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Introducing Primary and Tertiary Amino Groups into a Neutral Polymer: A Simple Way to Fabricating Highly Efficient Nonviral Vectors for Gene Delivery

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ABSTRACT: In this work, a brushed polycationic polymer with primary and tertiary amino groups was designed and synthesized for gene delivery. The backbone polymer was poly(*N*-hydroxyethylacrylamide) (PHEAA) by the atom transfer radical polymerization (ATRP), and then 3,3′-diaminodipropylamine (DPA) was grafted onto the PHEAA by the reaction between hydroxyl and the secondary amine. A brushed PHEAA-DPA cationic polymer was achieved with primary and tertiary amino groups and the ratio was 2 : 1. The $PHEAA_{100}$-DPA and $PHEAA_{200}$-DPA could effectively condense plasmid DNA (pDNA) at the weight ratio of vector/DNA of 0.6 and 0.4, respectively. The cytotoxicity of PHEAA-DPA/pDNA to COS-7 cells and HepG-2 cells within the weight ratio of vector/DNA of 16 : 1 was lower than that of PEI25k, and cell viability decreased with the increment of the weight ratio. Although the cytotoxicity of $PHEAA_{100}$-DPA/pDNA was lower than $PHEAA_{200}$-DPA/pDNA, the latter possessed higher transfection efficiency at the same weight ratio both in COS-7 cells and HepG-2 cells, compared with PEI25k, the transfection efficiency of $PHEAA_{200}$-DPA/pDNA was better in COS-7 cells and HepG-2 cells with the weight ratio of 12 : 1 and 10 : 1, respectively. These results showed that the PHEAA-DPA with less cytotoxicity and higher gene transfection efficiency has a broad perspective in gene therapy. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 40468.

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INTRODUCTION

Over the past decade, gene therapy which is capable of delivering appropriate, exogenous DNA into cells to express, enhance, or suppress a targeted gene has been regarded as a promising cure for life-threatening disease.[1–3] A delivery vector with low cytotoxicity and high transfection efficiency is a significant factor to achieve successful gene therapy.[4–6] Nonviral vectors such as cationic polymers would be promising vehicles for gene therapy due to their easy manipulation, low cytotoxicity, good biocompatibility, and high flexibility corresponding to the size of the transgene delivered.[7–9] All cationic polymers for gene delivery contain protonable amines such as primary, secondary, tertiary, or quaternary amino groups, which can condense plasmid DNA (pDNA) by electrostatic interaction under physiological conditions.[10,11] Usually, the effect of various amino groups on gene vector is different. The primary amine groups with a high pKa promote to condense DNA effectively and protect DNA, while the secondary and tertiary amine groups with a low pKa are speculated that buffers at a low pH in the endolysosomal compartments and potentially induces ruptures of the endolysosomal membrane to facilitate the release of pDNA into the cytoplasma through proton sponge mechanism.[12,13] For the poly(L-lysine)(PLL), the number of primary amino is significant for forming stable complexes with DNA.[14] On the other hand, all primary amino groups of PLL are protonated at physiological pH, yielding a structure with no buffering capacity to aid in endosomal escape.[15] The branched polyethyleneimine (PEI) which contains a 1 : 2 : 1 ratio of primary/secondary/tertiary aminos possesses nitrogen at every third atom, resulting in a high charge density capacity. As a result, PEI lends itself to protonation, with the charge density proportional to the pH of the biological environment.[16,17] Recently, Shen et al.[18] reported a poly-D, L-succinimide (PSI)-based polymer which mimicked the structure of branched PEI, and investigated the influence of primary, secondary, and tertiary amino group ratio on transfection efficiency and biocompatibility. The results showed that the DNA binding ability of PSI derivatives depended on the amount of tertiary amines, and exhibited better transfection ability and

biocompatibility than PEI *in vitro* and *in vivo* biological assay with the same amino group ratio of branched PEI.

Polyamidoamine (PAMAM) dendrimers also contain multivalent amine with primary amine groups at the surface and tertiary amine groups in the interior, and showed efficient transfection due to primary amine groups participating in DNA binding and tertiary amine groups exerting endosome buffering effect.[19–21] For poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA), it can mediate efficient transfection in COS-7 and OVCAR-3 cells although it only contains tertiary amines and possessed high buffer capacity at endosomal pH.[22,23] Zhu et al.[24] incorporated primary amino into DMAEMA-based copolymers which contain primary and tertiary amino side groups for enhancing gene transfection. The copolymers remained a good buffer capacity at endosomal pH and had a higher transfection activity than PDMAEMA due to the introduction of primary amino.

In this study, we designed a cationic polymer with primary amino and tertiary amino for gene delivery. The backbone polymer was neutral poly(*N*-hydroxyethylacrylamide) (HEAA) prepared by the atom transfer radical polymerization (ATRP), and then 3,3′-diaminodipropylamine (DPA) was grafted onto the PHEAA. The product PHEAA-DPA possessed primary amine and tertiary amine and the ratio is 2 : 1. The physicochemical properties of PHEAA-DPA/DNA complexes, cytotoxicity as well as *in vitro* transfection in COS-7 cells and HepG-2 cells were investigated. In addition, the effect of molecular weight on both the gene transfection efficiency and cytotoxicity was also evaluated by precisely controlled the molecular weight of PHEAA backbone over ATPR and the same DPA grafting ratio.

