Triblock Poly(lactic acid)-*b*-Poly(ethylene glycol)-*b*-Poly(lactic acid)/Paclitaxel Conjugates: Synthesis, Micellization, and Cytotoxicity

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ABSTRACT: A triblock poly(lactic acid)-*b*-poly(ethylene glycol)-*b*-poly(lactic acid) (PLA–PEG–PLA)/paclitaxel (PTX) conjugate was synthesized by the reaction of carboxyl-terminated copolymer PLA–PEG–PLA with PTX in the presence of dicyclohexylcarbodiimide and dimethylaminopyridine. Carboxyl-terminated copolymer PLA–PEG–PLA was prepared by the reaction of the hydroxyl end groups in copolymer PLA–PEG–PLA with succinic anhydride. Its structure was confirmed by NMR and gel permeation chromatography. The PLA–PEG–PLA/PTX conjugates could self-assemble into micelles in aqueous solutions with a low critical micelle concentration. Dynamic light scattering and environmental scanning electron microscopy analyses of the PLA–PEG–PLA/PTX micelles revealed their spherical structure and size of 220 nm. The antitumor activity of the conjugate against woman Hela cancer cells, evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide method, showed that the conjugates had an antitumor activity similar to that of pure PTX. The obtained PLA–PEG–PLA/PTX conjugates are expected to be used in clinical practice. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 105: 2271–2279, 2007

Key words: polyesters; ring-opening polymerization; synthesis

INTRODUCTION

In recent years, polymer chemistry has been dedicated to the synthesis, characterization, evaluation, and modification of new biocompatible and biodegradable polymers, which are potential carriers for drug delivery systems (DDSs). An ideal DDS can effectively control the rate of drug release, administrate at a low dosage, improve site specificity, and increase therapeutic benefit.[1–3] Interest in polymer conjugation with antitumor drugs has increased remarkably as such conjugates are preferably accumulated in solid tumors and can reduce systemic toxicity. In the past, several polymeric prodrugs have been developed and clinically evaluated for the delivery of anticancer agents.[4–8]

Paclitaxel (PTX) is a microtubule stabilizing drug and a potent chemotherapeutic agent that has shown substantial clinical efficacy for various solid tumors, including ovarian, breast, colon, head, neck, and non-small-cell lung cancer.[9–17] Furthermore, it has been shown *in vitro* that the cytotoxicity of PTX is more dependent on the exposure time than on increased PTX concentration.[18] Although PTX has high activity against many kinds of cancers, great efforts have been devoted to the development of new delivery systems to overcome the main troubles encountered in its use, including very low water solubility and hypersensitivity reactions associated with the traditional Cremophor EL based formulation.[19–21]

Biodegradable block copolymers containing poly (lactic acid) (PLA) and poly(ethylene glycol) (PEG) segments exhibit good potential for formulating DDSs.[22–25] Many groups have investigated PEG–PLA nanoparticles and micelles as DDSs because of the ease of preparation, high drug loading, and possibility for sustained drug release. However, to the best of our knowledge, there are few reports in the literature describing polymer conjugate drug-containing PEG–PLA.[26]

In a previous article,[19] we have reported a new conjugate of diblock copolymer monomethoxy-poly(ethylene glycol)-poly(lactic acid) (MPEG–PLA) with PTX. The antitumor activity of the conjugate against human liver cancer H7402 cells, evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method, showed that PTX can be released from the conjugate without losing cytotoxicity. How-

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ever, the self-assembly behaviors in water were not investigated. Moreover, the PTX molecule was connected to one terminal of the block copolymer, so the content of PTX in the conjugate was limited.

In this study, a novel conjugate of PTX with the triblock copolymer poly(lactic acid)-*b*-poly(ethylene glycol)-*b*-poly(lactic acid) (PLA–PEG–PLA) was synthesized. Because the two terminal hydroxyl groups were fully used, the PTX content in the conjugate could be up to 2 times that reported in the previous article.[19] The self-assembly behavior of the conjugate in aqueous solutions was investigated by fluorescence spectroscopy, dynamic light scattering (DLS), and environmental scanning electron microscopy (ESEM). The antitumor activity of the conjugate against woman Hela cancer cells, evaluated by the MTT method, shows that the conjugate has antitumor activity similar to that of pure PTX. The three components of the conjugate possess their own advantages. The PEG block acts as a hydrophilic and protein-resistive component; it makes self-assembly of the conjugate in water possible. The biodegradation of PLA segments allows the metabolization and elimination of the material after its function has been accomplished and could control the release rate of PTX. PTX is used as an antineoplastic agent. Therefore, potential medical applications are expected for this conjugate.

