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Polycations with excellent gene transfection ability based on PVP-g-PDMAEMA with random coil and micelle structures as non-viral gene vectors

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The low transfection efficiency of polycations is still a major problem for successful gene therapy. To address this issue, in this study, hydrophilic poly(vinyl pyrrolidone)-*graft*-poly[2-(*N,N*-dimethylamino)ethyl methacrylate] (PVP-g-PDMAEMA) and amphiphilic poly(vinyl pyrrolidone)-*graft*-poly[2-(*N,N*-dimethylamino)ethyl methacrylate]-*block*-poly(methylmethacrylate) (PVP-g-PDMAEMA-b-PMMA) were synthesized *via* atom transfer radical polymerization (ATRP) method, and their properties as gene vectors were investigated subsequently. PVP-g-PDMAEMA formed random coil in water and PVP-g-PDMAEMA-b-PMMA self-assembled into spherical core-shell micelle with a very low critical micelle concentration of only 6.3×10^{-3} mg mL^{-1}. PVP-g-PDMAEMA-b-PMMA/pDNA polyplexes performed excellent gene transfection efficiency, which showed not only much higher gene transfection efficiency than PVP-g-PDMAEMA/pDNA polyplexes, but obviously surpassed 25k PEI at low N/P ratio around 3 on 293T cell lines. Hence, the results suggested that PVP-g-PDMAEMA-b-PMMA could be a highly efficient gene vector.

Introduction

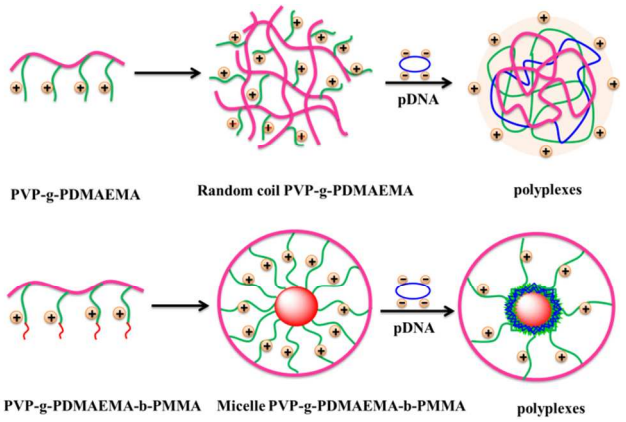
Gene therapy has been proved to be a promising approach to treat inherited and acquired diseases.[1-3] In general, gene therapy is defined as the delivery of genes into patient's host cells to produce or silence functional proteins to cure diseases.[4, 5] The clinical success of gene therapy greatly depends on the development of effective delivery vehicles with low cytotoxicity and high transfection efficiency.[6] Viral vectors show high transfection efficiency due to natural evolution. However, current concerns on viral vectors are their strong immunogenicity and potential infectivity for the practical application.[7-9] Therefore, various types of non-viral vectors are drawing considerable attention because they are easily to be handled and weaken undesired responses.[10, 11] Among them, cationic polymers (polycations) as an important type of non-viral vectors are believed to be able to protect genes from enzymatic degradation and facilitate their cellular uptake.[12-14] Moreover, their structures can be easily modified, which makes them particularly attractive for gene delivery.[15] So far, a wide range of polycations have been investigated such as poly(ethyleneimine) (PEI),[16-18] poly-L-lysine (PLL),[19-21] poly[2-(*N,N*-dimethylamino)ethyl methacrylate] (PDMAEMA), poly(amino-*co*-ester)s (PAEs),[22-24] chitosan,[25, 26] cyclodextrin (CD) oligomer,[27, 28] and so on.[29-31] Although a lot of polycations exhibit attractive properties in many aspects, the low gene transfection efficiency is still a bottleneck for their application as gene carrier.

In order to improve the gene delivery efficiency, a lot of methods have been proposed through chemical modification of polycations.[32-34] PDMAEMA with well-defined structure prepared by ATRP has been widely used as a model for studying the relationship between the structure and functions of gene delivery.[35-37] Guo[38, 39] synthesized a series of amphiphilic polycations based on polycaprolactone-*graft*-poly[2-(*N,N*-dimethylamino)ethyl methacrylate] to delivery genes, which showed gene transfection efficiency *in vitro* comparable to Lipofectamine at N/P ratio of 10 on HepG2 cells. In addition, Zhang[40] designed a hybrid material with inorganic nanodiamond core and brush-like PDMAEMA layer as a new nano-carrier for enhancing gene transfection, the level of luciferase expression of which was higher than 25k PEI on COS-7 cells at the N/P ratio of 6. Dai[41] prepared a polysulfobetaine cationic methacrylate copolymer, namely 2-(dimethylamino) ethyl methacrylate-*block*-(*N*-(3-(methacryloyl amino) propyl)-*N,N*-dimethyl-*N*-(3-sulfopropyl) ammonium hydroxide) *via* ATRP, which showed comparable efficiency to

25k PEI on COS-7 cells at weight ratio 8. Those researches suggest that PDMAEMA has great potential to be modified to become excellent gene carrier.

