J Mater Sci: Mater Med (2010) 21:2817–2825
DOI 10.1007/s10856-010-4140-3

Self-assembled micellar aggregates based monomethoxyl poly(ethylene glycol)-*b*-poly(ε-caprolactone)-*b*-poly(aminoethyl methacrylate) triblock copolymers as efficient gene delivery vectors

Ming Ma · Feng Li · Xiu-hong Liu · Zhe-fan Yuan · Fu-jie Chen · Ren-xi Zhuo

Received: 12 April 2010 / Accepted: 29 July 2010 / Published online: 21 September 2010

Abstract Amphiphilic triblock copolymers monomethoxyl poly(ethylene glycol) (mPEG)-*b*-poly(ε-caprolactone) (PCL)-*b*-poly(aminoethyl methacrylate)s (PAMAs) (mPECAs) were synthesized as gene delivery vectors. They exhibited lower cytotoxicity and higher transfection efficiency in COS-7 cells in presence of serum compared to 25 kDa bPEI. The influence of mPEG and PCL segments in mPECAs was evaluated by comparing with corresponding diblock copolymers. The studies showed the incorporation of the hydrophobic PCL segment in triblock copolymers affected the binding capability to pDNA and surface charges of complexes due to the formation of micelles increasing the local charges. The presence of mPEG segment in gene vector decreased the surface charges of the complexes and increased the stability of the complexes in serum because of the steric hindrance effect. It was also found that the combination of PEG and PCL segments into one macromolecule might lead to synergistic effect for better transfection efficiency in serum.

1 Introduction

Gene therapy has gained significant attention over the past two decades as a potential method for the treatment of human disease by the transfer of genetic material into specific cells of the patient [1–3]. Initial research concentrated on viral vectors for their high gene transfection efficiency. However, as the drawbacks associated with viral vectors were discovered, the focus has gradually diverted to nonviral gene delivery systems [4, 5].

Cationic polymers is an important class of nonviral gene delivery systems, including poly(ethylenimine) (pEI) [6], and poly-L-lysine (pLL) [7], poly(2-dimethylaminoethyl methacrylate) (pDMAEMA) [8], and so on [9–11]. These cationic polymers studied frequently could compact DNA effectively via electrostatic interactions between the positive charges of the polycations and the negatively charged phosphate groups of DNA to form nanoparticles capable of transfecting a variety of cell types. However, most of cationic polymers often cause distinct cytotoxicity due to their high positive charges, and their complexes with DNA are easily cleaned out from the circulation system by the reticuloendothelial system because of the association with plasma proteins and other cellular components after intravenous injection. Therefore, the conjugation of cationic polymers with hydrophilic and nonionic polymers, such as poly(ethylene glycol) (PEG), poly(2-hydroxyethyl methacrylate) (PHEMA), provides a potential modification strategy to create a steric barrier against self-aggregation, and to shield cationic charge groups [12–16]. Unfortunately, the incorporation of PEG segments also might lead to reducing DNA complexation and cell uptake to a certain extent [17, 18].

Recently, several research groups reported that the introduction of biocompatible hydrophobic segments into the gene delivery vectors could enhance the interaction of vectors with cells [19–21]. For instance, copolymers composed of poly(ethylene oxide) and poly(propylene oxide) (PPO) were found to possess the unique ability of penetrating into cell membranes as a result of the presence of the hydrophobic PPO chain [22]. In addition, Yang demonstrated that hydrophobic segments in cationic triblock amphiphilic oligopeptides could enhance the ability of carriers to condense DNA [23].

M. Ma · F. Li (✉) · X. Liu · Z. Yuan · F. Chen · R. Zhuo
Key Laboratory of Biomedical Polymers of Ministry of Education, College of Chemistry and Molecular Science, Wuhan University, Wuhan 430072, People's Republic of China
e-mail: lfsj2004@hotmail.com

In the present work, we designed and synthesized ABC-type triblock copolymers composed of mPEG, hydrophobic PCL and cationic PAMA blocks. Here, amphiphilic mPEG–PCL–PAMAs are supposed to form micelles with the hydrophobic PCL core and the hydrophilic shell consisting of nonionic PEG chains and cationic PAMA chains. It is expected that when these micelles are mixed with DNA in an aqueous solution, the cationic PAMA segments of the shell could first bound to DNA via electrostatic interaction and then aggregate with hydrophobic PCL chains to form a larger insoluble core, while the nonionic water-soluble PEG chains serve as a hydrophilic shell stabilizing the complexes. Finally, the influence of mPEG and PCL segments on DNA binding capability, size, charge, and gene transfection efficiency were investigated by comparing mPECAs with diblock copolymer mPEA and PCA.

2 Materials and method

2.1 Materials

Poly(ethylene glycol) monomethylether with M_n of 1900 (PEG–OH), ε-caprolactone, 1,1,4,7,7-pantamethyldiethylenetriamine (PMDETA), and 2-bromopropionyl bromide were obtained from Sigma-Aldrich. Copper(I) bromide (CuBr, Beijing Chemical Co.) was purified by washing with acetic acid and ethanol in turn. Methacryloyl chloride, ethanolamine, *N*,*N*-dimethylformamide (DMF), dichloromethane (DCM) and ether were all purchased from Beijing chemical company. *N-tert*-butoxycarbonyl-aminoethanol was synthesized according to the literature [24]. Monomethoxyl poly(ethylene glycol)-*b*-poly(ε-caprolactone) and cholesterol end-capped poly(ε-caprolactone) were synthesized according to the previous reports [25, 26]. Bovine serum albumin, Dubelcco's Modified Eagle's Medium (DMEM), penicillin–streptomycin, trypsin, and Dubelcco's phosphate buffered saline (PBS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich. The reporter plasmids pGL3-Luc was purchased from Promega. The plasmid DNA was stored at −20°C prior to use.