EXPERIMENTAL

Materials

Poly(ethylene glycol) with a molecular weight of 4600 (PEG4600) was purchased from Aldrich (Milwaukee, WI). Before use, PEG was dried by azeotropic distillation in toluene. PTX was purchased from Xi'an Baosai Biotechnology, Inc. (Xian, China). Dicyclohexylcarbodiimide (DCC) and dimethylaminopyridine (DMAP), supplied from GL Biochem, Ltd. (Shanghai, China), were used as received. Dichloromethane was treated with hexamethylene diisocyanate for 5 h at 50°C and distilled to remove any traces of amine and alcohol. Toluene was dried and distilled from sodium/benzophenone under a nitrogen atmosphere before use. Other reagents were commercially available and used as received.

Measurements

^{1}H-NMR spectra were recorded on a Bruker AV300M in $CDCl_3$ at 25°C. Chemical shifts were given in parts per million from that of tetramethylsilane as an internal reference. Gel permeation chromatography (GPC) measurements were conducted with a Waters 410 GPC instrument (Milford, MA) equipped with a Waters Styragel HT6E column and a differential refractometer detector. Tetrahydrofuran (THF) was used as the eluent at a flow rate of 1 mL/min at 35°C. The molecular weights were calibrated with polystyrene standards.

Synthesis of PLA–PEG–PLA

Triblock copolymer PLA–PEG–PLA was easily prepared by the ring-opening polymerization of L-lactide (LA) in the presence of PEG and stannous octoate [$Sn(Oct)_2$] according to a previous procedure.[19] The yield was 84%.

Synthesis of carboxyl-terminated copolymer PLA–PEG–PLA

Carboxyl-terminated copolymer PLA–PEG–PLA was prepared with succinic anhydride in the presence of DMAP and trimethylamine (TEA) according to the literature.[27,28] PLA–PEG–PLA (1.0 g), succinic anhydride (0.023 g), DMAP (0.029 g), and triethylamine (0.030 mL) were dissolved in 1,4-dioxane (10 mL) and left overnight at room temperature. The filtered solution was precipitated by the addition of diethyl ether, and the polymer precipitate was purified by dialysis with a cellulose membrane [cutoff number-average molecular weight (M_n) = 2000] and dried *in vacuo* for 3 days at room temperature. The yield was 85%.

Synthesis of the PLA–PEG–PLA/PTX conjugate

The conjugate was synthesized according to the previous literature[19] and purified by dialysis with a cellulose membrane (cutoff M_n = 3500) for 2 days. The yield was 80%.

Preparation of the micelles

The micelles were prepared with a solvent displacement method with a THF/H_2O system. The conjugate micelles were prepared as follows: a conjugate (0.025 g) was first dissolved in THF (10 mL) in a 100-mL volumetric flask, and 40 mL of doubly distilled water was added with gentle agitation. The THF was tardily removed at the ambient temperature over 2 h by rotary evaporation to get the micelles. To study the effect of the PTX conjugate on the properties of the micelles, the micelles of the PLA–PEG–PLA copolymer were also prepared for comparison.

Measurement of the critical micelle concentration (cmc)

The formation of the micellar structures was confirmed by a fluorescence technique with pyrene as a probe. Steady-state fluorescence spectra were obtained with a PerkinElmer LS50B luminescence spectrometer

(Norwalk, CT). The sample solutions were prepared first by the addition of known amounts of pyrene in acetone to a series of flasks. After the acetone had evaporated completely, micelle solutions with various concentrations of the copolymer were added to each of the flasks and mixed by vortexing. The concentration of pyrene in the final solution was 6×10^{-7} mol/L, similar to the saturation solubility of pyrene in water at 22°C. The flasks were thermostated at 40°C for about 2 h to equilibrate the pyrene partition between the water and micelles and subsequently were cooled overnight to room temperature. For fluorescence emission spectra, the emission wavelength was 391 nm for excitation spectra, and the excitation bandwidth was 4 nm. The spectra were recorded with a scanning rate at 240 nm/min.

Characterization of the micelle morphology and size

DLS

The hydrodynamic radius and the size distribution of the micelles were determined by DLS with a vertically polarized He–Ne laser (Dawn Eos, Wyatt Technology, Santa Barbara, CA). The scattering angle was fixed at 90°, and the measurement was carried out at a constant temperature of 25°C. The sample solutions were diluted in filtered, double-distilled water before analysis.

ESEM

The morphology and size of the micelles were investigated by ESEM. It was performed on an XL 30 ESEM FEG scanning electron microscope (Micrion FEI Philips, Hillsboro, OR). A drop of the micelle solution was deposited onto a silicon chip mounted on an aluminum stub. The sample was air-dried and coated with gold before the measurement.

