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A biodegradable triblock copolymer poly(ethylene glycol)-*b*-poly(L-lactide)-*b*-poly(L-lysine): Synthesis, self-assembly, and RGD peptide modification

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Abstract

A novel biodegradable triblock copolymer poly(ethylene glycol)-*b*-poly(L-lactide)-*b*-poly(L-lysine) (PEG–PLA–PLL) was synthesized by acidolysis of poly(ethylene glycol)-*b*-poly(L-lactide)-*b*-poly(ε-benzyloxycarbonyl-L-lysine) (PEG–PLA–PZLL) obtained by the ring-opening polymerization (ROP) of ε-benzyloxycarbonyl-L-lysine *N*-carboxyanhydride (ZLys NCA) with amino-terminated PEG–PLA–NH_2 as a macroinitiator, and the pendant amino groups of the lysine residues were modified with a peptide known to modulate cellular functions, Gly-Arg-Gly-Asp-Ser-Tyr (GRGDSY, abbreviated as RGD) in the presence of 1,1′-carbonyldiimidazole (CDI). The structures of PEG–PLA–PLL/RGD and its precursors were confirmed by ^{1}H NMR, FT-IR, amino acid analysis and XPS analysis. The cell adhesion and cell spread on the PEG–PLA–PLL/RGD film were enhanced compared to those on pure PLA film. Therefore, the novel RGD-grafted triblock copolymer is promising for cell or tissue engineering applications. Both copolymers PEG–PLA–PZLL and PEG–PLA–PLL showed an amphiphilic nature and could self-assemble into micelles of homogeneous spherical morphology. The micelles were determined by fluorescence technique, dynamic light scattering (DLS), and field emission scanning electron microscopy (ESEM) and could be expected to find application in drug and gene delivery systems.

Keywords: Poly(L-lactide); Poly(amino acid); RGD

1. Introduction

In the past three decades biodegradable polymers have become more and more important for pharmaceutical and biomedical fields [1,2]. Among them, polylactide (PLA), a very important synthetic biodegradable material, has been widely used in surgical repair, carriers in drug delivery, and temporary matrixes or scaffolds in tissue engineering [3–5] due to its biodegradability, biocompatibility, high mechanical properties, and excellent shaping and molding properties. However, an important problem is inadequate interaction between the polymers and cells, leading to in vivo foreign body reactions, such as inflammation, infections, local tissue necrosis, and implant encapsulation as well as thrombosis [6–8]. Moreover, due to lack of functional groups, they cannot be modified easily with biologically active moieties.

Recently, many investigations have attempted to improve the hydrophilicity of polyester and provide functional groups for polyesters. Poly(ethylene glycol) is often introduced for its hydrophilicity, nontoxcity, biocompatibility and nonimmunogenicity. Polypeptides are very important biological macromolecules and suitable for biomedical applications such as sutures, artificial tissues, implants and drug delivery [9–12] because of their excellent physical properties, biocompatibility

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and biodegradability. Among all polypeptides, poly(L-lysine) (PLL) is unique due to its hydrosolubility and functional side NH_2 groups which can help to improve its affinity to proteins and cells, or to covalently or ionically combine with drugs, antibodies or DNAs, and thus may lead to breakthrough in the fields of targeting drug delivery and gene delivery [13,14]. Therefore, chemical modifications of aliphatic polyesters are realized by preparing hyperbranched polyesters [15–17], star block copolymer [18], and preparing co-polyesters [19], such as PEG–polyester, polyester–poly(amino acid) [20–22].

Advances in tissue engineering require biofunctional scaffolds that can provide not only physical support for cells, but also chemical and biological cues needed in forming functional tissues. Since Pierschbacher and Ruoslahti found that the tripeptide arginine-glycine-aspatic acid (Arg-Gly-Asp or RGD) was the minimal cell-recognizable sequence in many extracellular matrix protein and blood protein [23], RGD-containing peptides have been incorporated into synthetic non-degradable polymers [24–31]. However, degradability may be very important so that implanted cells can eventually obtain a completely natural environment, thereby eliminating the possibility of long-term detrimental tissue responses [32]. Barrera et al. have synthesized biodegradable copolymer poly(L-lactic acid-*co*-L-lysine) and attached the peptide sequence GRGDY to the lysine residue in the copolymer [32]. But the yield of 3-(ε-benzyloxycarbonyl-L-lysine)–6-L-methyl-2,5-morpholinedione used to synthesize poly(L-lactic acid-*co*-L-lysine) is low and only 2% lysine can be incorporated into the copolymer [33]. Yamaoka et al. have prepared biodegradable malic acid-containing functional polymers poly(L-lactic acid-*co*-malic acid-*co*-glycolic acid) [34], however, the situation of low yield of monomer and low content of malic acid still exists. Recently, we have obtained a new biodegradable triblock copolymer, poly(ethylene glycol)-*b*-poly(L-lactide)-*b*-poly(L-glutamic acid), containing side carboxyl groups and modified it with RGD peptide [35,36].

In this paper, a novel structure of poly(ethylene glycol)-*b*-poly(L-lactide)-*b*-poly(L-lysine) (PEG-*b*-PLA-*b*-PLL) triblock copolymer containing side amino groups was synthesized with PEG-*b*-PLA-NH_2 diblock copolymer as a macroinitiator for the ring-opening polymerization (ROP) of NCA by a convenient way. The pendent amino groups of the lysine residues were modified with an RGD peptide in the presence of 1,1′-carbonyldiimidazole (CDI). The triblock copolymer combined the characters of polyester, PEG and poly(amino acid). Number of the grafted RGD peptide could be adjusted either by changing the length of PLL block or by changing the grafting rate of RGD. Therefore, this polymer is expected to have enhanced cell adhesion and can serve as biodegradable scaffold for cell and tissue engineering. Recently, much interest has been concentrated on the self-assembly behavior of the block copolymers containing polypeptide and hydrophilic block [37–43]. Here, the micelles behavior of triblock copolymer PEG–PLA–PZLL with PEG as hydrophilic block and PLA and PZLL as hydrophobic blocks, and PEG–PLA–PLL with PEG and PLL as hydrophilic blocks and PLA as hydrophobic block was investigated by fluorescence technique, DLS, and ESEM. Such micelles are potential biomedical materials and can be applied in carrier systems in drug and gene delivery.