Non-charged, non-toxic and biocompatible poly(vinyl pyrrolidone) (PVP)[42] is water soluble polymer with many interesting properties for its application in biomedical field.[43, 44] For example, PVP can facilitate the transport of gene into cells and enhance its expression, indicating the synergistic role when working with the cationic polymers in gene delivery.[45, 46] To date, several PVP-based carriers have been developed for drug and gene delivery. Park[47] synthetized galactosylated chitosan to conjugate with PVP for a hepatocyte-targeting gene carrier. Cook[48] used PEI coupled with PVP and lactose to improve its transfection efficiency and biocompatibility. However, the transfection efficiency is slightly better than 25k PEI/pDNA polyplexes at extremely high charge ratio of 40.

In this work, PVP was used to prepare hydrophilic PVP-g-PDMAEMA that can form random coil in water, as well as PVP-g-PDMAEMA-b-PMMA that can form micelle in water with the PMMA as hydrophobic blocks. Their properties of gene encapsulation and transfer *in vitro* were investigated subsequently. Excitingly, both of the polycations, especially amphiphilic PVP-g-PDMAEMA-b-PMMA, showed dramatically higher transfection efficiency than 25k PEI at low N/P ratio.



Scheme 1 Complex process of cationic polymers with pDNA.

Experimental

Materials and chemicals

2-(*N,N*-Dimethylamino)ethyl methacrylate (DMAEMA, 99%), azobisisobutyronitrile (AIBN) and PVP (Mw = 10000) were purchased from Aladdin. 2,2'-bipyridine was purchased from Beijing Shiying Reagent Manufactory. *N,N*-Dimethylformamide (DMF), Methylmethacrylate (MMA), *N*-bromosuccinimide (NBS, anhydrous) and carbon tetrachloride (anhydrous) achieved from Jiangtian Chemical Technology Co., Ltd were all analytical grade and used as received. CuBr was prepared in our laboratory.

High Glucose Dulbecco's modified Eagle's medium (H-DMEM), fetal bovine serum (FBS), and phosphate-buffered saline (PBS, pH 7.4) were purchased from Invitrogen Corporation (Carlsbad, CA). Ethidium bromide, dimethyl sulfoxide (DMSO), branch PEI (25k) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich. Agarose was purchased from GEN TECH (Shanghai, China). EGFP-N1 plasmid (5300 bp) was extracted from escherichia coli according to the protocol of plasmid extraction kit of TIANGEN.

Synthesis of PVP-g-PDMAEMA and PVP-g-PDMAEMA-b-PMMA

PVP-g-PDMAEMA was synthesized according to the reference.[49] PVP-g-PDMAEMA-b-PMMA was prepared similar to PVP-g-PDMAEMA. PVP-g-PDMAEMA (330 mg, 0.013 mmol) as the macroinitiator was dissolved in DMF (6 mL). Then CuBr (24 mg, 0.165 mmol) and 2,2'-bipyridine (49 mg, 0.338 mmol) were added to the Slenck tube purged by three repeated vacuum/nitrogen cycles. Next, MMA (0.285 mL, 0.003 mmol) was injected into the flask under nitrogen atmosphere. The polymerization was carried out at 60 ^{o}C for 20 h. Finally the reaction mixture was dialyzed against distilled and deionized water for 2 days and lyophilized to collect dry PVP-g-PDMAEMA-b-PMMA. ^{1}H NMR spectra were recorded on 500 MHz (Varian INOVA) NMR spectrometer with $D_2O/CDCl_3$ as solvent containing a small amount of TMS as internal standard. The molar composition in the PVP-g-PDMAEMA was calculated from these ^{1}H NMR spectra: δ (ppm) 2.6 (2H, -CH_2N<), 1.0 (3H, -CH_3), 1.7 (2H, -CH_2-), 2.3 (6H, -$N(CH_3)_2$), 4.1 (2H, -$COOCH_2$-). And the PVP-g-PDMAEMA-b-PMMA copolymer was examined in $CDCl_3$ by ^{1}HNMR: 2.3 (6H, -$N(CH_3)_2$), 2.6 (2H, -CH_2N<), 3.6 (3H, -$COOCH_3$), 4.1 (2H, -$COOCH_2$-).

Critical micelle concentration (CMC)

The CMC value of the copolymer was measured by fluorescence technique with pyrene as probe. The concentration of pyrene in each sample solution was 6 ×10^{-7} M after dilution to the calibration line. Then the acetone was removed perfectly. The copolymer solution and double-distilled water were desired to prepare copolymer solutions with concentrations from 1 × 10^{-7} mg mL^{-1} to 1 mg mL^{-1}.

All the samples were sonicated for 1 h at 60 ^{o}C, and cooled to room temperature overnight. Steady-state fluorescence spectra were obtained on a CARY Eclipse fluorescence spectrophotometer (VARIAN). The ratios of the peak intensities at 393 nm and 373 nm (I_{393}/I_{373}) of the excitation spectra were analyzed as a function of polymer concentration. All experiments were carried out at room temperature.