In vitro antitumor activity

The antitumor activity of the PTX conjugate was evaluated by the MTT method.[29] Woman Hela cancer cells were chosen as target cells. They were cultured in the growth medium Dulbecco's modified eagle's medium (DMEM), which contained 10% fetal bovine serum, 2.0 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin, and the cell density of the cell suspension was adjusted to 5×10^4 cells/mL. Two hundred microliters of aliquots of this suspension were added to the walls in a 96-well plate and incubated for 24 h in a humidified atmosphere containing 5% CO_2 at 37°C. The conjugate was made of PLA–PEG–PLA, and the weight content of PTX was 16%. It was dissolved in DMSO at the proper concentration. It was diluted 200-fold with the cell culture medium DMEM and added to the wells (200 μL per well). After 48 h of incubation, a 20-μL MTT solution (5 mg/mL) was added to each well of the plate. The incubation was continued for another 4 h. Then, the MTT derivative in the solution was dissolved in 150 μL of DMSO, and the solution was determined with a Thermo MK3 microplate reader (Waltham, MA) at 492 nm. The relative cell inhibition rate was calculated and averaged.

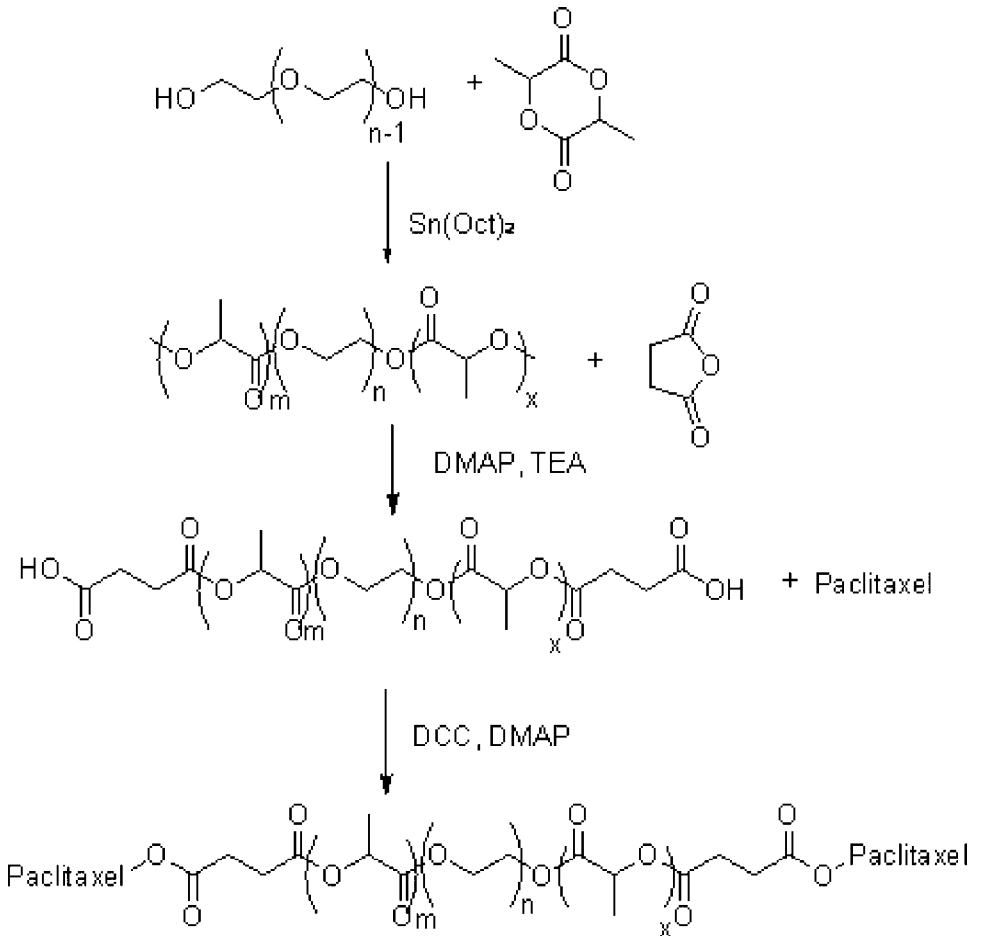
RESULTS AND DISCUSSION

Synthesis of the PLA–PEG–PLA copolymer

PLA–PEG–PLA was prepared by the ring-opening polymerization of LA with PEG as a macroinitiator and $Sn(Oct)_2$ as a catalyst in a toluene solution (Scheme 1). The block lengths of PEG and PLA could be adjusted by changes in the molecular weight of PEG and the molar ratio of LA to PEG. Starting with PEG4600 and taking the mass ratio in the feed of LA to PEG4600 as 1/1, we obtained triblock copolymer PLA–PEG–PLA with an average molecular of 8780 by ^{1}H-NMR, which is lower than the molecular weight determined by GPC, as shown in Table I and Figure 1. This discrepancy may be attributed to the difference in the hydrodynamic property between the PLA–PEG–PLA and polystyrene standard. In Figure 1(a), a single and sharp peak was found with the molecular weight distribution of 1.10. These results indicated that the PEG had reacted with LA successfully and that no homopolymer of LA was produced during the reaction.