2. Experimental

2.1. Materials

Monomethoxy-poly(ethylene glycol) with a molecular weight of 750 (PEG 750) was obtained from Aldrich. Prior to use, it was dried by an azeotropic distillation in toluene. L-Lactide (LA) was purchased from Purac and recrystallized in ethyl acetate for three times. *N-tert*-Butoxycarbonyl-L-phenylalanine (Boc-Phe) and dicyclohexylcarbodiimide (DCC) from GL Biochem (Shanghai) Ltd. were used as received. 1,1′-Carbonyldiimidazole (CDI) obtained from Fluka, 33 wt% solution of HBr in HAc supplied by Acros, and peptide Gly-Arg-Gly-Asp-Ser-Tyr (GRGDSY, abbreviated as RGD) purchased from CL (Xi'an) Bio-scientific Inc. were used without further purification. ε-Benzyloxycarbonyl-L-lysine *N*-carboxyanhydride (ZLys NCA) was prepared according to Daly's method [44]. Hexane, methylene chloride, dimethyl sulfoxide (DMSO), and chloroform were refluxed over CaH_2 and distilled under nitrogen. Tetrahydrofuran (THF) was dried and distilled in the presence of sodium immediately before use. DMF was dried over CaH_2 and distilled before use.

2.2. Measurements

FT-IR spectra were recorded on a Bio-Rad Win-IR instrument. ^{1}H NMR spectra were measured by an AV-400 NMR spectrometer at room temperature. Gel permeation chromatography (GPC) measurements were conducted on a Waters 410 GPC with THF as eluent (flow rate: 1 ml/min, at 35 °C). The molecular weights were calibrated against polystyrene (PS) standards. Water contact angle was measured by drop shape analysis (DSA 10, KRüSS GmbH) to investigate the wettability of the film samples.

2.3. Amino acid analysis

The sample was first hydrolyzed in 6 N HCl at 110 °C for 24 h. Then it was reacted with FDNB (1-fluoro-2,4-dinitrobenzene) and was analyzed by high pressure liquid chromatograph (HPLC) equipped with a C_{18} column and detected at 360 nm at room temperature to determine the amounts of glycine and aspartic acid. On the basis of these two contents, the amount of RGD and the coupling efficiency of RGD were calculated.

2.4. XPS analysis

Surface elemental compositions of the peptide-containing samples were analyzed on an Escalab MKII photoelectron spectrometer (VG Scientific). The XPS experiments were

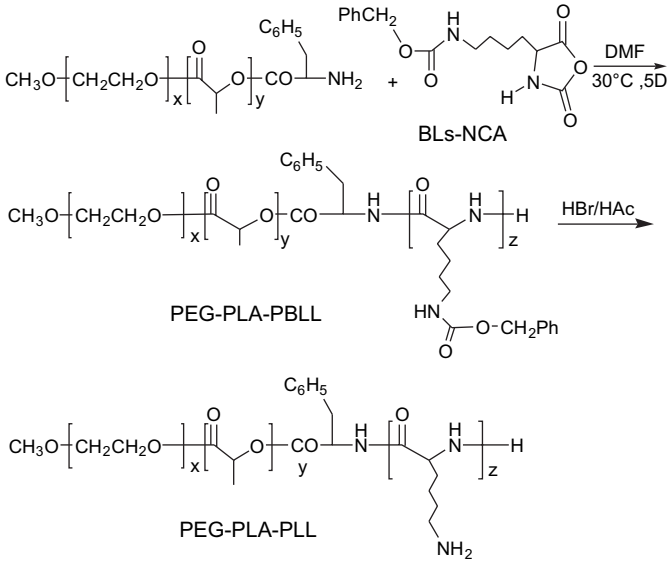
performed in the spectroscopy chamber using a standard Mg anode X-ray source (Mg Kα X-rays at 1253.6 eV) and a 150 mm hemispherical electron energy analyzer. The spectra were obtained for each sample using a 90° takeoff angle.

2.5. Syntheses of triblock copolymer PEG–PLA–PZLL

The PEG–PLA–PZLL was synthesized in three steps as shown in Scheme 1: (1) to prepare block copolymer PEG–PLA–OH by ROP of L-lactide in the presence of methoxy-poly(ethylene glycol) ($M_n = 750$) with stannous octanoate as catalyst; (2) to convert its end-group –OH into $-NH_2$, i.e., to prepare PEG–PLA–NH_2. The experimental details are described in Ref. [35]; and (3) to carry out ROP of ZLys NCA in the presence of PEG–PLA–NH_2 as macroinitiator. Following is a typical procedure for the third step. In a dried flask, 4.15 g (1.0 mmol) of PEG–PLA–NH_2 and 3.06 g (10 mmol) of ZLys NCA were dissolved in dried DMF (65 ml) and the solution was stirred for 72 h at 30 °C. The product mixture was precipitated with an excess of a mixture of acetic acid and methanol (1:3, v/v) under vigorous stirring to give a white solid while the unreacted PEG–PLA–OH remained dissolved in the mixture. After removal of the PEG–PLA–OH solution, purified PEG–PLA–PBGL was obtained under vacuum at 40 °C for 24 h.

2.6. Deprotection of PEG–PLA–PZLL

PEG–PLA–PZLL (0.5 g) was dissolved in 5 ml of CF_3COOH, then 4 equiv of a 33% solution of HBr in HAc with respect to the benzyl carbamate (Z) groups were added and the solution was stirred under argon for 1 h at 0 °C. After that, the reaction mixture was precipitated with an excess of diethyl ether to give a white product. The precipitate was dried in vacuo at 40 °C for 24 h.



Scheme 1. Synthesis of triblock copolymer PEG–PLA–PLL.

2.7. Syntheses of PEG–PLA–PLL/RGD

For the coupling of RGD, 0.1 g (0.018 mmol) of PEG–PLA–PLL and 12 mg of RGD (0.018 mmol) were dissolved in 2 ml of anhydrous DMSO. After adding 0.009 g of CDI (0.054 mmol), the reaction mixture was stirred at room temperature for 4 h. The product mixture was dialyzed with a cellulose membrane (cut-off M_n: 3500) for 3 days. After dialysis, the solution was immediately lyophilized for 2 days.

2.8. Test cells

L929 cells were chosen as the test cells. The cells were purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences and cultured with Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum (FBS, GIBCO), 1.0×10^5 U/L penicillin (Sigma) and 100 mg/L streptomycin (Sigma).

2.9. Cell adhesion and spreading

The copolymer PEG–PLA–PLL/RGD was dissolved in DMF, and cast on cover slides. The copolymer PEG–PLA–PLL/RGD and pure PLA films were prepared in a similar way and were use as the control. The slides were kept under vacuum for 48 h to remove the last traces of DMF, and exposed to UV light for 30 min for sterilization. The cover slides coated with the polymer films were placed in each well of 6-well tissue culture plates (NUNC). The L929 cells were seeded on the cover slides at a density of 2×10^5 cells/well, incubated in a humidified incubator at 37 °C and 5% CO_2. The pictures of each cover slide were taken with a digital camera (DXM1200F, Nikon) after 3 h, 20 h and 2 days, respectively.