Measurement of buffering capacity

The buffering capacity of the polymers was measured by acid-base titration. Briefly, PVP-g-PDMAEMA, PVP-g-PDMAEMA-b-PMMA and 25k PEI (10 nmol μL^{-1} of the final concentration of amine groups) were dissolved to 20 mL with

0.15 M NaCl solution. The pH of the polymers solution was initially adjusted to about 9 using 0.1 M NaOH, and then different volumes of 0.1 M HCl were added to the solution with stirring. The different pH values of the solution were measured using a microprocessor pH meter at the room temperature.

Preparation of polycations/pDNA polyplexes

PVP-g-PDMAEMA was dispersed in PBS (PH = 7.4) to prepare aqueous stock solution. PVP-g-PDMAEMA-b-PMMA was firstly dissolved in 1 mL of methanol and dropwise added to 20 mL of PBS. The methanol was removed by stirring the mixture for overnight. The final volume was adjusted to 50 mL and a final concentration of 1 nmol N μL^{-1} (N represents the amine groups). And then the plasmid DNA (pDNA) was diluted to 100 μL with PBS and the final concentration of 1 nmol P μL^{-1} (P represents the phosphate groups). Then the polycations/pDNA polyplexes at varied ratios were formulated by adding polycations of desired concentration to an equal volume of pDNA solution. The mixtures were incubated at room temperature for 30 min before further characterization.

Characterization of zeta potential and particle size

The zeta potential and particle size of both of the polycations/pDNA polyplexes were measured using a Zetasizer 3000HS (Malvern Instrument), which was at a wave length of 677 nm with a constant angle of 173° at room temperature. PVP-g-PDMAEMA-b-PMMA micelle and various N/P ratios of polyplexes suspensions containing 3 μg of pDNA were prepared and diluted with 0.8 mL of PBS before characterization.

Transmission electron microscopy

PVP-g-PDMAEMA-b-PMMA micelle and both of the polycations/pDNA polyplexes were examined by the Philips EM400 ST transmission electron microscopy at an accelerating voltage of 200 kV. Briefly, samples were made by dipping the 400-mesh glider copper TEM grids in the nanoparticles solution, and then they were took out and dried before taking images.

Agarose gel electrophoresis

To assess the pDNA loading ability of the two polyplexes, agarose gel electrophoresis was applied. Both of the polyplexes of different N/P ratios were prepared freshly as described above. 2 μL 6 × loading buffer was mixed with 10 μL polyplexes suspensions containing 1 μg pDNA, and analyzed by 1% agarose gel containing 5 μg mL^{-1} ethidium bromide (120 V, 15 min). The pDNA retardation was recorded at UV light wavelength of 254 nm with image master VDS thermal imaging system (Bio-Rad, CA).

To determine the stability of copolymers/pDNA polyplexes, the polyplexes with 1 μg pDNA at N/P = 3 were incubated for 30 min. And then the polyplexes were incubated for 2 h at room temperature after adding the increasing concentration of heparin sodium (0, 0.1, 0.5, 1, 3 mg mL^{-1}). Afterwards, added 2 μL 6 × loading buffer and the mixtures were run at 120 V for 20 min. Finally, pDNA was visualized with image master VDS thermal imaging system (Bio-Rad, CA).

Cytotoxicity assay

The *in vitro* cytotoxicity of polyplexes was determined by the viability of transfected cells with an MTT assay. 293T cells were seeded at 5×10^3 cells per well in 96-well plates cultured at 37 °C in 5% CO_2 for overnight, and subsequently treated with the polyplexes containing 0.2 μg pDNA for 24 h, each ratio was conducted in five parallel groups. Then the medium was replaced by 100 μL of MTT solution (0.5 mg mL^{-1} in H-DMEM) incubated for 3 h. Finally, the MTT solution was exchanged with 150 μL DMSO each well to dissolve the formazan crystals. The absorbance of each well was measured using an Infinite M200 microplate reader (Tecan, Durham, USA) at 570 nm with a reference wavelength of 630 nm. The cells untreated with polyplexes in medium were used as control.

Transfection *in vitro*

Gene transfection of the polyplexes was investigated in 293T cells using EGFP-N1 plasmid. The cells were seeded in 24-well plates at 5×10^4 cells per well and cultured in H-DMEM supplemented with 10% FBS, 100 U mL^{-1} penicillin and 100 mg mL^{-1} streptomycin for overnight. Before transfection, the cells were hungried for 1 h using Opti-MEM. Then the medium was replaced by the polyplexes suspensions diluted by Opti-MEM containing 1 μg pDNA each well. After cultured 4 h, the solution was exchanged with supplemented H-DMEM for another 44 h. The expression of EGFP-N1 plasmid in the cells was directly observed by an inverted fluorescence microscope (Olympus IX 70, Olympus, Tokyo, Japan) and the transfection efficiency was determined by using an Attune® acoustic focusing cytometer (Applied Biosystems, Life Technologies, Carlsbad, CA).