Synthesis of carboxyl-terminated copolymer PLA–PEG–PLA

Zhang et al.[19] reported on the synthesis of carboxyl-terminated copolymer PLA–PEG–PLA by the reaction of PEG–PLA with mono-*t*-butyl ester of diglycolic acid and subsequently by the removal of the *t*-butyl. It is complicated and inefficient. As reported by Kim,[27] carboxyl-terminated PLA–PEG–PLA was prepared successfully by reacting PLA–PEG–PLA with succinic anhydride with DMAP and TEA as catalysts and 1,4-dioxane as solvent. Figure 2 shows the ^{1}H-NMR spectra of PLA–PEG–PLA and carboxyl-terminated PLA–PEG–PLA. It is obvious that the proton signals of CH_2 formed by the reaction with succinic anhydride at 2.75 ppm appeared, whereas other proton signals were little changed. The molecular weight and its distribution changed little after the reaction of PLA–PEG–PLA with succinic anhydride (Table I and Fig. 1). From these analyses, it was found that the ring of the succinic anhydride had been opened by the terminal hydroxyl group of the PLA–PEG–PLA copolymer. Thus, a terminal carboxyl group was introduced into the PLA–PEG–PLA copolymer.



Scheme 1 Synthesis route of the PLA–PEG–PLA/PTX conjugate.

Synthesis of the PLA–PEG–PLA/PTX conjugate

As mentioned in previous articles, the 2′-hydroxy in PTX is more active than others, and thus esterification could take place between the carboxyl-terminated PLA–PEG–PLA copolymer and PTX.[30] The PLA–PEG–PLA/PTX conjugate was synthesized in the presence of DCC and DMAP at 0°C. The unreacted PTX was removed by dialysis. Figure 3 shows the ^{1}H-NMR spectra of the PTX and PLA–PEG–PLA/PTX conjugate. It shows that the characteristic peaks of PTX can all be found in the PLA–PEG–PLA/PTX conjugate. To lean more about the chemical nature of the PTX derivative, we obtained the ^{13}C-NMR spectra of the conjugates. Figure 4 presents the ^{13}C-NMR spectra of the PLA–PEG–PLA/PTX conjugate. In addition to signals a–d corresponding to the

TABLE I
Molecular Weights and Molecular Weight Distributions of Triblock Copolymer PLA–PEG–PLA and Its Derivatives

Polymer	M_n (NMR)	$M_n \times 10^4$ (GPC)	$M_w \times 10^4$ (GPC)	PDI (GPC)
PLA–PEG4600–PLA	8,780	1.55	1.70	1.10
Carboxyl-terminated PLA–PEG–PLA	8,980	1.57	1.73	1.10
PLA–PEG–PLA/PTX conjugate	10,700	1.75	1.93	1.08

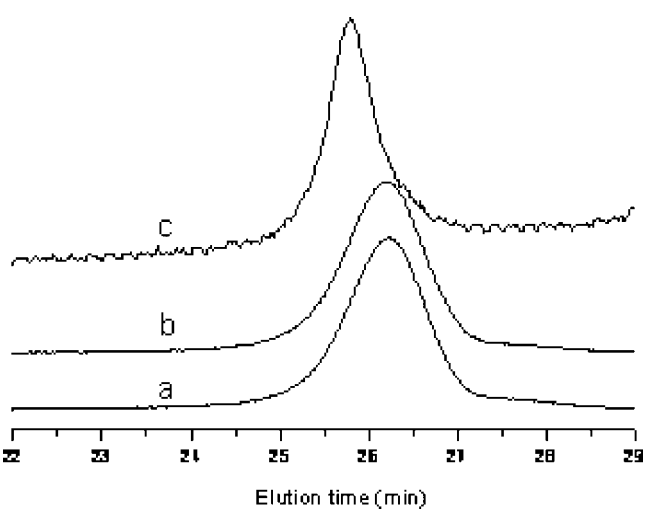


Figure 1 GPC traces of (a) PLA–PEG–PLA ($M_n = 1.55 \times 10^4$), (b) carboxyl-terminated PLA–PEG–PLA ($M_n = 1.57 \times 10^4$), and (c) PLA–PEG–PLA/PTX conjugate ($M_n = 1.75 \times 10^4$).

