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Synthesis of a novel biodegradable poly(ester amine) (PEAs) copolymer based on low-molecular-weight polyethyleneimine for gene delivery☆

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ABSTRACT

In this paper, a novel biodegradable poly(ester amine) (PEA) copolymer was successfully prepared from low-molecular-weight polyethyleneimine (PEI, Mn = 1800) and poly(ε-caprolactone)–Pluronic–poly(ε-caprolactone) (PCFC) copolymers. According to the results of agarose gel electrophoresis, particle sizes and zeta potential measurement and transfection efficiency, these PEA copolymers showed great ability to condense plasmid DNA effectively into nano-complexes with small particle size (≤200 nm) and moderate zeta potential (≥12 mV) at proper polymeric carrier/DNA weight ratio. Compared with low-molecular-PEI (Mn = 1800), the obtained PEAs exhibited higher transfection efficiency as well as lower cytotoxicity. These results indicated that such PEAs might have great potential application in gene delivery system.

1. Introduction

The development of carriers for the delivery of genes or oligonucleotides has experienced considerable progress in the past three decades. Gene therapy has been realized with almost 400 clinical trials using viral vectors owing to their intrinsic high transfection efficiency (Merdan et al., 2002). Although the viral vector has high transfection efficiency, they have many serious disadvantages, such as complicated preparation process, limited loading capability, immunogenicity and potential to induce cancer (El-Aneed, 2004). Compared to this, non-viral vector is an important supplement to viral vector. Non-viral gene carriers were easier to synthesize and capable of carrying large amounts of genetic material, which also performed non-immunogenic, non-infectious and non-malignant transformation, hence, many researchers took part in this filed. Non-viral gene carriers generally depend on their large number of positive charges to condense plasmid DNA or antisense oligonucleotide into compact and small complexes, which enables trafficking through the diverse barriers toward the nucleus of target cells where the gene can be expressed. Polyethyleneimine (PEI) is one successful and widely used non-viral gene delivery systems because of its proton sponge effect (Boussif et al., 1995; Godbey et al., 1999b). However, it has shown cytotoxicity in many cell lines. In the meantime, several authors reported that macromolecular weight of PEI has strong influence on its transfection efficiency and cytotoxicity. It is considered that PEI with high-molecular-weight PEI (≥25 kD) not only shows high transfection but also high cytotoxicity. On the contrary, low-molecular-weight PEI (≤2000) is proved to be non-toxic but displays very poor transfection activity (Fischer et al., 1999; Godbey et al., 1999a). Many scientists reported that when the low-molecular-weight PEI is crosslinked with biodegradable bonds, such as ester, amide, and so on (Kim et al., 2007), the transfection efficiency can be enhanced while the cytotoxicity remains low.

Poly(ε-caprolactone) is a semi-crystalline linear absorbable aliphatic polyester, which has been widely used in biomedical field due to its great biocompatibility and biodegradability. But its degradation rate is very slow due to its great hydrophobicity (Pitt et al., 1990). Poly(ethylene glycol)–poly(propylene glycol)–poly(ethylene glycol) copolymer (PEG–PPG–PEG), commercially known as "Pluronic", "Lutrol", or "Poloxamer" has been extensively studied as potential drug delivery vehicle due to its excellent biocompatibility and environmental sensitivity (Xiong et al., 2003). These copolymers have many applications such as

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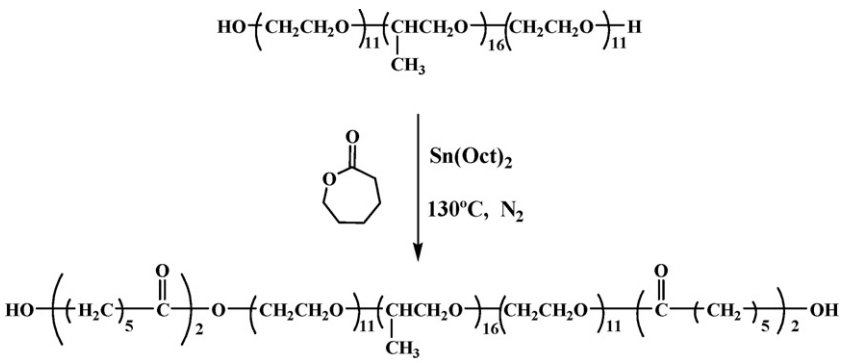

Fig. 1. Synthesis scheme of PCL–Pluronic–PCL (PCFC).

emulsifiers, wetting agents, solubilizers, etc. (Rangelov et al., 2005). Recently, Kabanov et al. (2002) reported that Pluronic-graft-PEI displayed higher transfection activity with lower cytotoxicity than PEI (Mw = 25KD) owing to its property of sticking to the cell membrane with Pluronic. It is expected that the copolymers based on PEI, Pluronic and PCL will be non-toxic along with increased transfection efficiency (Arote et al., 2007).

Due to the combined great advantages of Pluronic and PCL, PCL–Pluronic–PCL copolymer might have great potential application in biomedical fields. In the present study, PCL–Pluronic–PCL block copolymer was successfully prepared from ε-caprolactone and low-molecular-weight Pluronic (L35, BASF) by ring-opening polymerization of ε-caprolactone. In this paper, a novel biodegradable PEA based on PCL–Pluronic–PCL and PEI was prepared and characterized. We expect that the obtained PEAs might be optimized with enhanced transfection rate, great biodegradability and lower cytotoxicity compared to PEI (Mn = 1800).

