Synthesis and Characterization of Poly(ethylene glycol)-*b*-poly(ε-caprolactone)-*b*-poly(2-(2-aminoethyl amino)ethyl methacrylate) Triblock Copolymers as Efficient Gene Delivery Vectors

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ABSTRACT: In this study, ABC-type triblock copolymers, poly(ethylene glycol)-*b*-poly(ε-caprolactone)-*b*-poly(2-(2-aminoethyl amino) ethyl methacrylate)s (PEG-PCL-PAEAEMAs), composed of novel poly(2-(2-aminoethyl amino) ethyl methacrylate) (PAEAEMA) that have a primary and a secondary amino group in each monomeric unit, were synthesized successfully. It was found that the length of PAEAEMA segments did not have obvious influence on the DNA-binding capacity and other biophysical properties (size and zeta potential values of polymer/pDNA complexes) , but longer PAEAEMA chains led to a better buffering capacity. The triblock copolymers could mediate efficient gene expression that was similar to branched 25-kDa polyethylenimine (25 kDa PEI) in the absence of serum and even superior to 25 kDa PEI in the presence of serum in COS-7 cells. Low cytotoxicity of these polymers was also found in COS-7 cells. As a result, PEG-PCL-PAEAEMAs are attractive candidates as serum-tolerable gene carriers in biomedical field. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 121: 666–674, 2011

Key words: block copolymers; atom transfer radical polymerization; gene delivery

INTRODUCTION

Gene therapy has been promoted as a potential method for curing cancer and inherited diseases.[1] Polymer-based non-viral gene carriers have been widely used because of their easy fabrication, low immunogenicity, safety, and reproducibility compared to viral vectors.[2] Many cationic polymers have been deeply investigated such as polyethylenimines (PEI), poly(L-lysine) (PLL), and poly(2-dimethylaminoethyl methacrylate) (pDMAEMA).[3–8]

However, there still remain drawbacks such as unfavorable biocompatibility and low transfection efficiency for most of the polycationic vectors. Therefore, many works have been done to overcome these problems. Introducing the hydrophilic segments such as poly(ethylene glycol) (PEG) into cationic polymers is a common strategy, which can decrease the cytotoxicity of the polymers, increase the stability of the polymer/DNA complexes, and prolong circulation time of the complexes.[9–12] In addition, the introduction of hydrophobic segments into polycations may enhance the interactions between polymers and cells and further affect the transfection activity, although the effect of structure–function relationship on the transfection activity is still poorly understood. For instance, Seow reported that hydrophobic segments in amphiphilic triblock oligopeptides could promote better DNA binding and cell membrane penetration.[13] Zhu also reported that the transgene expression mediated by *N*-methylene phosphonic chitosan (NMPCS) was much higher (more than 100 folds) than that mediated by chitosan, which was ascribed to its hydrophilic/lipophilic property. The interaction of NMPCS with plasma membranes was able to make a perturbation or disruption on membrane organization and possibly created a specific room or space for successfully transporting DNA into cells and the nucleus.[14]

The recent development in synthetic route of polymer, such as new synthetic methods or the combination of two or more methods, has allowed for facile synthesis of well-defined macromolecules with precise control of structure and molecular weight.[15–18] Heise et al. reported the synthesis of block copolymer with variable compositions and architectures by

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combining ring-opening polymerization (ROP) and atom transfer radical polymerization (ATRP).[19–22] More recently, Zhang and our group have also used the same method to prepare amphiphilic triblock copolymers as gene delivery vectors.[23,24]

Considering these facts, we synthesized ABC-type triblock copolymers composed of monomethoxyl poly(ethylene glycol) (mPEG), hydrophobic poly(ε-caprolactone) (PCL), and novel cationic poly(2-(2-aminoethyl amino) ethyl methacrylate) (PAEAEMA) blocks that have a primary and a secondary amino group in each monomeric unit. It was reported that primary amine groups are mainly responsible for high affinity of DNA binding and toxicity. Some polymers like PLL with primary amino groups only are all protonated at physiological pH, yielding a structure without buffering capacity to aid in endosomal escape.[25] Therefore, we expect that the combination of primary and secondary amino groups in one monomeric unit may obtain good properties in both DNA-binding and buffering capacity.

In this work, the chemical structures and compositions of these polymers were fully characterized. The particle size, cellular uptake of polymer/DNA complexes, and DNA-binding capacity of the polymers were investigated by dynamic light scattering (DLS), confocal laser scanning microscopy (CLSM), and agarose gel retardation assay, respectively. The results showed that these triblock copolymers could condense plasmid DNA (pDNA) into 170–190 nm aggregates with positive zeta potentials approximately 7–34 mV at N/P ratios of 10–30. It demonstrated that PEG-PCL-PAEAEMAs could bind pDNA, translocate pDNA into cytoplasm, and release pDNA in nucleus of COS-7 cells effectively. It also was found that the length of PAEAEMA did not have obvious influence on the DNA-binding capacity and other biophysical properties (size and zeta potential of polymer/pDNA complexes), but longer length of PAEAEMA led to a better buffering capacity. Reporter gene expression efficiency of polymers was investigated in COS-7 cell lines. It was demonstrated that PEG-PCL-PAEAEMA mediated gene expression at levels that were comparable with 25 kDa PEI and even superior in the presence of serum. Moreover, low cytotoxicity of them was also found in COS-7 cells. Therefore, PEG-PCL-PAEAEMAs may be potential to be a safe, efficient non-viral vector in the field of gene therapy.