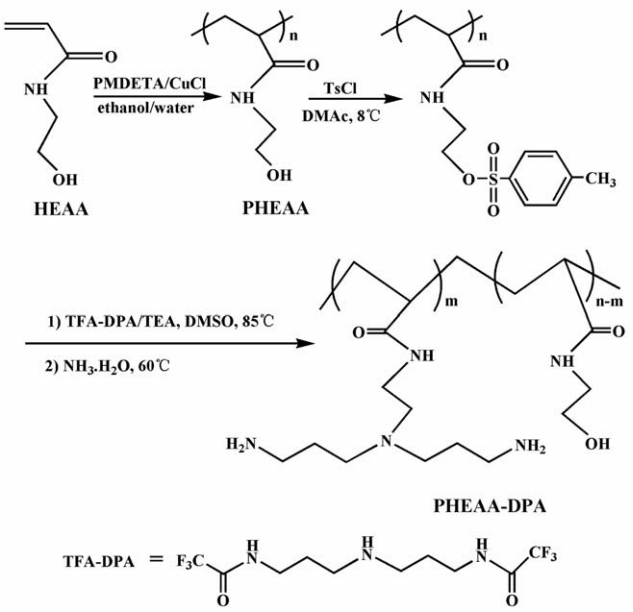
MATERIALS AND METHODS

Materials

N-hydroxyethylacrylamide (HEAA), 3,3′-diaminodipropylamine (DPA), ethyl-2-bromoisobutanoate (EBiB, 98%), *N,N,N′,N″,N‴*-pentamethyldiethylenetriamine (PMDETA, 99%), CuCl (99.999%) and ethyl trifluoroacetate were supplied by Alfa Aesar. Branched polyethylenimine (PEI, 99%, $M_w = 25{,}000$) and Poly(L-lysine) (PLL, 98% $M_n = 8000$) were obtained from Sigma Aldrich. Dimethylacetamide (DMAc), triethylamine (TEA) and dimethyl sulfoxide (DMSO) were purchased from Kewei Company (Tianjin University, Tianjin China). Plasmid pGL3-control with SV40 promoter and enhancer sequences encoding luciferase (pDNA) was obtained from Promega, Madison, WI. The plasmid was amplified in Escherichia coli and purified by the differential precipitation method. All other reagents were of analytical grades and used without further purification.

Synthesis of PHEAA by ATRP

The synthesis procedure and molecular structure of PHEAA were depicted in Scheme 1. The details of the preparation of $PHEAA_{200}$ were given below as a typical example. HEAA (20 mmol), CuCl (0.1 mmol), and ethanol/H_2O (v/v = 4/1, 10 mL) were mixed into a dry Schlenk flask. Then the Schlenk flask was degassed by three freeze-thaw cycles. After that, N_2-purged mixture of PMDETA (0.1 mmol), EBiB (0.1 mmol) and ethanol/H_2O (v/v = 4/1, 2 mL) was injected into



Scheme 1. Schematic illustration of synthesis of PHEAA and PHEAA-DPA.

the frozen reaction system. Then the mixture was degassed by another three freeze-thaw cycles. During the last cycle, the Schlenk flask was filled with nitrogen. The reaction was kept for 12 h at room temperature. Then the reaction product was dissolved in deionized water followed by dialysis in a Cellu SepH1-membrane (MWCO = 3500). Finally, the product was collected by freeze-drying overnight. PHEAA with different chain lengths were synthesized at varied molar ratios of monomer to initiator.

Preparation of PHEAA-DPA

The protection of primary amine of DPA is according to Mary's work.[25] Briefly, DPA (7 mmol) in acetonitrile (15 mL) was added to ethyl trifluoroacetate (24.6 mmol) and water (152 μL). The solution was refluxed for 24 h and the solvent was removed to give a buff-colored solid which was washed with dichloromethane (3 × 80 mL) yielding trifluoroacetylated DPA (TFA-DPA) as a pale yellow-colored solid. The compound was recrystallized from ethyl acetate (3 × 80 mL) to produce a white solid.

According to the literature,[26] the tosyl chloride was employed to active the hydroxyl. PHEAA (1.15 g), water-free LiCl (10 mmol) and DMAc were mixed and stirred at 80°C for complete dissolution of PHEAA in DMAc. Then, the solution of triethylamine (11 mL) in DMAc (11 mL) and tosyl chloride (40 mmol) in DMAc (15 mL) was dropped into the mixture at 8°C. The reaction was stirring at 8°C for 24 h. Finally, the product was isolated by precipitation in 500 mL ice water, and then washed with ice water (2 × 200 mL) and isopropanol (3 × 200 mL) to remove the solvent and unreacted substance. Then the product was dried at 50°C under vacuum.

A solution of tosylated PHEAA (1 mmol), TFA-DPA (4 mmol), and TEA (8 mL) in DMSO (32 mL) was stirring for 72 h at 85°C under nitrogen. After that, the reactor was dialyzed for 2 days to remove the solvent and impurities. The solution was distilled to 30 mL and transferred into a three-neck flask. At the

same time, 7.5 mL aqueous ammonia was added. The reaction was stirred for another 48 h at 60°C with nitrogen. The product was dialyzed (MWCO = 3500) for 72 h by changing deionized water every 8 h. The product we obtained was freeze-drying overnight.

^{1}H-NMR spectra of PHEAA and PHEAA-DPA were measured with a UNITY plus-500 NMR spectrometer (Varian, USA) using D_2O as a solvent.

Buffering Capacity

The proton buffering capacities of the $PHEAA_{100}$-DPA and $PHEAA_{200}$-DPA were determined via acid-base titration methods according to the literature.[27–29] Briefly, 6 mg polymer was dissolved in 30 mL of 150 m*M* sodium chloride to give a final concentration of 0.2 mg/mL. Then the solution was adjusted to an initial pH of 10.0 by 0.1*M* NaOH. Subsequently, in order to make the basic polymer solution titrated to pH 2.2, the aliquots of 0.1*M* HCl was added with various volume increments. The pH values of all solutions were measured by a microprocessor pH meter after each addition. PEI and PLL were used as a control.

Preparation of PHEAA-DPA/pDNA Complexes

PHEAA-DPA was dissolved in ultrapure water. The PHEAA-DPA/pDNA complexes were formed at different weight ratios by adding PHEAA-DPA of desired concentrations to an equal volume of a defined pDNA solution. The complexes were homogeneously mixed and incubated for 30 min at room temperature.

