Synthesis and Self-Assembling Behaviors of Biotinylated Pluronic/Poly(lactic acid) Biocompatible Block Copolymers in Aqueous Solutions

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ABSTRACT: Four kinds of Biotinylated Pluronic/PLA block copolymers were synthesized by two-step reactions. Pluronic were firstly modified by biotin to obtain B-Pluronic-OH. Biotin-Pluronic-PLA block copolymers were then produced by ring-opening polymerization of the monomer L-lactide using Biotin-Pluronic-OH as the initiator and stannous octoate ($Sn(Oct)_2$) as the catalyst. The self-assembling behaviors of Biotin-Pluronic-PLA block copolymers in aqueous solutions were examined by fluorescence measurement, dynamic light scattering (DLS), and transmission electron microscopic (TEM) techniques. The size of Biotin-F127-PLA-61, Biotin-F87-PLA, and Biotin-P85-PLA nanoparticles were determined to be 198, 229, and 257 nm, respectively, and their morphologies were found to be spherical micelles. Biotin-F127-PLA-87 produces both spherical micelles and large compound micelles with the size of 127 and 906 nm. The cytotoxicity studies using human ovarian cancer cells OVCAR-3 indicate that Biotin-Pluronic-PLA block copolymers have good biocompatibility. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 115: 1573–1580, 2010

Key words: block copolymers; self-assembly; micelles; biocompatibility; drug delivery systems

INTRODUCTION

Amphiphilic block copolymers have been widely studied in the past few decades for their application in drug delivery systems because they are able to self-assemble into nanoparticles containing a hydrophobic core and a hydrophilic shell.[1–6] Hydrophobic poly(lactic acid) (PLA) are well-known biodegradable and biocompatible polyester. Pluronic block copolymers are one of the very few synthetic polymeric materials approved by the U.S. Food and Drug Administration for use as food additives and pharmaceutical ingredients. The biocompatible amphiphilic block copolymers PLA-Pluronic-PLA have been synthesized previously and their application in drug delivery systems have been studied in detail by Xiong et al.[7–12]

There has recently been a strong impetus to the development of polymeric nanoparticles with targeting ligands, which are able to increase the selectivity and efficiency of drug delivery to the target cells, leading to a better therapeutic efficacy as well as reduced side effects.[13–17] A versatile approach to prepare targeting polymeric nanoparticles is to take advantage of the avidin–biotin interaction, which is the strongest known noncovalent biological interaction (association constant $10^{15}M^{-1}$).[18] The avidin containing four binding sites for biotin can serve as a universal linkage between the biotinylated polymeric nanoparticles and targeting ligands.

Thus, in this study, biotin-Pluronic-PLA block copolymers were synthesized so as to obtain polymeric nanoparticles with biotin groups on the surface. The self-assembling behaviors and cytotoxicity of biotin-Pluronic-PLA copolymers in aqueous solutions were examined. To our knowledge, there are

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only few reports regarding the synthesis of similar block copolymers biotin-poly(ethylene oxide)-PLA (biotin-PEO-PLA) and no study at all on the synthesis of biotin-Pluronic-PLA copolymers.[13,18–20] Only the newly published paper among them published the micellization behavior of biotin-PEO-PLA copolymers.[13] It was reported that poly(ethylene imine)-Pluronic (PEI-Pluronic) block copolymer exhibited higher transfection efficiency toward plasmid DNA than PEI-PEO copolymer due to the amphiphilic property of Pluornic block copolymer.[21] This is the reason for selecting the amphiphilic PEO-PPO-PEO instead of hydrophilic PEO in this study.

EXPERIMENTAL SECTION

Materials

Pluronic F127 ($\overline{M}_n$ 12,600 Da), F87 ($\overline{M}_n$ 7700 Da), and P85 ($\overline{M}_n$ 4600 Da) were kindly supplied by BASF Corporation (Mount Olive, NJ). L-Lactide was purchased from Sigma-Aldrich (St. Louis, MO) and recrystallized twice from ethyl acetate (EtAc). The purified L-lactide was stored at 4–5°C under argon environment. D-Biotin, stannous octoate [$Sn(Oct)_2$], sodium phosphotungstate, and avidin were purchased from Sigma-Aldrich and used as received. 2-(4-Hydroxyphenylazo)benzoic acid (HABA) and Pyrene were purchased from Acros (Geel, Belgium) and used as received. *N*,*N*′-Dicyclohexylcarbodiimide (DCC) and 4-Dimethylaminopyridine (DMAP) were purchased from Acros (Beijing, China) Chemica and used as received. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Solarbio (Beijing, China) and used as received. Dulbecco's modified eagle's medium (DMEM) was purchased from Gibco (Australia) and used as received. All other chemicals were of reagent grade. Human ovarian cancer cells OVCAR-3 were purchased from CICAMS, Beijing.