BSA adsorption

Briefly, 200 μL of polymer solution (PVP-g-PDMAEMA, PVP-g-PDMAEMA-b-PMMA and PEI, 0.71 mg mL^{-1}) was mixed with equal volume BSA solution (2 mg mL^{-1}). After shaking 30 min, the three kinds of mixtures were measured the adsorption at 280 nm using a UV spectrophotometer. Subsequently, the samples were centrifuged at 13000 rpm for 10 min and the adsorption of supernatants were measured same as above, regarding as the calibration curve obtained from BSA solutions of known concentrations. The amount of BSA adsorbed on the polymer was calculated using the following equation:

$$q = \frac{(C_i - C_S)V}{m}$$

Where C_i represents the initial BSA concentration and C_s represents the BSA concentration in the supernatant; V represents the total volume of the solution (400 μL); m represents the total amount (0.144 mg) of the polymer in the solution.

Cellular uptake

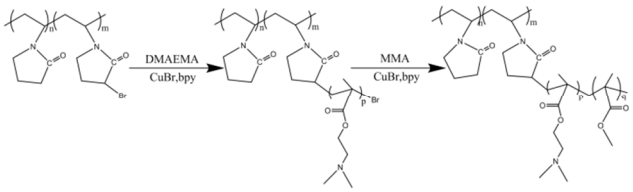
293T cells were plated at a density of 5×10^4 cells per well in 24-well plates and incubated for 24 h. Then, the polyplexes (1 μg, containing cy5-DNA/polycations) were added into each well. After 3 h incubation at 37 °C, the cells were washed with PBS there times. Subsequently, trypsin digestion was terminated in complete medium. Finally, the cells were introduced into a FACS Calibur flow cytometer.

Statistical analysis

Data are expressed as mean ± SD. For transfection results, the t-test was used to determine the different significance from each other. Statistical significance was regards as having $P < 0.05$.

Results and Discussion

Synthesis and Characterization of both the cationic polymers



Scheme 2 The synthetic route of PVP-g-PDMAEMA-b-PMMA.

The synthetic route of amphiphilic PVP-g-PDMAEMA-b-PMMA is shown in Scheme 2. Those two kinds of copolymers have about 100 DMAEMA units as determined by ^{1}H NMR shown in Fig. 1A, which is calculated from the peak integrals among the protons in the methylene groups of PVP (peak "a" at 1.7 ppm) and the protons of PDMAEMA (peak "i" at 2.6 ppm). PVP-g-PDMAEMA-b-PMMA has 20 MMA units by comparing the integral ratio between the peak "i" and peak "m" as shown in Fig. 1C. Amphiphilic copolymers in water could self-assemble into micelles with hydrophobic core and hydrophilic shell. In order to check whether MMA units were introduced into PDMAEMA chains, NMR spectroscopy of PVP-*g*-PDMAEMA-*b*-PMMA in D_2O and $CDCl_3$ were measured, respectively. As shown in Fig. 1C (^{1}H NMR spectrum of PVP-g-PDMAEMA-b-PMMA copolymer in $CDCl_3$), the characteristic peak of -$COOCH_3$ at 3.6 ppm confirms the successful introduction of MMA to the PVP-g-PDMAEMA copolymer. While the proton signals in hydrophobic PMMA segments are significantly weakened in Fig. 1B (^{1}H NMR spectrum of the PVP-g-PDMAEMA-b-PMMA copolymer in D_2O), indicating the formation of micelles with PMMA blocks as core.

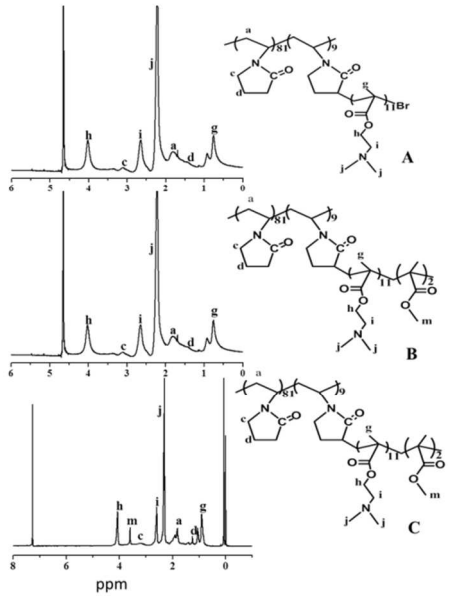


Fig. 1 ^{1}H NMR spectrum of PVP-g-PDMAEMA (A) and PVP-g-PDMAEMA-b-PMMA (B) in D_2O, and PVP-g-PDMAEMA-b-PMMA (C) in $CDCl_3$.