2. Experimental

2.1. Materials and methods

Branched polyethyleneimine (PEI) (Mn = 1800), ε-caprolactone, glycidyl methacrylate (GMA), Pluronic 105 (Mn = 1900), deoxyribonuclease I (DNase I), Dulbecoo's Modified Eagle's Medium (DMEM), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma, USA. Dimethyl sulfoxide (DMSO), methanol, petroleum ether, and anhydrous dichloromethane were purchased from Chengdu KeLong chemicals, China.

All the chemicals used in this work are analytical pure grade, and used as received.

2.2. Synthesis of poly(ε-caprolactone)–Pluronic–poly(ε-caprolactone) copolymer

Poly(ε-caprolactone)–Pluronic–poly(ε-caprolactone) (PCFC) was synthesized by ring-opening polymerization of ε-caprolactone initiated by Pluronic 105, according to the protocol reported previously (Gou et al., 2007; Hang et al., 2008). Briefly, PCFC copolymer was prepared by introducing calculated amount of caprolactone and Pluronic 105 under nitrogen atmosphere into a dry three-necked bottle, and several drops of $Sn(Oct)_2$ were added. The bottle was kept at 130 °C. Five hours later, the reaction was stopped and cooled to room temperature under nitrogen atmosphere. The just-obtained PCFC copolymer was first dissolved in methylene chloride and reprecipitated from the filtrate using excess cold petroleum ether. Then the mixture was filtered and vacuum dried to constant weight. The purified PCFC copolymer was kept in air-tight bags in desiccators before using it. The reaction scheme is shown in Fig. 1.

2.3. Synthesis of GMA–PCFC–GMA copolymers

PCFC was dissolved in anhydrous dichloromethane and calculated amount of glycidyl methacrylate (GMA) 97% was added, with DMAP used as catalyst. The reaction mixture was stirred for 48 h at room temperature. The obtained GMA–PCFC–GMA macromonomer precipitated from petroleum ether was filtrated

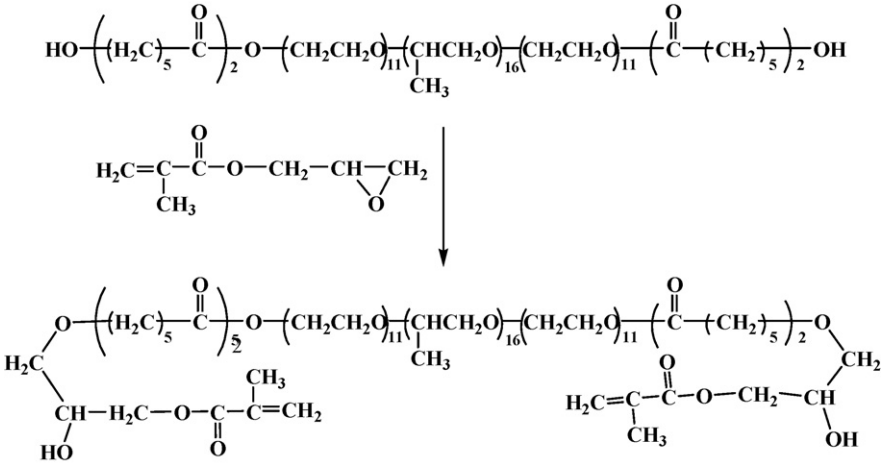

Fig. 2. Synthesis scheme of GMA–PCFC–GMA.