Synthesis of biotin-pluronic-OH

Pluronic F127, F87, and P85 were modified by biotin on one end, respectively. The synthesis procedure of a typical Biotin-F127-OH is as follows. Pluronic F127 (20 g, 1.59 mmol) was dissolved in 300 mL of dichloromethane, followed by the addition of biotin (0.47 g, 1.93 mmol) and DMAP (0.015 g, 0.12 mmol) to the reaction flask and cooling of the solution to 0°C. DCC (0.33 g, 1.60 mmol) was added dropwise via a dropping funnel over 30 min, and the reaction was carried out for 48 h at room temperature. The reaction mixture was then extracted with 10% $NaHCO_3$ solution to remove unreacted biotin. After this step, the organic phase was frozen overnight and the insoluble substances were removed by filtration. The organic solution was then precipitated twice in cold diethyl ether. The polymers were filtered and dried overnight under vacuum. ^{1}H-NMR (400 MHz, $CDCl_3$, TMS), δ (ppm): 1.13–1.15 (m, —OCH_2—$CH(CH_3)$—), 1.52–1.60 (m, —$(CH_2)_3$—CH_2—COO—), 3.40–3.82 (m, —OCH_2—CH_2—, and —OCH_2—$CH(CH_3)$—) [Fig. 1(A)].

Synthesis of biotin-pluronic-PLA block copolymers

PLA segment was attached to one end of Pluronic F127, F87, and P85 by ring-opening polymerization to obtain amphiphilic block copolymers Biotin-Pluronic-PLA, respectively. The synthesis procedure of a typical Biotin-F127-PLA-61 is as follows. Biotin-Pluronic-OH (3.5 g) was distillated by azeotropic distillation under argon. Toluene was then distilled off to give a final volume of 30 mL. LA (6.5 g) was added to the F127 solution at room temperature under argon and was followed by the addition of stannous octoate (about 0.1 wt % of LA). The mixture was stirred at 120°C for 6 h. Then, the reaction mixture was poured into cold ethyl ether to precipitate the product. Following this, the product was dissolved in methylene chloride and precipitated in methanol. The white product was then filtered and dried overnight under vacuum. ^{1}H-NMR (400 MHz, $CDCl_3$, TMS), δ (ppm): 1.13–1.15 (m, —OCH_2—$CH(CH_3)$—), 1.41–1.75 (m, —O—$CH(CH_3)$—CO— and HO—$CH(CH_3)$—CO—), 3.40–3.65 (m, —OCH_2—CH_2— and —OCH_2—$CH(CH_3)$—), 4.25–4.35 (m, HO—$CH(CH_3)$—CO— and —CO—OCH_2—CH_2—O—), 5.13–5.18 (m, —O—$CH(CH_3)$—CO—) [Fig. 1(B)]. The other Biotin-Pluronic-PLA block copolymers were similarly synthesized. In the designation for the two polymers Biotin-F127-PLA-61 and Biotin-F127-PLA-87, the numeric numbers 61 and 87 correspond to the weight fraction of PLA block in percent, determined from NMR.

Preparation of biotin-pluronic-PLA nanoparticles

The aggregates of a typical Biotin-F127-PLA-61 in aqueous solutions were prepared as follows: Biotin-F127-PLA-61 block copolymer (12 mg) was dissolved in THF and then the polymer solution was added dropwise to ultra pure water (15 g) under gentle stirring. The polymer aggregated solution was dialyzed against ultra pure water for 5 h using a cellulose membrane bag (molecular weight cut-off 12,000–14,000 Da) to remove THF outside Biotin-F127-PLA-87 aggregate. The water was exchanged at intervals of 1 h. The final concentration of Biotin-F127-PLA-87 aggregates is about 0.08 wt %. The other Biotin-Pluronic-PLA nanoparticles in aqueous were prepared in the same way.