2.2 Synthesis of *N-tert*-butoxycarbonyl-aminoethyl methacrylate (Boc-AMA)

N-tert-Butoxycarbonylaminoethanol (8 g, 0.05 mol) and anhydrous triethylamine (5 g, 0.05 mol) in 50 ml anhydrous DCM were added into a 250 ml dried flask equipped with a dropping-funnel and a stirring bar. After cooling to 0°C, methacryloyl chloride (6.5 g, 0.06 mol) in 20 ml dried DCM was added dropwise. The mixture was allowed to react at room temperature overnight. The precipitate was filtered and the filtrate was concentrated under vacuum. The residue was first precipitated into excess *n*-pentane, and then purified by silica gel column chromatography using *n*-hexane/acetic ether (v/v, 1/1) as the eluent. The solvent was removed by rotary evaporation, and the product was obtained with a yield of 80%.

2.3 Synthesis of mPEG–PCL–Br, mPEG–Br and PCL–Br macroinitiator

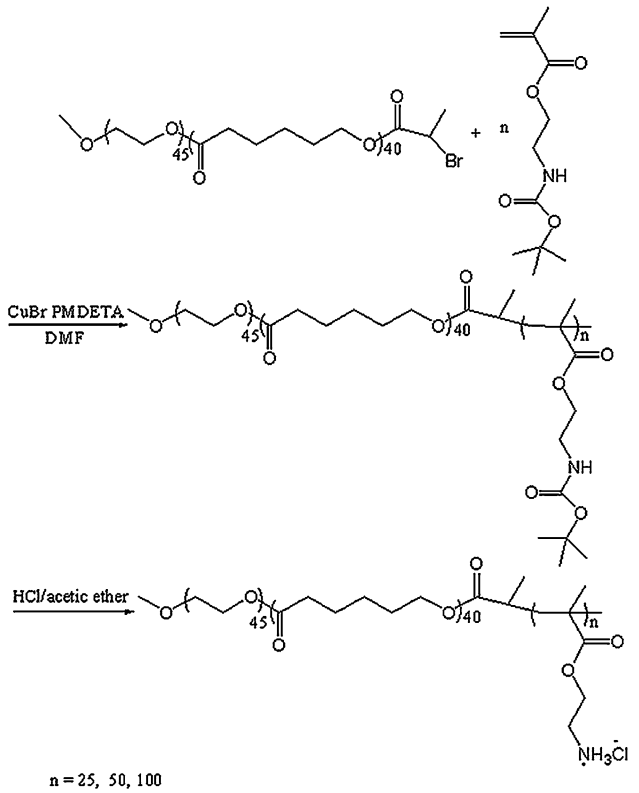
mPEG–PCL–OH (9 g, 1.5×10^{-3} mol) and triethylamine (0.2 g, 3×10^{-3} mol) were dissolved in 20 ml dried DCM and cooled to 0°C, then 2-bromopropionyl bromide (0.3 g, 1.5×10^{-3} mol) in 2 ml dried DCM was added slowly. The mixture was further stirred at room temperature overnight. After removing the precipitate, the filtrate was concentrated and precipitated in excess ether three times. The obtained product was dried in vacuum. mPEG–Br and PCL–Br macroinitiator were prepared following the similar procedure.

2.4 Synthesis of mPEG–PCL–P(Boc-AMA)s, mPEG–P(Boc-AMA) and PCL–P(Boc-AMA)

mPEG–PCL–P(Boc-AMA)s, mPEG–P(Boc-AMA) and PCL–P(Boc-AMA) were all prepared by atom transfer radical polymerization (ATRP). As a typical example of $mPEG_{45}$–PCL_{40}–$P(Boc\text{-}AMA)_{100}$, a brown reaction flask was charged with $mPEG_{45}$–PCL_{40}–Br macroinitiator (0.60 g, 0.1×10^{-3} mol) and Boc-AMA (2.45 g, 11×10^{-3} mol) and dried DMF (3.0 ml). The flask was degassed by three freeze–pump–thaw cycles, and then CuBr (0.014 g, 0.1×10^{-3} mol) and PMDETA (0.034 g, 0.2×10^{-3} mol) were added. After another three freeze–pump–thaw cycles, the polymerization was carried out under N_2 atmosphere at 70°C for 10 h. After that, the solution was poured into excess ether for precipitation. The precipitate was dissolved in DCM, purified using an alumina column to eliminate the used copper, and then precipitated again in ether three times.

2.5 Synthesis of mPEG–PCL–PAMAs, mPEG–PAMA and PCL–PAMA

mPEG–PCL–P(Boc-AMA), mPEG–P(Boc-AMA) and PCL–P(Boc-AMA) were added into anhydrous saturated HCl solution of ethyl acetate, respectively, and stirred at 0°C for 5 h. After the signal of Boc-protected groups disappeared in ^{1}H NMR spectra, the solution was dialyzed (8000, cutoff) for 2 days. The desired polymers were obtained by freeze-drying (Scheme 1).



Scheme 1 Synthesis pathway of mPEG-*b*-PCL-*b*-PAMAs

2.6 Polymer characterizations

^{1}H NMR spectroscopy (Mercury VX-300) was used to record the synthesized polymers, while molecular weight and molecular weight distribution were estimated by gel permeation chromatography (GPC) with THF as an eluent and PEG with narrow molecular distribution as the standards. Critical micellar concentration (cmc) was measured using LS55 luminescence spectrometer (Perkin-Elmer) according to the literature [27].

2.7 Cell culture

COS-7 cells were incubated in DMEM supplemented with 10% (v/v) FBS and antibiotics (penicillin–streptomycin, 10,000 U ml) at 37°C in a humidified atmosphere contain 5% CO_2. Cells were subcultured prior to confluence using trypsin–EDTA.