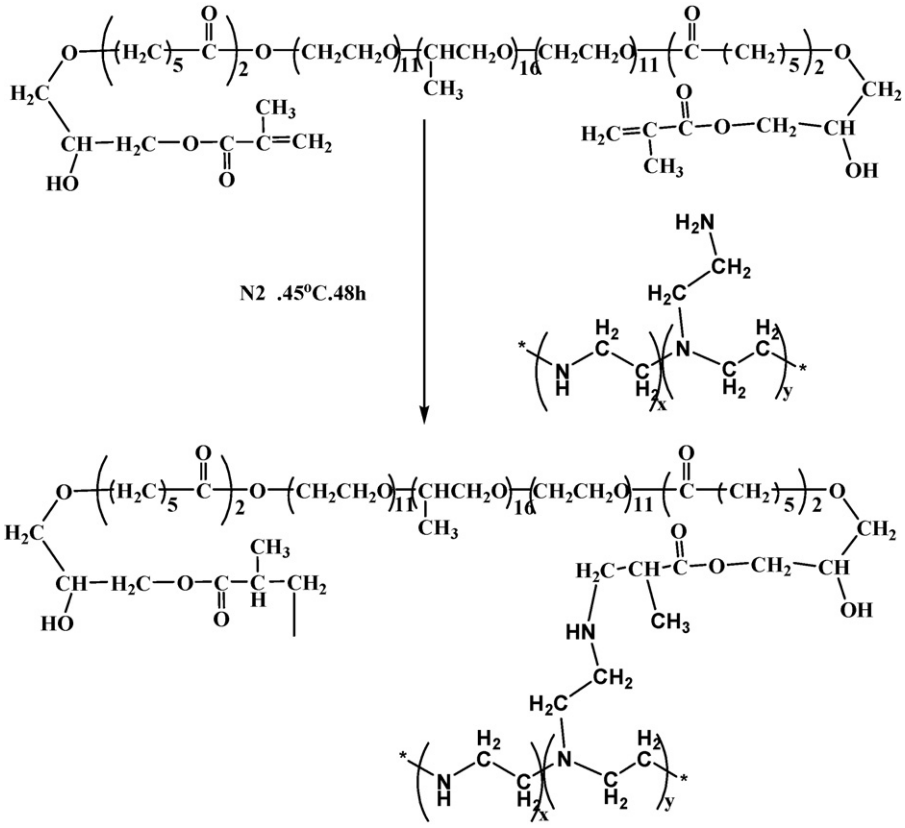

Fig. 3. Synthesis scheme of PEAs.

and dried in vacuum at 25 °C. The purified products were kept in air-tight bags in desiccators before use. The reaction scheme is shown in Fig. 2

2.4. Synthesis of branched PEAs

In this work, the PEAs were successfully synthesized by Michael addition reaction of low-molecular-branched PEI (Mn = 1800) and GMA–PCFC–GMA, according to scheme shown in Fig. 3. Briefly, PEI and GMA–PCFC–GMA were separately dissolved in anhydrous methanol. The solution of GMA–PCFC–GMA was added dropwise by syringe to the solution of PEI (Mn = 1800) with continuous stirring. The reaction mixtures were maintained at 45 °C under nitrogen atmosphere with constant shaking for 48 h. After completion of reaction, the vials were cooled down to room temperature and were vacuum dried for 2 days. The obtained PEAs were dissolved in distilled water and dialyzed using bag filter (Mw = 3500) against distilled water for 72 h. After the dialysis, the PEAs were lyophilized. The obtained purified PEAs were kept in air-tight bags before further use.

2.5. Characterization of the PCFC, GMA–PCFC–GMA and PEAs

The chemical structure of PCFC (in $CDCl_3$), GMA–PCFC–GMA (in $CDCl_3$) and PEAs (in D_2O) were characterized on ^1H Nuclear Magnetic Resonance (^1H NMR, 400 MHz, Varian, US).

2.6. Preparation of PEAs/DNA complexes

The whole coding sequence of green fluorescent protein (GFP) was inserted into pcDNA3.1 (Invitrogen, San Diego, CA) which contains a CMV promoter to construct the plasmid GFP expressing green fluorescent protein. And the pGFP solution was adjusted to 0.5 μg/μl. Complexes were prepared by mixing plasmid DNA with appropriate PEAs solution at desired charge ratio. The proportional amount of polymer were each diluted in 100 μl of distilled water and gently mixed. The polycomplex formulation was incubated at room temperature for 30 min before use.

2.7. Measurement of particles sizes and zeta potential

The particle size and zeta potential measurements of polymer/DNA complexes were measured using a Malvern Zetasizer 3000HS (Malvern, UK). Polymer/DNA complexes were prepared in 0.9% sodium chloride solution at various weight ratios. Before measurement, complexes were kept at room temperature for 10 min.

2.8. Agarose gel retardation assay

The agarose gel electrophoresis was used to determine the capability of condensing negatively charged nucleonic acid and protecting DNA from degradation. Plasmid DNA (pGFP-N1) was diluted to 0.5 μg/μl, cationic polymer solutions were then added to the plasmid solutions with the same volume at various [PEAs]/[pDNA] weight ratios and shortly vortexed. After 10 min incubation at room temperature, the complex formed then. And 10 μl of the polyplex solution was electrophoresised on a 0.9% (w/v) agarose gel in 0.5× Tris–boric acid–EDTA (TBE) buffer at 90 V for 30 min. The DNA bands stained with anthodium bromide were visualized by ultraviolet Trans illumination and photographed with a Lumi-Imager.

2.9. Cell viability assay

The cytotoxicity of branched PEAs and PEI (Mn = 1800) prepared at different concentrations was determined by MTT assay. Cells were seeded in 96-well plates at a density of 1×10^4 cells/well (HEK293) or 4×10^4 cells/well (A549) in 0.1 ml of growth medium and incubated for 24 h before replaced by 0.1 ml of fresh serum free DMEM medium containing different concentrations of PEAs and PEI (Mn = 1800) to each well. The cells were incubated with PEAs and PEI (Mn = 1800) for 24 h and then followed by an addition of 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml). After further incubation for 2–4 h, the MTT solution (0.5 mg/ml) was carefully removed from each well, and 150 μl DMSO was added to dissolve the MTT formazan crystals. The absorbance was recorded at 570 nm by an ELISA microplate reader (Bio-Rad). Polymer-untreated cells in media were used as a control.