EXPERIMENTAL

Materials

Poly(ethylene glycol) mono-methyl ether with M_n of 1900 (PEG-OH), ε-caprolactone, 1,1,4,7,7-pantamethyldiethylenetriamine (PMDETA), *N*-(2-hydroxyethyl) ethylenediamine, and 2-bromopropionyl bromide were purchased from Alfa Aesar Co. (Ward Hill, MA) Copper (I) bromide (CuBr, Beijing Chemical Co. Beijing, China) was purified by washing with acetic acid and ethanol in turn. Methacryloyl chloride, *N,N*-dimethylformamide (DMF), dichloromethane (DCM), triethylamine, and ether were all purified prior to use. Macroinitiator $mPEG_{45}$-PCL_{40}-Br was synthesized according to the literatures previously.[26,27] Bovine serum albumin (BSA), Dubelcco's Modified Eagle's Medium (DMEM), penicillin–streptomycin, trypsin, and phosphate-buffered saline (PBS), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Co. (St. Louis, MO). The reporter plasmids pGL3-Luc was purchased from Promega Co. (Madison, WI).

Monomer synthesis

N,N'-Di-(*tert*-butoxycarbonyl)-2-(2-aminoethyl amino) ethanol (M_1)

A solution of di-*tert*-butyl dicarbonate (156.51 mmol, 34.12 g) in DCM (25 mL) was added via syringe to a cooled (0°C) solution of 2-(2-aminoethyl amino) ethanol (77.39 mmol, 8.01 g) in DCM (75 mL).And the reaction mixture was stirred overnight at room temperature. The reaction solution was filtrated and then concentrated by evaporation. The residue was purified with column chromatography (silica gel, ethyl acetate/hexane (2/1) as eluent) to yield M_1 (51.4 mmol, 15.67 g) as a colorless oil. ^{1}H-NMR ($CDCl_3$): δ = 3.76 (m, C**H**$_2$O), 3.48-3.27 (m, NC**H**$_2$ and N**H**), 1.46 (s, one *tert*-butyl), and 1.43 (s, one *tert*-butyl).

N,N'-Di-(*tert*-butoxycarbonyl)-2-(2-aminoethyl amino) ethyl methacrylate (Boc-AEAEMA)

A solution of methacryloyl chloride (0.022 mol, 2.30 g) in dried DCM (3 mL) was added dropwise to an ice-cooled mixture of M_1 (0.02 mol, 6.08 g) and triethylamine (0.022 mol 2.23 g) in dry DCM (45 mL). The reaction mixture was stirred overnight at room temperature. The reaction solution was filtrated and washed with 10% $NaHCO_3$ solution and brine. The solution was concentrated by evaporation after dried over $MgSO_4$. Boc-AEAEMA was isolated after precipitation in ice-cooled hexane as a colorless crystal (5.21 g, 0.014 mol). The further purification was carried out by column chromatography (silica gel, ethyl acetate/hexane (1/1) as eluent). ^{1}H-NMR ($CDCl_3$): δ = 6.12 and 5.59 (d, C**H**$_2$=C), 4.26–4.18 (m, C**H**$_2$O), 3.50–3.27 (m, NC**H**$_2$ and N**H**), 1.46 (s, one *tert*-butyl), and 1.43 (s, one *tert*-butyl). IR (KBr): 3372 cm^{-1} (s, NH), 1719 cm^{-1} (vs, C=O), 1700 cm^{-1} (vs, C=O), 1638 cm^{-1} (m, C=C).

Polymer synthesis

$mPEG_{45}$-PCL_{40}-P(Boc-AEAEMA)s were prepared by ATRP. As a typical example of $mPEG_{45}$-PCL_{40}-

P(Boc-AEAEMA)$_{129}$, a reaction flask was charged with mPEG$_{45}$-PCL$_{40}$-Br macroinitiator (0.1 mmol, 0.60 g), Boc-AEAEMA (16 mmol, 5.95 g), and dry DMF (5.0 mL). After three freeze-pump-thaw cycles, CuBr (0.1 mmol, 0.014 g) and PMDETA (0.2 mmol, 0.034 g) were added. The polymerization was carried out under N_2 atmosphere at 70°C for 7 h and the termination was achieved by pouring the solution into excess THF. After passed through an alumina column to eliminate the copper, the solution was poured into excess ether for precipitation. The white powder was dried under vacuum.

The obtained mPEG$_{45}$-PCL$_{40}$-P(Boc-AEAEMA)s were then added into an anhydrous saturated HCl solution of ethyl acetate for deprotection. After stirred vigorously at 0°C for 6 h, the solution was dialyzed against water (M_w: 8000, cutoff) for 2 days. The desired polymers were obtained by lyophilization.

Polymer characterization

^{1}H-NMR signals were recorded with Mercury VX-300 (Varian Mercury Co. Palo Alto, CA) by using $CDCl_3$, DMSO-d_6, or D_2O as solvents and TMS as an internal. Infrared spectra were recorded on a Nicolet Avator 360 FTIR spectrometer (Thermo Nicolet Co. Madison, WI). The molecular weight and molecular weight distribution of the polymers were determined by gel permeation chromatography (GPC) equipped with a Waters 2690 separation module and a Waters 2410 refractive index detector (Waters Co., Milford, MA). Tetrahydrofuran (THF) was used as eluent at a flow rate of 0.5 mL min^{-1} with the temperature maintained at 30°C, and the results were calibrated against polystyrene standards.