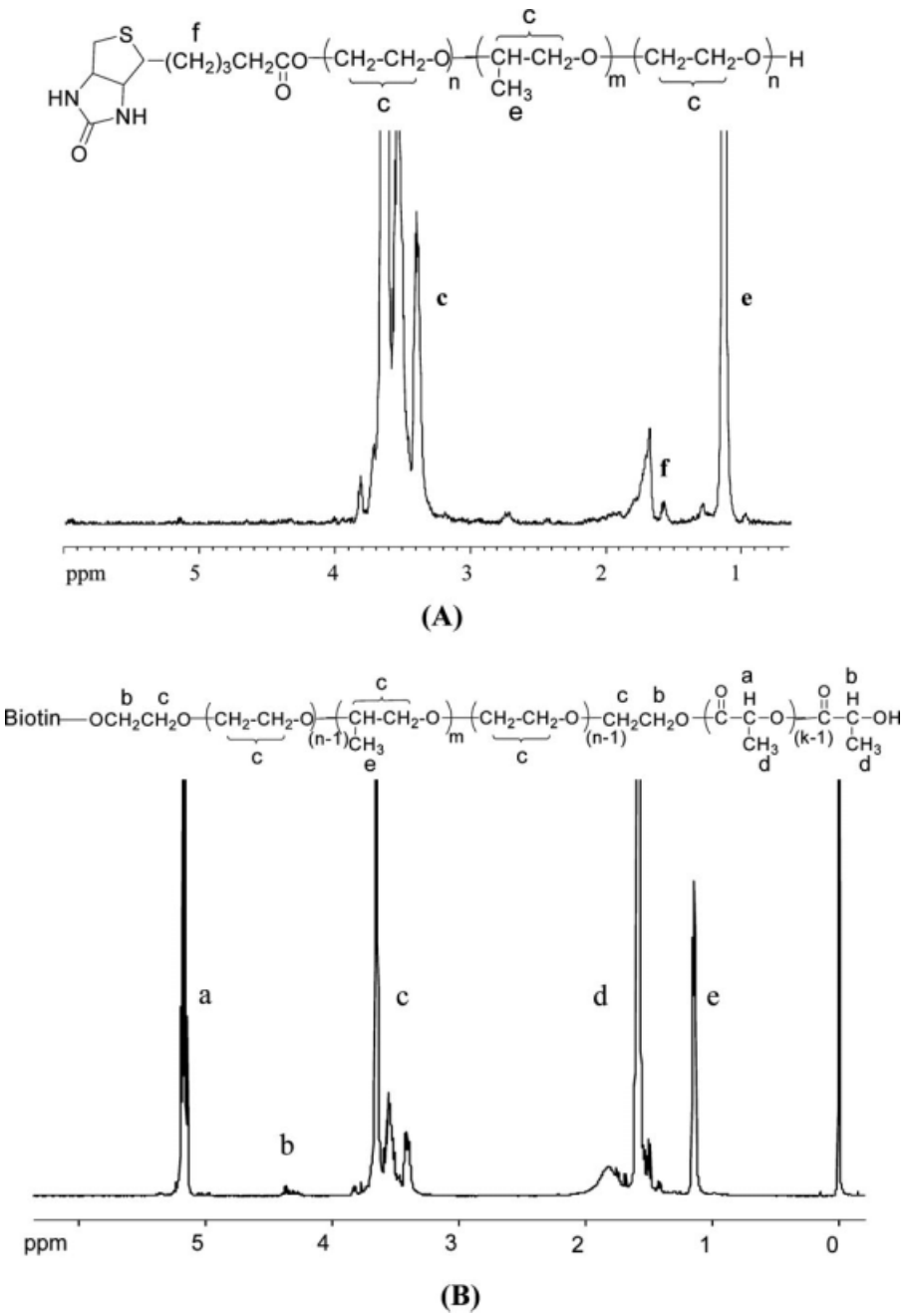


Figure 1 ^{1}H-NMR spectra of Biotin-F127-OH (A) and Biotin-F127-PLA-61 (B) block copolymer ($CDCl_3$).

Characterization

Nuclear magnetic resonance (NMR) spectra were recorded at room temperature using a Bruker ACF-400 (400 MHz) Fourier transform spectrometer. Chemical shifts (δ) were given in ppm using tetramethylsilane (TMS) as an internal reference. Molecular weight and molecular weight distribution of polymers were measured with a gel permeation chromatography (GPC) system equipped with a Waters 2414 refractive index detector, a Waters 515 binary HPLC pump, and three Waters Styragel Columns (HT2, HT4, and HT5) with THF as an eluent at a flow rate of 0.8 mL/min at 40°C. Mean diameters and size distributions of biotin-pluronic-PLA nanoparticles were determined by the dynamic light scattering (DLS) method using a PSS NICOMP 380 ZLS Particle Sizing Systems (CA, USA). The analysis lasted 310 s at 23°C with a detection angle of 90°.

