ACS APPLIED MATERIALS & INTERFACES

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Series of New β-Cyclodextrin-Cored Starlike Carriers for Gene Delivery

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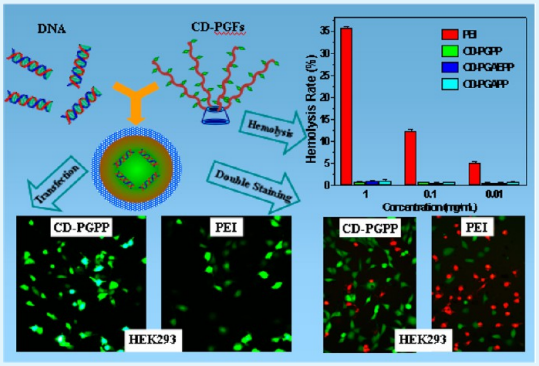
ABSTRACT: The development of safe and effective β-cyclodextrin (β-CD)-cored cationic star gene carriers has attracted considerable attention. In this work, a series of star-shaped hemocompatible CD-PGPP, CD-PGAEPP, and CD-PGAPP vectors composed of β-CD cores and piperazine (PP)-, *N*-(aminoethyl)piperazine (AEPP)-, or *N*-(3-aminopropyl)-2-pyrrolidinone (APP)-functionalized poly(glycidyl methacrylate) arms were successfully proposed and compared for highly efficient gene delivery. Such star carriers possess plentiful secondary amine, tertiary amine, and nonionic hydroxyl groups. CD-PGPP, CD-PGAEPP, and CD-PGAPP were effective in condensing plasmid DNA into nanoparticles, whose sizes were 100−200 nm and positive ζ potentials were 25−40 mV at nitrogen/phosphate (N/P) ratios of 10 and above. CD-PGPP, CD-PGAEPP, and CD-PGAPP showed significantly lower cytotoxicity than control poly(ethylenimine) (PEI; ∼25 kDa). At most N/P ratios, CD-PGAPP exhibited better gene transfection performance than CD-PGPP and CD-PGAEPP particularly in HepG2 cells. More importantly, in comparison with PEI, all of the CD-PGPP, CD-PGAEPP, and CD-PGAPP vectors did not cause undesirable hemolysis.



KEYWORDS: *gene delivery, β-cyclodextrin, piperazine, pyrrolidinone, star vector*

INTRODUCTION

The two basic requirements for gene-delivery vectors are low cytotoxicity and high transfection efficiency.[1] As the primary type of nonviral gene-delivery carriers, cationic polymers possess low host immunogenicity and can be produced massively. Many kinds of polycations such as poly(ethylenimine) (PEI)- and cyclodextrin (CD)-based cationic carriers were reported to transmit nucleic acids.[2−12] CD-based vectors had been widely investigated because CDs possess a large number of advantages including nonimmunogenicity, good biocompatibility, and low toxicity.[13] CDs also could improve gene bioavailability through enhancement of cell membrane absorption or stabilization of the gene in physiological media.[14,15] It was reported that poly(amidoamine) dendrimers[16,17] and PEI[18−20] incorporating CDs could provide improved efficiencies of delivering nucleic acids.

As nonviral gene vectors, CD-cored star polycations possess flexible molecular architectures for gene delivery.[21,22] We successfully used atom-transfer radical polymerization (ATRP) to prepare the β-CD-cored polycations with poly[(2-dimethylamino)ethyl methacrylate] (PDMAEMA) arms.[21,23] However, the PDMAEMA-based carriers still exhibited high cytotoxicity. Recently, it was found that the PGEA polycation prepared by functionizing poly(glycidyl methacrylate) (PGMA) with ethanolamine (EA) can possess abundant secondary amine and hydroxyl groups.[24,25] Such PGEA vectors exhibited high transfection efficiency and low toxicity in some cell lines. More recently, we prepared CD-cored star PGEA vectors, which combined the flexibility of star polymers and low toxicity of PGEA.[26,27]

Both piperazine (PP) and pyrrolidinone are cyclic compounds with biological properties. PP is a six-membered ring including two opposing nitrogen atoms, and its scaffolds are most widely used as backbones in medicinal chemistry and bioactive templates.[28,29] Pyrrolidinone is a five-membered ring, and its polymer derivative, poly(vinylpyrrolidinone) (PVP), had been intensively investigated as a biocompatible material.[30−33] PVP possessed properties similar to those of poly(ethylene glycol) (PEG) including water solubility, nontoxicity, and uncharged property.[30,31] PVP had better stability in blood than PEG. PVP also showed good ability to stabilize a protein drug.[32−34] To the best of our knowledge, such cyclic PP and pyrrolidinone had not been explored for the design of CD-

Received: November 23, 2013
Accepted: March 2, 2014
Published: March 2, 2014

   dx.doi.org/10.1021/am5005255 | ACS Appl. Mater. Interfaces 2014, 6, 3969−3978

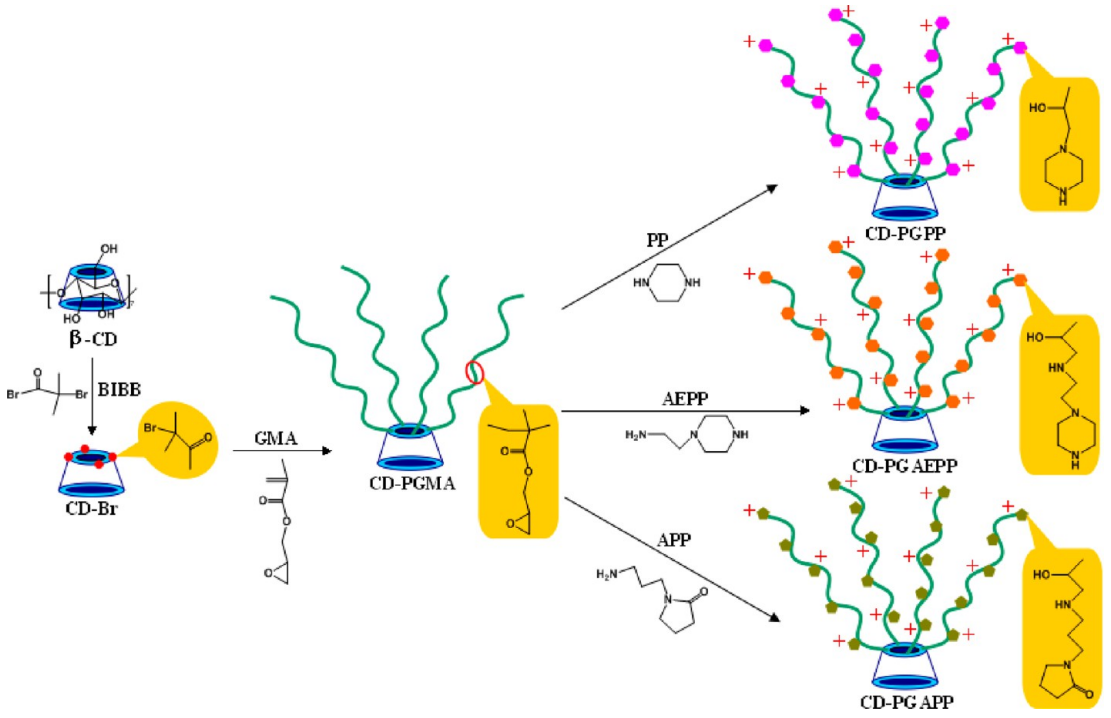


Figure 1. Schematic diagram illustrating the preparation processes of CD-PGPP, CD-PGAEPP, and CD-PGAPP.

cored gene-delivery systems. The introduction of cyclic compounds with biological properties to CD-cored polycations may enhance the transfection efficiency and even induce favorable biological effects.