The buffering capacity of the polymers was evaluated by titrating against 0.01*M* HCl by using a pH meter. The electrodes were calibrated with standards before use. Ten milliliters of polymer solutions (0.8 mg mL$^{-1)}$ was used for all experiments.

Particle size and zeta potential of polymer/DNA complexes

The polymer/DNA complexes were prepared by adding the polymer solution to 1 μg DNA (in 40 × 10^{-3}*M* Tris-HCl buffer) at various N/P ratios (nitrogen atoms of the polymer over phosphates of pDNA). The complexes were incubated for 30 min and diluted with 150 × 10^{-3}*M* NaCl solution or pure water to 1.0 mL volume for size or zeta-potential measurements on a Nano-ZSZEN3600 (Malvern Instruments Ltd., Malvern, UK) instrument at room temperature.

Agarose gel retardation assay

Agarose gel electrophoresis was conducted to investigate the DNA-binding ability of the polymers. The polymer/DNA complexes containing 0.1 μg DNA were formed at various N/P ratios. Six microliters of sample solution together with 1 μL loading buffer was added to the wells of 1.0% agarose gel containing ethidium bromide and subjected to migration (80 mV, 60 min). Imaging was captured with Cam2-com software under the irradiation of UV light.

Cytotoxicity assay

The cytotoxicity assessment was carried out in COS-7 cells by using the MTT assay. Hundred microliters of cell suspension containing 6 × 10^3 COS-7 cells were seeded into each well of a 96-well plate and the cells were incubated for 24 h. Then the cells were treated with polymers at various concentrations and a further incubation for 24 h was carried out. After that, all mediums were replaced with MTT reagent (20 μL in PBS, 5 mg mL^{-1}) and incubated for another 4 h. The medium in each well was carefully removed and replaced by 100 μL DMSO. When the purple solution was homogeneous, the absorbance at 570 nm was recorded by a microplate reader (Bio-Rad model 550, Bio-Rad Lab., Hercules, CA). Cell viability was calculated by

$$\text{Cell viability}\,(\%) = (A_{\text{treated}}/A_{\text{control}}) \times 100\% \qquad (1)$$

The data are shown as the average value ± standard deviation.

In vitro luciferase expression with or without serum

COS-7 cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and antibiotics (penicillin-streptomycin, 10,000 U mL$^{-1)}$ at 37°C with 5% CO_2. Cells were subcultured prior to confluence using trypsin-EDTA.

For transfection experiments, 1 mL of DMEM with 10% FBS containing 6 × 10^4 COS-7 cells were seeded into each well of a 24-well plate and the cells were cultured at 37°C with 5% CO_2 for 24 h. The medium was replaced with DMEM (serum free) freshly and then polymer/DNA complexes solution containing 1 μg of pGL3 plasmid DNA was added into each well. After 4 h, the medium was replaced with 1 mL of fresh DMEM (10% FBS) each well and the cells were incubated for a further 44 h before expression measurement. For the preincubation study with serum, DMEM containing 10% (v/v) FBS was added to the solutions of the complexes and incubated at 37°C for 30 min prior to the transfection study.

After incubation, 200 μL of reporter lysis buffer (1×) (Promega) was added into each well. The membrane lysis was completed with a freeze-thaw cycle. The cell suspension was centrifuged at 13,000

TABLE I
The Data of Diblock Macroinitiator and the Corresponding Triblock Copolymers

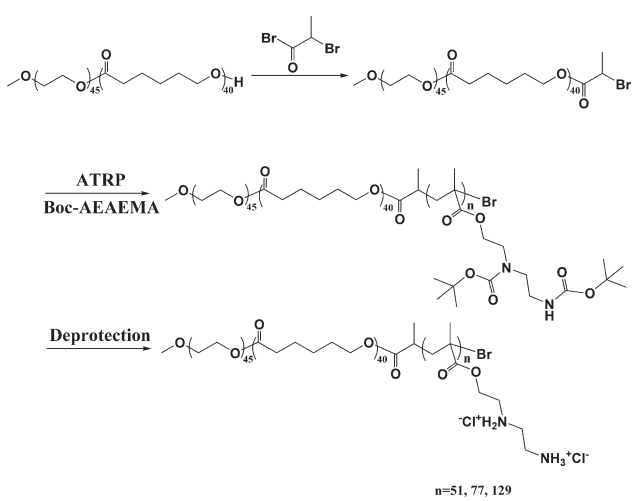
Sample	M_n[a] (g mol^{-1})	M_n[b] (g mol^{-1})	PDI[b]
mPEG$_{45}$-PCL$_{40}$-Br	6500	6100	1.28
mPEG$_{45}$-PCL$_{40}$-P(Boc-AEAEMA)$_{51}$	25,400	20,200	1.68
mPEG$_{45}$-PCL$_{40}$-P(Boc-AEAEMA)$_{77}$	35,100	25,700	1.65
mPEG$_{45}$-PCL$_{40}$-P(Boc-AEAEMA)$_{129}$	54,400	48,500	1.66

[a] Determined from ^{1}H-NMR.
[b] Determined from GPC results.

rotations per min for 3 min. The relative light units (RLU) were measured by detecting the light emission from a mixture of 20 μL of lysate supernatant extracts and 100 μL of a luciferase assay reagent (Promega) with a luminometer (Lumat LB9507, Berthold, Bad wildbad, Germany) and normalized against the protein concentration, which was measured with a protein assay kit (Pierce, Thermo Fisher Scientific, Rockford, IL).