HABA/avidin binding assay

The amount of biotin available on the surface of Biotin-Pluronic-PLA nanoparticles was quantified by a competitive binding assay (avidin/HABA).[22,23]

Biotin is able to displace the dye HABA binding to avidin in stoichiometric proportions due to its higher affinity for avidin. The biotin solutions in PBS with a series of concentration were added into avidin-/HABA-mixed PBS solution (avidin, 0.5 mg/mL; HABA, 0.3 m*M*) for about 2 h, respectively. Then, the absorbance of these solutions were measured by UV–Vis spectrophotometry (Perkin Elmer UV–Vis Spectrophotometer Lambda 35, USA) at 500 nm to obtain a calibration curve. Biotin-Pluronic-PLA nanoparticles in PBS solutions were also mixed with avidin/HABA solution and measured by UV. The change in absorbance can then be used to calculate the amount of biotin present.

Fluorescence measurements

The critical micellization concentration (CMC) of Biotin-Pluronic-PLA nanoparticles in PBS solutions was determined by fluorescence measurements (HITACHI F-4500) using pyrene as a fluorescence probe.[24,25] The pyrene stock solution in acetone (5 μL) was added into a series of test tubes, respectively, and the acetone was evaporated. Following this, the Biotin-Pluronic-PLA solutions (5 mL) were added to each test tube and then sonicated for 2 h. The final concentration of pyrene in the solutions was $6 \times 10^{-7} M$. For the measurement of pyrene excitation spectra, the slit widths for both excitation and emission sides were maintained at 5 nm, and the emission wavelength used was 390 nm.

Transmission electron microscope (TEM)

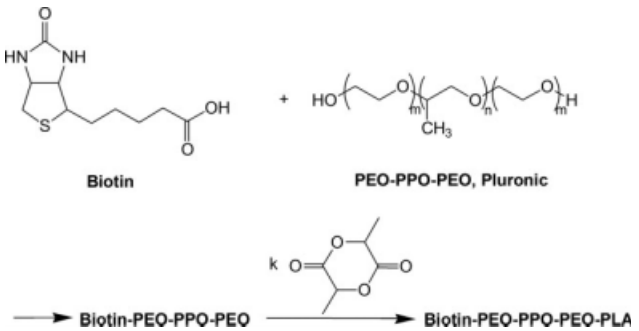
TEM was performed on a HITACHI H-600 electron microscope at an acceleration voltage of 75 kV. The copper grid (300 meshes) with a carbon film was used. The copper grid was immersed in a drop of the aqueous Biotin-Pluronic-PLA nanoparticles solution for 2 min and then removed and dried. A drop of sodium phosphotungstate (2 wt %) was placed on the copper grid for 2 min. The copper grid was then dried overnight at room temperature before measurement.

In vitro cytotoxicity studies

MTT assays were performed to investigate the biocompatibility of Biotin-Pluronic-PLA nanoparticles. OVCAR-3 cells were cultured in DMEM supplemented with 2.0 mmol/L glutamine, 10% fetal bovine serum (FBS), 100 μg/mL streptomycin sulfate, and 100 U/mL penicillin at 37°C in humidified 5% CO_2. Cells were seeded on three pieces of 96-well plates with a cell density of 2.5×10^5 cells/mL for 8 h. Following this, the medium was replaced by the Biotin-F127-PLA-61 unimers and nanoparticles in DMEM medium at concentrations between 1×10^{-7} and 0.01 wt % in a final volume of 200 μL. After predetermined time (4, 12, and 24 h) of incubation, one of the three 96-cell plates were taken out and 10 μL of the MTT solution (5 mg/mL) was added to each well and incubated for 4 h under normal growing conditions. Following this, all media was removed and 150 μL dimethylsulfoxide (DMSO) was added. After shaking the plate for 10 min, absorbance was immediately measured at 492 nm using a microplate reader (Thermo Labsystems MK3). The cell viability was expressed as a percentage compared with a control that had not been treated with the nanoparticles, using the following equation:

$$\text{Viability}(\%) = \frac{A(\text{test})}{A(\text{control})} \times 100\%$$

where A(test) and A(control) are the absorbance of surviving cells treated with nanoparticles and untreated cells, respectively.



Scheme 1 The synthesis scheme of Biotin-Pluronic-PLA block copolymers.

