other hand, genistein severely hindered the uptake, and an 83% reduction of positive cells was found. Thus, it is evident that the internalization of **P1**/DNA polyplex is caveolae-dependent. Because caveolae uptake is a nonacidic and nondigestive route of internalization, cargos in the caveosomes can be directly transported to the Golgi apparatus or endoplasmic reticulum. Therefore, as a positive effect, they would not experience endosomal entrapment and lysosomal degradation, which serve as major intracellular barriers against effective gene transfection.[49,52] Finally, nocodazole could also impede the cellular uptake, but the inhibitory effect was lower than genistein, and about a 35% decrease of positive cells was obtained. Reilly et al. found that the transfected DNA that escaped from the caveosomes into the cytoplasm en route to the nucleus require additional microtubules, which take effect synergically with caveolae.[52] In the absence of microtubules, DNA may be trapped in the caveosomes, and the escape would be inhibited.

Confocal microscopy was utilized to visually examine the internalization and intracellular location of Cy5-labeled DNA (red) at the optimal transfection weight ratio in U-2OS cells. The cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, blue). As shown in Figure 10, in the absence of serum, a considerable amount of labeled DNA were delivered to the perinuclear region after 4 h of incubation with polyplexes derived from both **P1** and bPEI. With the increase of serum concentration, the **P1**-mediated transfection was seldom affected, and all of the images were similar to the one obtained in the serum-free experiment. However, for bPEI, the Cy5-labeled DNA was very few from the serum concentration of 20%. Although good uptake was also found in the presence of 10% of the serum, the location of the guest DNA was not perinuclear but rather randomly distributed in the cytoplasm. The visible results from CLSM corresponded well with flow cytomety analysis (Figure 8). These assays all demonstrated good TE together with excellent serum tolerance of such polycationic gene vectors.

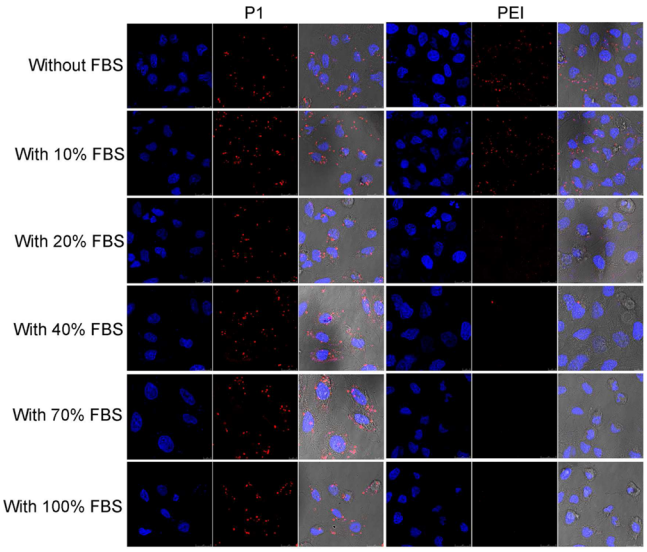

Figure 10. CLSM images of U-2OS cells transfected with Cy5-labaled DNA by **P1** at optimal weight ratio. bPEI was used as control (w/w = 1.4, N/*P* = 10). For each triad image: (left, blue) cell nuclei stained by DAPI, (middle, red) Cy5-labeled pDNA, and (right) merged image.

4. CONCLUSION

A series of novel hyperbranched cationic polymers were designed and synthesized by the ring-opening polymerization between diepoxide and several polyamines. These materials were applied in the gene transfection as nonviral vectors. The structures of starting polyamine **1**−**5** largely influenced the DNA condensation ability, buffering capacity, and transfection efficiency of the formed polyplexes. Polymers formed form branched or cyclic polyamine (**P1** and **P5**) exhibited higher buffering capacity and tighter DNA affinity, which might lead to a smaller size of polyplex, easier endocytosis, and subsequent higher TE. Compared to 25 kDa bPEI, **P1** could give 5 times higher TE (Figure 5A). More importantly, unlike that of bPEI, the TE of **P1** improved, even as the serum concentration reached 70%. Several assays, including BSA adsorption, flow cytometry, and confocal microscopy, demonstrated the excellent serum tolerance of such polycationic nonviral vectors. We consider that the structures of such polymers combine the advantages of bPEI (hyperbranched polyamine for DNA binding and buffering capacity) and PEG (oxygen-rich backbone for biocompatibility). This ring-opening polymerization from bis-epoxides may be an effective synthetic approach toward gene delivery materials with high biological activity.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Particle size of **P1**/DNA polyplexes in different concentrations of serum at weight ratios of 6 determined by DLS, detailed particle size data with PDI, stability assay against DNase I, fluorescent quenching assay, cytotoxicity with 24 h incubation, EGFP expression in U-2OS cells at optimal weight ratio, ^{1}H NMR spectra of target polymers. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

TE = transfection efficiency
PEI = polyethylenimine
BSA = bovine serum albumin
PEG = poly(ethylene glycol)
GPC = gel permeation chromatography
PDI = polydispersity
M_w = weight-average molecular weight

EB = ethidium bromide
TEM = transmission electron microscopy
DLS = dynamic light scattering
CCK-8 = Cell Counting Kit-8
HepG-2 = human liver hepatocellular carcinoma cell line
PBS = phosphate-buffered saline
DAPI = 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride
HEK = human embryonic kidney
U-2OS = human osteosarcoma
DMEM = Dulbecco's modified Eagle's medium
FBS = fetal bovine serum

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