Confocal laser scanning microscopy

COS-7 cells were seeded in a six-well plate and incubated at 37°C for 24 h. Polymer/pGL3 (labeled with YOYO-1) complexes were prepared at N/P = 10 and incubated for 30 min. The cells were treated with the labeled polymer/DNA complexes and incubated in serum-free DMEM for 4 h. After that, the medium was removed and the cells were washed two times with PBS. The nucleus dye H33258 solution (20 μL H33258 in 400 μL serum-free DMEM) was then added into each wells and the cells was incubated for another 20 min. After the nucleus dye solution was removed, the cells were washed two times with PBS and then DMEM containing 10% FBS was added immediately. The fluorescence was examined with CLSM (Nikon C1-si, Nikon, Melville, NY). A laser (408 nm excitation) was used to induce the blue fluorescence of H33258 and a laser (488 nm) was used to excite the green fluorescence of YOYO-1.



Scheme 1 The reactive route of mPEG$_{45}$-*b*-PCL$_{40}$-*b*-PAEAEMAs.

RESULTS AND DISCUSSION

Synthesis of mPEG$_{45}$-PCL$_{40}$-PAEAEMAs

In this study, triblock copolymers mPEG$_{45}$-PCL$_{40}$-PAEAEMAs were synthesized by ROP and ATRP (Scheme 1). Generally, mPEG$_{45}$-PCL$_{40}$-OH was synthesized via ROP of ε-caprolactone using mPEG$_{45}$-OH as the initiator. The terminal hydroxyl groups of the mPEG$_{45}$-PCL$_{40}$-OH were then modified by 2-bromopropionyl bromide to obtain mPEG$_{45}$-PCL$_{40}$-Br as the macroinitiator to prepare of mPEG$_{45}$-PCL$_{40}$-P(Boc-AEAEMA)$_{51}$, mPEG$_{45}$-PCL$_{40}$-P(Boc-AEAEMA)$_{77}$, and mPEG$_{45}$-PCL$_{40}$-P(Boc-AEAEMA)$_{129}$ (denoted as Boc-P$_{51}$, Boc-P$_{77}$, and Boc-P$_{129}$, respectively) by initiating Boc-AEAEMA via ATRP (Table I). Figure 1 shows the ^{1}H-NMR spectra of mPEG$_{45}$-PCL$_{40}$-Br and Boc-P$_{129}$. In Figure 1(a), the single peak at 3.65 ppm was ascribed to the methylene protons of PEG, and the peaks at 1.38, 1.65, 2.30, and 4.03 ppm were assigned to the protons of PCL. The small double peaks at 1.81–1.83 ppm (peak n) were corresponding to the protons of terminal methyl group. In Figure 1(b), we

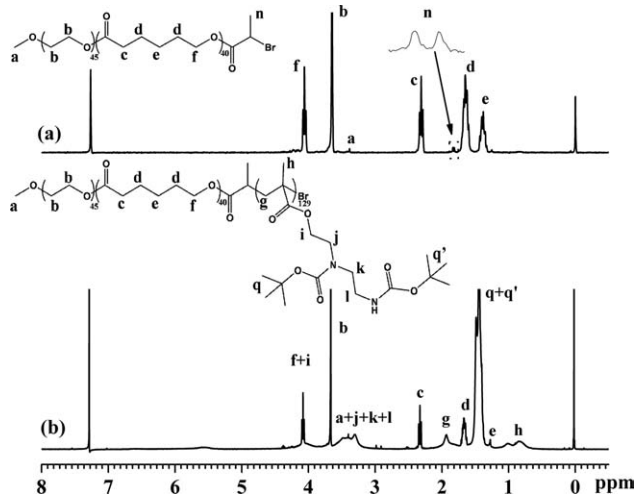


Figure 1 ^{1}H-NMR spectra of (a) macroinitiator mPEG$_{45}$-PEL$_{40}$-Br ($CDCl_3$); (b) mPEG$_{45}$-*b*-PCL$_{40}$-*b*-P(Boc-AEAEMA)$_{129}$ ($CDCl_3$).

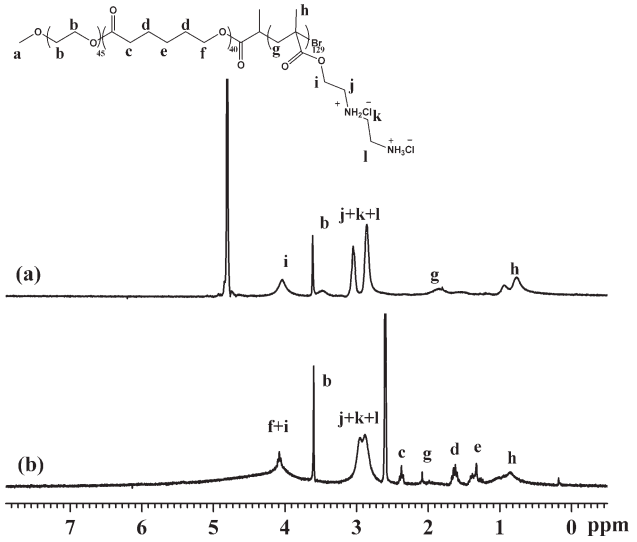


Figure 2 ^{1}H-NMR spectra of mPEG$_{45}$-*b*-PCL$_{40}$-*b*-PAEAEMA$_{129}$ in (a) D_2O and (b) DMSO-d_6.