2.10. Transfection assay in vitro

For transfection *in vitro*, HEK293 cells were cultured in DMEM medium containing Gln supplemented with 10% heated-inactivated fetal calf serum and antibiotics (100 units/ml penicillin, 100 units/ml streptomycin), and grown at 37 °C in humidified air containing 5% CO_2 and passage every 2–3 days. The HEK293 cells were seeded in 6-well plates at density of 1×10^5 cells per well and incubated for 24 h before transfection. Immediately before the initiation of transfection experiments, the medium was replaced by serum free media containing polymer/pGFP-N1 complexes at various weight ratios (carrier to gene) and additionally incubated for 6 h. Serum free medias were replaced by fresh media containing serum and incubated for additional 24 h. Relative light units (RLU) were measured with chemiluminometer (Autolumat, LB953, EG&G Berthold, Germany). RLUs were normalized to protein concentrate in the cell extract measured by BCA protein assay kit.

2.11. Abbreviations

PEI: polyethyleneimine; Pluronic: poly(ethylene glycol)–poly(propylene glycol)–poly(ethylene glycol) (PEG–PPG–PEG); ε-CL: ε-caprolactone (99%); GMA: glycidyl methacrylate (97%); DMEM: Dulbecoo's Modified Eagle's Medium; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO: dimethyl sulfoxide.

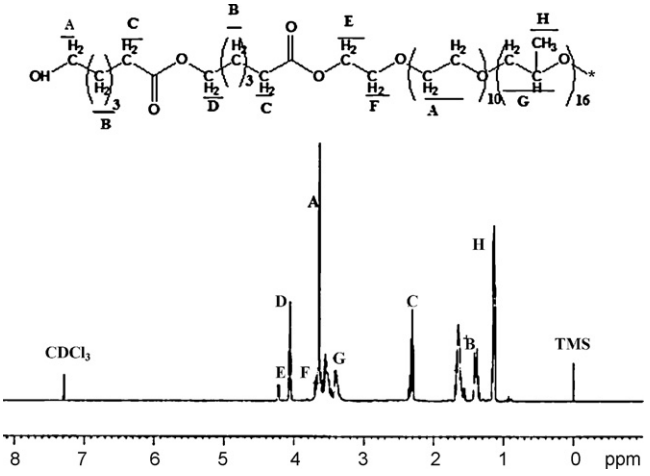

Fig. 4. Representative ^{1}H NMR spectrum (400 MHz) of PCL–Pluronic–PCL copolymer in $CDCl_3$.

3. Results and discussion

3.1. Preparation of PCL–Pluronic–PCL (PCFC)

According to Fig. 1, the PCL–Pluronic–PCL biodegradable block copolymers was successfully prepared by ring-opening polymerization of ε-caprolactone initiated by Pluronic and was confirmed by ^{1}H NMR spectroscopy in Fig. 4. The double peaks at 1.14 ppm, 3.42 ppm, and 3.50 ppm belong to protons of $-CH_3$, $-CH$, and $-CH_2$ in PPG unit of Pluronic block, respectively. The sharp peak at 3.65 ppm is attributed to methylene protons of $-CH_2CH_2O-$ in PEG unit of Pluronic block. Peaks at 1.40 ppm, 1.65 ppm, 2.30 ppm and 4.06 ppm are assigned to methylene protons of $-(CH_2)_3-$, $-OCCH_2-$, and $-CH_2OOC-$ in PCL blocks, respectively. The very weak peak at 4.23 ppm and 3.82 ppm are, respectively attributed to methylene protons of $-O-CH_2-CH_2-$ in PEG end unit linked with PCL blocks. ^{1}H NMR results indicated that the PCL–Pluronic–PCL block copolymers were prepared successfully with controlled macromolecular weight.

3.2. Characterization of GMA–PCFC–GMA

^{1}H NMR spectrum of GMA–PCFC–GMA macromonomer is shown in Fig. 5, which is very consistent with previously published results of our group. The signals at 6.13 ppm and 5.60 ppm cor-

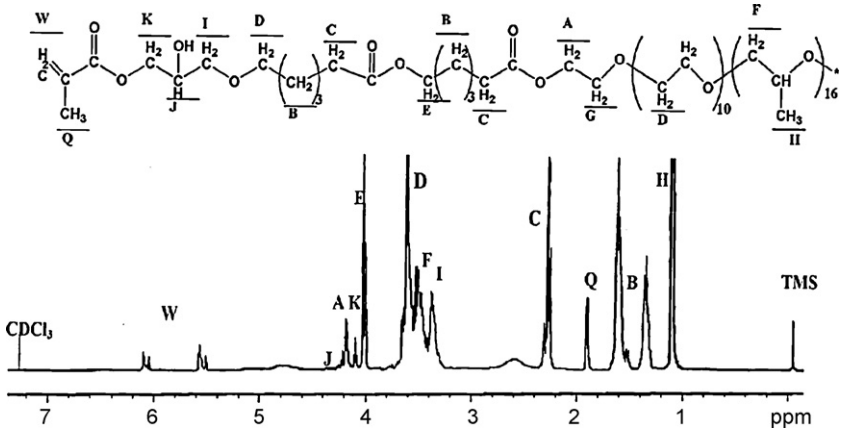

Fig. 5. Representative ^{1}H NMR spectrum (400 MHz) of GMA–PCL–Pluronic–PCL–GMA copolymer in $CDCl_3$.

