Synthesis of Poly(ethylene glycol)/Polypeptide/Poly(D,L-lactide) Copolymers and Their Nanoparticles

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Received 11 December 2010; accepted 9 April 2011
DOI: 10.1002/pola.24720
Published online 10 May 2011 in Wiley Online Library (wileyonlinelibrary.com).

ABSTRACT: Core-shell structured nanoparticles of poly(ethylene glycol) (PEG)/polypeptide/poly(D,L-lactide) (PLA) copolymers were prepared and their properties were investigated. The copolymers had a poly(L-serine) or poly(L-phenylalanine) block as a linker between a hydrophilic PEG and a hydrophobic PLA unit. They formed core-shell structured nanoparticles, where the polypeptide block resided at the interface between a hydrophilic PEG shell and a hydrophobic PLA core. In the synthesis, poly(ethylene glycol)-*b*-poly(L-serine) (PEG-PSER) was prepared by ring opening polymerization of *N*-carboxyanhydride of *O*-(*tert*-butyl)-L-serine and subsequent removal of *tert*-butyl groups. Poly(ethylene glycol)-*b*-poly(L-phenylalanine) (PEG-PPA) was obtained by ring opening polymerization of *N*-carboxyanhydride of L-phenylalanine. Methoxy-poly(ethylene glycol)-amine with a MW of 5000 was used as an initiator for both polymerizations. The polymerization of D,L-lactide by initiation with PEG-PSER and PEG-PPA produced a comb-like copolymer, poly(ethylene glycol)-*b*-[poly(L-serine)-*g*-poly(D,L-lactide)] (PEG-PSER-PLA) and a linear copolymer, poly(ethylene glycol)-*b*-poly(L-phenylalanine)-*b*-poly(D,L-lactide) (PEG-PPA-PLA), respectively. The nanoparticles obtained from PEG-PPA-PLA showed a negative zeta potential value of −16.6 mV, while those of PEG-PSER-PLA exhibited a positive value of about 19.3 mV. In pH 7.0 phosphate buffer solution at 36 °C, the nanoparticles of PEG/polypeptide/PLA copolymers showed much better stability than those of a linear PEG-PLA copolymer having a comparable molecular weight. © 2011 Wiley Periodicals, Inc. J Polym Sci Part A: Polym Chem 49: 2859–2865, 2011

KEYWORDS: block copolymers; nanoparticles; biodegradable; drug delivery systems

INTRODUCTION

Core-shell-type nanoparticles with modified surface by flexible hydrophilic chains have been extensively studied as drug carriers for targeted delivery with long-circulation time in blood after intravenous administration.[1–8] The hydrophilic surface properties of the nanoparticles increase the circulation time by reducing the adsorption of plasma proteins and the recognition of phagocytes, and thereby influence the distribution pattern in the organs. Polylactide (PLA) is widely used to form a core of the core-shell-type nanoparticle.[8–11] As a flexible hydrophilic chain, poly(ethylene glycol) (PEG) is outstanding.[12,13] The polymer shows essentially good water solubility to have high degree of hydration. When PEG chains are tethered densely at the surface of the hydrophobic nanoparticle, the chains stretch out from the surface to form a brush conformation. The hydrophilicity and brush conformation of PEG chains prevent adsorption of hydrophobic proteins to the nanoparticles via steric repulsion.

Hydrophobic drugs are loaded in the PEG-PLA nanoparticles through noncovalent interactions and released by a diffusion mechanism.[11,14,15] Hence, it is evident that the physicochemical properties of the interfacial region and the core of the nanoparticle has a great influence on the drug release behavior. There have been a number of efforts to modify physical properties of PLA by controlling the structural architecture. In particular, PLAs with branched structures such as star-shaped, comb-shaped, and hyperbranched polymers attracted considerable attention.[16–18] The physical stability of the nanoparticles is primarily dependent on the molecular weight of a PLA block. As the size of the PLA block increases, the hydrophobic core becomes more solid-like and stable. Several studies demonstrated that the stability of the nanoparticles could be further improved by chemical or physical crosslinking.[19,20]

In this study, we report the preparation of PEG/polypeptide/poly(D,L-lactide) (PLA) copolymers and their nanoparticles. The copolymers had a comb-like or a linear structure, where a hydrophilic PEG was linked to a hydrophobic PLA unit through a poly(L-serine) or a poly(L-phenylalanine) block. In their nanoparticles, the polypeptide blocks were expected to reside at the interface between a hydrophilic PEG shell and a hydrophobic PLA core and to form intermolecular hydrogen bonds.