In this work, a series of star-shaped CD-PGPP, CD-PGAEPP, and CD-PGAPP vectors consisting of β-CD cores and PP-, *N*-(aminoethyl)piperazine (AEPP)-, or *N*-(3-aminopropyl)-2-pyrrolidinone (APP)-functionalized PGMA arms were proposed and compared for highly efficient gene delivery (Figure 1). CD-PGPP and CD-PGAEPP possessed a large number of cationic PP groups, while CD-PGAPP possessed plentiful secondary amine groups and neutral pyrrolidinone groups. The CD-PGPP, CD-PGAEPP, and CD-PGAPP carriers were investigated and compared in detail through a series of experiments including DNA condensation capability, cytotoxicity, gene transfection, and hemolysis assay. The current work would provide valuable information for the development of better CD-cored starlike delivery systems.

EXPERIMENTAL SECTION

Materials. β-Cyclodextrin (β-CD; >98%, vacuum-dried at 100 °C overnight before use), *N*,*N*,*N*′,*N*′,*N*″-pentamethyldiethylenetriamine (PMDETA; 99%), copper(I) bromide (CuBr; 99%), glycidyl methacrylate (GMA; 97%, stabilized with 4-methoxyphenol), 2-bromoisobutyryl bromide (BIBB; 98%), anhydrous piperazine (PP; 99%), and *N*-(aminoethyl)piperazine (AEPP; 98%) were obtained from Sigma-Aldrich Chemical Co. *N*-(3-Aminopropyl)-2-pyrrolidinone (APP; 95%) was purchased from Acros Organics, Geel, Belgium. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), penicillin, and streptomycin were purchased from Sigma-Aldrich Chemical Co. HEK293, COS7, and HepG2 cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD).

Synthesis of CD-Cored PGMA via ATRP. In this work, about four initiation bromoisobutyryl sites were introduced to CD (CD-Br) for the preparation of CD-cored star PGMA (CD-PGMA) with four arms. Our previous publication described the detailed synthesis procedures and characterization of CD-Br.[21] The CD-*g*-P(GMA) (CD-PGMA) star polymers (Figure 1) with different molecular weights were prepared by controlling the dosage of GMA (Table 1)

Table 1. Characterization of the Polymers

sample[a]	feed monomer volume of GMA (mL)	reaction time (min)	M_n (g/mol)[b]	PDI[b]	monomer repeat units per arm[c]
CD-PGMA-1	3.5	5	1.61×10^4	1.36	25
CD-PGMA-2	4.5	5	2.02×10^4	1.31	32
CD-PGMA-3	5.5	5	2.55×10^4	1.27	42

[a]Synthesized using a molar feed ratio [CD-Br]/[CuBr]/[PMDETA] of 1:4:8 at room temperature in 5 mL of DMSO containing 0.3 g of CD-Br, which possesses about four initiation sites. [b]Determined from GPC results. PDI = weight-average molecular weight/number-average molecular weight or M_w/M_n. [c]Determined from M_n of CD-PGMA and the molecular weight of CD-Br (1.73×10^3 g/mol).

and adopting a molar feed ratio [CD-Br (0.3 g)]/[CuBr]/[PMDETA] of 1.0:4.0:8.0. First, dimethyl sulfoxide (DMSO), CD-Br, and PMDETA were dissolved completely in a 25 mL flask containing 3.5, 4.5, or 5.5 mL of GMA. Afterward, CuBr was introduced into the mixture with degassing by bubbling nitrogen, and then a rubber stopper with a sealant was used to seal the flask. The polymerization proceeded at room temperature for 5 min. The reaction was terminated by bubbling oxygen. The CD-PGMAs were precipitated in excess methanol. The crude polymer was reprecipitated twice in methanol and three more times in deionized (DI) water for purification, prior to lyophilization. The CD-PGMA yields (and the conversion of GMA) from ATRP containing 3.5, 4.5, and 5.5 mL of GMA were 1.6 g (35%), 2.0 g (36%), and 2.4 g (35%), respectively.

Functionalized CD-PGMA Vectors. The CD-PGMA-based carriers with different functionalization were synthesized by reacting CD-PGMAs with PP, AEPP, or APP (Figure 1). In a typical synthesis of functionalized CD-PGMA vectors, 0.2 g of CD-PGMA, 2.5 g of PP, AEPP, or APP, and 200 μL of triethylamine were added in 10 mL of *N*,*N*-dimethylformamide. The reaction mixture was stirred at 30 °C for 2 h and then at 80 °C for 40 min to produce the corresponding CD-

PGPP, CD-PGAEPP, or CD-PGAPP vectors. The final reaction mixtures were purified by precipitation and washing with excess diethyl ether followed by dissolution in 30 mL of DI water and dialysis against DI water (4 × 5 L) using a 3.5 kDa molecular weight cut-off dialysis membrane at room temperature for 48 h, prior to lyophilization.

Polymer Characterization. Gel permeation chromatography (GPC) and nuclear magnetic resonance (NMR) spectroscopy were used to determine the molecular weights of the polymers and chemical structures, respectively. GPC measurements were performed on a Waters GPC system equipped with a Waters 2414 refractive index detector, a Waters 2487 dual-wavelength (λ) UV detector, and Waters Styragel columns. The eluent was tetrahydrofuran at a flow rate of 1.0 mL/min. Monodispersed polystyrene standards were used to generate the calibration curve. Accumulation of 1000 scans at a relaxation time of 2 s was set up to measure ^{1}H NMR spectra on a Bruker ARX 300 MHz spectrometer, using $CDCl_3$ (for CD-PGMA) and D_2O (for CD-PGPP, CD-PGAEPP, and CD-PGAPP) as the solvent. The chemical shifts δ 7.28 and 4.70 referred to the solvent $CDCl_3$ and D_2O peaks, respectively.

Characterization of Polymer/Plasmid DNA (pDNA) Complexes. Plasmid pRL-CMV (encoding Renilla luciferase; Promega Co., Cergy Pontoise, France) was used in this work. All polymer stock solutions were prepared to achieve a nitrogen component concentration of 10 mM in DI water. Star-shaped polycation to pDNA ratios are expressed as molar ratios of nitrogen (N) in polycation to phosphate (P) in DNA (or as N/P ratios). Equal volumes of polycation and pDNA solutions were mixed to form all polycation/DNA complexes to achieve the desired N/P ratios, where the solutions were vortexed and then kept for 30 min at room temperature.

The ability of each polycation to bind pDNA at various N/P ratios was examined through agarose gel electrophoresis in a Sub-Cell system (Bio-Rad Laboratory, Hercules, CA) using procedures similar to those described earlier.[3] The particle sizes and ζ potentials of the polycation/pDNA complexes were measured by a Zetasizer Nano ZS (Malvern Instruments, Southborough, MA) based on the procedures as described earlier.[3] The polyplex morphology was visualized by an atomic force microscopy (AFM) system with a Nanoscope IIIa controller (Dimension 3100 model, Veeco, Santa Barbara, CA). The tapping mode with a setting of 512 pixels/line and a scan rate of 1 Hz was used to image the samples.