2.10. Preparation of micelles from PEG–PLA–PZLL and PEG–PLA–PLL/RGD

The triblock copolymer (40 mg) was first dissolved in 10 ml of DMF in a 100 ml volumetric flask, and then 15 ml of distilled water was slowly added in portions of 5 ml. The solution was transferred into a dialysis bag (cut-off M_n: 3500) and dialyzed against distilled water for 48 h at room temperature to tardily remove DMF and to get the micelles.

2.11. Micelle characterization

The critical micelle concentrations (CMC) were measured by a fluorescence technique using pyrene as a probe. Pyrene solution (0.1 mg/L in acetone) was added into a series of volumetric flasks in such an amount that the final concentration of pyrene in each sample solution was 6.0×10^{-7} mol/l (its solubility in water at 22 °C) after dilution to the calibration line, and then the acetone was removed completely. The polymer solution and doubly distilled water were added into the volumetric flasks containing the solid pyrene to obtain desired copolymer concentrations from 1×10^{-5} to 0.4 mg/ml.

Steady-state fluorescence spectra were obtained on a Perkin Elmer LS50B luminescence spectrometer using 1.0 cm × 1.0 cm quartz cells. For emission spectra, $\lambda_{ex} = 339$ nm, and for excitation spectra, $\lambda_{em} = 390$ nm. The scan rate was 100 nm/min and the slit opening was 5 nm. Size distribution of micelles was determined by dynamic light scattering (DLS) with a vertically polarized He–Ne laser (DAWN EOS, Wyatt technology). The scattering angle was fixed at 90° and the measurement was carried out at 25 °C. The morphology of micelles was examined by ESEM, a drop of micelle solution was deposited onto a silicon chip and air-dried before observation.

3. Results and discussion

3.1. Synthesis of the triblock copolymer PEG–PLA–PZLL

It is well known that primary amines, being more nucleophilic than basic, can be used as initiators for the ROP of NCA to prepare poly(α-amino acid)s, undergoing a nucleophilic addition to the carbonyl group of the NCA [45]. Therefore, we obtained PEG–PLA–PBLG with PEG–PLA–NH_2 as a macromolecular initiator [35]. Similarly, we synthesize triblock copolymer PEG–PLA–PZLL according to Scheme 1. The results are summarized in Table 1.

The structure of the triblock copolymer was confirmed by the ^{1}H NMR spectra (Fig. 1(B)) [46]. The peak i at 7.26 ppm is attributed to the benzene ring of the protecting group. The peaks at 4.96 ppm, 3.82 ppm, 2.94 ppm, and 1.24–1.90 ppm were assigned to protons of the PZLL block. The peaks at 3.51 ppm and 3.24 ppm are assigned to protons of the PEG block. The peaks at 5.20 ppm and 1.46 ppm are assigned to protons of the PLA block. DP_{PZLL} in the triblock copolymer can be obtained from the integral ratio of CH_3O– (3.24 ppm, a) to $-C_6H_5CH_2OCONHCH_2-$ (4.96 ppm, h) or $-C_6H_5CH_2OCONHCH_2-$ (2.94 ppm, g), as shown in the following formula, $DP_{PBLG} = 3h/2a$ or $3g/2a$.

Table 1
Feed composition and molecular characteristics of triblock polymer PEG–PLA–PZLL

Polymer	PEG–PLA–NH_2:NCA		$M_n \times 10^{-3}$ (^{1}H NMR)	$M_n \times 10^{-3}$ (GPC)	$M_w \times 10^{-3}$ (GPC)	M_w/M_n
	In feed	In copolymer[a]				
P1	1:5	1:4	5.2	5.9	6.5	1.09
P2	1:10	1:14	7.8	8.4	9.8	1.17
P3	1:20	1:22	10.1	11.0	12.4	1.13
P4	1:40	1:45	15.9	18.2	21.9	1.20

[a] Determined by ^{1}H NMR.

The IR spectra of PEG–PLA–OH and PEG–PLA–PZLL are shown in Fig. 2(A) and (B). The absorption peak at 3300 cm^{-1} was assigned to ν_{NH} stretch vibration, and the peaks at 1655 cm^{-1} (*amide I*) and 1542 cm^{-1} (*amide II*) were attributed to the amide group, indicating the formation of the polypeptide block. The absorptions at 697 cm^{-1} and 749 cm^{-1} from the phenyl group were characteristic of the PZLL block carrying protection groups. The peak at 1734 cm^{-1} (ν_{CO}) was characteristic of the PLA block. The peak at 1127 belonged to the PEG block. The peak at 1087 cm^{-1} (ν_{C-O-C}) was corresponding to PLA block.

The GPC (Fig. 3(B)) trace of the triblock copolymer showed a unimodal shape. This further indicates that the copolymerization is completed successfully and there is no homopolymer in the reaction product.

3.2. Deprotection

The benzyloxycarbonyl protective group of the copolymer can be removed by acidolysis with a 33% solution of HBr in

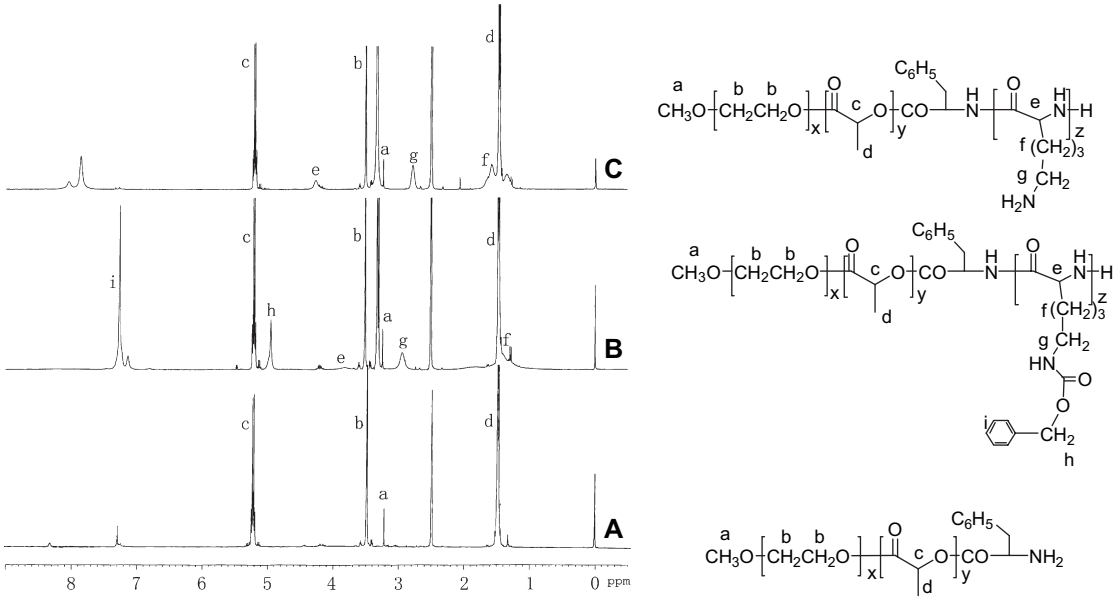


Fig. 1. The ^{1}H NMR spectra and their assignments of (A) PEG–PLA–NH_2, (B) PEG–PLA–PZLL, and (C) PEG–PLA–PLL in DMSO-d_6.