can find the characteristic signals of protons in P(Boc-AEAEMA) block besides the signals found in Figure 1(a). The peaks at 0.84–1.00 ppm and 1.81–1.95 ppm were ascribed to the protons on the backbone of P(Boc-AEAEMA) segment, and the peaks at 3.20–3.59 ppm were of the protons of methylene adjacent to secondary and tertiary amino groups. The peaks at 1.43 and 1.46 ppm were the characteristic signal of methyl protons of Boc-protected group. The composition of triblock copolymer mPEG$_{45}$-PCL$_{40}$-P(Boc-AEAEMA)s was calculated based on the integration values of the peaks at 3.65, 2.30, and 1.43–1.46 ppm, attributed to ethylene protons of PEG backbone, methylene protons neighboring to carbonyl group of PCL backbone, and methyl protons of Boc-protected group, respectively. Moreover,

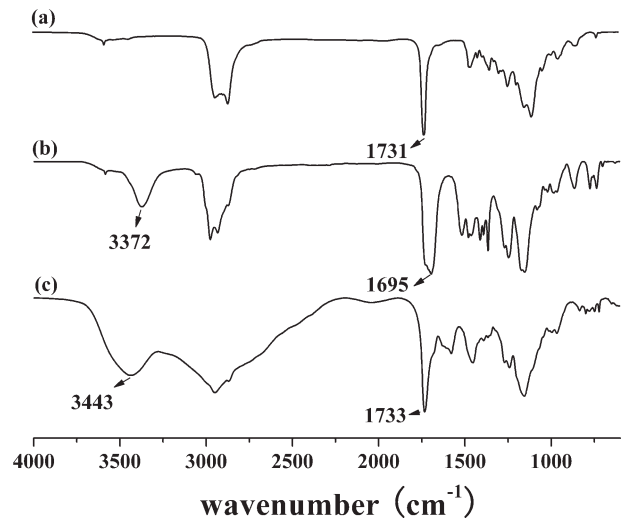


Figure 3 FTIR spectra of (a) mPEG$_{45}$-PCL$_{40}$-Br; (b) mPEG$_{45}$-*b*-PCL$_{40}$-*b*-P(Boc-AEAEMA)$_{129}$; and (c) mPEG$_{45}$-*b*-PCL$_{40}$-*b*-PAEAEMA$_{129}$.

the gradually shortening elution time with the increase of molecular weight of polymers and the unimodal of molecular weight distributions in GPC elution profiles also indicated that the well-defined polymers were achieved successfully (Supporting Information Fig. S1).

The deprotection reaction was carried out in anhydrous saturated HCl solution of ethyl acetate, the corresponding triblock copolymers containing primary and secondary amines groups were obtained after dialysis and lyophilization. The success of this process was evidenced by the disappearance of the signals of *tert*-butoxycarbonyl groups at 1.43 and 1.46 ppm and the maintenance of the peaks at 0.84–1.00 and 1.81–1.95 ppm, which is ascribed to the protons on the backbone of PAEAEMA [Fig. 2(a,b)]. The data of ^{1}H-NMR spectra also showed the integration values of mPEG, PCL, and PAEAEMA segments changed little after the deprotection process. mPEG$_{45}$-PCL$_{40}$-PAEAEMA$_{51}$, mPEG$_{45}$-PCL$_{40}$-PAEAEMA$_{77}$, and mPEG$_{45}$-PCL$_{40}$-PAEAEMA$_{129}$ were denoted as P$_{51}$, P$_{77}$, and P$_{129}$, respectively.

Figure 3 exhibited the FTIR spectra of mPEG-PCL-Br, Boc-P$_{129}$, and P$_{129}$. The peak at 1731 cm^{-1} was attributed to the carbonyl groups of PCL [Fig. 3(a)] and the broad peak at 1695 cm^{-1} was the overlap of carbonyl groups belonging to PCL, polymethacrylate, and *tert*-butoxycarbonyl groups, respectively [Fig. 3(b)]. After the *tert*-butoxycarbonyl groups were cut from of Boc-P$_{129}$, the single peak at 1733 cm^{-1} appeared [Fig. 3(c)]. Furthermore, the peak at 3372 cm^{-1} of N—H stretching bands of —NHCO— group in Boc-P$_{129}$ and the broad peak at 3443 cm^{-1} of N—H stretching bands of P$_{129}$ were observed after the triblock copolymers were formed.

Buffer capacity

The buffering capacity of a vector is an important factor for successful gene delivery. To evaluate these properties, P$_{51}$, P$_{77}$, and P$_{129}$ were all dissolved in

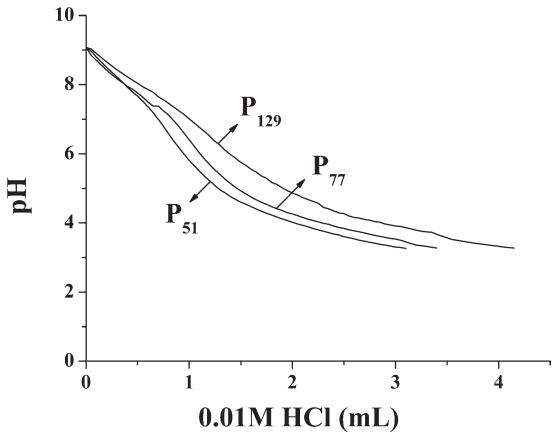


Figure 4 Titration curves of mPEG$_{45}$-*b*-PCL$_{40}$-*b*-PAEAEMAs (0.8 mg mL^{-1}) titrated with 0.01*M* HCl.