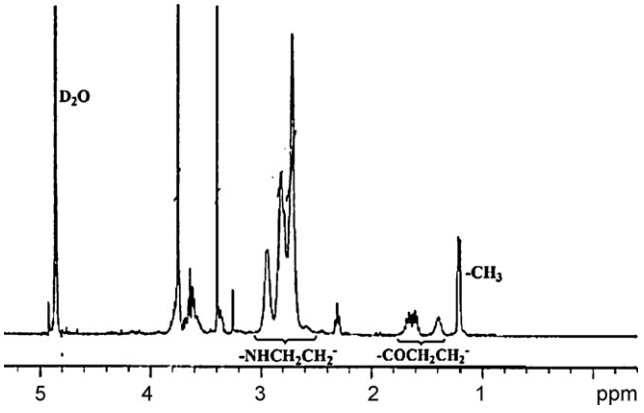

Fig. 6. Representative ^{1}H NMR spectrum (400 MHz) of PEAs copolymer in D_2O.

respond to protons of the double bonds. The double peaks at ca. 1.14 ppm, 3.42 ppm and 3.50 ppm belong to protons of $-CH_3$, $-CH$, and $-CH_2$ in PPG unit of Pluronic block, respectively. The sharp peak at 3.65 ppm is attributed to methylene protons of $-CH_2CH_2O-$ in PEG unit of Pluronic block. Peak at ca. 1.40 ppm, 1.65 ppm, 2.30 ppm and 4.06 ppm are assigned to methylene protons of $-(CH_2)_3-$, $-OCCH_2-$, and $-CH_2OOC-$ in PCL blocks, respectively. The very weak peaks at ca. 4.23 ppm and 3.82 ppm are attributed to methylene protons of $-O-CH_2-CH_2-$ in PEG end unit linked with PCL blocks, respectively.

3.3. Synthesis and characterization of PEAs copolymer

We aimed to synthesize a novel class of potentially safe non-viral gene delivery polymer. The hyper branched PEAs were obtained by the reaction of GMA–PCFC–GMA and low-molecular-weight branched PEI (Mn = 1800) as shown in Fig. 6. PEAs were synthesized through conjugate addition of amines to methacrylate groups which led to stoichiometrically dependant, broad statistical chain length distribution and molecular weight. Different types of amine groups might give rise to better transfection efficiencies like hyper branched PEI (Boussif et al., 1995; Kircheis and Wightman, 2001). Depending on the nucleophilicity of the amine groups, it will attack the vinyl groups of GMA–PCFC–GMA. The obtained polymers showed significant water solubility. The formation and the compositions of synthesized PEAs were confirmed through ^{1}H NMR spectroscopy. Chemical shifts at 2–3 ppm of the ^{1}H NMR spectrum are attributed to the protons of $-NHCH_2CH_2-$ of PEI. 3–4 ppm corresponded to the protons of Pluronic segment, 1–2 ppm and 4–5 ppm are attributed to the protons of PCL.

3.4. DNA condensation

The condensation of anionic DNA into small particles is an important prerequisite for gene carriers employing polycations (Bieber and Elsasser, 2001). In this study, to determine the optimal concentration for complete retardation of DNA, PEAs were incubated with plasmid DNA at different weight ratios keeping the amount of DNA (2 μg) constant, and retardation was analyzed by agarose gel electrophoresis. Fig. 7 shows the results of agarose gel electrophoresis of PEAs/DNA complexes at various weight ratios. The naked DNA was used as control. Increasing amounts of PEAs in DNA complexes lead to decreased electrophoretic mobility. The PEAs/DNA complexes showed complete retardation at weight ratio 0.5 (carrier to gene). It was demonstrated that the polymer can concentrate DNA at low weight and have protection ability against DNase I.

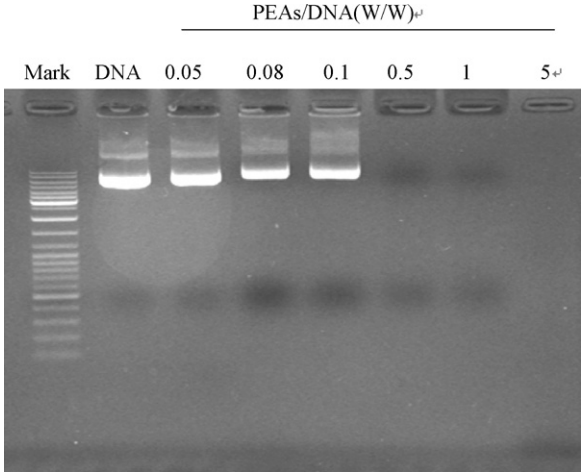

Fig. 7. Agarose gel electrophoresis of PEAs/DNA complexes at various weight ratios.