Characterization of PVP-g-PDMAEMA-b-PMMA copolymer micelles

CMC is an important parameter to describe the thermodynamic stability of self-assembled aggregates in aqueous solution, and that of PVP-g-PDMAEMA-b-PMMA copolymer was determined by the fluorescence probe technique using pyrene as a fluorescence probe. As shown in Fig. 2A, it can be seen that the intensity ratio I_{393}/I_{373} begins to increase dramatically when the concentration reaches to CMC. The CMC of PVP-g-PDMAEMA-b-PMMA was determined to be 6.3×10^{-3} mg mL^{-1}. The low CMC value of PVP-g-PDMAEMA-b-PMMA indicates that it possesses excellent stability in aqueous solution.[13]

The PVP-g-PDMAEMA-b-PMMA copolymer was prepared above the CMC. The size and zeta potential of the PVP-g-PDMAEMA-b-PMMA copolymer micelles were determined by DLS. From the results of Fig. 2C, it is shown that the micelles possess positive charges. Furthermore, it is apparent that the average size of micelles is around 318.2 nm in aqueous buffer and they are homogeneously dispersed as individual nanoparticles (Fig. 2B and Fig. 2D).

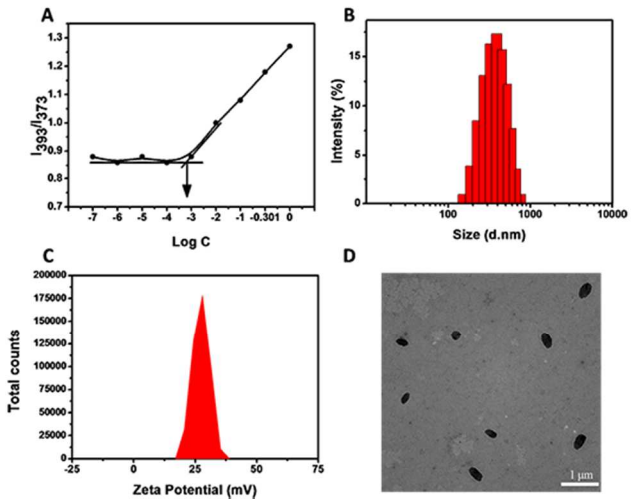


Fig. 2 Plot of I_{393}/I_{373} ratios as a function of logarithm of PVP-g-PDMAEMA-b-PMMA concentrations (A). The size distribution (B), zeta potential (C) and the TEM image (D) of PVP-g-PDMAEMA-b-PMMA copolymer micelles.

Buffer capacity

It is believed that gene vectors with good buffering capacity may help polyplexes escape from the endosome and consequently promote transfection efficiency. In order to assess the buffer capacity of the two kinds of polycations in the endosome, the titration curves were determined by acid-base titration. As can be seen from Fig. 3, the two kinds of polycations present better buffering capability than 25k PEI. In addition, PVP-g-PDMAEMA-b-PMMA has no significant difference in buffering capacity compared with PVP-*g*-PDMAEMA, indicating that the incorporation of MMA does not affect their ability to buffer capacity.

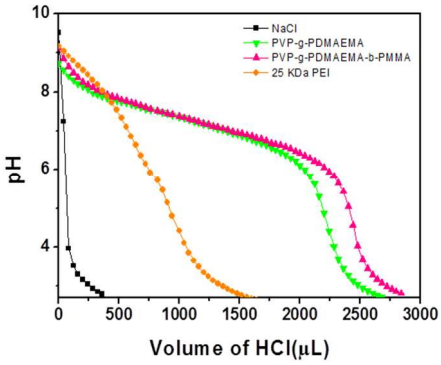


Fig. 3 Acid-base titration curves of NaCl, 25k PEI, PVP-g-PDMAEMA and PVP-g-PDMAEMA-b-PMMA. Measurements were taken using a pH meter.

Zeta potential Measurement

Zeta potential is an important factor affecting the cellular uptake of polyplexes, which were measured here for the two types of polyplexes to determine their surface charge at the N/P ratios ranging from 1 to 10. As shown in Fig. 4 A, the zeta potentials of both the polyplexes increases with the increasing N/P ratio. Compared with PVP-g-PDMAEMA/pDNA polyplexes, PVP-g-PDMAEMA-b-PMMA/pDNA polyplexes shows lower zeta potentials, which may also be attributed to the shielding effect by the micelle structure, entangling certain cationic charges of PDMAEMA in the linked blocks.

Particle size Measurement

Particle size plays an important role in the cellular uptake rate of polyplexes. The hydrodynamic diameters of the polyplexes at different N/P ratios were measured at room temperature. It is shown that the particle sizes of all the polyplexes decrease with increasing N/P ratio. Among them, the particle size of PVP-g-PDMAEMA/pDNA and PVP-g-PDMAEMA-b-PMMA/pDNA is around 200.2 nm and 169.7 nm at the N/P ratio of 3, respectively. As shown in Fig. 4 B, it can also be seen that amphiphilic PVP-g-PDMAEMA-b-PMMA/pDNA polyplexes tend to form smaller nanoparticles compared with hydrophilic PVP-g-PDMAEMA/pDNA polyplexes without hydrophobic segments at all N/P ratios, as a result of more compressed particles by hydrophobic segments in aqueous solution.[50]

Transmission Electron Microscopy (TEM)

The images of those two kinds of polyplexes were obtained at the N/P ratio of 3 by TEM. As shown in Fig. 4 C, both of the polyplexes have spherical structure. The diameters of PVP-g-PDMAEMA/pDNA polyplexes and PVP-g-PDMAEMA-b-PMMA/pDNA polyplexes are around 160 nm and 100 nm, respectively. In accordance with the results of DLS measurement, the particle sizes of PVP-g-PDMAEMA-b-PMMA/pDNA polyplexes are smaller than PVP-g-PDMAEMA/pDNA polyplexes.