Cell Viability. MTT assays were used to evaluate the cytotoxicity of the star-shaped polycations in HEK293, COS7, and HepG2 cell lines using procedures similar to those described earlier,[3] where Dulbecco's modified eagle medium (DMEM), replenished with 10% heat-inactivated fetal bovine serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin, was employed to culture cells at 37 °C. The sterile-filtered MTT stock solution in phosphate-buffered saline (PBS; 5 mg/mL) was used. The absorbance was measured using a Bio-Rad model 680 microplate reader (UK) at a wavelength of 570 nm. For each sample, the final absorbance was the average of those measured from six wells in parallel. The formula $[A]_{test}/[A]_{control} \times 100\%$ was used to calculate the cell viability (%), where $[A]_{test}$ and $[A]_{control}$ are defined as the absorbance values of the wells with the polycations and controls (without the polycations), respectively.

The cytotoxicity of the star-shaped polycations was also evaluated using a fast, simultaneous double-staining procedure of fluorescein diacetate (FDA) and propidium iodide (PI) in HEK293 and HepG2 cell lines. FDA-stained living cells are in green, and PI-stained dead cells are in red. A working solution of FDA, which was kept in the dark at 4 °C, was prepared by dissolving 25 mg of FDA in 1 mL of acetone and then mixed with 4 mL of a D-mannitol solution. A working solution of PI was prepared by dissolving 2 mg of PI in 4 mL of a 0.65 M aqueous solution of D-mannitol directly. The cells were seeded in a 24-well microtiter plate at a density of 5×10^4 cells/well and incubated in 500 μL of DMEM/well for 20 h. The culture media were replaced with fresh culture media containing a solution of polymer/pDNA at the predetermined N/P ratio, and the cells were incubated for 4 h. Then the culture media were exchanged with 500 μL of fresh media. Then, 10 μL of an FDA working solution and 8 μL of a PI working solution were added directly to each well in the dark. After 5 min, the cells were imaged using a Leica DMIL fluorescence microscope.

In Vitro Transfection Assay. Transfection assays were first performed with the reporter plasmid pRL-CMV in HEK293, COS7, and HepG2 cell lines using the procedures as described earlier.[3] The cells were seeded in 24-well plates at a density of 5×10^4 cells in 500 μL of medium/well. The N/P ratios of the star-shaped polycation/pDNA complexes varied from 10 to 30. A total of 1.0 μg of pDNA for one cell was used. After a total transfection time of 24 h, a commercial kit (Promega Co., Cergy Pontoise, France) and a luminometer (Berthold Lumat LB 9507, Berthold Technologies GmbH KG, Bad Wildbad, Germany) were used to quantify luciferase gene expression. Gene expression results were expressed as relative light units (RLUs) per milligram of cell protein lysate (RLU/mg of protein).

The typical polycation-mediated gene transfection was also evaluated at the optimal N/P ratios using a reporter plasmid pEGFP-N1 encoding green fluorescent protein (GFP; BD Biosciences, San Jose, CA) in HEK293 and HepG2 cell lines. A Leica DMIL fluorescence microscope was used to image the transfected cells. The percentage of enhanced GFP (EGFP) positive cells was determined by flow cytometry (FCM; Beckman Coulter, Pasadena, CA).

Hemolysis Assay. Red blood cells (RBCs) were isolated from a mixture of 5 mL of a rabbit blood sample and 10 mL of PBS by centrifugation at 1500 rpm for 10 min. The supernatant containing plasma and platelets was discarded. Washing with PBS was continued until the supernatant was clear. The resultant RBC suspension (about 1 mL) was diluted to 50 mL of PBS, producing a stock RBC solution with about 2% RBC suspensions. Herein, RBC incubation with DI water and PBS was expressed as positive and negative controls, respectively. The CD-PGPP, CD-PGAEPP, and CD-PGAPP samples were weighed and dissolved in PBS for 24 h. A total of 2 mL of dilute 2% RBC suspension was added to 2 mL of polycation solutions with different concentrations. The resultant mixtures were incubated at 37 °C for 3 h and then centrifuged at 1500 rpm for 10 min. The absorbance of the supernatant caused by the release of hemoglobin was measured at 545 nm.[35] The percentage of hemolysis was calculated as follows:

$$\%\ \text{hemolysis} = [(OD_{test} - OD_{neg})/(OD_{pos} - OD_{neg})] \times 100$$

where OD_{test}, OD_{neg}, and OD_{pos} are the absorbance values of the test sample, negative control (PBS), and positive control (water), respectively. All of the hemolysis experiments were carried out in triplicate.

RESULTS AND DISCUSSION

Preparation and Characterization of PP-, AEPP-, and APP-Functionalized CD-PGMA Vectors. In this work, CD-Br with four initiation sites was prepared by esterification between the four hydroxyl groups on the outside surface of β-CD and BIBB (Figure 1). As reported, all 21 hydroxyl groups of β-CD could be transformed into initiation sites.[36,37] CD-Br with four initiation sites was used to allow some flexibility for gene delivery.[21,26,27] The detailed characterization of CD-Br was described in our previous paper.[21] Well-defined CD-PGMA was subsequently synthesized via ATRP of GMA from CD-Br. The PGMA arm lengths can be controlled by adjusting the molar feed ratios of the monomers. Table 1 summarizes the GPC results of three well-defined CD-PGMAs [CD-PGMA-1, ~101 GMA units, $M_n = 1.61 \times 10^4$ g/mol, polydispersity index (PDI) = 1.36; CD-PGMA-2, ~130 GMA units, $M_n = 2.02 \times 10^4$ g/mol, PDI = 1.31; CD-PGMA-3, ~167 GMA units, $M_n = 2.55 \times 10^4$ g/mol, PDI = 1.27]. CD-PGMAs were reacted with excess PP, AEPP, or APP to produce the corresponding CD-PGPP, CD-PGAEPP, or CD-PGAPP carriers.

The representative structures of CD-PGMA, CD-PGPP, CD-PGAEPP, and CD-PGAPP were characterized by ^{1}H NMR spectra, as shown in Figure 2. For CD-PGMA (Figure 2a), the