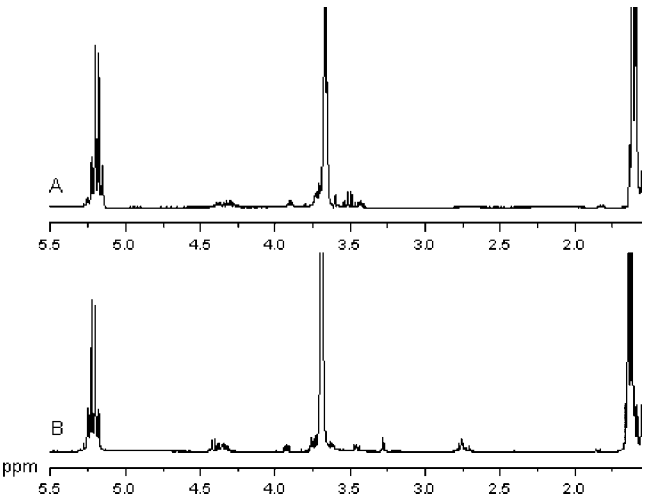


Figure 2 ^{1}H-NMR spectra of (A) PLA–PEG–PLA and (B) carboxyl-terminated PLA–PEG–PLA in $CDCl_3$.

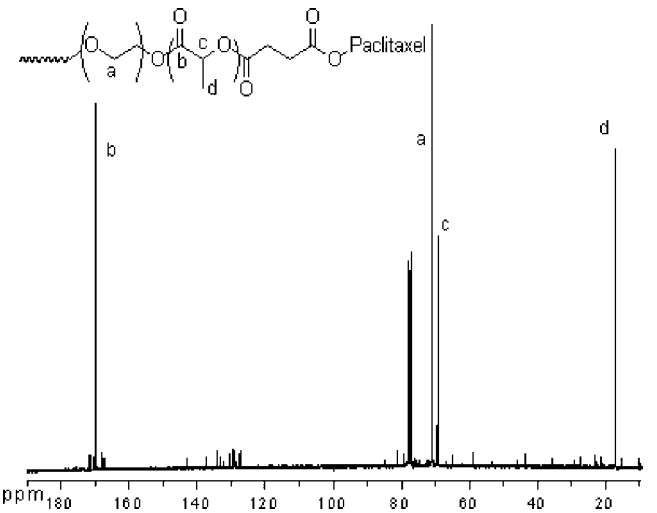


Figure 4 ^{13}C-NMR spectrum of PLA–PEG–PLA/PTX in $CDCl_3$.

PLA–PEG–PLA unit, signals for the PTX unit also appear, such as the signals at 128–135 ppm for the carbon in the benzene ring. Furthermore, as expected, the molecular weight determined by GPC becomes larger than that of PLA–PEG–PLA, whereas the molecular weight distribution is lower that of PLA–PEG–PLA. All these results indicate that PTX was conjugated with PLA–PEG–PLA.

As stated in the introduction, the main reason for the use of the triblock copolymer is that the PTX content in the conjugate can be up to 2 times that reported in the previous article. In the previous work, the conjugate was made of MPEG (M_n = 5000)–PLA (M_n = 2000), and its weight content of PTX was 10%. Herein, the conjugate was made of PEG (M_n = 4600)–PLA (M_n = 4200), and its weight content of PTX was 16%. It is obvious that the content of PTX increases with the triblock copolymer.

Formation of the micelles

The amphiphilic nature of the PLA–PEG–PLA/PTX conjugate provides an opportunity to form micelles in water. As shown in Figure 5, the water-soluble PEG chains serve as the hydrophilic shell, stabilizing the nanoparticle, and PLA/PTX constitutes the hydrophobic core. A pyrene probe was used to prove the micelle formation of the PLA–PEG–PLA/PTX conjugate and to measure its cmc.

Excitation spectra of pyrene in the PLA–PEG–PLA/PTX solutions are shown in Figure 6. As can be seen, the fluorescence intensity increases with an increasing concentration of PLA–PEG–PLA/PTX. Concomitantly with the increase in the fluorescence intensity, a redshift from 333 to 335 nm

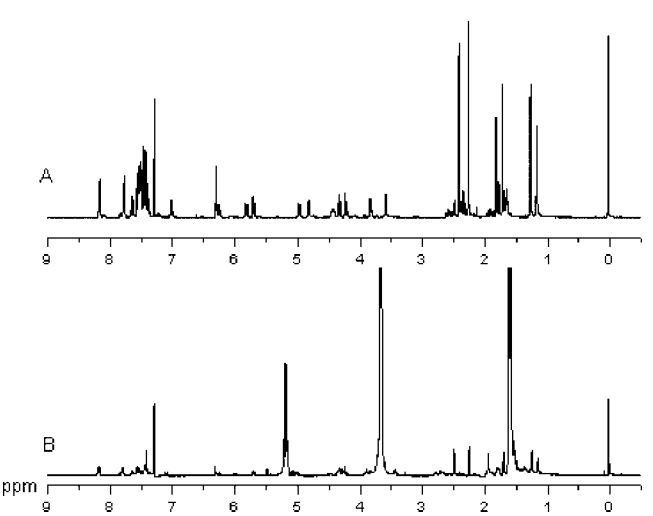


Figure 3 ^{1}H-NMR spectra of (A) PTX and (B) PLA–PEG–PLA/PTX in $CDCl_3$.