The ability of PHEAA-DPA binding pDNA was assessed by the agarose gel electrophoresis assay. The PHEAA-DPA/pDNA complexes with varied weight ratios were prepared as mentioned above. Totally, 10 μL complex solutions were loaded in a well on a 1% agarose gel with EB staining and Tris-acetate (TAE) running buffer at 100 V for 30 min. The images were captured through Bio-Imaging Systems (UVP).

The morphology and size of the PHEAA-DPA/pDNA complexes at the selected weight ratios of 5 : 1, 10 : 1, and 15 : 1 were observed by JEOL JEM-100CXII TEM. Briefly, a drop of the complex solution was transferred on a carbon-coated grid for 5 min and stained with 1.5 wt % phosphotungstic acid. Then the complexes were recorded on films with TEM.

Zeta potentials of PHEAA-DPA/DNA complexes at varied weight ratios were tested by Beckman Coulter Zeta analyzer (Delsa^{TM}Nano Zeta Potential). The process of complexing was the same method mentioned above. The final concentration of pDNA was 5 μg/mL.

Cell Culture and Cytotoxicity Assay

African green monkey kidney cells (COS-7) and HepG-2 purchased from Peking Union Medical College (Beijing, China) were incubated in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C in 5% CO_2 humidified atmosphere.

COS-7 cells and HepG-2 cells were seeded in a 96-well plate at 2×10^4cells/well and incubated for 24 h at 37°C in 5% CO_2 humidified atmosphere. PHEAA-DPA with the increasing concentrations from 0 to 48 μg/mL was added into each well and incubated for 24 h before replacing the medium with fresh complete medium (200 μL/well). After incubation for another 24 h, 20 μL/well 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide stock solution (MTT, 5 mg/mL in PBS) was added to each well, and the plate was further incubated for 4 h. Then, all media were removed and 150 μL/well DMSO was added, followed by shaking for 30 min at room temperature. The absorbance of each well was measured at 570 nm on a $\sum$960 plate-reader (Metertech) with pure DMSO as a blank. Non-treated cell (in DMEM) was used as a control and the relative cell viability (mean% ± SD, $n = 3$) was expressed as follows:

$$\text{Cell viability}\,(\%) = \text{Abs}_{\text{sample}} / \text{Abs}_{\text{control}} \times 100\%$$

In Vitro Transfection and Luciferase Assay

The in vitro transfection efficiency of PHEAA-DPA vector was evaluated in COS-7 cells and HepG-2 cells. Typically, the cells were seeded at a density of 5×10^4cells/well in 24-well plates and incubated for 24 h at 37°C, in 5% CO_2 humidified atmosphere. Before transfection, the medium in each well was replaced with 450 μL DMEM containing 10% FBS. PHEAA-DPA/pDNA complexes (50 μL, containing 1 μg DNA) at various weight ratios were then added to each well ($n = 3$ for each ratio). After incubating at 37°C for 24 h, the complexes that were not internalized were removed by replacing with fresh medium. The transfected cells were incubated for an additional 24 h. After incubation, the culture medium was removed and the cells were washed with PBS twice. The cells in each well were treated for 15 min with 150 μL of reporter lysis buffer (RLB, Promega) followed by freeze-thaw cycles to ensure complete lysis. The lysate was centrifuged for 3 min at 13,000 rpm and the supernatant was collected for luminescence measurements. The luminescence of each sample was measured by 1420 Multilabel counter (Wallac, USA) using Bright-GloTM luciferase assay system (Promega, USA) according to the manufacturer's protocol. The results were expressed as relative light units (RLU) per milligram of cell protein, and the protein concentration of each well was measured by a BCA protein assay (Pierce, Rockford, IL). PEI25k served as a positive control. PEI25k/pDNA complex with a weight ratio of 2 : 1 was formed based on the optimization of transfection efficiency and toxicity as suggested protocol.[30]

Statistical Analysis

Data are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using two population Student's *t*-test to evaluate the cytotoxicity and transfection efficiency with $P < 0.05$.

RESULTS AND DISCUSSION

The Structure of PHEAA and PHEAA-DPA

Synthesis procedure was illustrated in Scheme 1. First, the PHEAA was prepared by ATRP. PHEAA polymerized by the ATRP was regarded as the nonionic, water-soluble, and hydrolysis-resistant polymer with a well-defined molecular weight, low toxicity and excellent biocompatibility.[31,32] It has been reported that PHEAA-based materials with different polymeric architecture forms were extensively used in biomedical

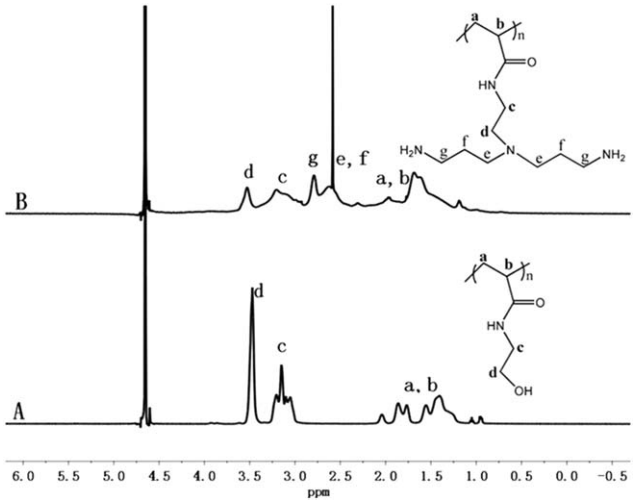


Figure 1. ^{1}H-NMR spectra of $PHEAA_{100}$ and $PHEAA_{100}$-DPA (D_2O).

field, including nonviral vector.[33,34] The hydroxyls of PHEAA can react with amino, carboxyl, and hydroxyl group by 1,1′-carbonyldiimidazole or tosyl chloride activation. To make sure that the primary amino can be preserved and the secondary amine of DPA was employed to react with hydroxyls of PHEAA, the tosylated PHEAA was synthesized by the reaction of PHEAA with tosyl chloride in DMAc. After that, the tosylated groups of tosylated PHEAA which could be solved in DMSO were substituted by trifluoroacetylated DPA. Finally, PHEAA-DPA was prepared by the deprotection of primary amines in ammonia water.