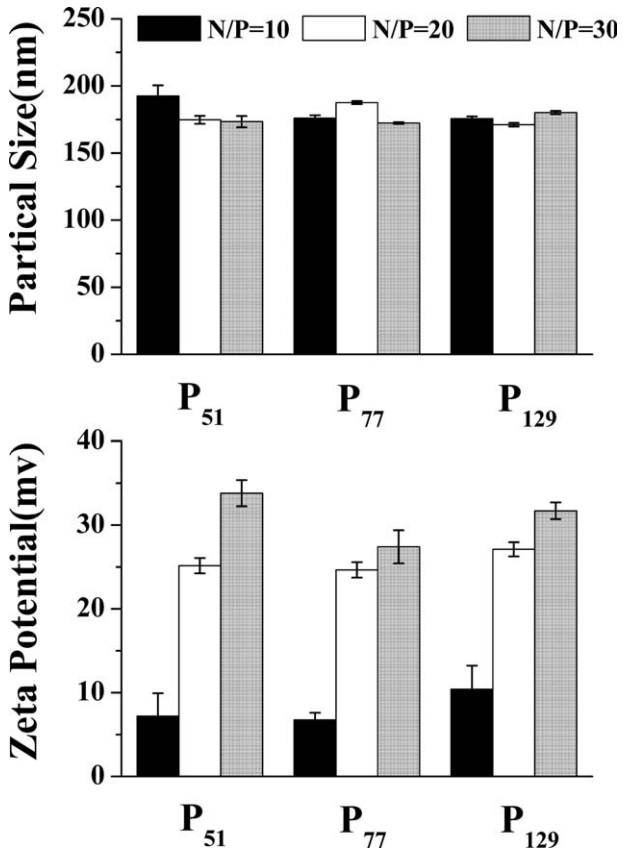


Figure 5 Particle sizes and zeta potential of P_{51}/DNA complexes, P_{77}/DNA complexes, and P_{129}/DNA complexes.

water (0.8 mg mL^{-1}) and titrated against 0.01*M* HCl. All the titrations were carried out under the same conditions. In Figure 4, it was observed that all the polymers had buffer capacity in pH ranging from 9.0 to 4.0, and the volume of HCl required for acidifying the polymer solutions from 9.0 to 4.0 were in the descending order of P_{129}, P_{77}, and P_{51}. This indicated that all the polymers studied did have buffer capacity, and P_{129} was the best.

Particle size and zeta potential of polymer/DNA complexes

The particle sizes of polymer/DNA complexes at various N/P ratios in 150×10^{-3}*M* NaCl solution and zeta potential of polymer/DNA complexes in pure water were examined by DLS and shown in Figure 5. The hydrodynamic diameters of the complexes were 170–190 nm at N/P ratios in range from 10 to 30. No obvious size change was observed with the increase of the length of the PAEAEMAs or the N/P ratios.

A positive surface charge of untargeted polyplexes is necessary for the attachment to anionic cell membrane surface via electrostatic interaction. However, too high zeta potential of polymer/DNA complexes also leads to serious cytotoxicity and unstability in the presence of serum.[28] As shown in Figure 5, all the complexes showed the similar results, i.e., the surface charge of the complexes increased as the increasing of N/P ratio and all the zeta potentials were below 35 mV. The surface charge of the complexes was positive but relatively low because of the shielding effect of PEG segment.[11] Therefore, $mPEG_{45}$-PCL_{40}-PAEAEMAs are expected to be suitable candidates for effective gene expression owing to their comparable particle sizes and surface charges.

Agarose gel retardation assay

The DNA-binding ability of three polymers was evaluated by agarose gel retardation assay. As shown in Figure 6, P_{51} could completely retard DNA at N/P = 5 while both P_{77} and P_{129} could do so at N/P = 4. There was a little influence of the length of PAEAEMA segment on the DNA-binding