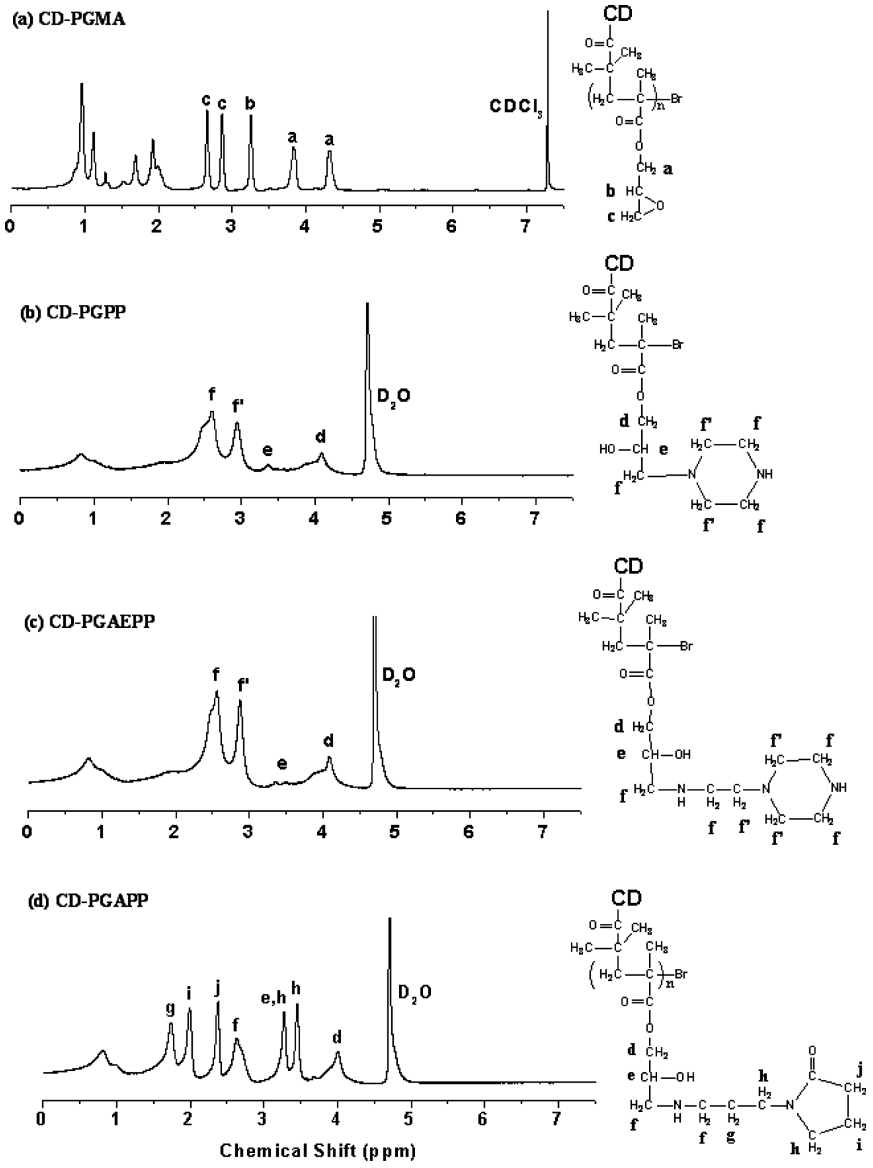


Figure 2. 300 MHz ^{1}H NMR spectra of (a) CD-PGMA, (b) CD-PGPP, (c) CD-PGAEPP, and (d) CD-PGAPP.

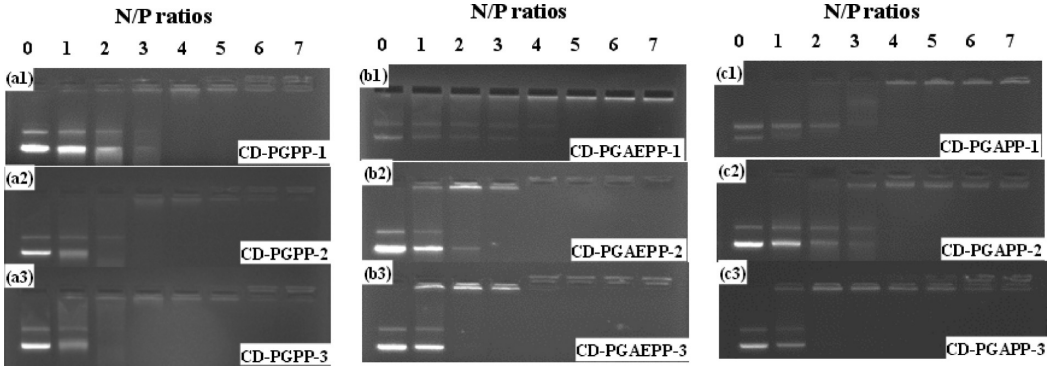


Figure 3. Electrophoretic mobility of pDNA in the complexes of the cationic polymers (a1−a3, CD-PGPP; b1−b3, CD-PGAEPP; c1−c3, CD-PGAPP) at various N/P ratios.

methylene protons adjacent to the oxygen moieties of the ester linkages (a, $CH_2OC{=}O$) corresponded to the signals at δ 3.8 and 4.3. The peaks at δ 3.2 and δ 2.6 and 2.8 could be assigned to the $CH_2CH(O)CH_2$ (b) methylidyne and $CHCH(O)CH_2$ (c) methylene protons of the epoxy ring, respectively. The area ratio of peaks a−c was about 2:1:2, manifesting that the epoxy groups in the CD-PGMA were not damaged throughout ATRP.

After the ring-opening reactions of CD-PGMA with PP, AEPP, and APP, the peaks (b and c in Figure 2a) representing the epoxy groups of CD-PGMA disappeared completely. The

dx.doi.org/10.1021/am5005255 | ACS Appl. Mater. Interfaces 2014, 6, 3969−3978

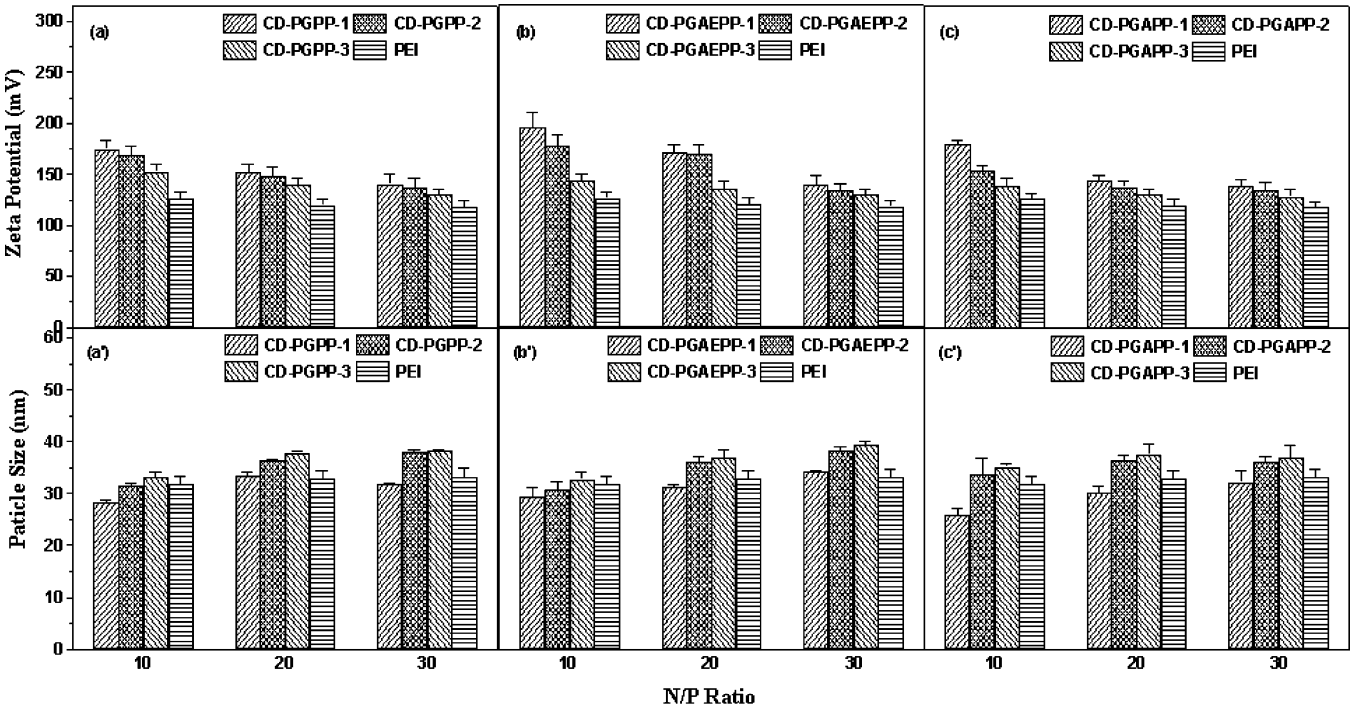


Figure 4. Particle sizes (a–c) and ζ potentials (a′–c′) of the cationic polymer (CD-PGPP, CD-PGAEPP, CD-PGAPP, and PEI)/pDNA complexes at various N/P ratios.