The obtained polymer was only soluble in aqueous solution. Six gel permeation chromatography (GPC) instruments with different models were employed but failed to determine the molecular weights of the synthesized polymers because of the electrostatic interaction between the cationic polymers and the chromatographic column. So in order to measure the conversion rate of HEAA monomer, a certain amount of ATRP product was characterized using ^{1}H-NMR before dialysis. The conversion rate is 55% calculated from the integral area of the peak. Thus the molecular weight of $PHEAA_{100}$ and $PHEAA_{200}$ are 6.3 kDa and 12.7 kDa, respectively. Figure 1 showed the ^{1}H-NMR spectra of $PHEAA_{100}$ and $PHEAA_{100}$-DPA. In Figure 1(A), the characteristic peaks of $PHEAA_{100}$ are present: δ1.2–1.8 (-CH_2CH_2-), δ3.2 (-NHCH_2-), δ3.5 (-CH_2OH).[31] Figure 1(B) shows the feature signals of $PHEAA_{100}$-DPA. Except for the peaks of PHEAA, δ2.8, and δ2.3-2.6 are assigned to -CH_2NH_2 and -CH_2CH_2-, respectively. It indicates that the DPA has been linked to the PHEAA. The degree of substitution (DS), defined as the percentage of DPA coupled to the $PHEAA_{100}$, is calculated using the following formula:

$$DS_{DPA} = \frac{(CH_2)_3/3}{(CH_2)_2/2} \times 100\%$$

Where DS_{DPA} is the degrees of substitution of DPA, $(CH_2)_2$ is the integral of the ethyl peaks of $PHEAA_{100}$ locating at 3.2–3.5 ppm, while $(CH_2)_3$ is the integral of the propyl groups peaks of DPA locating at 2.3–2.8 ppm. To inspect the effect of molecular weight of the polymers on transfection efficiency, the DS_{DPA} value of $PHEAA_{100}$-DPA and $PHEAA_{200}$-DPA was fixed equally at 38%.

Buffer Capability

The buffer capability of gene vectors is very crucial for the complexes to assist DNA to escape from the endosomal compartments, thus improving transfection efficiency.[35] Generally, cationic polymers will undergo a drop in pH from neutral to 5.0 when entering cells by endocytic pathways.[36] The proton buffering capacities of the $PHEAA_{100}$-DPA and $PHEAA_{200}$-DPA are shown in Figure 2. It is obvious that $PHEAA_{100}$-DPA and $PHEAA_{200}$-DPA have similar buffer capabilities which are lower than that of PEI25k and are higher than that of PLL. Some studies reported that the buffer capability of polycations mainly depended on the presence of primary, secondary and tertiary amines groups.[18,24,37] Both $PHEAA_{100}$-DPA and $PHEAA_{200}$-DPA have primary and tertiary amino groups in polymer chains. The similar buffer capability exhibited by $PHEAA_{100}$-DPA and $PHEAA_{200}$-DPA is probably due to the little different density of amine groups on them. It is well known that PLL has no buffer capability at physiological pH because all primary amino groups with a high pKa are protonated.[5] The existence of tertiary amine in $PHEAA_{100}$-DPA and $PHEAA_{200}$-DPA may improve the buffer capability leading to the better buffer capability than PLL. Although the two polycations possess lower buffer capability than PEI25k due to the lower positive charge density than PEI, they will demonstrate the higher or comparable transfection efficiencies in comparison with PEI25k, as shown in the following experiment. It is acknowledged that the proper buffer capability can promote the transfection efficiency since pDNA could be released easily from the complexes in cells.[38]

pDNA Condensation by PHEAA-DPA

For efficient gene delivery, the ability of cationic polymers to condense pDNA is a prerequisite. The agarose gel electrophoresis assay was performed at various weight ratios, aiming at evaluating the ability of PHEAA-DPA induced pDNA condensation (Figure 3). $PHEAA_{100}$-DPA and $PHEAA_{200}$-DPA can completely retard pDNA at the weight ratio of 0.6 and 0.4, respectively. There are primary amino and tertiary amino groups in

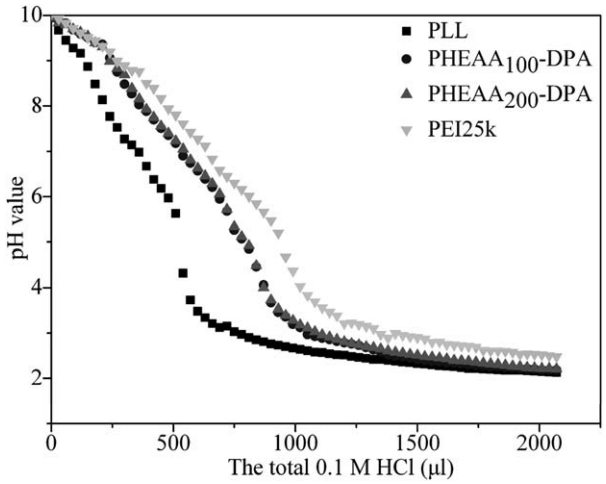


Figure 2. Acid–base titration profiles of $PHEAA_{100}$-DPA and $PHEAA_{200}$-DPA in 0.15*M* NaCl.