EXPERIMENTAL

Materials and Instruments

Triphosgene (98%), poly(vinyl alcohol) (MW = 31,000–50,000, 87–89% hydrolyzed), *O*-(*tert*-butyl)-L-serine, L-

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phenylalanine, and tin(II) 2-ethyl hexanoate were purchased from Aldrich and used as received. Methoxy-poly(ethylene glycol)-amine (MW = 5000) and D,L-lactide (3,6-dimethyl-1,4-dioxane-2,5-dione) were purchased from SunBio and TCI, respectively, and used as received. Tetrahydrofuran and toluene were dried over sodium metal and distilled. Other reagent-grade solvents were used as received. ^{1}H NMR spectra were recorded on a Bruker Avance-300 spectrometer. ^{13}C NMR spectra were recorded on a Bruker Avance-500 spectrometer. Elemental analysis was performed using EA1110 (CE Instrument, Italy). FTIR measurements were recorded on a PERKIN ELMER Spectrum GX I using KBr pellets. The particle sizes and zeta potentials of the nanoparticles were measured using an ELS-Z (Otsuka Electronics, Japan) spectrometer. A typical result was obtained based on the average from 5 runs. SEM images were taken by a JEOL JSM6330F microscope. UV–vis spectra were obtained with a SCINCO S-3100 spectrometer.

Synthesis of *O*-(*tert*-Butyl)-L-serine *N*-Carboxyanhydride

A solution of triphosgene (3.31 g, 11.2 mmol) in THF (10 mL) was added to a suspension of *O*-(*tert*-butyl)-L-serine (1.50 g, 9.31 mmol) in THF (20 mL) at 50 °C, and the resulting solution was stirred for 2 h at the same temperature.[21] After evaporation of the solvent, the crude product was isolated by precipitation in *n*-hexane. The product was purified by recrystallization from THF/*n*-hexane [yield: 1.33 g (76.0%)].

Anal. Cald for $C_8H_{13}NO_4$: C, 51.33; H, 7.00; N, 7.48; found: C, 51.36; H, 6.99; N, 7.44; ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 168.07, 152.68, 74.67, 60.98, 58.97, 27.44; ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 8.94 (s, NH, 1H), 4.56 (t, CH, 1H), 3.63–3.46 (m, CH_2, 2H), 1.09 (s, —CCH_3, 9H); FTIR (KBr, cm^{-1}): 3311 (N—H), 2976 (C—H), 1851 (C=O), 1787 (C=O).

Synthesis of Poly(ethylene glycol)-*b*-poly [*O*-(*tert*-butyl)-L-serine] Diblock Copolymer

To a solution of *O*-(*tert*-butyl)-L-serine *N*-carboxyanhydride (1.33 g, 7.10 mmol) in DMF (30.0 mL), a solution of methoxy-poly(ethylene glycol)-amine (1.41 g, 0.28 mmol, MW = 5000) was added in DMF (20.0 mL). The reaction mixture was stirred for 2 days at 40 °C. After evaporation of the solvent, the solid was dissolved in chloroform (40.0 mL). The polymer was isolated by precipitation into diethyl ether (500 mL) and further purified by reprecipitation from the polymer solution in chloroform into diethyl ether twice.

Yield, 0.87 g. ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 7.82 (NH), 4.0–4.5 (CH and CH_2 of polyserine), 3.50 (OCH_2CH_2), 1.10 (CCH_3).

Deprotection of *tert*-Butyl Group

To a solution of poly(ethylene glycol)-poly[*O*-(*tert*-butyl)-L-serine] (0.87 g, 0.15 mmol) in dichloromethane (50 mL), a solution of hydrogen bromide in acetic acid (33 wt %, 0.26 g) was added slowly. The reaction mixture was refluxed for 24 h. After concentration by evaporation of the solvent, the polymer was isolated by precipitation into diethyl ether (500 mL) and further purified by reprecipitation of the polymer solution in dichloromethane into diethyl ether.

Yield, 0.855 g. ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 7.99 (NH), 4.0–4.5 (CH and CH_2 of polyserine), 3.50 (OCH_2CH_2).