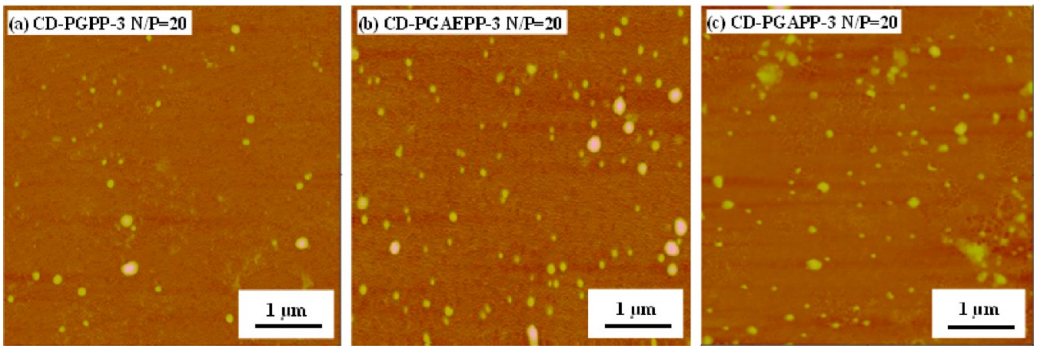


Figure 5. AFM images of the (a) CD-PGPP-3/pDNA, (b) CD-PGAEPP-3/pDNA, and (c) CD-PGAPP-3/pDNA complexes at a N/P ratio of 20.

peaks at δ 3.8 and 4.3 (a in Figure 2a) merged into a peak at δ 4.1 (d in Figure 2b–d). The chemical shift of δ 3.3 mainly corresponded to the resultant methylidyne protons adjacent to the hydroxyl groups (e, C*H*OH). The signals at δ 2.6 and 2.9 were mainly attributable to the methylene protons adjacent to the amine groups [f, CH(OH)CH_2NH, CH(OH)CH_2N, and NCH$_2$CH_2NH; f′, NCH_2CH$_2$NH]. As shown in Figure 2b,c, the area ratios of peaks d–f and f′ were about 2:1:6:4 and 2:1:8:6, respectively, indicating that each PP and AEPP linked with an epoxy ring with a secondary and a tertiary amine, respectively. For CD-PGAPP (Figure 2d), the peaks at δ 1.7 and 2.0 were mainly attributable to the methylene protons adjacent to two methylenes (g, NHCH$_2$CH_2CH$_2$NC═O; i, NCH$_2$CH_2CH$_2$C═O). The peaks at δ 3.3 and 3.5 mainly corresponded to the methylene protons adjacent to the nitrogen moieties of the amide linkage (h, CH$_2$CH_2NC═O). The signal at δ 2.4 belonged to the methylene protons adjacent to the carbonyl moieties of the amide linkage (j, CH_2C(O)N). The area ratio of peaks d, f, i, j, and g and peaks e and h was about 1:2:1:1:1:2.5, which was consistent with the chemical structure of CD-PGAPP. These NMR results thus indicate that the PP-, AEPP-, and APP-functionalized CD-PGMA vectors have been successfully prepared.

Biophysical Characterization of Polymer/pDNA Complexes. For efficient gene delivery, cationic polymers have to condense pDNA into polymer/plasmid nanoparticles small enough for cellular uptake and subsequent transfection. In this work, agarose gel electrophoresis, particle size, ζ potential measurements, and AFM imaging were used to confirm the ability of PP-, AEPP-, and APP-functionalized CD-PGMA vectors to condense pDNA. Figure 3 shows the gel retardation results for the polycation (CD-PGPP, CD-PGAEPP, and CD-PGAPP)/pDNA complexes with increasing N/P ratios. At the same conditions, they exhibited similar condensation capability and compacted pDNA completely at the N/P ratios of 4.0 or 5.0 (for CD-PGPP1, CD-PGAEPP1, and CD-PGAPP1) and 2.0 or 3.0 (for CD-PGPP3, CD-PGAEPP3, and CD-PGAPP3). The above gel retardation results indicated that CD-PGPP, CD-PGAEPP, and CD-PGAPP showed enhanced capability to condense pDNA with an increase in their molecular weights, because of the increasing density of amino groups in the star polymers.

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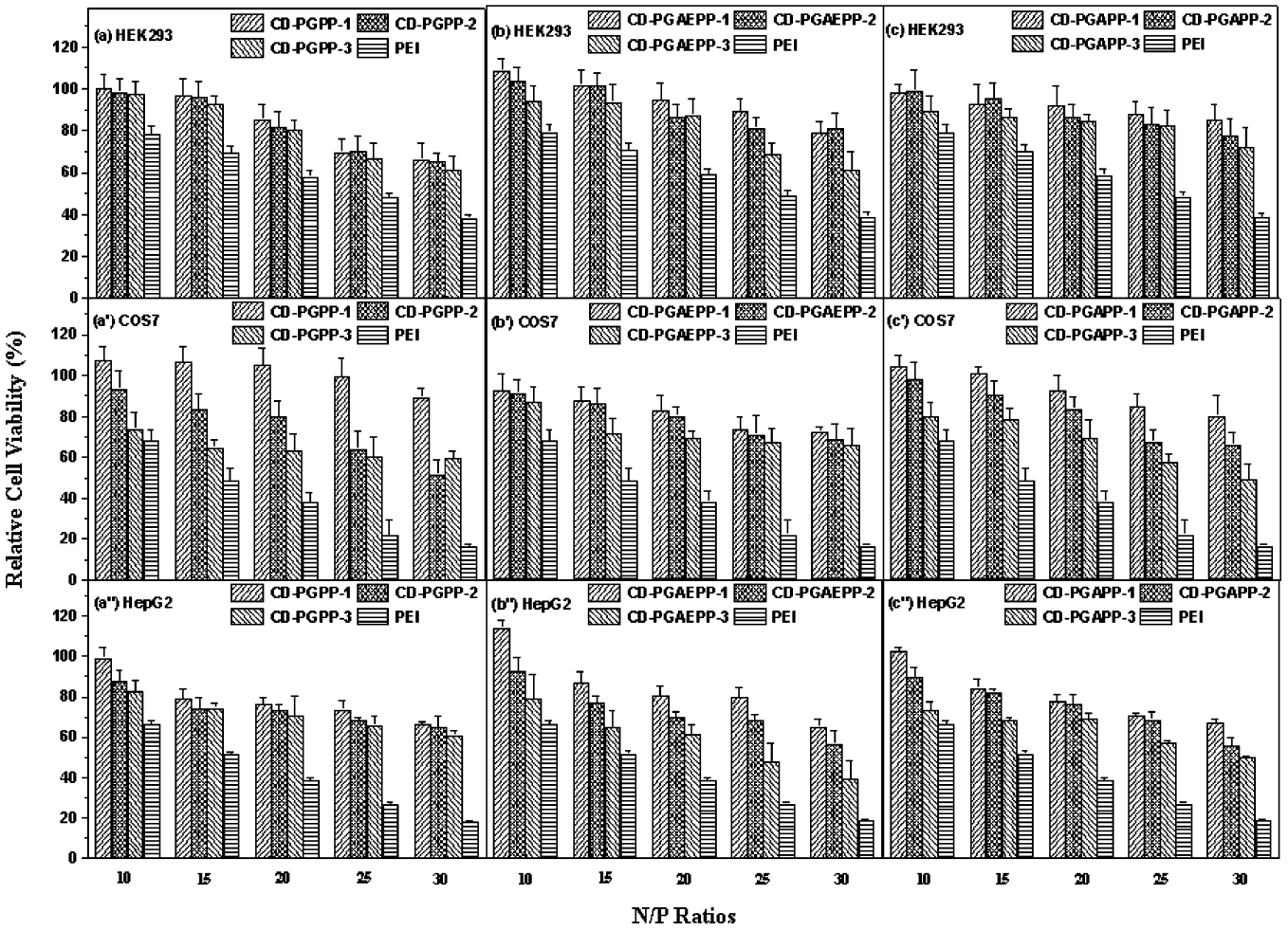