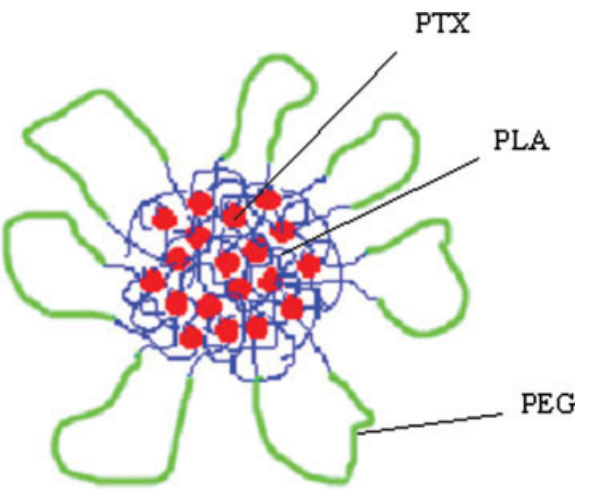


Figure 5 Micelle formed from PLA–PEG–PLA/PTX. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

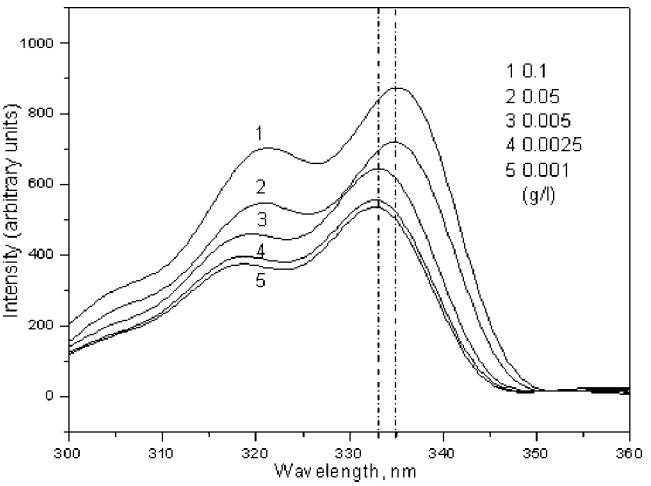


Figure 6 Fluorescence excitation spectra of pyrene in aqueous PLA–PEG–PLA/PTX solutions. The emission wavelength was 391 nm, and the temperature was 20°C.

takes place. These results are ascribed to the micellization of PLA–PEG–PLA/PTX. When the intensity ratio I_{335}/I_{333} is plotted against the polymer concentration (C) in Figure 7(B), the cmc value can be obtained.

The same redshift from 333 to 335 nm was observed when the same experiments were carried out for PLA–PEG–PLA micelles. However, when I_{335}/I_{333} is plotted against the polymer concentration, as shown in Figure 7(A), a larger cmc value than that of the PLA–PEG–PLA/PTX micelles can be observed. The change in the cmc value may be the enhancement of hydrophobicity by the conjugation of highly hydrophobic PTX.

Micelle morphology and size

The size and morphology of the PLA–PEG–PLA/PTX and PLA–PEG–PLA micelles in aqueous solutions were examined by DLS and ESEM. Figure 8 presents the size of the micelles obtained by DLS. The mean diameter of the PLA–PEG–PLA and PLA–PEG–PLA/PTX micelles is about 180 and 210 nm, respectively. As we know, the size of the micelles mainly depends on the hydrophobic block in the