Synthesis of Poly(ethylene glycol)-*b*-[poly (L-serine)-*g*-poly(D,L-lactide)]

Poly(ethylene glycol)-poly(L-serine) block copolymer (0.20 g) and D,L-lactide (1.22 g) were dissolved in dry toluene (75 mL). To remove moisture in the monomer and the macroinitiator, the solution was further dried by azeotropic distillation for 1 day. The solution (50 mL) and tin(II) 2-ethylhexanoate (0.5 wt %) were put into a flame-dried polymerization tube (100 mL). After three freeze-thaw cycles, the tube was sealed under vacuum condition. The polymerization was carried out at 110 °C for 24 h. After evaporation of the solvent, the solid residue was dissolved in dichloromethane. The polymer was isolated by precipitation into an excess amount of diethyl ether and further purified by reprecipitation from the polymer solution in dichloromethane into diethyl ether twice.

Yield, 0.23 g. ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 8.20 (NH), 5.19 (CH of polylactide), 4.0–4.8 (CH and CH_2 of polyserine), 3.50 (OCH_2CH_2), 1.46 (CH_3 of polylactide); FTIR (KBr, cm^{-1}): 3300 (N—H), 2930–2870 (C—H), 1760 (C=O), 1630 (amide I), 1550 (amide II), 1190 (C—O).

Synthesis of Poly(ethylene glycol)-*b*-Poly(D,L-lactide)

The linear poly(ethylene glycol)-*b*-Poly(D,L-lactide) (PEG-PLA) diblock copolymer was synthesized by ring opening polymerization of D,L-lactide using methoxy-poly(ethylene glycol)-OH (MW = 5000) as an initiator according to the literatures.[4,22]

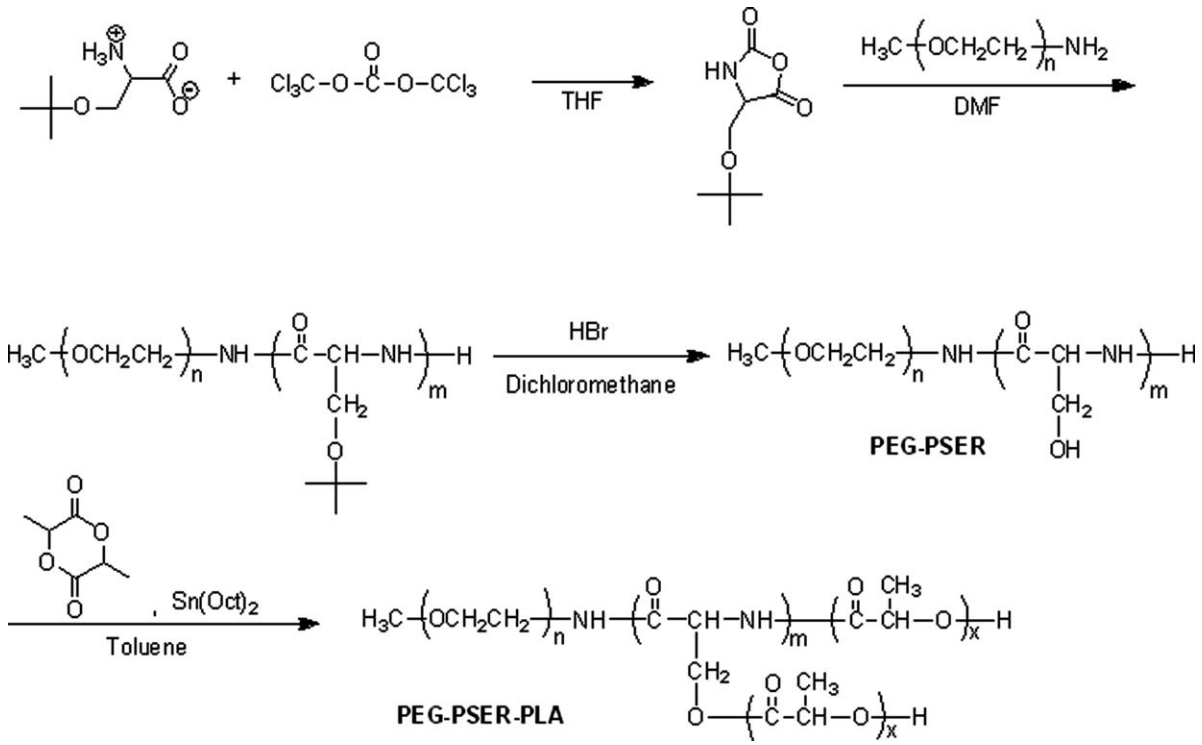
Synthesis of L-Phenylalanine *N*-Carboxyanhydride

This compound was prepared in the same manner as the preparation of *O*-(*tert*-butyl)-L-serine *N*-carboxyanhydride. From L-phenylalanine (2.0 g, 12.1 mmol) and triphosgene (4.65 g, 15.7 mmol), the product (1.63 g, 8.52 mmol) was obtained in 70.4% yield.