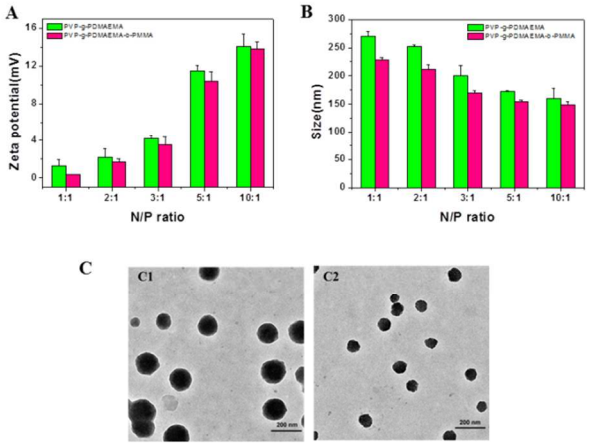


Fig. 4 Zeta potential of polyplexes at different N/P ratios (A), Size of polyplexes at different N/P ratios (B), TEM images of PVP-g-PDMAEMA/pDNA polyplexes (C1), PVP-g-PDMAEMA-b-PMMA/pDNA polyplexes (C2) at N/P ratio of 3. All measurements of polyplexes were performed after the polyplexes were incubated for 30 min at room temperature (mean ± SD, n = 3).

Agarose gel electrophoresis

The abilities of PVP-g-PDMAEMA and PVP-g-PDMAEMA-b-PMMA to condense pDNA were studied by agarose gel electrophoresis. As shown in the Fig. 5, both of PVP-g-

PDMAEMA and PVP-g-PDMAEMA-b-PMMA display excellent pDNA compacting abilities at the extremely low N/P ratio of 0.8 and 0.5, respectively. Compared with PVP-g-PDMAEMA, PVP-g-PDMAEMA-b-PMMA can totally retard at a relative lower N/P ratio, because the micelle structure can condense pDNA more effectively.

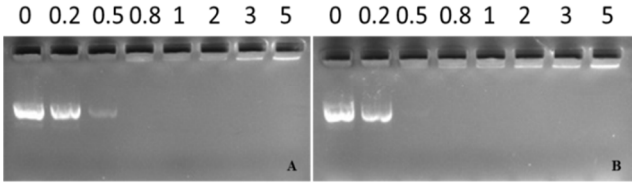


Fig. 5 Agarose gel electrophoresis of pDNA complexed with PVP-g-PDMAEMA (A), PVP-g-PDMAEMA-b-PMMA (B) at different N/P ratios.

Stability Measurement

The appropriate stability of polycation/pDNA polyplexes is one of the key factors to affect the transfection efficiency. In order to assess the stability of polyplexes, the agarose gel electrophoreses were performed in the presence of heparin sodium with increasing concentration. As can be seen from Fig. 6, the threshold concentration of heparin at which the disruption of polyplexes is 0.5 mg mL^{-1}, 1 mg mL^{-1} and 3 mg mL^{-1} for PVP-g-PDMAEMA/pDNA, PVP-g-PDMAEMA-b-PMMA/pDNA and PEI/pDNA, respectively. It indicates that PVP-g-PDMAEMA-b-PMMA is more stable than PVP-g-PDMAEMA in agreement with the data of polyplexes size and the agarose gel electrophoresis results, indicating that the smaller size has better stability. It is generally accepted that overly stable polycation/pDNA binding may be a limited factor for the highly efficient gene expression.[51, 52] Compared to PEI/pDNA polyplexes, the looser structure of PVP-g-PDMAEMA/pDNA and PVP-g-PDMAEMA-b-PMMA/pDNA polyplexes may contribute to easy release of pDNA.

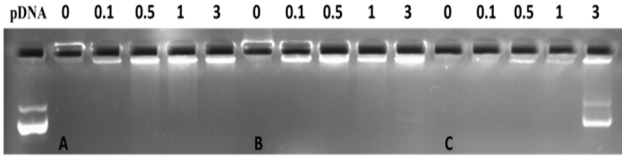


Fig. 6 Agarose gel electrophoreses of polyplexes in the presence of various amount of heparin sodium. All the vectors/pDNA polyplexes were formed at N/P ratio of 3. A, B and C represent PVP-g-PDMAEMA/pDNA, PVP-g-PDMAEMA-b-PMMA/pDNA and PEI/pDNA polyplexes, respectively.