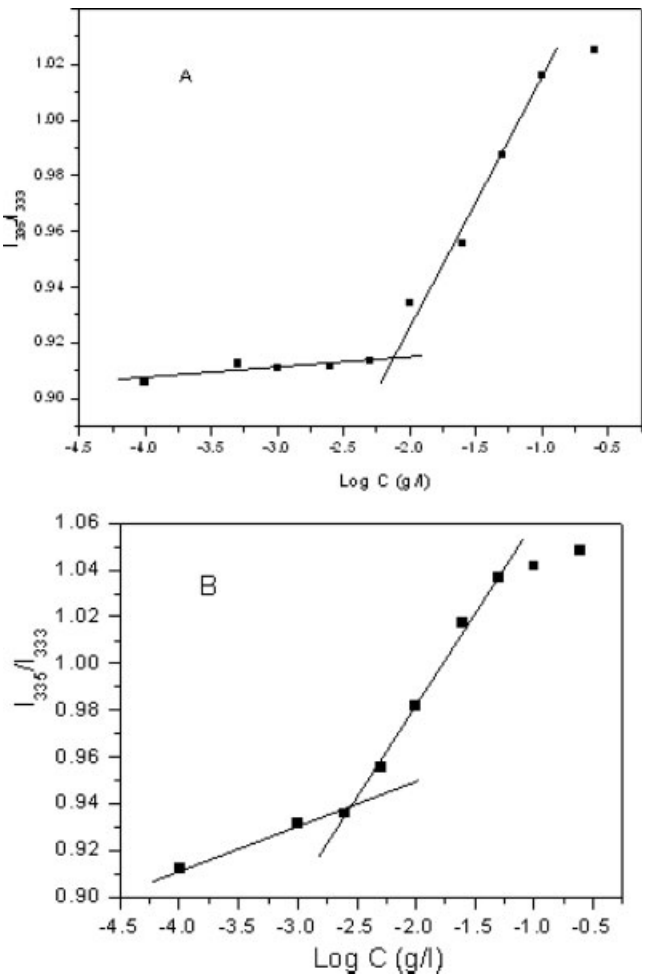


Figure 7 Plots of I_{335}/I_{333} versus log C of (A) the PLA–PEG–PLA micelle and (B) the PLA–PEG–PLA/PTX micelle.

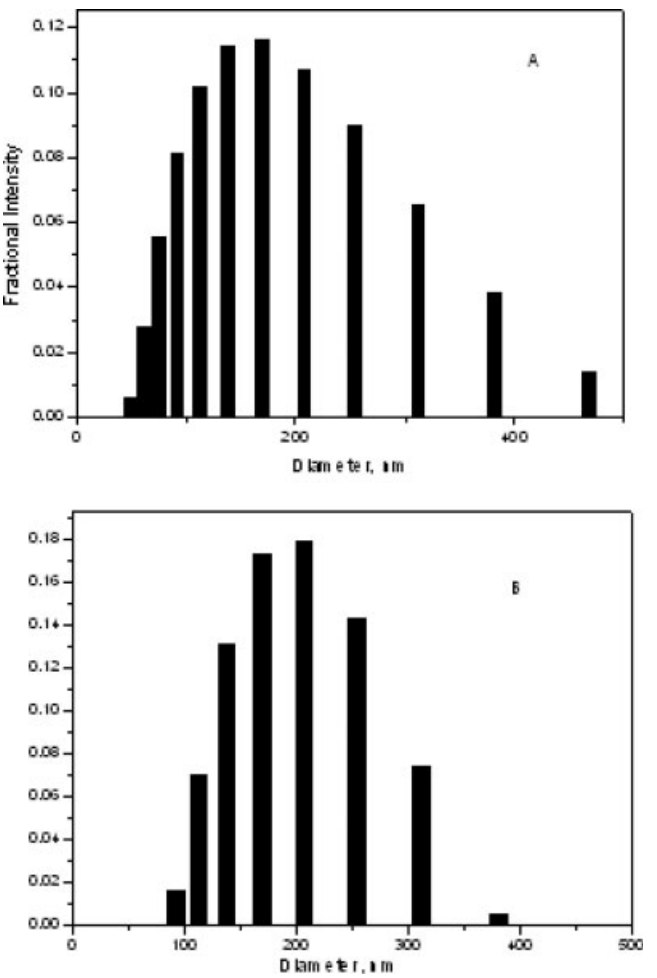


Figure 8 Size distributions of (A) PLA–PEG–PLA micelles and (B) PLA–PEG–PLA/PTX micelles.