2.8 Particle size and zeta potential measurements

The particle size and zeta potential were performed on a Nano-ZSZEN3600 (Malvern) instrument at room temperature. The polycations/pDNA complexes were prepared by adding appropriate volume of copolymers solution (in 150×10^{-3} M NaCl) to 1 μg DNA (0.1 mg ml^{-1} in 40×10^{-3} M Tris–HCl buffer) at N/P ratios ranging from 10 to 30. Then the polyplexes were incubated at room temperature for 30 min. After that, the polyplexes were diluted with 150×10^{-3} M NaCl solution or pure water to 1.0 ml volume for size and zeta potential measurements, respectively.

2.9 DNA binding

DNA condensation ability of these polymers was assessed by means of an agarose gel retardation assay. The polycations/DNA complexes with different N/P ratios (nitrogen atoms of the polymer over phosphates of pDNA) from 0 to 3.5 were prepared by adding designed aqueous solutions of polymer into pDNA solution. Then 6 μl of samples containing 1 μl ethidium bromide and 0.1 mg DNA were electrophoresed through a 1% agarose gel using Tris–borate–EDTA buffer at 80 mV for 80 min. DNA retardation was observed by irradiation with UV light and assayed with Cam2com software.

2.10 Confocal laser scanning microscopy

For confocal microscopy experiments, COS-7 cells were seeded in 6-well plate and incubated at 37°C for 24 h. $mPEG_{45}$–PCL_{40}–$PAMA_{100}$/pGL-3 labeled by YOYO-1 complexes were prepared at N/P = 20 for 30 min. After the complexes were incubated in serum-free DMEM for 4 h at 37°C, the serum-free DMEM was replaced by nucleus dye H33258 solution (20 μl H33258 in 400 μl serum-free DMEM) for 20 min. Then the nucleus dye H33258 solution was replaced by DMEM containing 10% FBS after the cells were washed three times by PBS. The fluorescence was examined with confocal laser scan microscope (Nikon C1-si). 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.

2.11 In vitro transfection

For transfection experiments, the pGL3 plasmid DNA was used to evaluate the transfection efficiency of the polycations in COS-7 cells. COS-7 cells were seeded in 24-well plate at the density of 6×10^4/well and cultured with 1 ml DMEM containing 10% FBS for 1 day at 37°C in a humidified atmosphere of 95% air and 5% CO_2 until the cells reached about 70% confluence, respectively. The polycations/DNA complexes were formed at different N/P ratios ranging from 10 to 30 according to conditions described above (containing 1 μg DNA in each N/P ratio). The complexes were added to each well and incubated in DMEM at 37°C for 4 h, and then the medium was replaced

with 1 ml of fresh FBS, and the cells were further incubated for 48 h. After incubation, cells were permeabilized with 200 μl/well of cell lysis buffer. The luciferase activity was measured by detecting the light emission from an aliquot of cell lysate incubated with 100 μl of luciferin substrate (Promega) in a luminometer (Lumat LB9507, Berthold). The light units (LU) measured using protein assay kits (Pierce) were normalized against protein concentration in the cell extracts. Luciferase activity was expressed as relative light units (RLU/mg protein). The data were reported for triplicate samples.

The procedure of transfection experiment in serum was the same as described above except that the medium was replaced by the DMEM with 10% FBS (fetal bovine serum).

2.12 Cytotoxicity assay

The cytotoxicity assay was carried out on the basis of an MTT assay in COS-7 cells. Cells were seeded in 96-well plate at an initial density of 6000 cells/well and cultured 24 h in 100 μl of DMEM. After polymer was added for 24 h, the medium was replaced with MTT reagent (20 μl in PBS, 5 mg ml^{-1}), and then incubated for another 4 h. Thereafter, MTT was removed and 200 μl DMSO was added until crystals dissolved. The absorbance at 570 nm of the solution in each well was recorded using a Microplate Reader (Bio-Rad model 550). Cell viability was calculated according to the following equation: cell viability (%) = $(OD_{sample} - OD_{blank})/(OD_{control} - OD_{blank}) \times 100$, where OD_{sample} is the absorbance of the solution of the cells cultured with the polymer or PEI; OD_{blank} is the absorbance of the medium; and $OD_{control}$ is the absorbance of the solution of the cells cultured with the medium only.

2.13 Absorption of bovine serum albumin (BSA)

The polymer/DNA complexes at N/P ratio of 30 were formed according to described above, and then incubated with BSA (2 mg ml^{-1}) at 37°C with 140 r min^{-1}. 1 ml of mixed solution was removed at determined time. After centrifugation at 8000 r min^{-1}, the upper clear solution was measured at 280 nm with a Lambda Bio40 UV–vis spectrometer (Perkin-Elmer).

3 Results and discussion

3.1 Synthesis of $mPEG_{45}$–PCL_{40}–PAMAs (mPECAs), $mPEG_{45}$–$PAMA_{105}$ ($mPEA_{105}$) and PCL_{38}–$PAMA_{95}$ (PCA_{95})

In order to synthesize triblock copolymers mPECAs, $mPEG_{45}$–OH was firstly used to polymerize ε-caprolactone by ring-opening polymerization. And the resultant diblock copolymer $mPEG_{45}$–PCL_{40}–OH was then modified by 2-bromopropionyl bromide to obtain $mPEG_{45}$–PCL_{40}–Br macroinitiator (data not shown). Finally, the macroinitiator was used to initiate monomer Boc-AMA at 70°C via ATRP to prepare the tribolck copolymers $mPEG_{45}$–PCL_{40}–P(Boc-AMA)s. As shown in ^{1}H NMR spectrum of $mPEG_{45}$–PCL_{40}–$P(Boc\text{-}AMA)_{100}$, the single peak at 3.55 ppm was attributed 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, respectively. 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-AMA) segment, and the peak at 3.38 ppm was of the protons of methylene adjacent to amino group. The sharp peak at 1.45 ppm was the characteristic signal of methyl protons of Boc-protected group (Fig. 1c). After the deprotection reaction in anhydrous saturated HCl solution of ethyl acetate, the signal of methyl protons of Boc-protected group in 1.45 ppm disappeared, and the integration values of PEG, PCL and PAMA changed little, which confirmed the successful synthesis of $mPEG_{45}$–PCL_{40}–$PAMA_{100}$ (Fig. 1b).