Anal. Cald for $C_{10}H_9NO_3$: C, 62.82; H, 4.74; N, 7.33; found: C, 62.85; H, 4.74; N, 7.29; ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 168.92, 152.21, 134.05, 129.41, 128.19, 59.04, 37.96; ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 9.07 (s, NH, 1H), 7.17–7.35 (m, —C_6H_5, 5H), 4.78 (t, CH, 1H), 3.01 (d, CH_2, 2H); FTIR (KBr, cm^{-1}): 3301 (N—H), 3030 (C—H from phenyl), 2923 (C—H), 1836 (C=O), 1777 (C=O).

Synthesis of Poly(ethylene glycol)-*b*-poly(L-phenylalanine)

To a solution of L-phenylalanine *N*-carboxyanhydride (0.4 g, 0.68 mmol) in DMF (10.0 mL), a solution of methoxy-poly(-ethylene glycol)-amine (0.2 g, 0.04 mmol, MW = 5000) was added in DMF (10.0 mL). The reaction mixture was stirred for 3 days at 50 °C. After evaporation of the solvent, the residue was dissolved in dichloromethane (10.0 mL). The polymer was isolated by precipitation into diethyl ether (400 mL) and further purified by reprecipitation from the polymer solution in dichloromethane into diethyl ether twice.



SCHEME 1 Synthesis of poly(ethylene glycol)-*b*-[poly(L-serine)-*g*-poly(D,L-lactide)] (PEG-PSER-PLA).

Yield, 0.16 g. ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 8.14 (NH), 7.15 (C_6H_5), 4.51 (CH), 3.50 (OCH_2CH_2), 2.90 (CH_2).

Synthesis of Poly(ethylene glycol)-*b*-poly(L-phenylalanine)-*b*-poly(D,L-lactide)

This polymer was prepared in the same manner as the preparation of poly(ethylene glycol)-*b*-[poly(L-serine)-*g*-poly(D,L-lactide)] (PEG-PSER-PLA). From poly(ethylene glycol)-*b*-poly(L-phenylalanine) (PEG-PPA) (0.20 g) and D,L-lactide (0.9 g), the copolymer (0.24 g) was obtained.

^{1}H NMR (DMSO-d_6, 300 MHz): δ = 8.22 (NH), 7.14 (C_6H_5), 5.19 (CH of polylactide), 4.50 (CH of polyphenylalanine), 3.50 (OCH_2CH_2), 2.91 (CH_2), 1.47 (CH_3); FTIR (KBr, cm^{-1}): 3289 (N—H), 2996–2887 (C—H), 1758 (C=O), 1634 (amide I), 1548 (amide II), 1114 (C—O).

Fabrication of Nanoparticles

Nanoparticles were prepared by an emulsion-solvent evaporation method.[23] Typically, a block copolymer (40 mg) was dissolved in dichloromethane (2 mL). The solution was dropped into deionized water (20 mL) containing 0.7% (w/v) poly(vinyl alcohol) with stirring under 600 rpm. After stirring for 9 h, the nanoparticles were isolated by centrifugation at 5000 rpm for 15 min and washed twice with deionized water. The nanoparticles were then resuspended in deionized water (5 mL), filtered with a 0.80-μm pore size filter, and freeze-dried.

Stability Test of Nanoparticles

The nanoparticles (1 mg) were suspended in pH 7.0 phosphate buffer solution (5 mL) at 36 °C. The particle size was measured by dynamic light scattering (DLS) at particular time intervals.