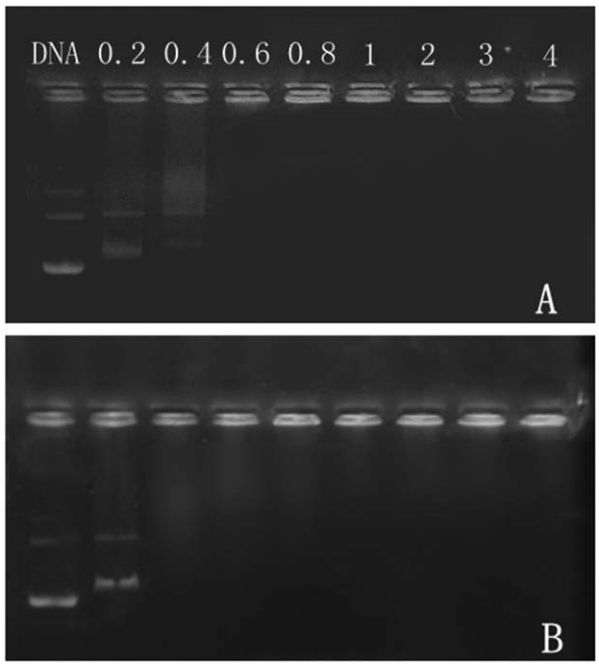


Figure 3. Agarose gel electrophoresis retardation assay of $PHEAA_{100}$-DPA/pDNA complexes (A) and $PHEAA_{200}$-DPA/pDNA complexes (B).

PHEAA-DPA. The existence of these protonable amines makes the PHEAA-DPA form complexes with pDNA by electrostatic interactions between positively charged of PHEAA-DPA and negatively charged phosphate backbone of pDNA. Apparently, $PHEAA_{200}$-DPA is more efficient in condensing pDNA than $PHEAA_{100}$-DPA. For cationic polymers, the DNA binding ability is affected by multiple parameters such as the amount of primary amines and tertiary amines, the ionization degree and the charge density of the carrier, etc.[18,24,39,40] For example, the number of primary amine is critical for PLL to form stable complexes with pDNA because only the PLL with molecular weight more than 3000 Da can condense pDNA effectively.[14] While for the two cationic polymers, the similarity is the existence of primary amines and tertiary amines, and the ratio of primary amines to tertiary amines is fixed at 2 : 1 in $PHEAA_{200}$-DPA and the $PHEAA_{100}$-DPA. Meanwhile, the number of DPA units which provide all the amine groups is the same between $PHEAA_{200}$-DPA and $PHEAA_{100}$-DPA at the same weight. However, the number of charged amine groups in $PHEAA_{200}$-DPA may be greater than that of $PHEAA_{100}$-DPA due to larger spatial separation between the charged groups. So the $PHEAA_{200}$-DPA with more charged amine groups can condense pDNA more efficiently.

The size of polycation/DNA complexes is an important parameter influencing endocytosis and transfection efficiency.[41] Herein, TEM was employed to observe the morphology of $PHEAA_{100}$-DPA/pDNA and $PHEAA_{200}$-DPA/pDNA complexes at selected weight ratios of 5 : 1, 10 : 1, and 15 : 1. As shown in Figure 4, $PHEAA_{100}$-DPA and $PHEAA_{200}$-DPA can condense pDNA into spherical nanoparticles with similar diameters between 20 nm and 30 nm. According to the size distribution of the complexes, the average particle diameters in the TEM images A-F are 28.8 nm, 28.7 nm, 24.4 nm, 24.7 nm, 20.6 nm, and 19.7 nm, respectively. Overall, the particle size of $PHEAA_{200}$-DPA/pDNA complexes is a slightly smaller than $PHEAA_{100}$-DPA/pDNA complexes at the same weight ratio.

We have measured the size of PHEAA-DPA/pDNA complexes at various weight ratios in water (Table I) and in 150 m*M* NaCl solution (Table II) by laser particle size analyzer (DLS). It is obvious that the average particle sizes of $PHEAA_{100}$-DPA/pDNA and $PHEAA_{200}$-DPA/pDNA complexes decreases with the increment of the weight ratio both in water and 150 m*M* NaCl solution, and the particle size of $PHEAA_{200}$-DPA/pDNA complexes shows a smaller diameter than that of $PHEAA_{100}$-DPA/pDNA complexes at same weight ratio which is in accordance with the tendency in TEM images. Compared with in water, the sizes of the complexes formed by $PHEAA_{100}$-DPA and $PHEAA_{200}$-DPA increase dramatically in 150 m*M* NaCl solution. It indicated that the salt ions can screen the electrostatic repulsion between individual complexes and cause them to aggregate as the literature reported.[42,43] Nonetheless, these sizes of complexes could be internalized into cells and achieved efficient gene transfection. The simlar results have also been reported in our previous work.[42]

The size of the complex is attributed to the physical properties of the cationic polymer, and it has been correlated with the molecular weight of the polymer and the content of protonable amines.[5] For different cationic polymers, the effect of molecular weight on particle size of complex varies. It is reported that PLL with low molecular weight forms DNA complex with smaller nanoparticles than high molecular weight species,[44] while low-molecular-weight PEI/DNA complexes have much larger size than those of high-molecular-weight PEI.[45,46] For PHEAA-DPA cationic polymers, they have the same tendency with PEI. There are more charged amine groups in the $PHEAA_{200}$-DPA due to its larger spatial separation with lower electrostatic repulsion between the charged groups and provide more positive charges; as a result, the interaction between the $PHEAA_{200}$-DPA and pDNA is stronger and smaller nanoparticles are formed.