The composition of triblock copolymer $mPEG_{45}$–PCL_{40}–PAMAs was calculated based on the integration values of the peaks at 3.55, 2.30 and 3.38 ppm, attributed to ethylene protons of PEG backbone, methylene protons

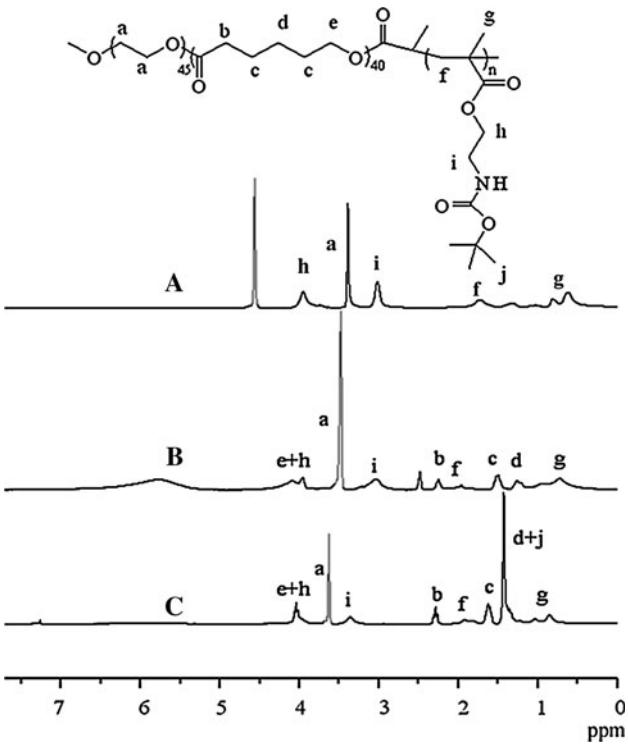


Fig. 1 ^{1}H NMR spectra of $mPEG_{45}$-*b*-PCL_{40}-*b*-$PAMA_{100}$ in D_2O (**a**), $mPEG_{45}$-*b*-PCL_{40}-*b*-$PAMA_{100}$ in DMSO-d_6 (**b**) and $mPEG_{45}$-*b*-PCL_{40}-*b*-$P(BOC\text{-}AMA_{100})$ in $CDCl_3$ (**c**)

Table 1 Characterization of mPECAs

Sample	Mn[a] (g mol^{-1})	Mn[b] (g mol^{-1})	PDI	cmc (mg L^{-1})
mPEC	6500	8400	1.30	
$mPECA_{25}$	12200	20300	1.54	151
$mPECA_{50}$	17900	25500	1.61	167
$mPECA_{100}$	29400	36300	1.57	179

[a] Determined from ^{1}H NMR

[b] Determined from GPC results

neighboring to carbonyl group of PCL backbone and methyl group adjacent to amino group of PAMA, respectively. Therefore, these three triblock polymers were named as $mPEG_{45}$–PCL_{40}–$PAMA_{25}$, $mPEG_{45}$–PCL_{40}–$PAMA_{50}$ and $mPEG_{45}$–PCL_{40}–$PAMA_{100}$ (denoted as $mPECA_{25}$, $mPECA_{50}$ and $mPECA_{100}$, respectively). $mPEG_{45}$–$PAMA_{105}$ ($mPEA_{105}$) and PCL_{38}–$PAMA_{95}$ (PCA_{95}) were synthesized and analyzed via the similar way (data not shown). The GPC results of mPECAs were also listed in Table 1, which further confirmed the successful synthesis of these copolymers.

3.2 Characterization of self-assemblies of mPECAs

The amphiphilic triblock copolymer mPECAs could self-assemble into nano-sized micelles in aqueous solution. It was believed that hydrophobic PCL segments were locked in the dense inner core of micelles, while the hydrophilic PEG and PAMA chains formed the corona shell for their highly hydrophilic nature [28]. As shown in Fig. 1a, the ^{1}H NMR spectrum of $mPECA_{100}$ in D_2O revealed the signals of PCL protons were suppressed obviously but the signals of the other components remained, proving the formation of micelles. Furthermore, based on fluorescence technique using pyrene as the probe, cmc of mPECAs was obtained and listed in Table 1. $MPECA_{100}$ showed the highest cmc about 179 mg l^{-1} for its longest hydrophilic segment.

3.3 Gel retardation assay of mPECAs

The DNA binding ability of $mPECA_{25}$, $mPECA_{50}$ and $mPECA_{100}$, was studied through gel retardation assay. As shown in Fig. 2a–c, three polymers could bind DNA efficiently, and the required N/P ratios were 1.5, 1.5 and 1 for $mPECA_{25}$, $mPECA_{50}$ and $mPECA_{100}$ to completely retard DNA, respectively. There was not significantly difference in DNA binding capability among all of them although the number of the repeat units of PAMA varied from 25 to 100. As is mentioned above, mPECAs could self-assemble into micelles in aqueous solution, which might lead to the aggregation of positive charges to a certain extent and weaken the influence of the length of PAMA subsequently [23]. As a result, when mPECAs mixed with DNA in the aqueous solution with a concentration above cmc, polymeric micelles could condense DNA efficiently.