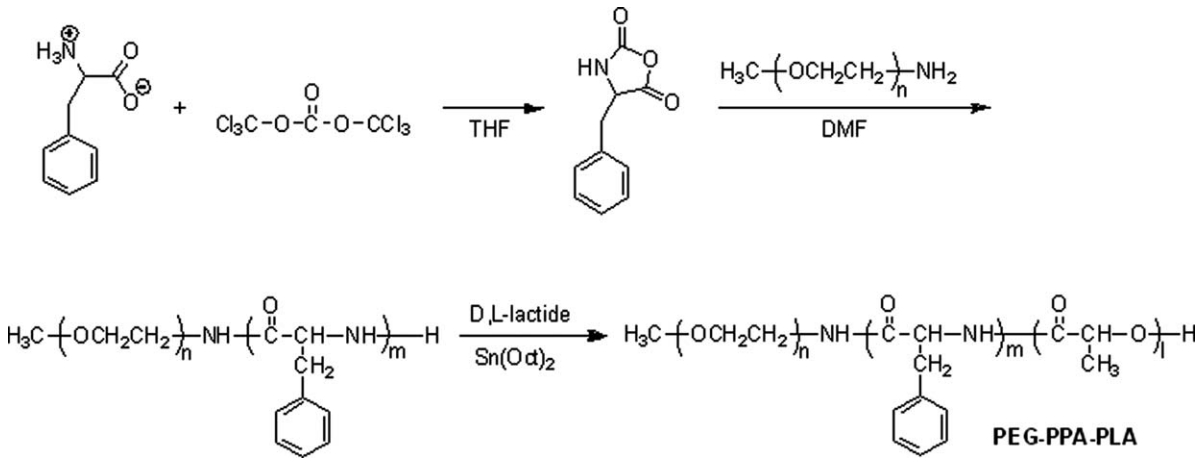
1-Aminopyrene-Loaded Nanoparticles

From a solution of 1-amionopyrene (8 mg) and a block copolymer (40 mg) in dichloromethane (2 mL), 1-aminopyrene-loaded nanoparticles were prepared as described above. Loading efficiency of the nanoparticles was determined as follows: 1-Aminopyrene loaded nanoparticles (5 mg) were dissolved in DMF (2 mL). A concentration of 1-aminopyrene in the DMF solution was calculated by measuring the absorbance at 370 nm with a UV–vis spectrometer. The molar extinction coefficient at 370 nm was 15,000 M^{-1} cm^{-1}. The loading efficiency was calculated using the following equation.

$$\text{Loading efficiency} = \frac{\text{Weight of } 1-\text{aminopyrene in nanoparticles}}{\text{Weight of nanoparticles}} \times 100$$

Release of 1-Aminopyrene from the Nanoparticles

A suspension of 1-amonopyrene-loaded nanoparticles (4 mg) in 1 mL of pH 3 or pH 7 phosphate buffer solution was put in a dialysis tube (cutoff M_n = 3500) and then placed in a glass vial filled with 15 mL of the phosphate buffer solution. At particular time intervals at 36 °C, the concentration of 1-aminopyrene in the glass vial was calculated by measuring the absorbance at 370 nm with a UV–vis spectrometer, and the phosphate buffer solution was exchanged with a fresh one.



SCHEME 2 Synthesis of poly(ethylene glycol)-*b*-poly(L-phenylalanine)-*b*-poly(D,L-lactide) (PEG-PPA-PLA).

RESULTS AND DISCUSSION

Synthesis of Copolymers

Scheme 1 shows the synthetic route of PEG-PSER-PLA. Poly(ethylene glycol)-*b*-poly(L-serine) (PEG-PSER) was prepared by ring-opening polymerization of *N*-carboxyanhydride of *O*-(*tert*-butyl)-L-serine using methoxy-poly(ethylene glycol)-amine (CH_3O-PEG-NH_2) with a MW of 5000 as an initiator. In the ^{1}H NMR spectrum, methylene protons of PEG and *tert*-butyl protons of polyserine appeared at 3.50 and 1.10 ppm, respectively. *tert*-Butyl groups were removed with hydrogen bromide to generate hydroxyl groups on the polymer backbone, which was confirmed by ^{1}H NMR spectroscopy. The hydroxyl groups were used for the initiation of the ring-opening polymerization of D,L-lactide in the presence of tin(II) 2-ethylhexanoate as a catalyst to give PEG-PSER-PLA having a comb-like structure. In the ^{1}H NMR spectrum, the peaks corresponding to methine and methyl protons of the PLA segment appeared around 5.19 and 1.46 ppm, respectively.

The number of PLA chains and its average degree of polymerization were estimated to be 7 and 10, respectively, based on the ^{1}H NMR spectrum. The peak from PEG (MW = 5000) at 3.50 ppm was used as an internal standard. For the comparison, PEG-PLA was also synthesized by ring-opening polymerization of D,L-lactide using MeO-PEG-OH with a MW of 5000 as an initiator. The M_n determined by ^{1}H NMR spectroscopy was 8900.