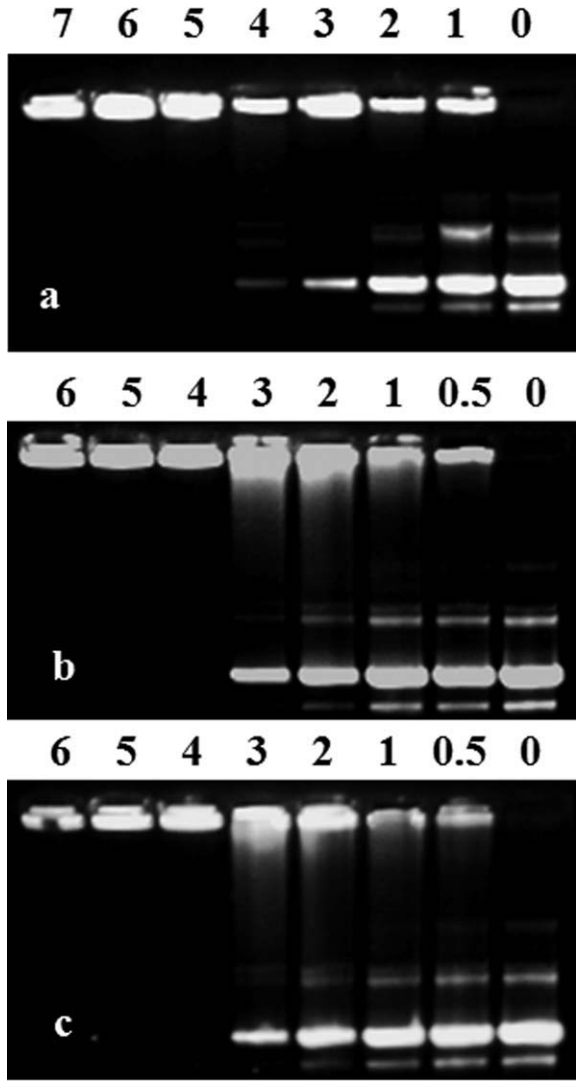


Figure 6 DNA-binding ability of polymers. Electrophoretic mobility of plasmid DNA in (a) P_{51}, (b) P_{77}, and (c) P_{129} complexes at the N/P ratios specified.

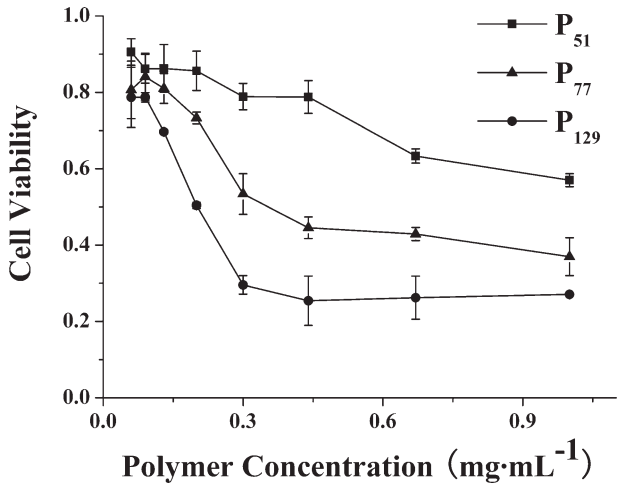


Figure 7 Relative cell viability of COS-7 cells coincubated with polymers for 24 h.

capability of the triblock copolymers although the number of the monomer units of PAEAEMA varied from 51 to 129, which might be due to the introduction of PEG and PCL segments. The hydrophobic PCL segments of these triblock copolymers always tend to aggregate in aqueous solution, which might lead to the aggregation of positive charges to a certain extent and weaken the influence of the length of PAEAEMA subsequently. The hydrophilic PEG segments could shield the surface charge of complexes to keep back the further electrostatic bonding of DNA.[23,24]

Cytotoxicity assay

MTT assay was utilized to measure the cytotoxicity of polymers in COS-7 cells. As shown in Figure 7, a dose-dependent cytotoxic effect was observed for all the polymers. P_{51} exhibited the lowest cytotoxcity and the cell viability was in excess of 56% even the concentration of polymer reached up to 1 mg mL^{-1}. The lethal concentration 50 (LC50) of P_{77} and P_{129} were about 0.3 and 0.2 mg mL^{-1}, respectively. In previous studies, Wolfert found that the cytotoxicity of PLL increased with the increase of the molecular weight.[29] Hennink also made the similar conclusion for PDMAEMA.[30] In our study, there was apparent decrease of cell viability due