RESULTS AND DISCUSSION

Synthesis and characterization of biotin-pluronic-OH block copolymers

Biotin-Pluronic-PLA block copolymers were synthesized by two steps. Pluronic block copolymers were firstly modified by biotin. Figure 1(A) shows a ^{1}H-NMR spectrum of a typical Biotin-F127-OH in $CDCl_3$. The small peak at δ of 1.52–1.60 ppm belongs to methylene protons of biotin, indicating that biotin has been attached to the end of F127 block copolymers.

Biotin-Pluronic-PLA block copolymers were then synthesized by ring-opening polymerization of the monomer L-lactide using Biotin-Pluronic-OH as the initiator and stannous octoate ($Sn(Oct)_2$) as the catalyst (Scheme 1). The possible byproduct biotin-Pluronic-biotin generated in the first step can be removed by methanol in the second step. The polymer composition, structure, and molecular weight

TABLE I
Characterizations of Biotin-Pluronic-PLA Block Copolymers

Sample	W_{PLA} (%)[a]	$\overline{M}_n$ (NMR)	$\overline{M}_w/\overline{M}_n$ (GPC)	CMC ($\times 10^{-4}$ wt %)
Biotin-F127-PLA-61	61	32,300	1.13	4.0
Biotin-F127-PLA-87	87	98,800	1.08	2.5
Biotin-F87-PLA	83	45,600	1.06	1.8
Biotin-P85-PLA	88.6	40,500	1.04	0.79

[a] W_{PLA} stands for the weight fraction of PLA blocks in Biotin-Pluronic-PLA block copolymers.

were characterized by NMR and GPC techniques. Figure 1(B) shows a ^{1}H-NMR spectrum of a typical Biotin-F127-PLA-61 in $CDCl_3$. The small peak at δ of 4.30 ppm belongs to methylene protons of PLA-CO—OCH_2—CH_2—O—PEO— segment, indicating the successful synthesis of Biotin-F127-PLA-87 block copolymer. The absence of a peak at δ of 4.9–5.0 ppm which could have been contributed by the methine proton of the PLA—O—CH(CH_3)—COOH group, suggest that there was negligible or no PLA homopolymer in the Biotin-F127-PLA-87 block copolymer. The peaks belonging to biotin group were hard to see in Figure 1(B) due to the high-molecular weight of Biotin-F127-PLA-87 block copolymer.

The degree of polymerization (n) of PLA in Biotin-Pluronic-PLA$_k$ copolymers was calculated from the peak intensity ratio of methyl protons of PLA (O—CH(CH_3)—CO—: δ = 1.58 ppm) and methyl protons of Pluronic (—OCH_2—CH(CH_3)—: δ = 1.14 ppm). The number-average molecular weight ($\overline{M}_n$) of the Biotin-Pluronic-PLA$_k$ copolymer was obtained by using the following expression:

$$\overline{M}_n = \overline{M}_n(\text{Pluronic}) + 72k + 226$$

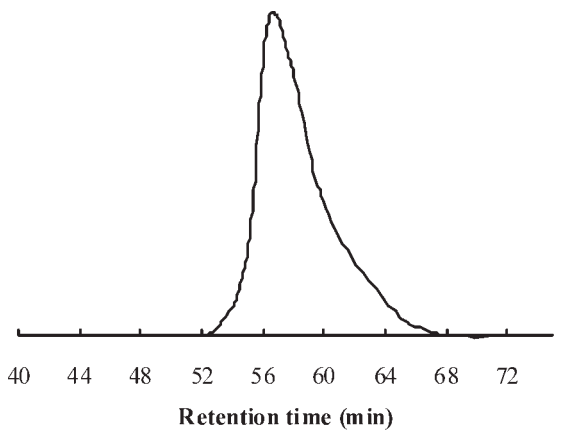


Figure 2 The GPC trace of Biotin-F127-PLA-87 block copolymer.