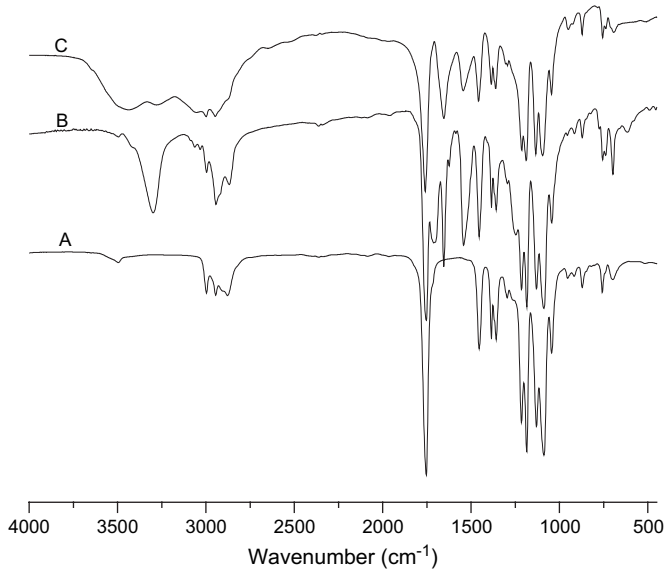


Fig. 2. The IR spectra of (A) PEG–PLA–OH, (B) PEG–PLA–PZLL, and (C) PEG–PLA–PLL.

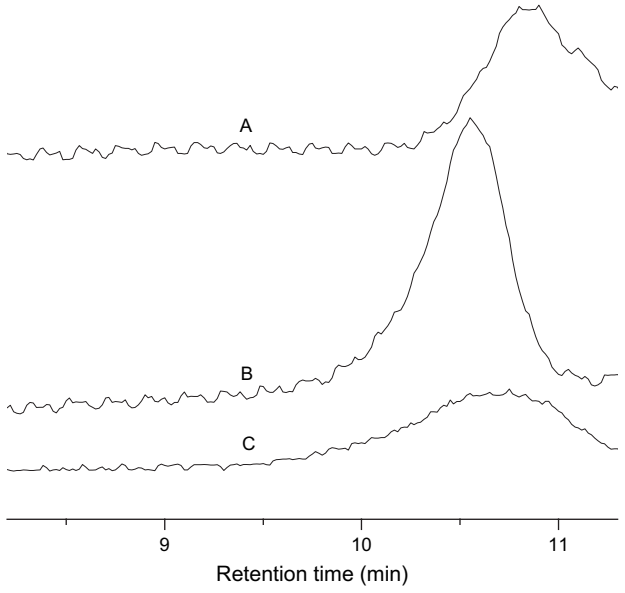


Fig. 3. The GPC traces of (A) PEG–PLA–NH_2, (B) PEG–PLA–PZLL, and (C) PEG–PLA–PLL.

HAc [46]. The deprotection was confirmed with ^{1}H NMR and FT-IR as shown in Figs. 1(C) and 2(C), respectively. In the ^{1}H NMR spectrum, the disappearance of the benzyl peaks at 4.96 ppm and 7.26 ppm, and the peak shift of the PLL block resonances (4.26 ppm, 2.79 ppm, 1.25–1.57 ppm) compared with those in PEG-*b*-PLA-*b*-PZLL (Fig. 1(B)) indicate that the protective groups in the polymer were removed completely. In the FT-IR spectrum, the δ_{CH} vibration of the benzyl at 749 cm^{-1} and 697 cm^{-1} disappeared and the vibration of amino group at 3480 cm^{-1} was strengthened greatly after acidolysis (Fig. 2(C)). The result clearly confirms that the benzyloxycarbonyl groups have been removed completely. GPC

Table 2
Water contact angles of PEG–PLA–PZLL and PEG–PLA–PLL

Polymer	Water contact angle (degree)	
	Protected copolymer	Deprotected copolymer
P1	71.3	50.4
P2	69.5	53.2
P3	78.0	47.9
P4	72.5	23.9

trace of the deprotected copolymer is shown in Fig. 3(C). Compared to that of PEG-*b*-PLA-*b*-PZLL (Fig. 3(B)), its peak shifted to lower molecular weight side to some extent due to the removal of the protective groups, and it became a little bit wider. It implied that the main-chain degradation of the polymer was negligible.

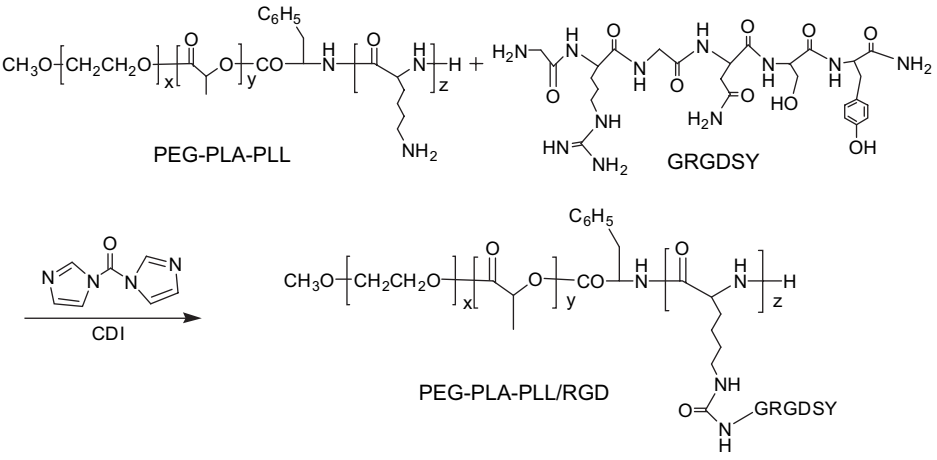
The water contact angle data are summarized in Table 2 for various PEG–PLA–PLL and PEG–PLA–PZLL samples. Typically, contact angle of sample P1 is 71.3° before deprotection and it becomes 50.4° after deprotection. Other samples also show lower contact angles after deprotection. This is because after deprotection the hydrophobic benzyloxycarbonyl groups have left and they are replaced by hydrophilic amino groups. Moreover, from sample P1 to P4, the degree of polymerization undergoes the same variation, but the PEG–PLA–PLL group shows a wider contact angle range (53–23°) than the PEG–PLA–PZLL group (78–69°). This is ascribed to contribution of the amino groups of the lysine residues. These data clearly demonstrate the promising hydrophilicity of PEG–PLA–PLL as well as the possibility of adjusting the hydrophilicity of PEG–PLA–PLL by changing the lysine content in the copolymer.