The net charge of complexes is also an important factor affecting gene transfection. In most cases, the complexes with positive surface charge can bind to negatively charged cell membrane surfaces.[47,48] Herein, the zeta potentials of $PHEAA_{100}$-DPA/pDNA and $PHEAA_{200}$-DPA/pDNA complexes were evaluated in order to inspect the surface charges of complexes. As shown in Figure 5, the zeta potentials of the both complexes are increased with the increment of weight ratio. This variation trend is similar to that reported previously.[49,50] An explanation is that the positive charges of PHEAA-DPA are increased with weight ratio, and there are more surplus positive charges on the surface of complexes after interaction with negatively charged phosphate backbone of pDNA. Obviously, the zeta potentials of $PHEAA_{200}$-DPA/pDNA complexes are higher than those of $PHEAA_{100}$-DPA/pDNA complexes at the same weight ratio. This phenomenon is in accordance with the ability of cationic vectors to condense DNA. It is evident that $PHEAA_{200}$-DPA with more charged amine groups can provide more positive charges.

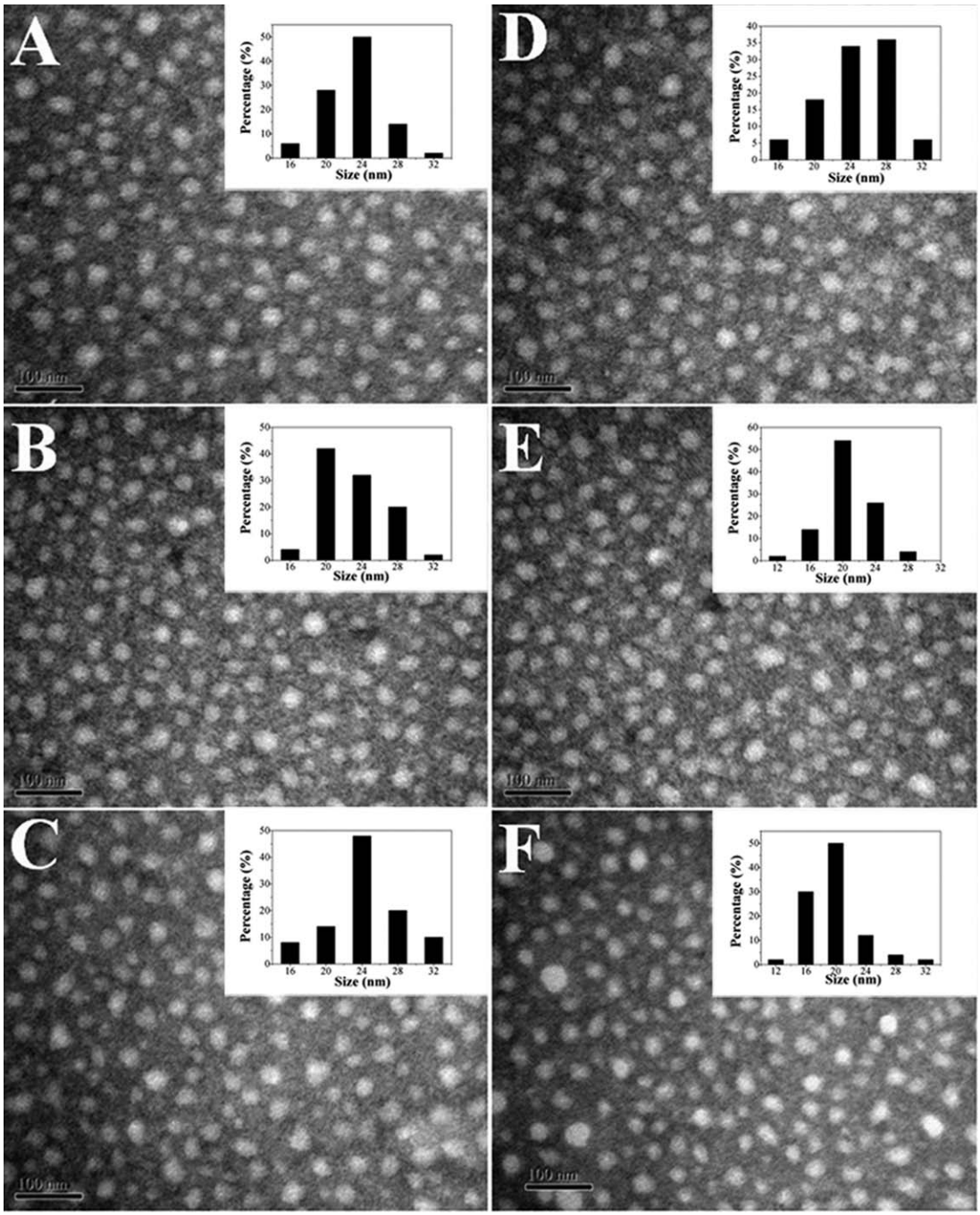


Figure 4. TEM images of $PHEAA_{100}$-DPA/pDNA complexes at selected ratios of 5 : 1 (A), 10 : 1 (B), 15 : 1 (C); and $PHEAA_{200}$-DPA/pDNA complexes at selected ratios of 5 : 1 (D), 10 : 1 (E), 15 : 1 (F). Scale bar = 100 nm.

Cell Viability Evaluation

As a potential gene delivery system, it must possess a low cytotoxicity and excellent biocompatibility. Herein, the cytotoxicity of PHEAA-DPA to COS-7 cells and HepG-2 cells was evaluated by MTT assay (Figures 6 and 7). The results show that more than 80% COS-7 cells and HepG-2 cells are yet viable for PHEAA-DPA when the weight ratio goes up to 16 : 1, and the cell viability of PHEAA-DPA is nearly comparable to that of PEI25k at the weight ratio of 2 : 1. It demonstrates that PHEAA-DPA has low cytotoxicity. However, increasing the weight ratio tends to result in the decrease of cell viability because of the augment of positive charge density. As shown both in Figures 6 and 7, $PHEAA_{100}$-DPA has a lower cytotoxicity than $PHEAA_{200}$-DPA at the same weight ratio. It is most probable that the low molecular weight is the main reason for the reduced cytotoxicity of $PHEAA_{100}$-DPA when compared with $PHEAA_{200}$-DPA. Similarly, the increased cytotoxicity of cationic vectors with higher molecular weight was also observed for PLL and poly(amidoamines).[51] It is well established that the irreversible cell membrane damage caused by