3.4 Measurement of particle size and zeta potential of mPECA/DNA complexes

The particle sizes of polyplexes at various N/P ratios in 150×10^{-3} M NaCl solution were examined by dynamic light scattering (DLS). All three mPECAs could condense

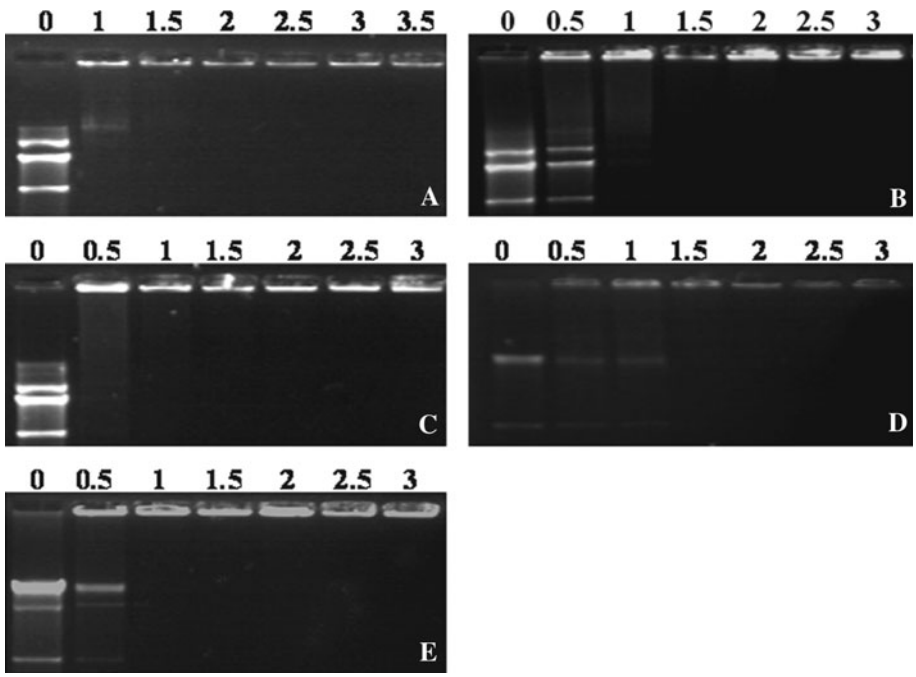


Fig. 2 DNA binding ability of polymers. Electrophoretic mobility of plasmid DNA in **a** $mPECA_{25}$, **b** $mPECA_{50}$, **c** $mPECA_{100}$, **d** $mPEA_{105}$ and **e** PCA_{95} complexes at the N/P ratios specified

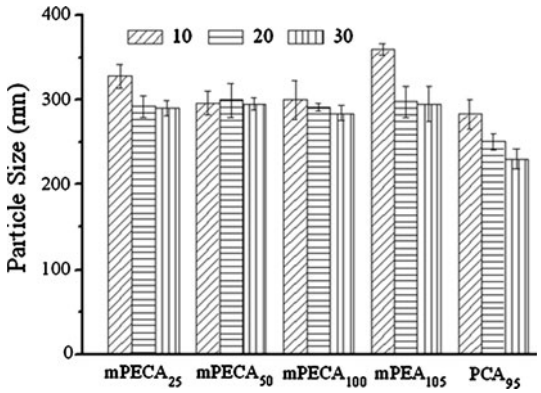


Fig. 3 Particle sizes of mPECA/DNA complexes, $mPEA_{105}$/DNA complexes and PCA_{95}/DNA complexes (▨: N/P = 10; ▭: N/P = 20; ▯: N/P = 30) measured by DLS in 150 mM NaCl solution

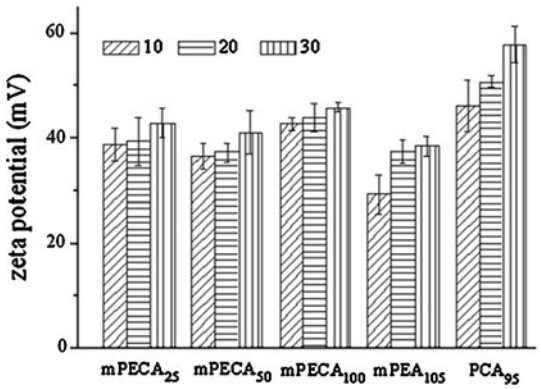


Fig. 4 Zeta potential of mPECA/DNA complexes. $mPEA_{105}$/DNA complexes and PCA_{95}/DNA complexes (▨: N/P = 10; ▭: N/P = 20; ▯: N/P = 30) measured by DLS in pure water

DNA into nanoparticles of 300 ± 20 nm in diameter at N/P ratios in range from 10 to 30. As shown in Fig. 3, the particle sizes of complexes decreased slightly as the N/P ratios increased. At the same time, no obvious size change of these complexes was observed with an increase in the length of PAMA segments.

Zeta potential of the polyplexes were measured in pure water and shown in Fig. 4. For mPECAs, the zeta potentials increased a little with increasing N/P ratios and the complexes of $mPECA_{100}$ exhibited the highest zeta potential at same N/P ratio. Generally, the zeta potentials of mPECA/DNA complexes were all above 30 mV and varied in a narrow range of 10 mV, suggesting that the surface charge of the polyplexes was also not obviously affected by the length of PAMA chains.

3.5 Cellular uptake

As well be known, the nuclear entry of plasmid DNA was the ultimate obstacle to achieve a high gene expression. Most of complexes could translocate plasmid DNA into cytoplasm but only not more than 0.001–0.1% plasmid DNA could be transcribed [29–31]. For better understanding of cellular location of plasmid DNA in COS-7 cells, the cellular uptake of $mPECA_{100}$/YOYO-1 labeled plasmid DNA complexes was investigated using a confocal microscope. As presented in Fig. 5, the fluorescent labeled complexes were observed to be internalized the cells and some of them were translocated into nucleus successfully, which implied $mPECA_{100}$ did affect the cellular uptake of the complexes could translocate DNA into nucleus.

3.6 Cytotoxicity assay

MTT assay was utilized to measure the cytotoxicity of mPECAs in COS-7 cells. As revealed in Fig. 6, a dose-dependent cytotoxic effect was observed for mPECAs, and their cytotoxicity enhanced with increasing concentration of the polymers. It was obvious that the length of PAMA could greatly affect the cytotoxicity of mPECAs. More importantly, mPECAs showed a low cytotoxicity as compared with bPEI.