3.3. PEG–PLA–PLL/RGD

It was synthesized by reacting RGD with the activated PEG–PLA–PLL, as shown in Scheme 2. In ^{1}H NMR of PEG–PLA–PLL/RGD, RGD signals could not be identified easily because the signals from the PEG–PLA–PLL overwhelmed the RGD signals. However, appearance of the two doublets at 6.63 ppm and 7.0 ppm (p and q) belonging to the characteristic protons on RGD (Fig. 4) confirmed successful synthesis of PEG–PLA–PLL/RGD. The FT-IR spectrum showed two enhanced peaks at 1653 cm^{-1} (*amide I*) and 1548 cm^{-1} (*amide II*) (Fig. 5(B), compared to 5(A)) due to the formation of the ureylene linkage between the side amino group and the amino group of RGD. Therefore, the RGD had been attached to the copolymer PEG-PLA-PGL. The water contact angles of PEG–PLA–PLL/RGD are shown in Table 3, they are in the same range as PEG–PLA–PLL. It indicates that incorporation of the peptides do not change the wettability of the polymer significantly.

3.4. Amino acid analysis

The amino acid analysis was conducted to determine the contents of glycine and arginine and the coupling efficiency of RGD in the PEG–PLA–PLL/RGDs synthesized. As shown



Scheme 2. Synthesis of copolymer PEG–PLA–PLL/RGD.

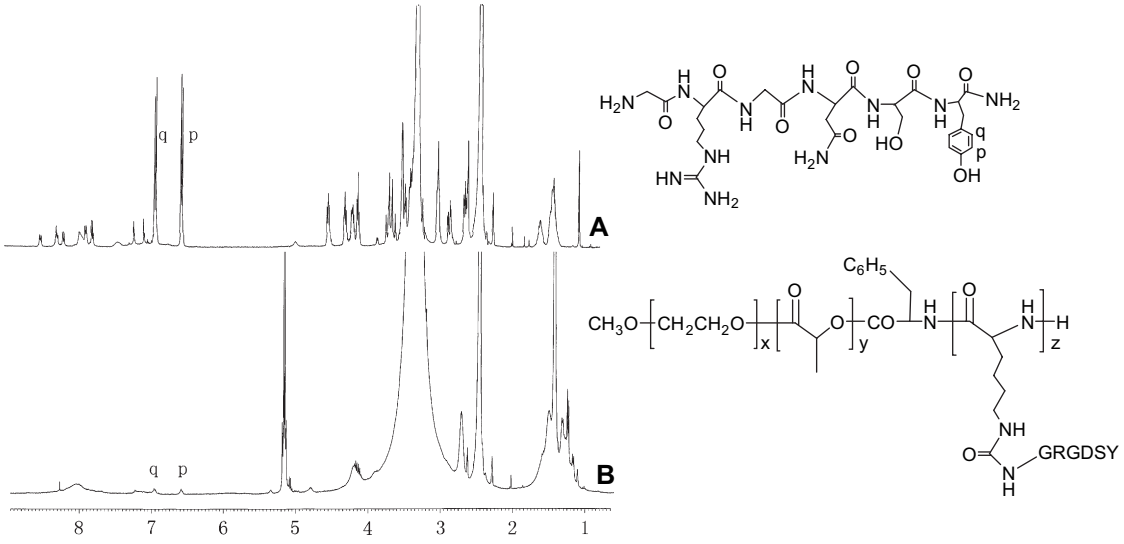


Fig. 4. The ^{1}H NMR spectra of (A) GRGDSY and (B) PEG-*b*-PLA-*b*-PLL/RGD in DMSO-d_6.

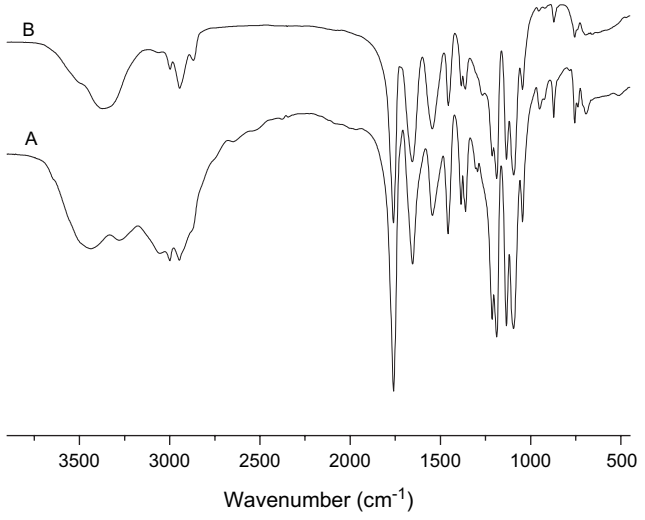


Fig. 5. IR spectra of (A) PEG–PLA–PLL and (B) PEG–PLA–PLL/RGD.

Table 3
Amino acid analysis of the RGD-grafted triblock coplymers

Sample	Feed ratio ($n_{RGD}/n_{polymer}$)	Lysine (μmol/l)	Glycine (μmol/l)	Aspartic acid (μmol/l)	Coupling ratio[a] (mol%)
1	1:1	3827.7	229.3	121.6	3.1
2	4:1	1735.4	130.1	79.8	4.2
3	8:1	2028.9	403.5	180.8	9.4

[a] Coupling efficiency = (Gly/2Lys + Asp/Lys)/2.

in Table 4, the coupling efficiency was improved with increasing RGD ratio in the reaction feed [47]. When the molar ratio of RGD to polymer increased from 1:1 to 8:1, the coupling ratio increased from ca. 3.1% to ca. 9.4%.