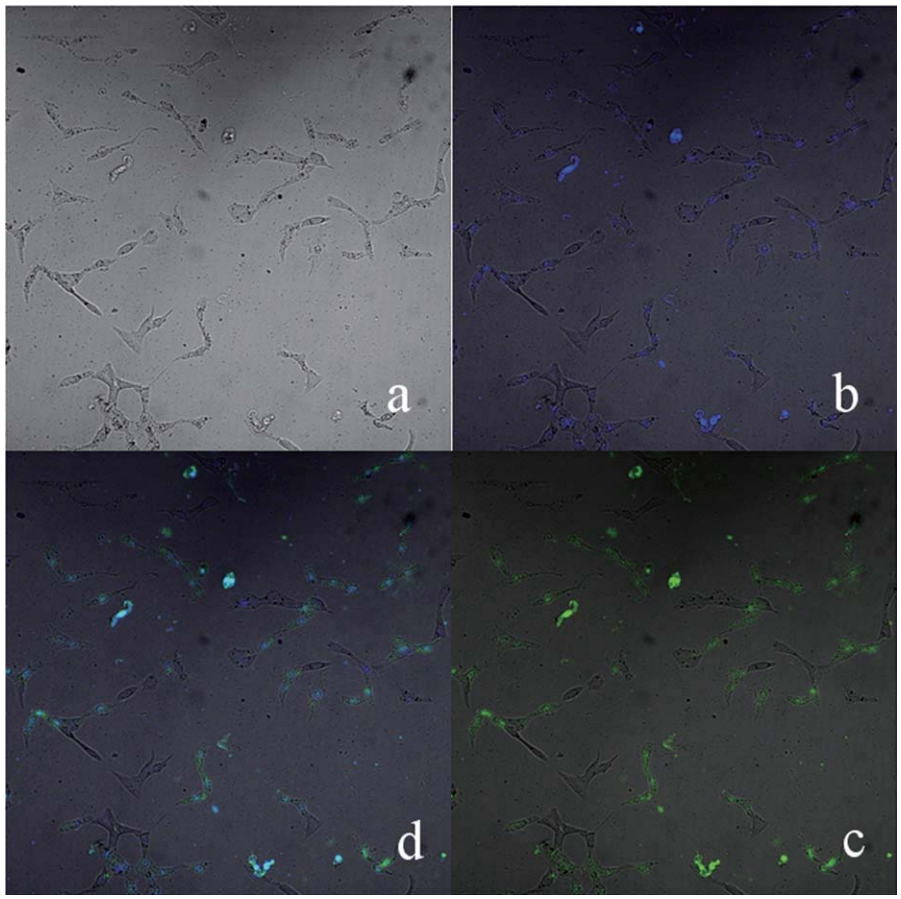


Figure 8 Cellular uptake of YOYO-1 labeled DNA from P_{77}/DNA complexes in COS-7 cells: (a) COS-7 cells; (b) H33258 labeled nucleus (blue) (c) YOYO-1 labeled DNA (green); (d) an overlay of (a)–(c). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

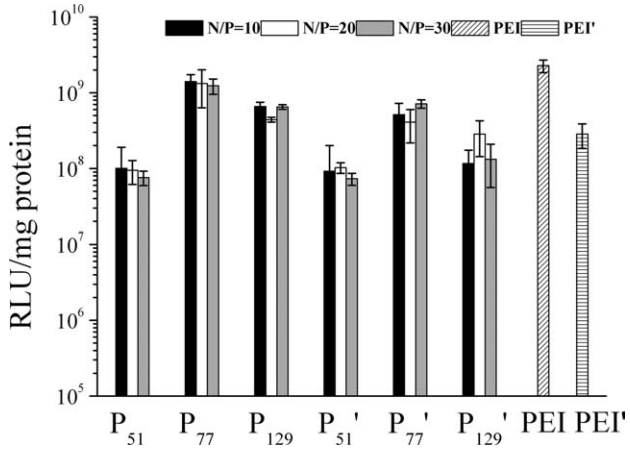


Figure 9 Luciferase expression in COS-7 cells mediated by P_{51}, P_{77}, P_{129}, and PEI in serum-free DMEM; P'_{51}, P'_{77}, P'_{129} and PEI′ in serum-supplemented DMEM.

to the increase of the length of PAEAEMA blocks, which also may be due to the increase of molecular weight.

Cellular uptake and in vitro transfection efficiency

As well be known, the nuclear entry of plasmid DNA efficiently was the ultimate obstacle to achieve high gene expression. Therefore, the cellar uptake of P_{77}/DNA (fluorescent dye YOYO-1 labeled) complexes was investigated by confocal microscopy. As shown in Figure 8, the green fluorescent labeled complexes were obviously observed to be internalized into the COS-7 cells and much of them were located at the nucleus. This proved that P_{77} could mediate the DNA into the cells and introduce the nuclear entry of plasmid DNA efficiently.

COS-7 cells were used to assess the ability of the polymers to mediate the luciferase expression. As revealed in Figure 9, P_{77} showed the highest expression efficiency up to 1.41×10^{-9} RLU per mg protein at N/P ratio 10, which was comparable to 2.28×10^{-9} RLU per mg protein of 25 kDa PEI in the medium without serum. P_{51} mediated the lowest expression level up to 7.58×10^{-7} RLU per mg protein at N/P ratio 30 among the three polymers. This may be explained by the relative lower buffering capacity and DNA-binding ability of P_{51} compared to that of P_{77} and P_{129}. Moreover, we observed that the increase of PAEAEMA blocks did not lead to a linear growth of the expression efficiency and P_{77} with medium molecular weight exhibited the highest expression efficiency. It was reported that high molecular weight chitosan also performed low expression efficiency.[31] A reason suggested was that the high molecular weight species interacted too strongly with DNA to release them.[32]

The similar tendency was observed in the presence of serum. It is worth noting that the transfection efficiency of the three polymers in serum had a minor decrease while an obvious decrease of magnitude in the luciferase expression occurred for 25 kDa PEI. As shown in Figure 9, P_{77} showed expression efficiency up to 7.16×10^{-8} RLU per mg protein at N/P ratio 30 that was 57.8% of expression level in the absence of serum compared to 2.84×10^{-8} RLU per mg protein of PEI that was 12.3% of expression level in the absence of serum while expression level of P_{51} decreased just 10%. It suggested that PEG-PCL-PAEAEMAs might be used as serum-tolerable non-viral gene vectors.

CONCLUSIONS

Novel ABC-type triblock copolymers PEG-PCL-PAEAEMAs that have a primary and a secondary amino group in each cationic monomeric unit were synthesized via ROP and ATRP. The results demonstrated that PEG-PCL-PAEAEMAs could bind pDNA, translocate pDNA into cytoplasm, and release pDNA in nucleus of COS-7 cells effectively. They were also able to mediate efficient *in vitro* luciferase expression that was similar to 25-kDa PEI in the absent of serum and even superior to PEI in the present of serum in COS-7 cells. Low cytotoxicity of these polymers was also found in the COS-7 cells. Therefore, PEG-PCL-PAEAEMAs may be potential to be safe, efficient non-viral vectors in the field of gene therapy.

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