3.7 In vitro transfection efficiency

In order to evaluate their transfection activity, the luciferase expression of mPECAs at the determined N/P ratios was investigated in COS-7 cells by using pGL-3 reporter gene. Branched 25 kDa PEI, which was considered to be the most efficiency gene vector, was used as control. As revealed in Fig. 7a, gene transfection efficiency of mPECAs depended on the length of PAMA segment. Among of them, $mPECA_{100}$ showed the highest gene expression up to 3.2×10^8 RLU/mg proteins at N/P ratio 20, while $mPECA_{25}$ that possessed the shortest PAMA segment, also exhibited high transfection activity.

Since therapeutic gene transfection is in vivo, plasma proteins and serum in blood have a great impact on application of gene delivery vectors, especially for cationic gene vectors. Transfection activity of gene vectors carrying out in presence of serum would be more convincing. Here, we used DMEM with 10% FBS to simulate in vivo environment and evaluated transfection efficiency of mPECAs in the medium (Fig. 7b). It was worth noting that all mPECAs retained their transfection efficiency in serum. For example, $mPECA_{100}$ showed luciferase expression of 5.3×10^8 RLU/mg proteins at N/P ratio of 30, which was about three times to that of bPEI at N/P ratio of 10 under the same condition.

MPECAs had shown potential to be an efficient gene vector for their higher transfection efficiency and less cytotoxicity compared to bPEI in serum. It was supposed that its unique properties were related to the structure of

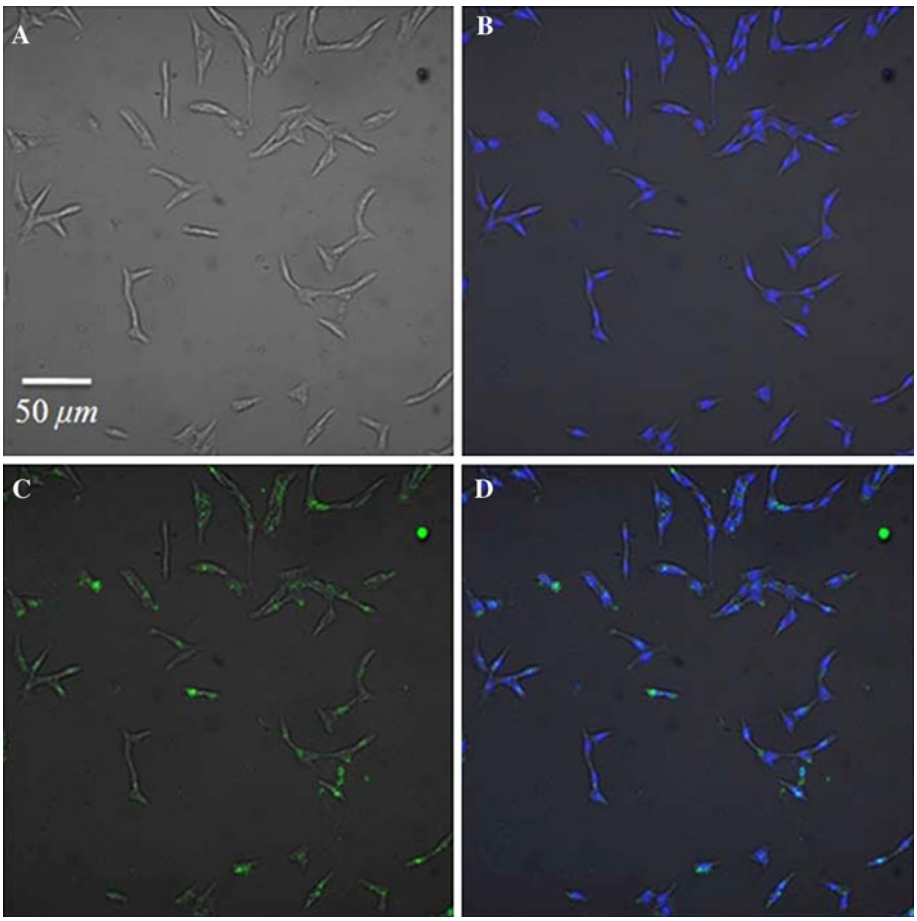


Fig. 5 Cellular uptake of YOYO-1 labeled DNA from $mPECA_{100}$/DNA complexes in COS-7 cells: **a** COS-7 cells; **b** H33258 labeled nucleus (*blue*); **c** YOYO-1 labeled DNA (*green*); **d** an overlay of **a**–**c** (Color figure online)

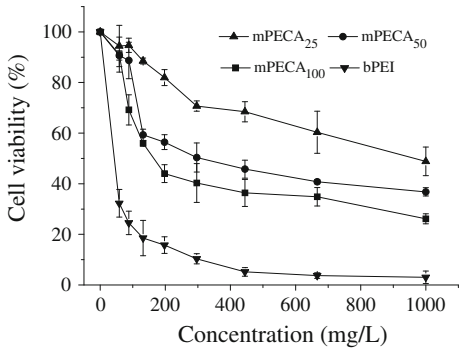


Fig. 6 Relative cell viability of COS-7 cells co-incubated with polymers for 24 h