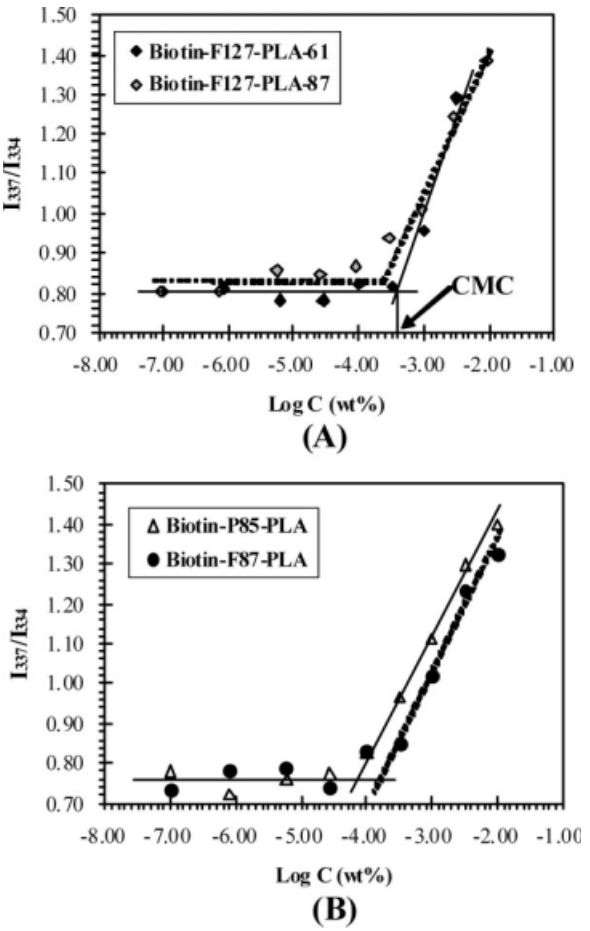


Figure 3 The concentration dependencies of peak intensity ratio (I_{337}/I_{334}) in the excitation spectra of pyrene for Biotin-Pluronic-PLA block copolymers.

Four kinds of Biotin-Pluronic-PLA block copolymers were synthesized. The molecular weights and weight fractions of Biotin-Pluronic-PLA copolymers were calculated and summarized in Table I.

Figure 2 shows the GPC trace of a typical Biotin-F127-PLA-87 block copolymer. The molecular weight distributions ($\overline{M}_w/\overline{M}_n$) of Biotin-Pluronic-PLA block copolymers were determined by GPC measurements (Table I).

The bioavailability of biotin presented from Biotin-Pluronic-PLA nanoparticles to its protein receptor was evaluated by avidin/HABA assay. The addition of Biotin-Pluronic-PLA nanoparticles to avidin–HABA complexes resulted in the decrease of the absorbance, showing the effective displacement of HABA from the complex. The absorbance did not decrease when F127 alone was added into avidin–HABA complexes. The content of polymer containing biotin end groups was calculated to be about 70%, 50%, and 40% for Biotin-F127-PLA-61, Biotin-P85-PLA, and Biotin-F87-PLA nanoparticles, respectively. These results show that it is hard to

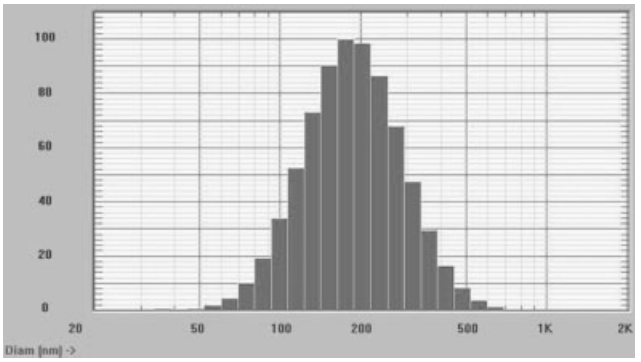


Figure 4 The intensity-weight Gaussian distribution for Biotin-F127-PLA-61 nanoparticles.

graft biotin groups to the ends of all the polymer chains. There are about 60% of block copolymers containing biotin groups obtained by other research groups.[18,23]

Characterization of biotin-pluronic-PLA nanoparticles

The CMC values of Biotin-Pluronic-PLA block copolymers were determined by a fluorescence spectroscopy measurement. A redshift can be observed for the excitation spectra of pyrene when it was transferred from the aqueous phase to the hydrophobic environment provided by the polymer aggregates. The peak intensity ratio (I_{337}/I_{334}) in the excitation spectra of pyrene for Biotin-Pluronic-PLA block copolymers was plotted against the polymer concentration as shown in Figure 3 and the CMC values determined were summarized in Table I. Compared with Biotin-F127-PLA-87, the CMC value of Biotin-F127-PLA-61 is higher due to the lower weight content of hydrophobic PLA block. It was observed that the CMC of Biotin-P85-PLA was the lowest among the four Biotin-Pluronic-PLA block copolymers. This should be due to the higher weight contents of both hydrophobic PPO and PLA blocks. The pretty low CMC values of Biotin-Pluronic-PLA block copolymers indicate the good stability of their nanoparticles and thus augur well for their potential applications as carriers for drug delivery.