Figure 6. Cell viability of the polycation (CD-PGPP, CD-PGAEPP, CD-PGAPP, and PEI)/pDNA complexes at different N/P ratios in (a−c) HEK293, (a′−c′) COS7, and (a″−c″) HepG2 cell lines.

As shown in Figure 4, the particle sizes and ζ potentials of the polycation (CD-PGPP, CD-PGAEPP, and CD-PGAPP)/pDNA complexes are compared with those of the PEI/pDNA complexes at various N/P ratios. All of the star polycations were confirmed to tightly compact pDNA into small nanoparticles. Generally, the hydrodynamic sizes of the complexes decrease when the N/P ratio increases within a certain range. With the N/P ratio varied from 10 to 30, all of the star polycations, as well as PEI, could compress pDNA into nanoparticles of 100−200 nm in diameter, and the generated complexes could readily undergo endocytosis. Figure 5 shows the representative AFM images of the CD-PGPP-3/pDNA, CD-PGAEPP-3/pDNA, and CD-PGAPP-3/pDNA complexes at a N/P ratio of 20. The AFM images distinctly revealed that most of the tight complexes existed in the form of uniform spherical nanoparticles within 200 nm diameter on average, which was consistent with the results of the particle size measurement.

A nanoparticle with a positively charged surface can electrostatically interact with negatively charged cell surfaces and promote cellular uptake.[38] The net surface positive charge of CD-cored polycation/pDNA complexes at the same N/P ratio increased slightly as the molecular weight increased (Figure 4). All of the polycation/pDNA complexes were strongly positive and varied within a narrow range of 25−40 mV. In addition, at higher N/P ratios, the excess polycation portions probably did not cause obvious effects on the complex particle sizes and ζ potentials. Such a phenomenon was consistent with that reported in the literature.[39]

Cytotoxicity Assay. Cytotoxicity is an important factor that should be given prior consideration in selecting polymeric gene carriers. The in vitro MTT assay results of CD-PGPPs, CD-PGAEPPs, CD-PGAPPs, and PEI in HEK293, COS7, and HepG2 cell lines are shown in Figure 6. In general, the cell viability of polycation/pDNA complexes decreased as the N/P ratios and the density of amino groups increased. At higher N/P ratios, the increased free cationic polymers induced the increasing cytotoxicity. At the same N/P ratio, the cell viability was associated with the arm length of the polycation. CD-PGPP3, CD-PGAEPP3, and CD-PGAPP3 with the longest arms exhibit the most toxicity. In general, higher molecular weight leads to the increased cytotoxicity of the polycations.[24,25] Compared with the CD-cored polycation/pDNA complexes, the control PEI-mediated complexes showed much higher cytotoxicity. PEI is mainly constituted of secondary amine groups. CD-PGPP, CD-PGAEPP, and CD-PGAPP possessed plentiful flanking nonionic hydrophilic hydroxyl units, which probably well weaken the positive charges of the cationic vectors.[24,25] In addition, the introduction of biocompatible CD cores not only lowered the relative concentration of the amino groups, which could lead to high cytotoxicity at a high concentration,[40,41] but also provided biocompatible

dx.doi.org/10.1021/am5005255 | ACS Appl. Mater. Interfaces 2014, 6, 3969−3978

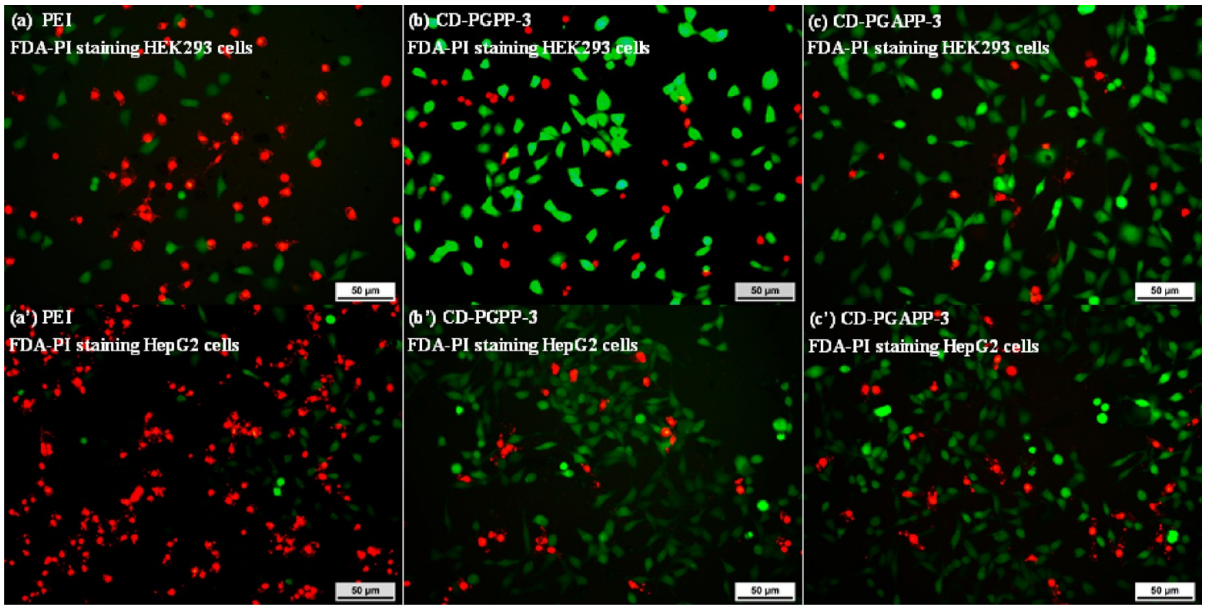

Figure 7. Representative images of FDA-PI staining HEK293 (a−c) and HepG2 (a′−c′) cell lines mediated by PEI, CD-PGPP-3, and CD-PGAPP-3 at a N/P ratio of 25.