mPECAs. In order to evaluate the influence of PEG segment and PCL segment in mPECAs, diblock copolymers PCA_{95} and $mPEA_{105}$ were chosen to compare with $mPECA_{100}$. In detailed, gel retardation assay showed PCA_{95} possessed better DNA binding capability (Fig. 2d, e) and higher zeta potential (Fig. 4) than $mPEA_{105}$. These results demonstrated that the incorporation of hydrophobic PCL indeed could increase the local concentration of positive charges via self-assembly in aqueous solution, resulting in better DNA binding ability [23]. Meanwhile, lower zeta potential of $mPEA_{105}$ may indicate PEG could shield the cationic surface charge [14]. Absorption of bovine serum albumin assay was further used to investigate the function of mPEG segments that could protect the complexes against self-aggregation for its steric effect. Here, the absorptive capacity of $mPECA_{100}$, $mPEA_{105}$ and PCA_{95} to BSA was investigated. As shown in Fig. 8, the complexes of $mPECA_{100}$ showed the least absorption rate of BSA among these three copolymers, indicating PEG segment of mPECAs could effectively reduce the interaction with plasma protein. Finally, the effect of PEG and PCL segments on the transfection ability was also evaluated in the medium with or without serum (Fig. 7). PCA_{95} in DMEM displayed much higher transfection efficiency than the other PEGylated PAMA copolymers, which illustrated that PEGylation maybe led to an inhibitive effect on the cellular uptake of the polyplexes [17]. However, in DMEM with 10% FBS, PCA_{105} exhibited sharp reduced transfection. It might be because PCA_{95}/DNA complexes aggregated in serum more easily due to their higher zeta potentials than the others. On the contrary, $mPEA_{105}$ could keep parallel transfection efficiency in FBS as compared to that in

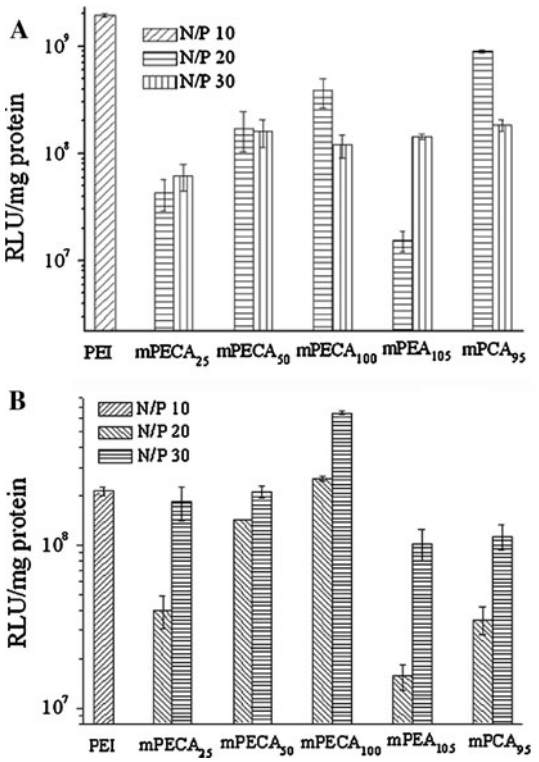


Fig. 7 Luciferase expression in COS-7 cells mediated by mPECAs, $mPEA_{105}$ and PCA_{95} in DMEM media without (**a**) or with 10% FBS (**b**)

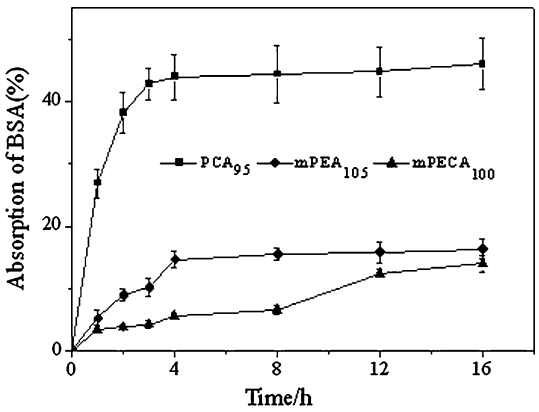


Fig. 8 Absorption of bovine serum albumin by PCA_{95}/DNA complexes (**a**), $mPEA_{105}$/DNA complexes (**b**) and $mPECA_{100}$/DNA complexes (**c**) at N/P ratio of 30

DMEM without FBS, indicating PEGylation did enhance the stability of polyplexes in serum. It is worth mentioning that $mPECA_{100}$ showed better luciferase expression than both PCA_{95} and $mPEA_{105}$ in DMEM with 10% FBS, which implied that the combination of PEG and PCL segments in one macromolecule might lead to synergistic effect to improve the transfection efficiency of $mPECA_{100}$ in serum (Fig. 7b).

4 Conclusions

In this study, we synthesized amphiphilic cationic triblock copolymers mPEG–PCL–PAMAs and explored their potential application for gene delivery. The results demonstrated that micellar aggregates formed by these copolymers could bind pDNA, translocate pDNA into cytoplasm and release pDNA in nucleus of COS-7 cells effectively. The studies also showed the incorporation of hydrophobic PCL segment in triblock copolymers could enhance the binding capability to pDNA and increase surface charges of complexes due to the formation of micelles increasing the local charges. The presence of mPEG in gene vector decreased the surface charges of the complexes and increased the stability of the complexes in serum due to the steric hindrance effect.

Acknowledgments This work was financially supported by National Natural Science Foundation of China (20704032), and National Basic Research Program of China (2009CB930300).