TABLE 1 Molecular Weights and Compositions of the Copolymers

		Weight Fraction		
Copolymer	M_n	PEG	Polypeptide	Polylactide
PEG-PSER-PLA	10,200	0.49	0.05	0.46
PEG-PPA-PLA	16,800	0.30	0.20	0.50
PEG-PLA	8,900	0.56	–	0.44

Poly(ethylene glycol)-*b*-poly(L-phenylalanine)-*b*-poly(D,L-lactide) (PEG-PPA-PLA) was synthesized according to Scheme 2. *N*-carboxyanhydride of L-phenylalanine was polymerized using CH_3O-PEG-NH_2 with a MW of 5000 as an initiator to give poly(ethylene glycol)-*b*-poly(L-phenylalanine) (PEG-PPA). PEG-PPA had a primary amino group at the end of the polymer chain, which was used for the initiation of the polymerization of D,L-lactide.[24] In the ^{1}H NMR spectrum, the peak for phenyl protons of poly(L-phenylalanine) appeared at 7.15 ppm. The average degrees of polymerization for PPA and PLA blocks were 22 and 119, respectively, which were estimated by ^{1}H NMR spectroscopy. The molecular weights and compositions of the copolymers are summarized in Table 1.

Preparation of Nanoparticles

The nanoparticles of the copolymers were prepared by an emulsion-solvent evaporation method.[9,23] A solution of a block copolymer in dichloromethane was dropped into deionized water containing poly(vinyl alcohol) with stirring. The average diameters of the nanoparticles measured by DLS, were 208 ± 34 nm (PEG-PSER-PLA) and 210 ± 96 nm (PEG-PPA-PLA) (Table 2). The average diameter of the nanoparticles obtained from linear PEG-PLA was 128 ± 91 nm. It is noticeable that PEG-PSER-PLA produced larger particles than PEG-PLA, although weight fractions of PLA chains of these two copolymers were comparable. We presume that the comb-like structure of PEG-PSER-PLA and the rigidity of the polyserine block prevented the close packing of hydrophobic PLA chains during the formation of nanoparticles. Figure 1 shows a SEM image of the nanoparticles obtained from PEG-PSER-PLA. The freeze-dried nanoparticles had a spherical shape and were readily dispersed in water.

TABLE 2 Sizes and Zeta Potentials of the Nanoparticles

Polymers	Size (nm)	Zeta Potential (mV)
PEG-PSER-PLA	208 ± 34	19.3 ± 0.37
PEG-PPA-PLA	210 ± 96	−16.6 ± 0.45
PEG-PLA	128 ± 91	−8.3 ± 0.75

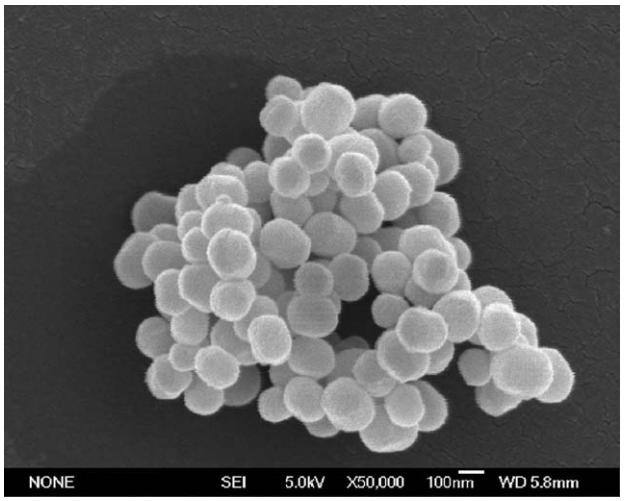


FIGURE 1 FE-SEM image of freeze-dried nanoparticles obtained from PEG-PSER-PLA.

Figure 2 shows a schematic drawing of the nanoparticle. Because the hydrophilic PEG chain of PEG-PSER-PLA or PEG-PPA-PLA was linked to the hydrophobic PLA through a peptide block, we expected that the polypeptide block should reside at the interface between a hydrophilic PEG shell and a hydrophobic PLA core. The core-shell structures of the nanoparticles were examined by zeta potential measurement. It has been reported that PLA nanoparticles have a negative zeta potential value of below −20 mV at pH 7 because of the terminal carboxyl group.[25] The absolute value decreases as the surface is coated by hydrophilic PEG chains in the core-shell structure of PEG-PLA. The nanoparticles obtained from PEG-PLA and PEG-PPA-PLA showed negative zeta potential values, exhibiting −8.3 and −16.6 mV, respectively (Table 2).