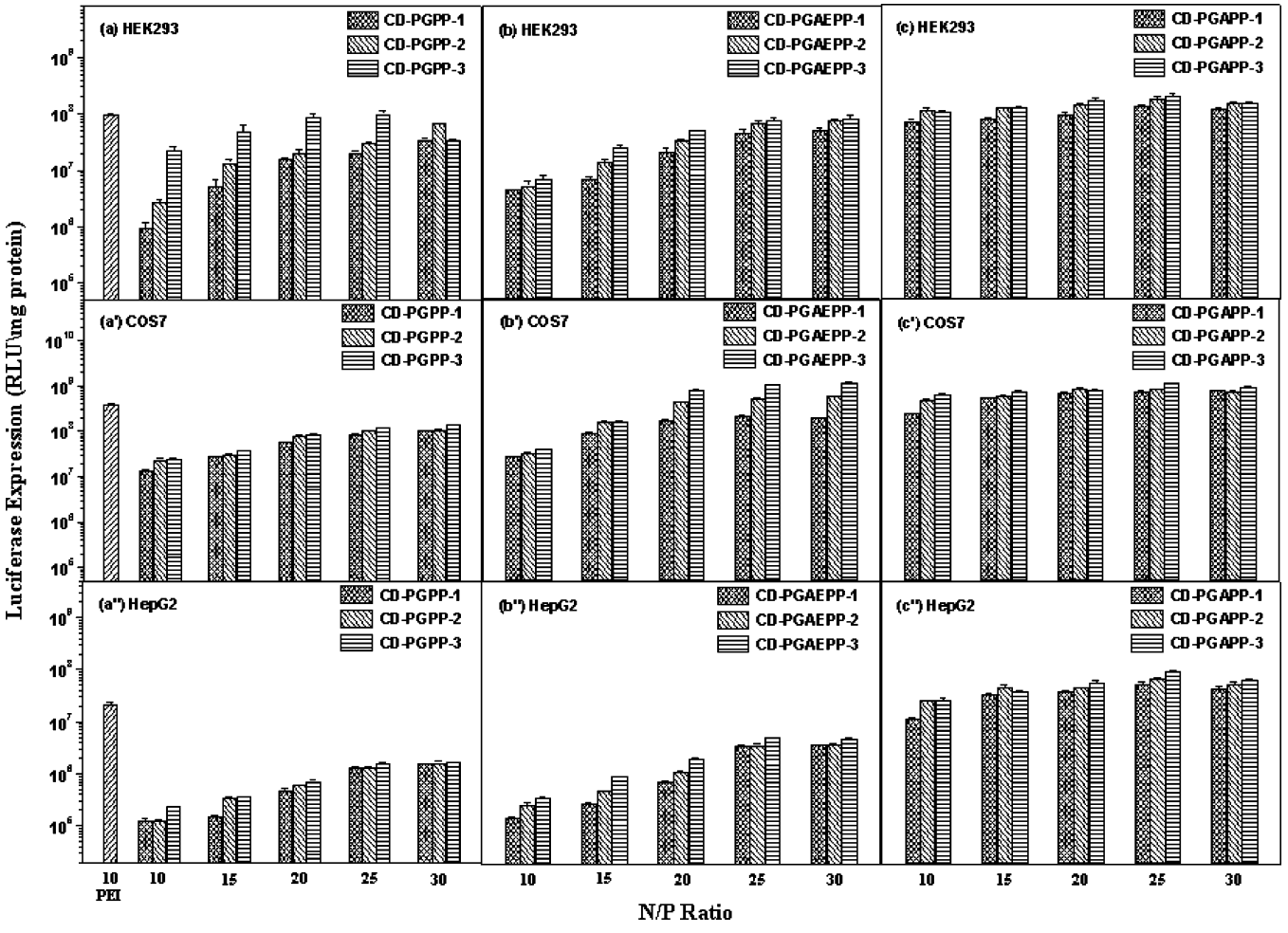

Figure 8. In vitro gene transfection efficiency mediated by CD-PGPP, CD-PGAEPP, and CD-PGAPP at various N/P ratios in comparison with that of PEI (25 kDa) (at the optimal N/P ratio of 10) in (a−c) HEK293, (a′−c′) COS7, and (a″−c″) HepG2 cells.

characteristics to the cationic carriers. It was also observed that there are no significant differences among the CD-PGPP, CD-PGAEPP, and CD-PGAPP vectors at the same N/P ratio in these different cell lines.

dx.doi.org/10.1021/am5005255 | ACS Appl. Mater. Interfaces 2014, 6, 3969−3978

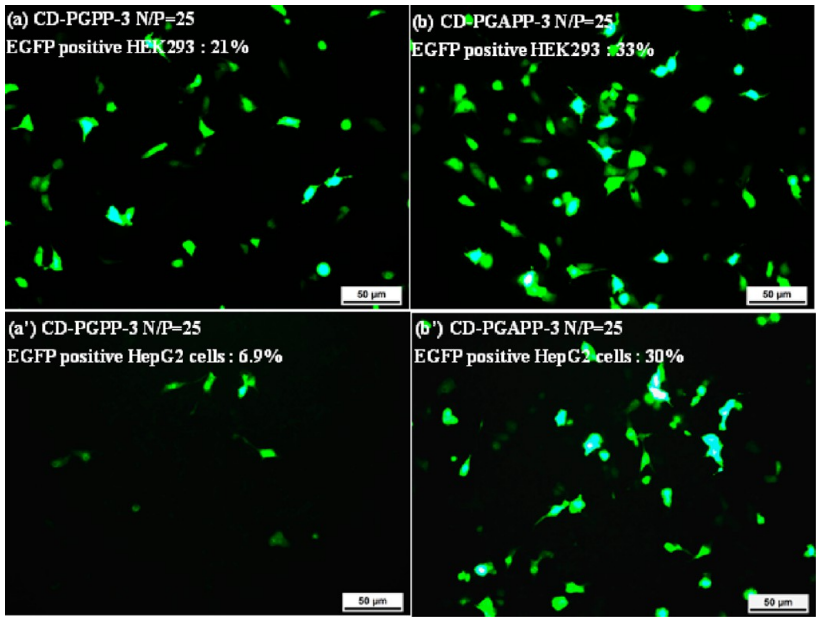

Figure 9. Representative images of EGFP expression mediated by (a and a′) CD-PGPP-3 and (b and b′) CD-PGAPP-3 at the optimal N/P ratio of 25 in HEK293 and HepG2 cells.

The cytotoxicity of the star-shaped polycations was also visibly evaluated via FDA-PI double-staining HEK293 and HepG2 cell lines mediated by CD-PGPP-3 and CD-PGAPP-3 at a ratio of 25. FDA, a nonpolar ester, stains viable cells exclusively. FDA can be hydrolyzed by intracellular esterases to produce polar fluorescein, which could pass slowly through a living cell membrane and exhibit green fluorescence when excited by blue light. Injured and dead cells are stained by PI, which intercalates with DNA and RNA to produce a bright-red fluorescent complex in the nuclei of dead cells after passing through damaged cell membranes. With FDA-PI, live and nonviable cells would be stained bright green and red, respectively.[42] As shown in Figure 7, the percentages of spherical cells stained red (nonviable cells) mediated by CD-PGPP-3 and CD-PGAPP-3 were significantly less than those of HEK293 and HepG2 cells treated by PEI. A significant higher number of living cells were observed for CD-PGPP-3 and CD-PGAPP-3, revealing lower cytotoxicity of CD-PGPP and CD-PGAPP. The results of FDA-PI staining assay were in agreement with the MTT assay results.