References

1. Mulligan RC. The basic science of gene therapy. Science. 1993;260:926–32.
2. Luo D, Saltzman WM. Synthetic DNA delivery systems. Nat Biotechnol. 2000;18:33–7.
3. Morille M, Passirani C, Vonarbourg A, Clavreul A, Benoit JP. Progress in developing cationic vectors for non-viral systemic gene therapy against cancer. Biomaterials. 2008;29:3477–96.
4. Lehrman S. Virus treatment questioned after gene therapy death. Nature. 1999;401:517–8.
5. Mintzer MA, Simanek EE. Nonviral vectors for gene delivery. Chem Rev. 2009;109:259–302.
6. Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, Behr JP. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc Natl Acad Sci USA. 1995;92:7297–301.
7. Wagner E, Cotten M, Foisner R, Birnstiel ML. Transferrin-polycation–DNA complexes: the effect of polycations on the structure of the complex and DNA delivery to cells. Proc Natl Acad Sci USA. 1991;88:4255–9.
8. Cherng JY, Wetering VP, Talsma H, Crommelin DJ, Hennink WE. Effect of size and serum proteins on transfection efficiency of poly((2-dimethylamino) ethyl methacrylate) plasmid nanoparticles. Pharm Res. 1996;13:1038–42.
9. Sato T, Ishii T, Okahata Y. In vitro gene delivery mediated by chitosan. Effect of pH, serum and molecular mass of chitosan on the transfection efficiency. Biomaterials. 2001;22:2075–80.
10. Haensler J, Szoka FC Jr. Polyamidoamine cascade polymers mediate efficient transfection of cells in culture. Bioconjug Chem. 1993;4:372–9.
11. Hwang SJ, Bellocq NC, Davis ME. Effects of structure of β-cyclodextrin-containing polymers on gene delivery. Bioconjug Chem. 2001;12:280–90.
12. Hugger ED, Novak BL, Burton PS, Audus KL, Borchardt RT. A comparison of commonly used polyethoxylated pharmaceutical excipients on their ability to inhibit p-glycoprotein activity in vitro. J Pharm Sci. 2002;91:1991–2002.

13. Hwang SJ, Davis ME. Cationic polymers for gene delivery: designs for overcoming barriers to systemic administration. Curr Opin Mol Ther. 2001;3:183–91.
14. Vinogradov SV, Bronich TK, Kabanov AV. Self-assembly of polyamine–poly(ethylene glycol) copolymers with phosphorothioate oligonucleotides. Bioconjug Chem. 1998;9:805–12.
15. Nguyen HK, Lemieux P, Vinogradov SV, Gebhart CL, Guerin N, Paradis G, Bronich TK, Alakhov VY, Kabanov AV. Evaluation of polyether-polyethyleneimine graft copolymers as gene transfer agents. Gene Ther. 2000;7:126–38.
16. Ochietti B, Lemieux P, Kabanov AV, Vinogradov S, St-Pierre Y, Alakhov V. Inducing neutrophil recruitment in the liver of ICAM-1-deficient mice using polyethyleimine grafted with Pluronic P123 as an organ-specific carrier for transgenic ICAM-1. Gene Ther. 2002;9:939–45.
17. Merdan T, Kunath K, Petersen H, Bakowsky U, Voigt KH, Kopecek J, Kissel T. PEGylation of poly(ethylene imine) affects stability of complexes with plasmid DNA under in vivo conditions in a dose-dependent manner after intravenous injection into mice. Bioconjug Chem. 2005;16:785–92.
18. Mishra S, Webster P, Davis ME. PEGylation significantly affects cellular uptake and intracellular trafficking of non-viral gene delivery particles. Eur J Cell Biol. 2004;83:97–111.
19. Alvarez-Lorenzo C, Barreiro-Iglesias R, Concheiro A, Iourtchenko L, Alakhov V, Bromberg L, Temchenko M, Deshmukh S, Hatton TA. Biophysical characterization of complexation of DNA with block copolymers of poly(2-dimethylaminoethyl) methacrylate, poly(ethylene oxide), and poly(propylene oxide). Langmuir. 2005;21:5142–8.
20. Gebhart CL, Sriadibhatla S, Vinogradov S, Lemieux P, Alakhov V, Kabanov AV. Design and formulation of polyplexes based on pluronic-polyethyleneimine conjugates for gene transfer. Bioconjug Chem. 2002;13:937–44.
21. Wang D, Narang AS, Kotb M, Gaber AO, Miller DD, Kim SW, Mahato RI. Novel branched poly(ethylenimine)–cholesterol water-soluble lipopolymers for gene delivery. Biomacromolecules. 2002;3:1197–207.
22. Batrakova EV, Miller DW, Li S, Alakhov VY, Kabanov AV, Elmquist WF. Pluronic P85 enhances the delivery of digoxin to the brain: in vitro and in vivo studies. J Pharmacol Exp Ther. 2001;296:551–7.
23. Seow WY, Yang YY. A class of cationic triblock amphiphilic oligopeptides as efficient gene delivery vectors. Adv Mater. 2009;21:86–90.
24. Shendage DM, Frohlich R, Haufe G. Highly efficient stereoconservative amidation and deamidation of alpha-amino acids. Org Lett. 2004;6:3675–8.
25. Hsu SH, Tang CM, Lin CC. Biocompatibility of poly(epsilon-caprolactone)/poly(ethylene glycol) diblock copolymers with nanophase separation. Biomaterials. 2004;25:5593–601.
26. Yu L, Zhang H, Cheng SX, Zhuo RX, Li H. Study on the drug release property of cholesteryl end-functionalized poly(ε-caprolactone) microspheres. J Biomed Mater Res B Appl Biomater. 2006;77B:39–46.
27. Wei H, Zhang XZ, Cheng C, Cheng SX, Zhuo RX. Self-assembled, thermosensitive micelles of a star block copolymer based on PMMA and PNIPAAm for controlled drug delivery. Biomaterials. 2007;28:99–107.
28. Zhang WL, He JL, Liu Z, Ni PH, Zhu XL. Biocompatible and pH-responsive triblock copolymer mPEG-b-PCL-b-PDMAEMA: synthesis, self-assembly, and application. J Polym Sci A Polym Chem. 2010;48:1079–91.
29. Capecchi MR. High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. Cell. 1980;22:479–88.
30. Pollard H, Remy JS, Loussouarn G, Demolombe S, Behr JP, Escande D. Polyethylenimine but not the cationic lipids promotes transgene delivery to the nucleus in mammalian cells. J Biol Chem. 1998;273:7507–11.
31. Dowty ME, Williams P, Zhang G, Hangstrom JE, Wolff JA. Plasmid DNA entry into post-mitotic nuclei of primary rat myotubes. Proc Natl Acad Sci USA. 1995;92:4572–6.