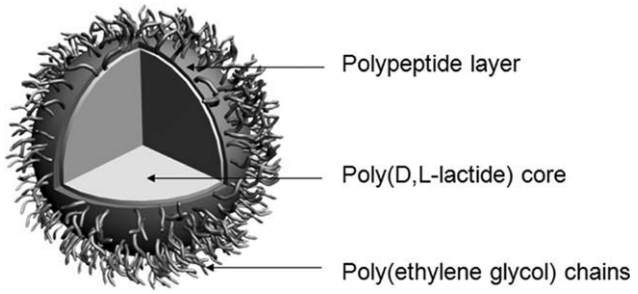


FIGURE 2 Schematic drawing of the nanoparticle structure.

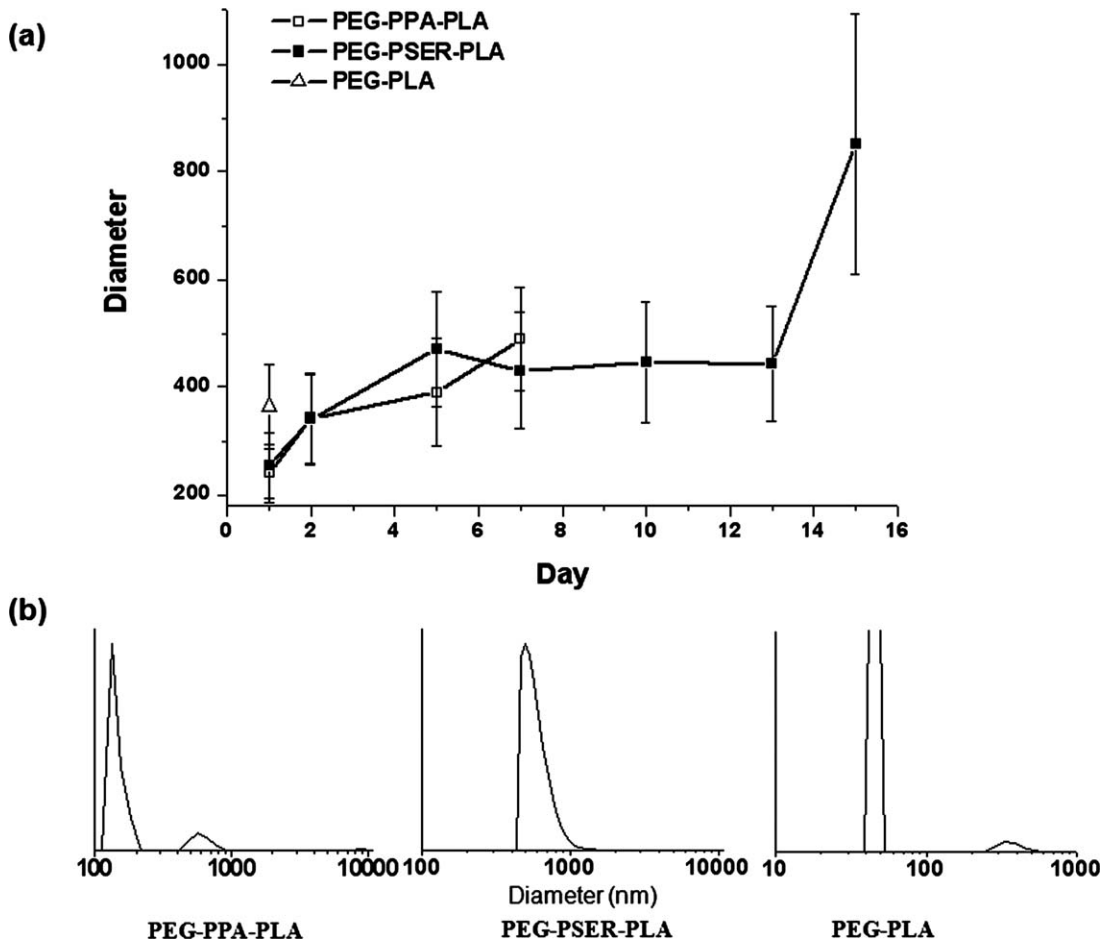


FIGURE 3 Size changes of the nanoparticles as a function of time in a pH 7.0 phosphate buffer solution at 36 °C (a) and diameter profiles of the nanoparticles measured after 15 days (b).