The particle size and size distribution of Biotin-Pluronic-PLA nanoparticles were measured by the DLS method using PSS Particle Sizing Systems. For Biotin-F127-PLA-61, Biotin-F87-PLA, and Biotin-P85-PLA nanoparticles, the intensity-weight Gaussian distribution was used for size analyzing and the unimodal size distributions were observed (Fig. 4). However, for Biotin-F127-PLA-87 nanoparticles, relative broad distributions and large particle sizes were observed. Therefore, the intensity-weight Nicomp distribution was used for size analyzing of Biotin-F127-PLA-87 and two kinds of particle sizes were obtained. The diameters of Biotin-Pluronic-PLA nanoparticles obtained were summarized in Table II.

The morphologies of Biotin-Pluronic-PLA nanoparticles were examined by TEM. The nanoparticles were negatively stained by sodium phosphotungstate. Figure 5 shows the TEM pictures of Biotin-Pluronic-PLA nanoparticles. It was good to see that the particle sizes and size distributions observed from TEM for the four Biotin-Pluronic-PLA nanoparticles were consistent with those from DLS analysis. The morphology of Biotin-F127-PLA-61, Biotin-F87-PLA, and Biotin-P85-PLA nanoparticles is spherical core-shell micelles. For Biotin-F127-PLA-87 nanoparticles, both spherical micelles and large compound micelles were observed. The existence of large compound micelles is in correspondence with the pretty large particle size (906 nm) obtained from DLS. Compared with the other three block copolymers, high $\overline{M}_n$ could be the main reason for the formation of large compound micelles for Biotin-F127-PLA-87 block copolymer. The possible microstructure of Biotin-Pluronic-PLA nanoparticles was proposed and shown in Figure 6. The core of Biotin-Pluronic-PLA nanoparticles was composed of hydrophobic PLA block. The shell is composed of Pluronic block, whose PPO block may tend to staying close to the hydrophobic PLA core due to its weak hydrophobic property. The biotin group should exist on the surface of nanoparticles due to its hydrophilic property.

In vitro cytotoxicity studies

The cytotoxicity of Biotin-F127-PLA-61 block copolymer was evaluated using MTT assay. The dependence of cell viability on the concentrations of Biotin-F127-PLA-61 block copolymer and the incubation time was investigated as shown in Figure 7. It was observed that over 83% of OVCAR-3 cells were viable after incubated for 24 h for Biotin-F127-PLA-61 block copolymer upto 0.01 wt %. PLA homopolymer and Pluronic F127 are well-known biocompatible polymers. The earlier results indicate

TABLE II
Characterizations of Biotin-Pluronic-PLA Nanoparticles

Sample	Diameter (nm) (DLS)[a]	Morphology (TEM)
Biotin-F127-PLA-61	198 ± 5.6	spheres
Biotin-F127-PLA-87	126.6 ± 1.6	spheres
	906 ± 0.8	large compound micelles
Biotin-F87-PLA	228.7 ± 1.5	spheres
Biotin-P85-PLA	256.7 ± 9.3	spheres

[a] The diameters of Biotin-Pluronic-PLA nanoparticles are expressed as mean ± standard deviation of three tests.