Gene Transfection Assay. Luciferase was first used as a gene reporter to evaluate the in vitro gene transfection efficiency of the polycation/pDNA complexes in HEK293, COS7, and HepG2 cell lines. Figure 8 shows the gene transfection efficiencies mediated by CD-PGPP, CD-PGAEPP, and CD-PGAPP at N/P ratios from 10 to 30 in comparison with that of PEI at its optimal N/P ratio of 10. The transfection efficiencies mediated by CD-cored vectors were dependent on their molecular weights, particularly for CD-PGPP and CD-PGAEPP. At the same N/P ratio, the CD-PGPP3, CD-PGAEPP3, and CD-PGAPP3 vectors with the longer arms exhibited higher transfection efficiencies than their corresponding counterparts, CD-PGPP1, CD-PGAEPP1, and CD-PGAPP1. The long polycation arms induce a higher transfection efficiency by improving the binding ability and complex stability.

In general, all of the polycations had optimal N/P ratios to exhibit the highest transfection efficiency. At the optimal N/P ratios, the appropriate concentration of amino groups contributed to improving the ability to penetrate the cell membranes. At higher N/P ratios, the further increased polycation concentration induced increasing cytotoxicity. In this work, the transfection efficiencies of CD-PGPP and CD-PGAEPP increased significantly first and then remained nearly unchanged at N/P ratios of 25 and above, probably arising from the low cytotoxicity of PP groups. The CD-PGAPPs exhibited a good performance in all of the cell lines, where no significant difference was observed in the transfection efficiency within the test N/P ratios.

The transfection efficiencies of CD-PGAPP were much higher than those of CD-PGPP and CD-PGAEPP, especially at lower N/P ratios in HEK293 and COS7 cells. The possible reason was that the CD-PGAPP containing pyrrolidinone residues possessed higher biocompatibility and greater affinity to pDNA than CD-PGPP and CD-PGAEPP. At higher N/P ratios of 25 and above, CD-PGPP and CD-PGAEPP exhibited transfection efficiencies comparable to those of CD-PGAPP. It is particularly noted that the transfection efficiencies mediated by CD-PGAPP in HepG2 cells were much higher than those mediated by CD-PGPP and CD-PGAEPP at various N/P ratios, possibly relating with the antitumor efficacy of the pyrrolidinone groups.[30]

Direct visualization of EGFP by gene expression in HEK293 and HepG2 cells was also carried out to further confirm the gene-delivery capabilities of CD-cored polycations. Figure 9 shows representative images of EGFP gene expression of carriers at their optimal N/P ratios. Fluorescence signals in delivering pEGFP mediated by CD-PGAPP-3 were much stronger than those mediated by CD-PGPP-3. The optimal percentages (determined using FCM) of the EGFP-positive HEK293 (or HepG2) cells for CD-PGPP-3 and CD-PGAPP-3 were 21% (or 6.9%) and 33% (or 30%), respectively. The more obvious difference among these polycations was observed in HepG2 cells. The above results were consistent with those of luciferase expression (Figure 8).

Hemolysis Assay. Under certain conditions including direct contact with water, RBCs swell to the critical bulk and then the cell membranes break up, leading to hemolysis, which may be aggravated in the presence of an extrinsic material. The following assembly of blood platelets can be intensified by the released adenosine diphosphate from the broken RBCs, which could expedite the formation of clotting and even thrombus. Thus, hemolysis of RBCs is quite a big problem related to the bioincompatibility of a material.[43] The hemolysis test results of PEI, CD-PGPP, CD-PGAEPP and CD-PGAPP at different concentrations are shown in Figure 10. In comparison with that

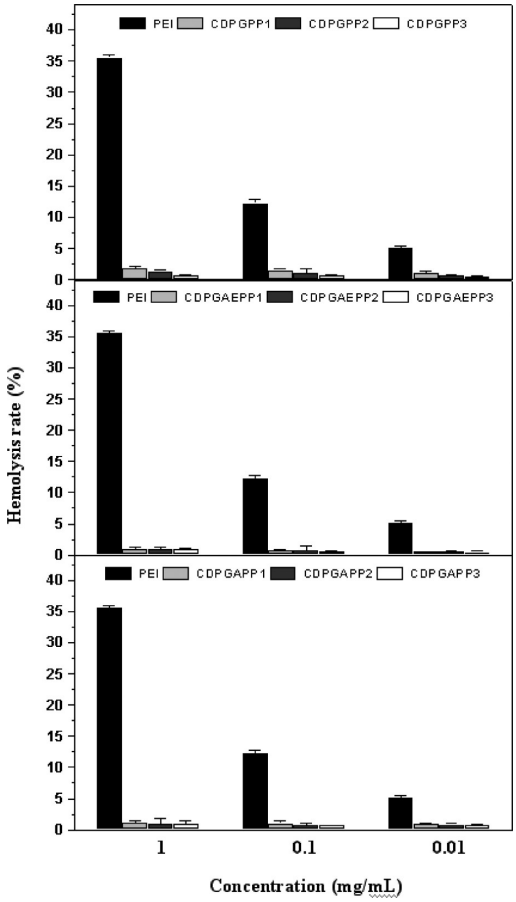

Figure 10. Hemolysis test results of PEI, CD-PGPP, CD-PGAEPP, and CD-PGAPP at different concentrations, where the hemolysis values of the positive control (water) and negative control (PBS) were 1 and 0, respectively.

(about 32%) of the PEI sample at a concentration of 1 mg/mL, all three kinds of CD-cored polycations exhibited extremely low hemolysis degrees below 2%. It was reported that the maximally permissible degree of hemolysis for biomaterials is 5%.[44] The CD-cored samples can be applied as biomaterials without inducing obvious hemolysis. The negligible hemolysis degrees may contribute to the lower cytotoxicity of CD-cored vectors (Figure 6) and their plentiful nonionic hydrophilic hydroxyl units. In addition, CD-PGAPP contains abundant pyrrolidinone groups, which could remain stable in blood.[30]

CONCLUSIONS

Different well-defined star-shaped cationic CD-PGPP, CD-PGAEPP, and CD-PGAPP containing β-CD cores were successfully prepared and systematically compared. CD-PGPP, CD-PGAEPP, and CD-PGAPP with different arm lengths could effectively bind pDNA to produce nanoparticle complexes of 100−200 nm with positive ζ potentials of 25−40 mV. CD-PGPP, CD-PGAEPP, and CD-PGAPP exhibited significantly lower cytotoxicity than control PEI (25 kDa). In most cases, CD-PGAPP exhibited a better gene transfection performance than CD-PGPP and CD-PGAEPP particularly in HepG2 cells. Furthermore, CD-PGPP, CD-PGAEPP, and CD-PGAPP caused extremely low hemolysis, which indicated that they may possess good blood compatibility. Therefore, structural tailoring of starlike CD-cored polymers with functionalization provided a universal method for the design of novel efficient gene vectors for gene therapy applications.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (Grants 21074007, 51173014, 51221002, 51325304, 51373017, and 51302009) and Research Fund for the Doctoral Program of Higher Education of China (Project 20120010120007).

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