In vitro cytotoxicity

To evaluate the cytotoxicity of the polyplexes, the cell viability on 293T cells by MTT assay was applied. As shown in Fig. 7, both types of polyplexes exhibit low cytotoxicity at N/P ratio from 1 to 3. Especially, PVP-g-PDMAEMA/pDNA polyplexes show comparable cell viability to commercial 25k PEI. PVP-g-PDMAEMA-b-PMMA/pDNA polyplexes show a little lower cell viability compared with PVP-g-PDMAEMA/pDNA polyplexes. The enhanced cytotoxicity of PVP-g-PDMAEMA-b-PMMA/pDNA polyplexes may result from the introduction of hydrophobic MMA segments.[53, 54]

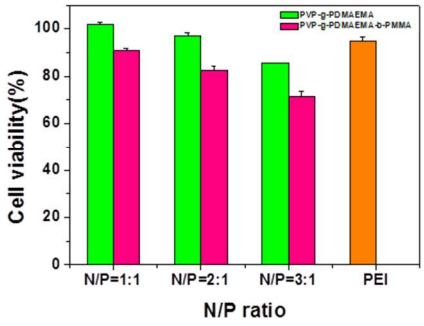


Fig. 7 *In vitro* cytotoxicity of 293T cells was treated by PVP-g-PDMAEMA/pDNA polyplexes and PVP-g-PDMAEMA-b-PMMA/pDNA polyplexes. The results were determined by MTT assay. The values represent percentage cell viability (mean ± SD, n = 3).

Transfection *in vitro*

To investigate gene transfection ability of the two kinds of polycations *in vitro*, 293T cells were transfected with PVP-g-PDMAEMA and PVP-g-PDMAEMA-b-PMMA. As shown in Fig. 8 A1 and A2, PVP-g-PDMAEMA-b-PMMA exhibits improved transfection efficiency compared to PVP-g-PDMAEMA at all N/P ratios. On the one hand, this is believed that PVP-g-PDMAEMA-b-PMMA/pDNA can compact more stable nanoparticles than PVP-g-PDMAEMA/pDNA due to the presence of hydrophobic segment, which benefits to endocytosis.[50] On the other hand, the amphiphilic copolymer can improve compatibility with the plasma membrane interactions in order to enhance gene transfection efficiency.[53] It is noteworthy that the transfection efficiency of PVP-g-PDMAEMA-b-PMMA is beyond 60% at N/P ratios of 2 and 3 at 24 h. The value is obviously higher than 25k PEI. Since both of the two kinds of polycations exhibited good transfection efficiency at N/P ratio of 3, the transfection time of both polyplexes was prolonged to check if they can enhance transfection efficiency further which was sequentially observed at N/P ratios of 2.5, 3 and 3.5 at 48 h. As shown in Fig. 8 B1 and B2, the transfection efficiency of the polycations enhances obviously when the transfection time is prolonged. Notably, the transfection efficacy of PVP-g-PDMAEMA-b-PMMA is higher than 81% and the efficacy of PVP-g-PDMAEMA is also higher than 60% at each N/P ratio. In addition, the optimal N/P ratio of PVP-g-PDMAEMA and PVP-g-PDMAEMA-b-PMMA is around 3:1, which is much lower than that of 25k PEI (10:1).

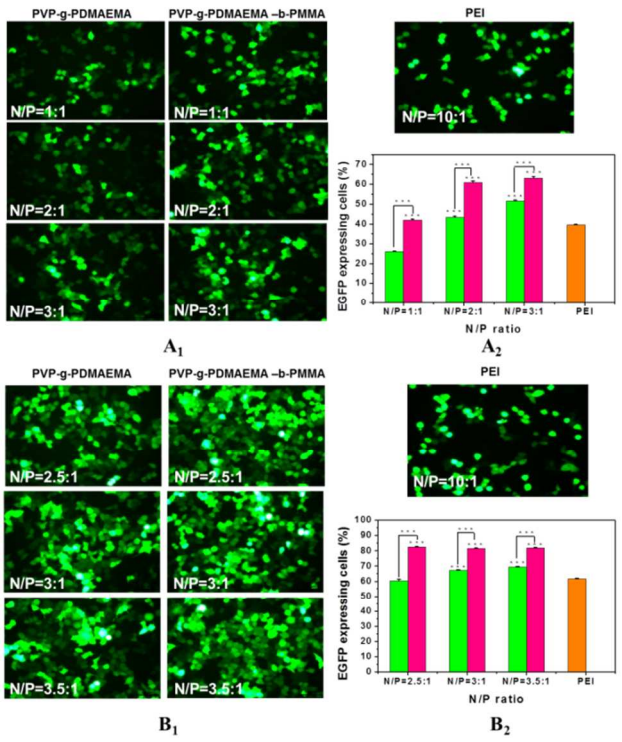


Fig. 8 The transfection efficiencies of PVP-g-PDMAEMA (green bar) and PVP-g-PDMAEMA-b-PMMA (red bar) complexed with pEGFP as reporter gene were determined by flow cytometer at 24 h (A_2) and 48 h (B_2) in 293T cells (mean ± SD, n = 3). Fluorescence images of 293T cells transfected by the two kinds of polyplexes at 24 h (A_1) and 48 h (B_1). 25k PEI were used as positive controls. Bar = 50 μm. *** $p < 0.001$ *vs* PEI.