3.5. XPS analysis

The XPS was used to determine the elemental composition of the polymers synthesized. According to the molecular

Table 4
Water contact angles of copolymer PEG–PLA–PLL/GRGDSY

Sample	Water contact angle (degree)
PEG–PLA–PLL	53.2
PEG–PLA–PLL/GRGDSY (3.1%)	56.9
PEG–PLA–PLL/GRGDSY (4.2%)	59.1
PEG–PLA–PLL/GRGDSY (9.4%)	55.7

Table 5
Surface elemental analysis

Sample	C (mol%)	O (mol%)	N (mol%)	N/C
PEG–PLA–PLL	64.25	33.72	2.03	0.0316
PEG–PLA–PLL/GRGDSY (3.1%)[a]	76.34	8.59	15.07	0.1125
PEG–PLA–PLL/GRGDSY (4.2%)[a]	66.22	24.38	9.39	0.1419
PEG–PLA–PLL/GRGDSY (9.4%)[a]	68.73	21.07	10.20	0.1484

[a] The percent in brackets was the coupling ratio of RGD.

formulas shown in Scheme 1, nitrogen is only present in the PLL segment and the grafted peptide, therefore, the atomic ratio of nitrogen to carbon can provide a qualitative measure of the amount of grafted peptide at the material surface. As indicated in Table 5, the N/C ratio increases from 0.0316 for PEG–PLA–PLL to 0.1125–0.1484 for PEG–PLA–PLL/RGDs depending on the coupling ratio of RGD, indicating the successful coupling of RGD.

3.6. Cell adhesion and spreading

Cell adhesion of various films was evaluated by culturing L929 cells in a culture medium of DMEM containing 10% of FBS (fetal bovine serum). The test sample was copolymer PEG–PLA–PLL/RGD (coupling ratio 3.1%) and the control samples were copolymer PEG–PLA–PLL and pure PLA. Fig. 6 showed the cell morphology during the incubation. After incubating for 3 h, the cells on the copolymers PEG–PLA–PLL and PEG–PLA–PLL/RGD are much more than those on pure PLA, moreover, most of the cells on the copolymer PEG–PLA–PLL/RGD began to spread, which indicated that PLL were of benefit to cell adherence, and RGD could be good for cell spreading. After incubating for 20 h, almost all the cells on the copolymer PEG–PLA–PLL/RGD spread very well, and they were fatter and more than those on the PEG–PLA–PLL and PLA film. It should be noticed that the cells on copolymer PEG–PLA–PLL became less and showed obvious shrinkage, which suggested that PLL was quite harmful to the cells. After incubating for 2 days, the cells on the copolymer PEG–PLA–PLL/RGD almost occupied the whole surface. The cells adhered and spread better and proliferated faster on the RGD-containing film than on the control films. This indicates that the copolymer PEG–PLA–PLL/RGD is a promising biodegradable material for cell and tissue engineering.

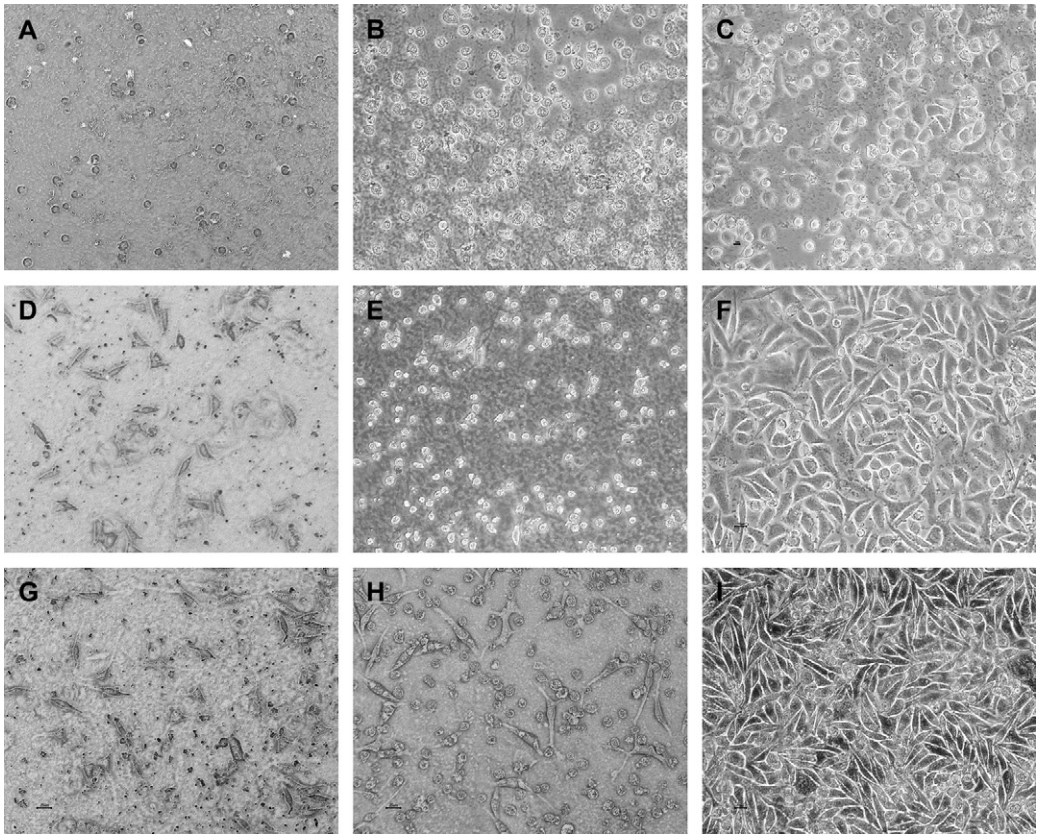


Fig. 6. Microscopic images of adhered and spread L929 cells. Film sample: (A, D and G) PLA, (B, E and H) PEG–PLA–PLL, and (C, F and I) PEG–PLA–PLL/RGD. Incubation time: A, B and C, 3 h; D, E and F, 20 h; G, H and I, 2 days.

3.7. Characterization of PGL–PLA–PGL and RGD/PGL–PLA–PGL/RGD micelles

It is well known that amphiphilic block copolymers can form micelles through self-assembling under suitable conditions. Recently, polymer micelles containing poly(amino acid)s such as poly(lysine), poly(aspartic acid) and poly(γ-benzyl-glutamic acid) have attracted more and more attention. An important application of the micelles formed by the polypeptide block copolymer is for drug, gene, and protein delivery systems [48–52]. Lee et al. obtained a novel copolymer poly(L-lactic acid)–poly(ethylene glycol)–poly(L-histidine)–biotin and designed a super pH-sensitive multifunctional polymeric micelle with it. The micelle showed pH-dependent dissociation, causing the enhanced release of doxorubicin from the carrier in early endosomal pH [48]. Miyata et al. prepared block catiomer polyplexes with regulated densities of charged and disulfide cross-linking directed to enhance gene expression using poly(ethylene glycol)–poly(L-lysine) [50]. Tian et al. designed a novel amphiphilic biodegradable cationic hyperbranched poly(ethylene glycol)–polyethylenimine–poly(γ-benzyl-L-glutamate) block copolymer to develop new properties of polymeric nanocarriers for drug release and gene delivery [52]. The triblock copolymers in the present study, PEG–PLA–PZLL and PEG–PLA–PLL, are composed of hydrophilic blocks PEG as well as PLL and hydrophobic block PLA as well as PZLL. Therefore, they can be self-assembled into micelles in aqueous solution. The micellar structure of PEG–PLA–PZLL is first confirmed by fluorescence technique using pyrene as a probe. The excitation spectra of pyrene of 6.0×10^{-7} mol/l in the presence of PGL-PLA-PGL at various concentrations are shown in Fig. 7(A). A red shift from 333 nm to 336 nm is observed with increasing concentration of PEG–PLA–PZLL, indicating the formation of micelles because pyrene is preferentially partitioned into the hydrophobic cores of the micelles with a change of the photophysical properties of pyrene molecules. The results of fluorescence analysis on the micellization of PEG–PLA–PLL copolymer with various ratios are shown in Fig. 7(B). Enhanced fluorescence intensity and a red shift from 332 nm to 335 nm were observed with increasing concentration of the copolymer PEG–PLA–PLL, indicating that the micellization occurred above the critical polymer concentration.