3.5. Size and zeta potential of the complexes

The size and zeta potential of the complex are important factors for cell uptake (Godbey et al., 1999a). It is already known that particles smaller than 200 nm in diameter could take advantages of endocytosis of various types of mammalian cells (Guy et al., 1995). In this paper, the particle sizes of PEAs/DNA complexes were analyzed by dynamic light scattering to investigate the degree of compaction with DNA. As is shown in Fig. 8, the particle sizes of PEAs/DNA complexes differed while prepared 0.9% sodium chloride solution at various weight ratios. In this experiment, we used 0.9% sodium chloride solution as the medium to dilute and to mix PEAs and DNA. Many factors could impact the result such as the concentration of DNA and polycation, the volumes of the solution before and after mixing, the different sequence of the reagents addition and also the speed of mixing. Finally, this part was carried out under the same conditions and was practiced to the protocol of transfection in cell culture, except the media. From Fig. 8, we found that the complexes of PEAs/DNA, when weight ratio was below 60, the size of PEAs/DNA nanoparticles was larger than 246 nm. And at weight ratio of 80, the diameters decreased to 192 nm. The decrease in size resulting from the increased weight ratio was found significant. Some reports have indicated that polyplexes in this size range are efficiently endocytosed by cells (Guy et al., 1995).

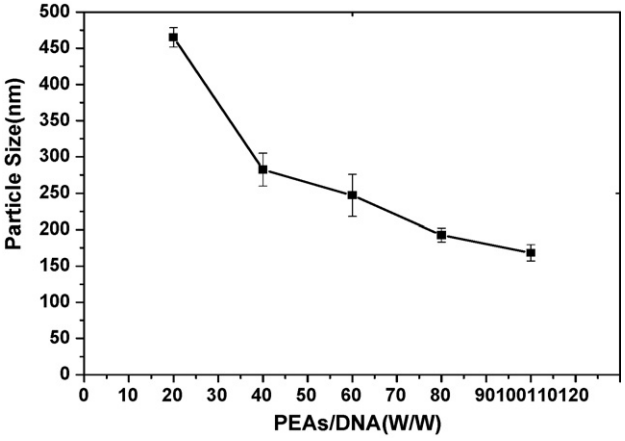

Fig. 8. Particle sizes of PEAs/DNA complex at various weight ratios.

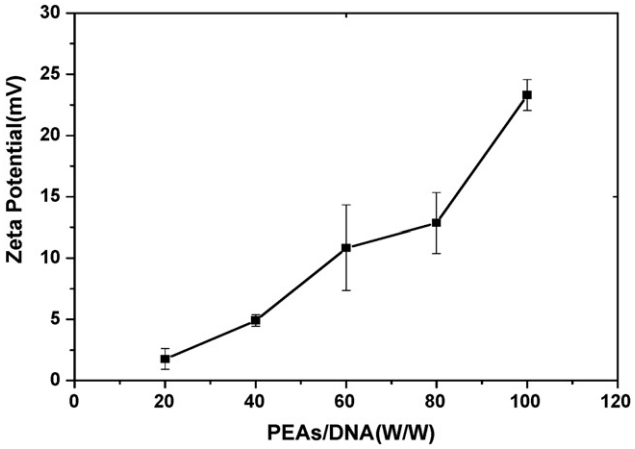

Fig. 9. Zeta potentials of PEAs/DNA complex at various weight ratios.

Zeta potentials of polymer/DNA complexes are closely related with cellular uptake (Mislick and Baldeschwieler, 1996; Putnam et al., 2003). Also, strong cationic charges of the complexes often cause great cytotoxicity. Therefore, PEGylation has been performed to reduce the polycation-mediated cell death (Park et al., 2004). Fig. 9 indicates that the majority of PEAs condensed DNA into nanoparticles with significant surface charges. As expected, the zeta potentials of complexes with PEAs were rather high. Zeta potentials rapidly increased with increasing weight ratio (carrier to DNA) from 20 to 100. The average zeta potentials of complexes PEAs/DNA ranged from 1.75 to 23.3 mV. Therefore PEAs are expected to be suitable candidates for effective transfection owing to their ideal particle sizes and significant surface charges.

3.6. Cell viability assay

Cytotoxicity is a major hurdle for clinical feasibility of polycationic gene carriers (Lv et al., 2006). Many researchers have reported that a high-molecular-weight PEI is more toxic than a low-molecular-weight one. Bieber and Elsasser (2001) also suggested the correlation between cytotoxicity and molecular weight. Relatively, our attempt is focused on realizing low cytotoxicity and increasing transfection efficiency of the carriers compared with PEI (Mn = 1800). Therefore, the hyper branched PEAs composed of PCFC and low-molecular-weight PEI (Mn = 1800) were designed to reduce the cytotoxicity and increase transfection efficiency. The cytotoxicity of PEAs and PEI (Mn = 1800) were carried out on the basis of an MTT assay on HEK293, HepG-2 and A549. The cell viability was observed with increasing concentration of