Cellular uptake

The analysis results from fluorescence-activated cell sorting (FACS) were shown in Fig. 9. It can be seen that the cellular uptake efficacy of PVP-g-PDMAEMA, PVP-g-PDMAEMA-b-PMMA and PEI polyplexes is almost 100%. However, the mean fluorescence intensity of the polyplexes exhibits obvious variation. Compared with PEI, our designed polymers show higher mean fluorescence intensity, which may be one of the reasons for the higher transfection efficiency (Fig. 9 C).

BSA adsorption

Serum protein would combine to polycation/pDNA polyplexes easily in a nonspecific way. This will result in the aggregation of polyplexes due to the electrolyte effect where the negative charges of the protein surround the positively charged polyplexes and screening their charges.[55] Therefore, the low protein adsorption of polymers will improve the stability of polyplexes to enhance the transfection efficiency. Considering the above problem, we further designed the experiment about the BSA adsorption assay. As can be seen in Fig. 9 D, the resistance against the BSA adsorption of our designed polymers is obviously better than 25k PEI. It is mainly because PVP can shield the positive charges by steric effect, while PEI with excess positive charges can combine with more negative charged BSA.[55, 56] In addition, PVP-g-PDMAEMA-b-PMMA is more stability than PVP-g-PDMAEMA due to the presence of hydrophobic segment. It can decrease the density of positive charges to abate the protein adsorption onto the polymer surface in accord with the zeta potential results.

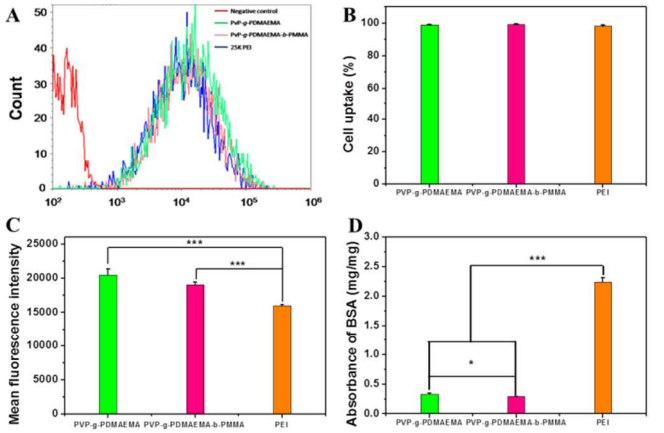


Fig. 9 Internalization of polyplexes by 293Tcells measured by fluorescence activated cell sorting (FACS).Cy5 fluorescence intensity and corresponding counts (A) Percentages of cell uptake of polyplexes (B) and mean fluorescence intensity (C). Protein adsorption of PVP-g-PDMAEMA, PVP-g-PDMAEMA-b-PMMA and PEI with BSA (D). *** $p < 0.001$.

Conclusions

PVP-g-PDMAEMA and PVP-g-PDMAEMA-b-PMMA copolymers were prepared to possess architectures of random coil and micelle nanoparticles through ATRP, respectively. It is showed that the incorporation of PMMA hydrophobic segments could enhance the stability of polyplexes by forming cationic micelle nanoparticles and condense pDNA more effectively. In addition, PVP-g-PDMAEMA-b-PMMA as an amphiphilic copolymer had a good buffer capacity and exhibited enhanced gene transfection efficiency than the hydrophilic copolymer of PVP-g-PDMAEMA. All the results suggested that PVP-g-PDMAEMA-b-PMMA copolymer would be a promising candidate for gene delivery *in vitro*.

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Notes and references

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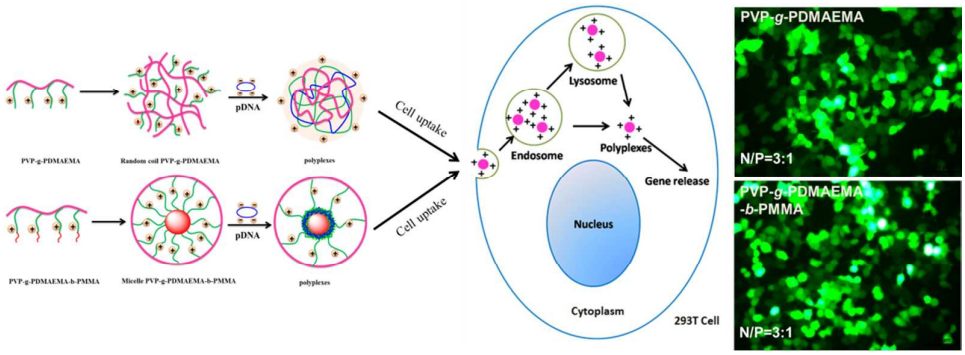

PVP-g-PDMAEMA formed random coil in water and PVP-*g*-PDMAEMA-*b*-PMMA self-assembled into spherical core-shell micelle. Both of them displayed excellent pDNA compacting abilities at the extremely low N/P ratio and showed good buffer capacity ability. Meanwhile, PVP-*g*-PDMAEMA-*b*-PMMA/pDNA polyplexes performed excellent gene transfection efficiency, which showed not only much higher gene transfection efficiency than PVP-*g*-PDMAEMA/pDNA, but obviously surpassed 25k PEI.