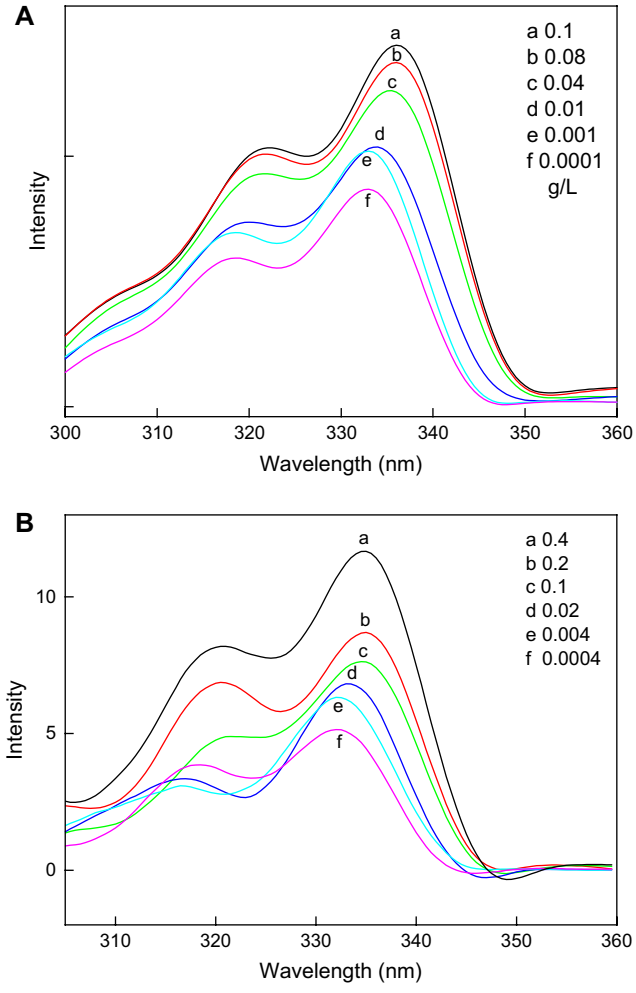


Fig. 7. (A) Excitation spectra of pyrene as a function of PEG–PLA–PZLL concentration in water. (B) Excitation spectra of pyrene as a function of PEG–PLA–PLL concentration in water.

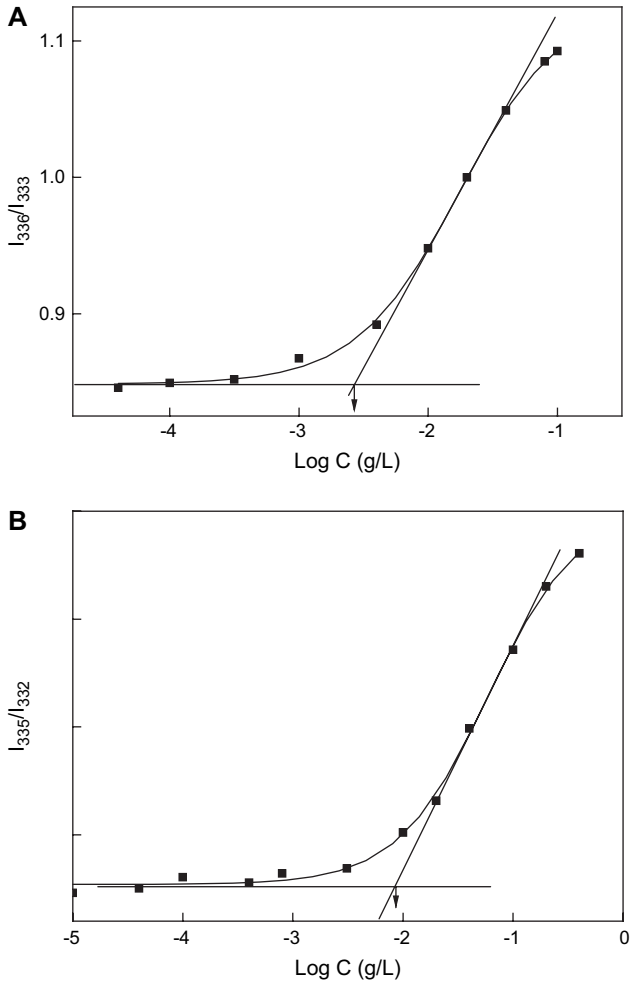


Fig. 8. (A) Plot of I_{336}/I_{333} versus logarithm of PEG–PLA–PZLL concentration. (B) Plot of I_{335}/I_{332} versus logarithm of PGL-PLA-PGL concentration.

The onset of micellization and the critical micelle concentration (CMC) can also be obtained from the studies of excitation spectra [53]. For the copolymer PEG–PLA–PZLL, 333 nm and 336 nm are chosen as the peak wavelength of the (0,0) band in the pyrene excitation spectra in the aqueous phase and in the entirely hydrophobic core of polymeric micelle, respectively. In Fig. 8(A) the pyrene fluorescence intensity ratios (I_{336}/I_{333}) are plotted against the logarithm of block copolymer concentration. Below a certain concentration, I_{336}/I_{333} is constant, above this concentration, I_{336}/I_{333} increases with increasing log C and finally reaches a plateau. From this plot, the critical micelle concentration (CMC) of 2.69 mg/L was obtained from the intersection of two straight lines: the base line and the rapidly rising I_{335}/I_{333} line. A typical plot of I_{335}/i_{332} versus log C for the copolymer PEG–PLA–PLL is shown in Fig. 8(B). Similar to the results of copolymer PEG–PLA–PZLL, negligible changes in the value of I_{335}/I_{332} were found at the low concentration of PEG–PLA–PLL and increased dramatically in a sigmoidal manner at a certain concentration (i.e., the CMC), indicating the formation of micelles. The CMC of 8.34 mg/L was also obtained from this plot, which was a little higher than that of PEG–PLA–PZLL as a result of the electrostatic repulsion of molecular chain.