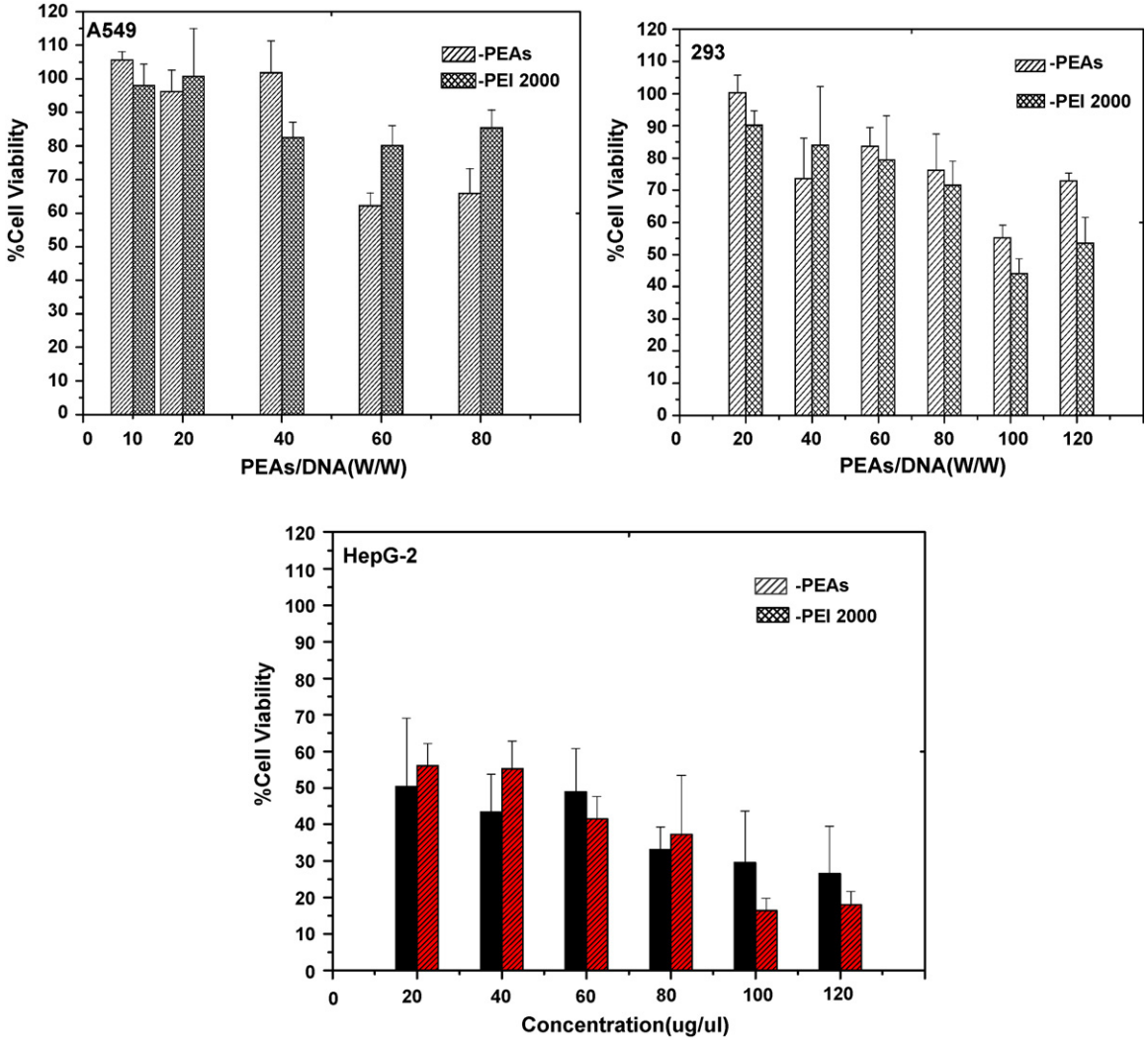

Fig. 10. Cytotoxicity of PEAs and PEI (Mn = 1800) at various weight ratios in two cell lines HEK293 and A549. Cell were seeded in 96-well plates at an initial density 1×10^4 (HEK293) or 2×10^4 (A549) cells/well in 0.2 ml growth media. Cell viability were detected by MTT assay (mean ± SD $n = 6$).