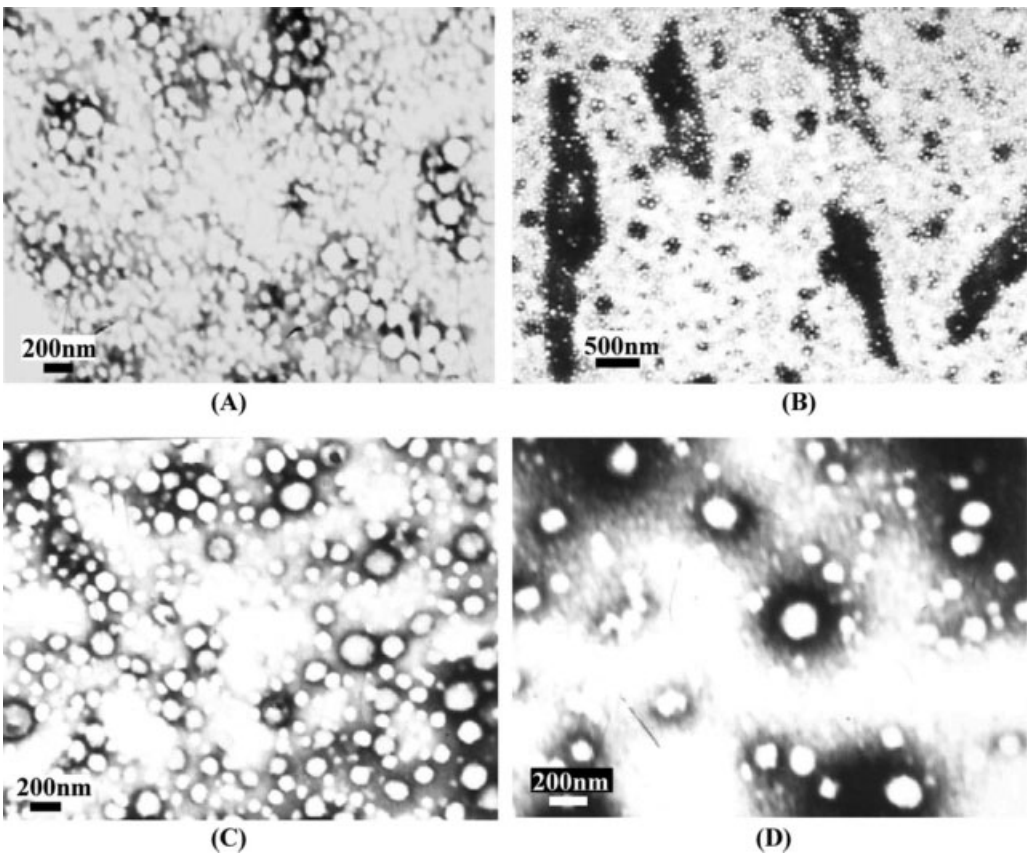


Figure 5 TEM micrographs of nanoparticles formed from Biotin-F127-PLA-61 (A), Biotin-F127-PLA-87 (B), Biotin-F87-PLA (C), and Biotin-P85-PLA (D) block copolymers in water.

that Biotin-F127-PLA block polymer also has very good biocompatibility.

CONCLUSIONS

In this study, four kinds of biotin-Pluronic-PLA block copolymers were synthesized by two-step reactions. The self-assembling behaviors of biotin-Pluronic-PLA block copolymers in aqueous solutions were examined. The CMC of biotin-Pluronic-PLA block copolymers is pretty low, indicating the good stability of biotin-Pluronic-PLA nanoparticles. The morphology of Biotin-F127-PLA-61, Biotin-F87-PLA, and Biotin-P85-PLA nanoparticles is spherical micelles. Two kinds of morphologies, spherical micelles and large compound micelles, were found for Biotin-F127-PLA-87 nanoparticles, which could be related to its high-molecular weight. Biotin-Pluronic-PLA block copolymers were found to have

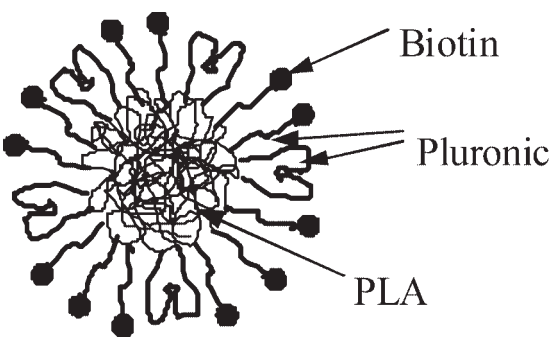


Figure 6 The possible microstructure of Biotin-Pluronic-PLA nanoparticles in water.

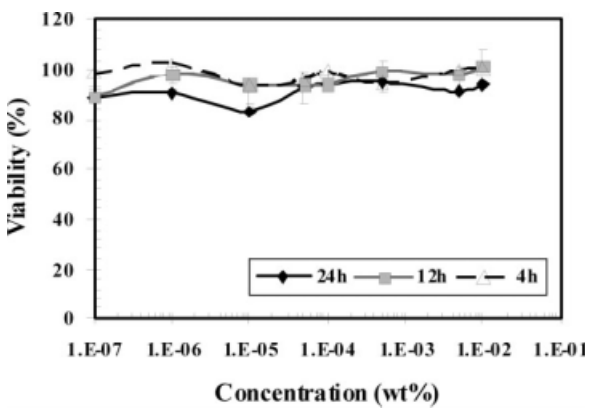


Figure 7 Cytotoxicity of Biotin-F127-PLA-61 unimers and nanoparticles in OVCAR-3 cell lines. Each point represents the mean ± SD of three cultures.

good biocompatibility by MTT assay using human ovarian cancer cells OVCAR-3. The study of Biotin-Pluronic-PLA block copolymers on the application of targeted drug delivery systems is on progress.

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