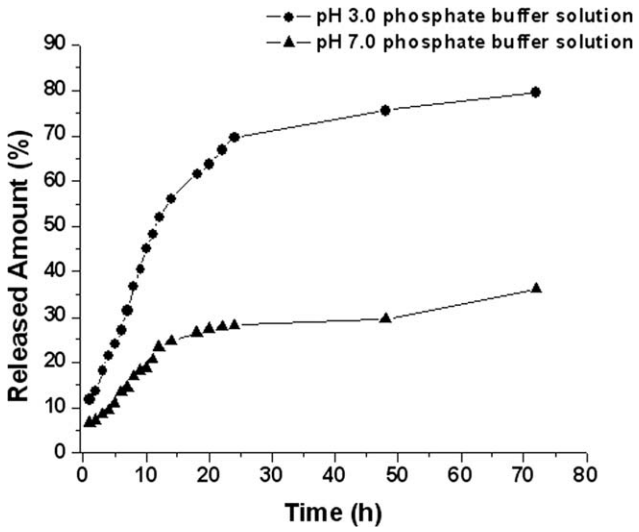


FIGURE 4 Release profiles of 1-aminopyrene from PEG-PSER-PLA nanoparticles in pH 3 (●) and pH 7 (▲) phosphate buffer solutions.

On the contrary, the nanoparticles of PEG-PSER-PLA had a positive value of about 19.3 mV. As only a few neutral organic polymers such as polyethylenimine exhibit positive zeta potentials,[26] this result strongly indicates that the surface of the nanoparticles was covered with polypeptide chains.

The stability of the nanoparticles was examined by measuring changes of the nanoparticle diameters in pH 7 phosphate buffer solution at 36 °C by DLS.[27] Figure 3 shows the stability test results. Nanoparticles of PEG-PLA degraded in 1 day. The relatively fast degradation of these nanoparticles is attributable to a low molecular weight of the PLA block. The nanoparticles of PEG-PPA-PLA and PEG-PSER-PLA showed much improved stability although they had a similar weight of the PLA unit to that of PEG-PLA. The nanoparticles of PEG-PPA-PLA and PEG-PSER-PLA were stable until 5 and 12 days, respectively. In the diameter profiles measured after 15 days, the nanoparticles of PEG-PPA-PLA and PEG-PLA showed the multimodal shapes, suggesting that they became degraded and then aggregated into larger particles. Nanoparticles of PEG-PSER-PLA exhibited a more uniform size distribution than the other two polymers [Fig. 3(b)].

The release profile of the nanoparticles obtained from PEG-PSER-PLA was studied by using 1-aminopyrene as a model drug. The nanoparticles were prepared by an emulsion-solvent evaporation method[9,23] in the presence of 1-aminopyrene. The loading efficiency was 6.7%, which was estimated by measuring the concentration of 1-aminopyrene with a UV–vis spectrometer after dissolving the nanoparticles in DMF. The release test of 1-aminopyrene from the nanoparticles was performed at 36 °C in pH 3 and pH 7 phosphate buffer solutions (Fig. 4). 1-Aminopyrene was more rapidly released at pH 3 than at pH 7. After 24 h, more than 70% of loaded 1-aminopyrene was released in pH 3 phosphate buffer solution, whereas a cumulative release amount was below 30% in pH 7 buffer solution. This result is attributable to the fact that the polypeptide chains at the surface of the hydrophobic core as well as 1-aminopyrene were protonated under acidic conditions.

CONCLUSIONS

We demonstrated the preparation of core-shell structured nanoparticles having polypeptide interlayers from the comb-like copolymer, PEG-PSER-PLA and the linear copolymer, PEG-PPA-PLA. Their nanoparticles showed better stability than those of a linear PEG-PLA copolymer having a comparable molecular weight. PEG-PSER-PLA had a positive zeta potential value, differently from the nanoparticles obtained from linear PEG-PLA, suggesting that the polypeptide chains formed an interface layer between a hydrophilic PEG corona and a hydrophobic PLA core. There have been numerous reports about the overexpression of proteases in tumor cells.[28,29] If the polypeptide chains in the interface layer contained a specific amino acid residue, the overexpressed proteases could facilitate the degradation of nanoparticles on accumulation in tumor cells. Our future studies will explore this possibility.

This study was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health and Welfare, Republic of Korea (Project No. A062254).

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