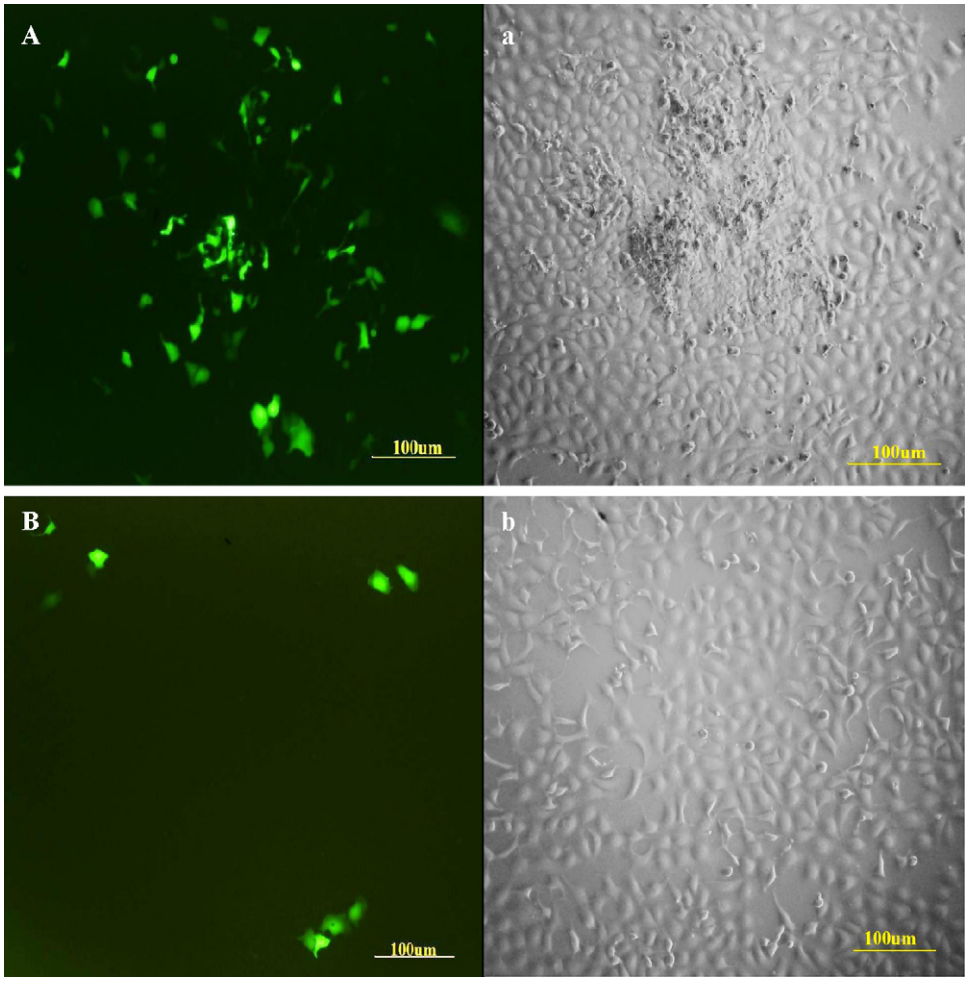

Fig. 11. Transfection images of PEAs (A) and PEI (B) were shown in two cell lines HEK293. Cells were incubated with PEAs/DNA and PEI (Mn = 1800) complexes at weight ratio 80 (carrier to gene) for 24 h. The green fluorescent protein (GFP) expression was observed under fluorescent microscope at 5× magnification.

PEI (Mn = 1800) and PEAs complexes. The results were shown in Fig. 10. 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 and 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.

On the three cell lines, the tolerance of the gene delivery vector had obvious distinction on a different density. In 293 cell lines and A549, the PEAs and PEI 1800 showed above 70% viability at 120 μg/μl and 75% at 80 μg/μl. In contrast, cell viability dropped by 20–40% in the HepG-2, the cell viability of PEAs and PEI 1800 was about 50% at 20 μg/μl and 20% at 120 μg/μl. It was obvious that these synthesized PEAs showed significantly the same cell viability as compared with control PEI (Mn = 1800).

3.7. Transfection efficiency

In this work, we successfully synthesized a novel PEAs copolymer that was cross-linked with low-weight-molecular bPEI (Mn = 1800) by multiblock poly(ε-caprolactone)–Pluronic–poly(ε-caprolactone) (PCFC). The aim of this study is to increase transfection efficiency and cell permeability by grafting Pluronic with biological activities. The transfection efficiency was increased compared with bPEI (Mn = 1800). PEAs effectively reduced toxicity on HEK293, and retained considerable transfection efficiency *in vitro*. The analysis of the size and zeta potential of PEAs/DNA complexes suggest that transfection efficiency depended on not only the molecular weight of the polycation, but also the structure and biological activity agent. The Pluronic block of this cationic complex may be a very good reference on gene transferring both *in vitro* and *in vivo* (Kim et al., 2007).

Many factors could have affected transfection efficiency such as cell line and weight ratio. For rapid examination, polyplexes formed of PEAs/DNA and PEI (Mn = 1800)/DNA of weight ratios (polymeric carrier to gene, W/W ratio from 10/1 to 100/1) were added to HEK293 cell cultures, followed by incubation. The maximum fluorescent emission was observed when the ratio reached 80/1 after 24 h. The transfection efficiency reached 17%, while the transfection efficiency of the PEI (Mn = 1800) was only 0.5% in Fig. 11. Also, as a Pluronic grafted copolymer, Pluronic 105 contained in the PCFC complex could be degraded in acid. It has been reported that Pluronic block copolymer exhibited valuable biological activities such as enhancing sealing of cell membranes permeabilized by ionizing radiation and electroporation, thus prevented cellular necrosis (Lee et al., 1999; Hanning et al., 2000), and it could also enhance polycation-mediated gene transfer *in vitro* (Astafieva et al., 1996). Due to these advantages, though the content of primary amine was decreased, the transfection efficiency did not decrease significantly as the result show.

4. Conclusion

In this work, we have successfully synthesized PCFC block copolymer PEAs which crossed link with low-weight-molecular

bPEI (1800) by multiblock poly(ε-caprolactone)–Pluronic–poly(ε-caprolactone) (PCFC). The aim of this study is to increase the transfection efficiency and decrease the cytotoxicity. Compared to low-molecular-PEI (Mn = 1800), the transfection efficiency of the non-viral PEAs was higher than that of PEI (Mn = 1800) on HEK293 *in vitro*. The analysis of the size and zeta potential of PEAs/DNA complexes suggest that transfection efficiency depended on many factors, not only the molecular weight of the polycation but also the structure and biological activity agent. The PCFC block of this cationic complex may be a very good reference on gene transferring both *in vitro* and *in vivo*.

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