Table I. Size of Vector/DNA Complexes in Water

Weight ratios	Average particle size (nm)	
	$PHEAA_{100}$-DPA/pDNA	$PHEAA_{200}$-DPA/pDNA
5 : 1	231.5 ± 18.7	215.3 ± 12.3
10 : 1	187.1 ± 16.5	143.9 ± 11.3
15 : 1	151.4 ± 21.8	118.7 ± 17.6
20 : 1	125.7 ± 23.8	103.3 ± 15.6
25 : 1	102.9 ± 14.3	92.1 ± 9.5
30 : 1	99.2 ± 15.1	88.9 ± 12.3

Table II. Size of Vector/DNA Complexes in 150 m*M* NaCl Solution

Weight ratios	Average particle size (nm)	
	$PHEAA_{100}$-DPA/pDNA	$PHEAA_{200}$-DPA/pDNA
5 : 1	493.1 ± 35.6	438.2 ± 38.5
10 : 1	401.2 ± 26.3	367.1 ± 28.9
15 : 1	336.2 ± 32.8	239.8 ± 38.5
20 : 1	323.6 ± 25.6	219.4 ± 19.5
25 : 1	275.5 ± 34.3	215.8 ± 27.2
30 : 1	230.6 ± 24.1	194.3 ± 22.8

cationic polymers is the main reason of cytotoxicity.[52,53] For $PHEAA_{200}$-DPA, the number of charged amine groups is larger due to lower electrostatic repulsion between the charged groups at the same number of DPA units, resulting in the higher positive charge density. Thus the interaction between cationic molecules and plasma membranes increases, which may cause damage to cells.[54]

In Vitro Gene Transfection

The gene transfection of PHEAA-DPA/pDNA complexes was assessed in COS-7 cells and HepG-2 cells using pGL-3 plasmid as a luciferase reporter gene. The gene transfection of COS-7 cells is shown in Figure 8. The transfection efficiency of

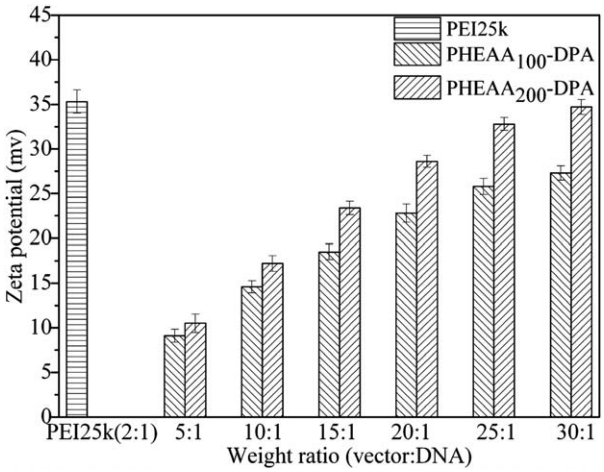


Figure 5. Zeta potentials of $PHEAA_{100}$-DPA/pDNA and $PHEAA_{200}$-DPA/pDNA complexes obtained at various weight ratios.

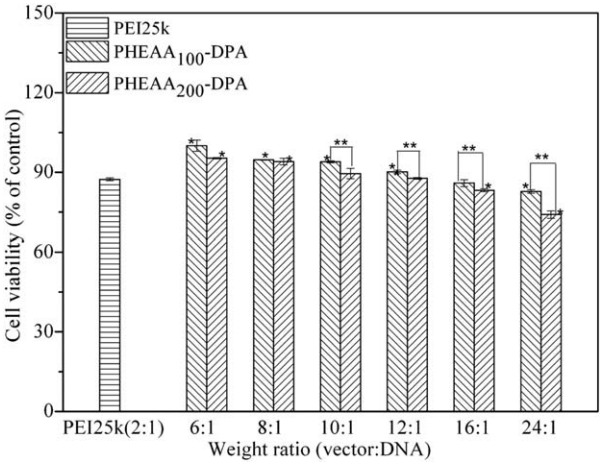


Figure 6. Cytotoxicity of $PHEAA_{100}$-DPA and $PHEAA_{200}$-DPA at different concentrations for COS-7 cells. Data represent mean ± SD ($n = 3$). $P^* < 0.05$ (compared with the control) and $P^{**} < 0.05$ (compared the two complexes at the same weight ratio).

PHEAA-DPA/pDNA complexes firstly increases and then decreases with the weight ratio. Moreover, the transfection efficiency of $PHEAA_{200}$-DPA/pDNA complexes is higher than that of $PHEAA_{100}$-DPA/pDNA complexes at the same weight ratio. It indicates that the $PHEAA_{200}$-DPA has better transfection efficiency for COS-7 cells. Notably, the $PHEAA_{200}$-DPA/pDNA complexes at weight ratio of 12 : 1 achieves luciferase a gene expression level of 6.05×10^7 RLU/mg protein in COS-7 cells, which is higher than that of PEI25k. However, the transfection efficiency of $PHEAA_{200}$-DPA decreases at 16 : 1.

For the HepG-2 cells, the same tendency of gene transfection as COS-7 cells is obtained at weight ratios from 8 : 1 to 16 : 1 (Figure 9). In contrast, the gene transfection efficiency of $PHEAA_{200}$-DPA/pDNA complexes is higher than PEI25k at the weight ratio of 10 : 1. The maximal transfection efficiency can reach luciferase gene expression level of 2.33×10^6 RLU/mg protein, which was 1.6-fold that of PEI25k.