The size and size distribution of micelles was measured by DLS. As shown in Fig. 9, the mean diameter of PEG–PLA–PZLL micelles was about 150 nm, while the mean diameter of PEG–PLA–PLL micelles was about 200 nm. The size of PEG–PLA–PLL was bigger than that of PEG–PLA–PZLL, which may attribute to the electrostatic repulsion of amino groups after deprotecting.

The micelle morphology and size were studied by ESEM. The micelles of copolymer PEG–PLA–PZLL and PEG–PLA–PLL aggregated into homogeneous spheres with a diameter of about 120 nm and 150 nm, respectively. (Fig. 10(A) and (B)). The diameter data were smaller than those determined by DLS, because the DLS showed the size of micelles in aqueous solution, while the ESEM gave the result after drying.

4. Conclusion

Starting from PEG, a novel triblock copolymer PEG–PLA–PLL was obtained by acidolysis of PEG–PLA–PZLL that was synthesized from the ROP of ZLys NCA with amino-terminated PEG–PLA–NH_2 as a macroinitiator. The pendant amino groups of the lysine residues were modified

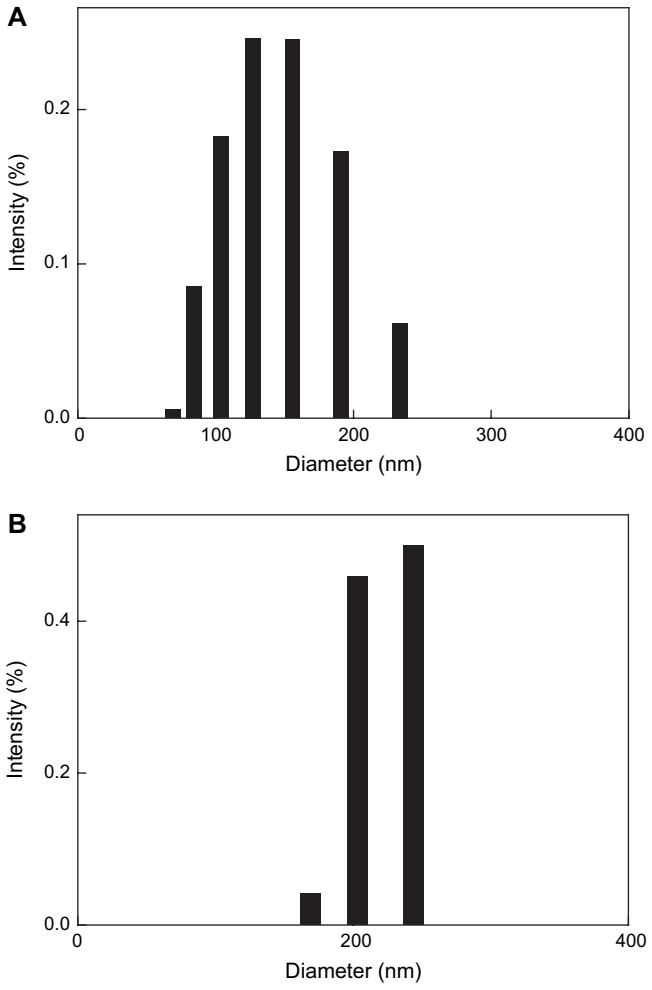


Fig. 9. The size distribution of (A) PEG–PLA–PZLL and (B) PEG–PLA–PLL micelles measured by DLS.

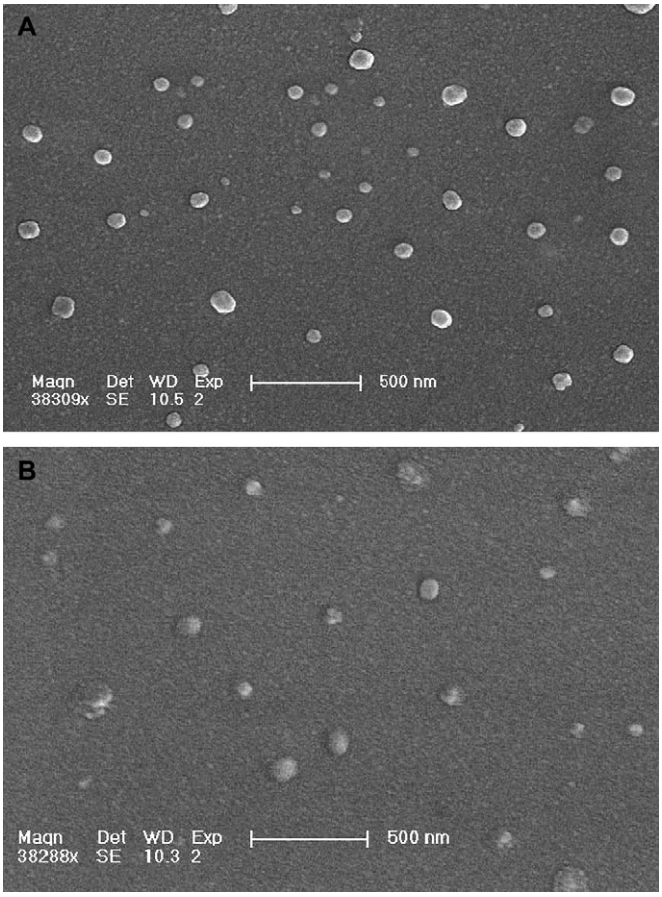


Fig. 10. ESEM micrographs of (A) PEG–PLA–PZLL micelles and (B) PEG–PLA–PLL micelles.

with RGD peptide in the presence of CDI. ^{1}H NMR, FT-IR, GPC, DSA, amino acid analysis and XPS analysis manifested that the intermediate and final products were successfully synthesized. The triblock copolymer combined the characters of polyester, PEG and poly(amino acid). Moreover, by changing the copolymer composition and by changing the RGD-grafting ratio, the amount of grafted RGD could be adjusted. The results of cell experiment suggested that this polymer can be used as biodegradable scaffolds for cell and tissue engineering. The micellization behaviors of copolymer PEG–PLA–PZLL and PEG–PLA–PLL were investigated by fluorescence spectroscopy, DLS and ESEM. The results of fluorescence spectroscopy confirmed that PEG–PLA–PZLL could form micelles easily in aqueous solution and the deprotected copolymer PEG–PLA–PLL could increase the critical micelle concentration. The results of DLS and ESEM showed that the micelles of PEG–PLA–PZLL and PEG–PLA–PLL were almost homogeneous spherical morphology with unimodal distribution. The average diameter of deprotected copolymer PEG–PLA–PLL was bigger than that of PEG–PLA–PZLL, which may ascribe to the electrostatic repulsion of side amino group of PLL after deprotecting.

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