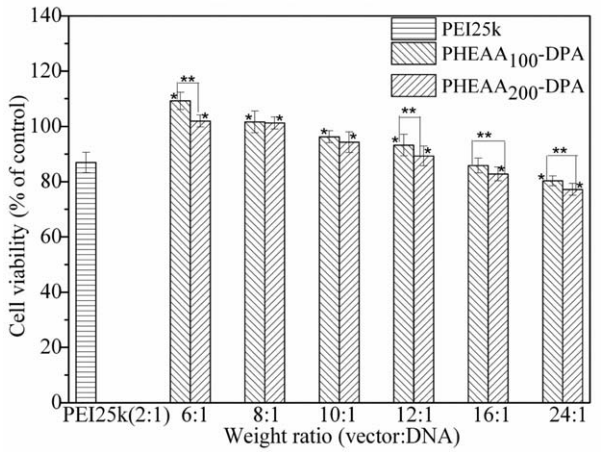


Figure 7. Cytotoxicity of $PHEAA_{100}$-DPA and $PHEAA_{200}$-DPA at different concentrations for HepG-2 cells. Data represent mean ± SD ($n = 3$). $P^* < 0.05$ (compared with the control) and $P^{**} < 0.05$ (compared the two complexes at the same weight ratio).

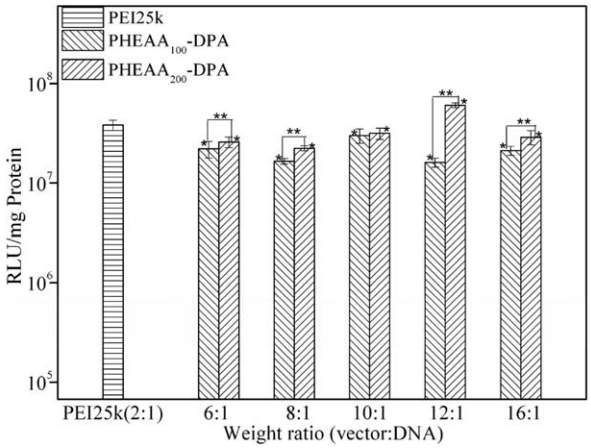


Figure 8. *In vitro* gene transfection efficiency of $PHEAA_{100}$-DPA/pDNA and $PHEAA_{200}$-DPA/pDNA complexes at different weight ratios in comparison with that of PEI25k (weight ratio = 2 : 1) in COS-7 cells. Data represent mean ± SD ($n = 3$). $P^* < 0.05$ (compared with the control) and $P^{**} < 0.05$ (compared the two complexes at the same weight ratio).

The transfection efficiency of the both PHEAA-DPA/pDNA complexes firstly increases and then decreases with the weight ratio. This phenomenon is attributed to two influencing factors. One is the increasing cytotoxicity with the weight ratio as Figures 6 and 7 shown. The other is the strong interaction between polycation and DNA at the higher weight ratio prevents the release of pDNA.[30] The transfection efficiency is also a function of multiple parameters such as the chemical structure, composition, and surface charge of the complexes.[11,18,55,56] For the PHEAA-DPA, the chemical structure and the composition are the same. The difference is the surface charge of the complexes caused by the distinction of molecular weight as discussed above. It has been mentioned that the $PHEAA_{200}$-DPA/pDNA complexes has smaller size and higher zeta potentials than $PHEAA_{100}$-DPA/pDNA. So $PHEAA_{200}$-DPA is capable of condensing DNA more efficiently, thereby providing more positive charges to condensing pDNA tightly to avoid the degradation of pDNA leading the higher transfection efficiency.

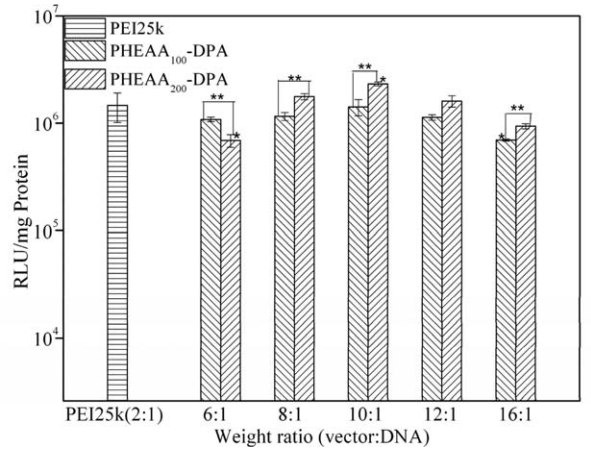


Figure 9. *In vitro* gene transfection efficiency of $PHEAA_{100}$-DPA/pDNA and $PHEAA_{200}$-DPA/pDNA complexes at different weight ratios in comparison with that of PEI25k (weight ratio = 2 : 1) in HepG-2 cells. Data represent mean ± SD ($n = 3$). $P^* < 0.05$ (compared with the control) and $P^{**} < 0.05$ (compared the two complexes at the same weight ratio).

The transfection efficiency of PHEAA-DPA/pDNA complexes in COS-7 cells and HepG-2 cells indicates that $PHEAA_{200}$-DPA is more suitable for gene delivery than $PHEAA_{100}$-DPA.

CONCLUSIONS

A brushed polycationic polymer with primary and tertiary amino groups was designed and synthesized for gene delivery. PHEAA polymerized by ATRP was employed as backbone, and then equipped with primary and tertiary amino groups by grafting DPA. The higher stability of polyplex formation and the improving buffering capacity were expected by the introduction of primary and tertiary amine. PHEAA-DPA could effectively condense pDNA. The cytotoxicity of $PHEAA_{200}$-DPA was lower than that of PEI25k until the weight ratio more than 16 : 1. Higher molecular weight of PHEAA grafting with DPA resulted in higher gene transfection efficiency, although it possessed higher cytotoxicity to COS-7 cells and HepG-2 cells. In addition, the transfection efficiency of $PHEAA_{200}$-DPA/pDNA was better than that of PEI25k in both COS-7 cells and HepG-2 cells at the respective weight ratio of 12 : 1 and 10 : 1. It is supposed that PHEAA-DPA with good biocompatibility hold a great potential as a new polycation for gene delivery system.

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