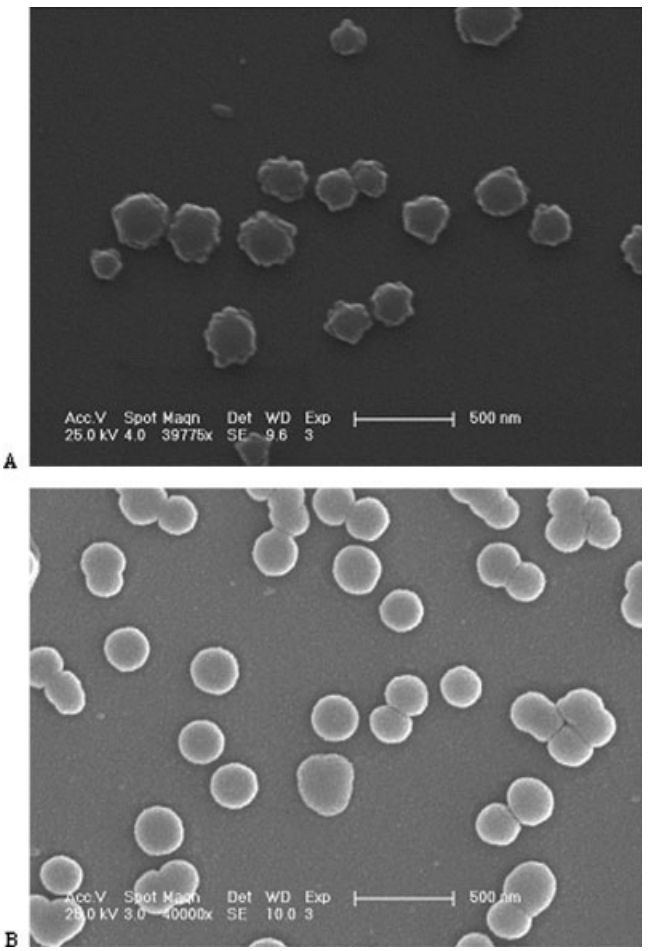


Figure 9 ESEM images of (A) PLA–PEG–PLA micelles and (B) PLA–PEG–PLA/PTX conjugate micelles.

amphiphilic copolymer. When PLA–PEG–PLA was conjugated with PTX, the hydrophobic segment became longer than that in PLA–PEG–PLA, whereas the hydrophilic segment remains unchanged.

Figure 9 shows the ESEM images of PLA–PEG–PLA and PLA–PEG–PLA/PTX micelles. They are all spherical. Their apparent particle sizes are 200 and 220 nm, respectively, being larger than those determined by DLS (Fig. 8). Compared with the PLA–PEG–PLA micelles, the PLA–PEG–PLA/PTX micelles have a larger average diameter and smoother surfaces. The larger diameters determined by ESEM and the rough surfaces of the PLA–PEG–PLA micelles indicate that micelle aggregation may occur to some extent during the specimen preparation for ESEM.

These results suggest that the PTX was immobilized in the micelle core because of the conjugation. Moreover, the size of the PLA–PEG–PLA/PTX micelles was larger than that of the PLA–PEG–PLA micelles. These results were consistent with the analysis of DLS.

In vitro antitumor activity

The antitumor activity of the PTX conjugate against the woman Hela cancer cells was evaluated with the MTT method.[29] Five different samples were studied in detail for comparison, including the pure PTX, the conjugate, the block copolymer, the conjugate micelles, and the block copolymer micelles. Figure 10 shows the cell inhibition rate after 48 h of incubation at different concentrations. At the same drug content, the conjugate exhibits almost the same antitumor activity as pure PTX. For example, the cell inhibition rates are 90.8 ± 1.8 and 90.3 ± 2.6% at a drug concentration of 10 ng/mL for 48 h, respectively. In the case of the block polymer, the sample does not display any cytotoxicity to Hela cancer cells. Furthermore, because the conjugate can form micelles in water, the antitumor activity of the conjugate micelles was also studied. It is clear in Figure 10 that the conjugate micelles show a high cell inhibition rate and that the block copolymer micelles do not. In the PTX concentration range of 0.1–100 ng/mL, PTX and its conjugate show a higher cell inhibition rate when the concentration is over 10 ng/mL; a further increase in the PTX concentration does not result in a further increase in the cell inhibition rate under the experimental conditions. However, as far as the cell inhibition rate is concerned, the difference between 10 ng/mL and 1 ng/mL is statistically significant. Therefore, 10 ng/mL may be considered the lowest effective PTX concentration.

The cell morphologies of Hela cancer cells treated by different samples are shown in Figure 11. The cells cultured with block copolymer PLA–PEG–PLA and its micelles grow normally and occupy the

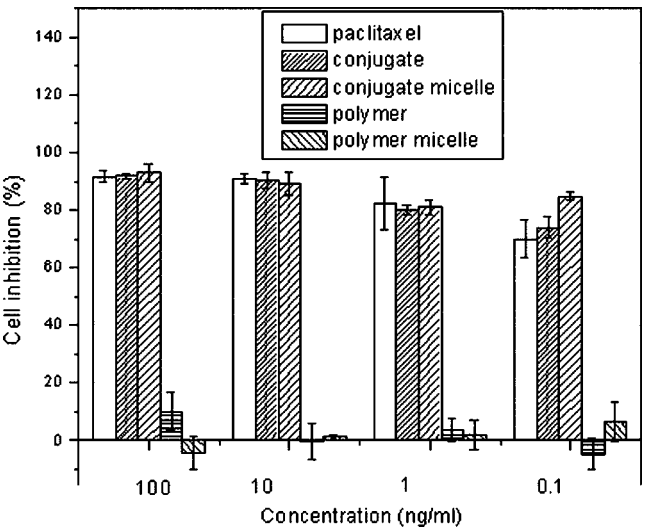


Figure 10 *In vitro* cytotoxicity of the PLA–PEG–PLA/PTX conjugate against human cancer cells. The general test procedures are described in the text. The cell density was 5×10^4 cells/mL.

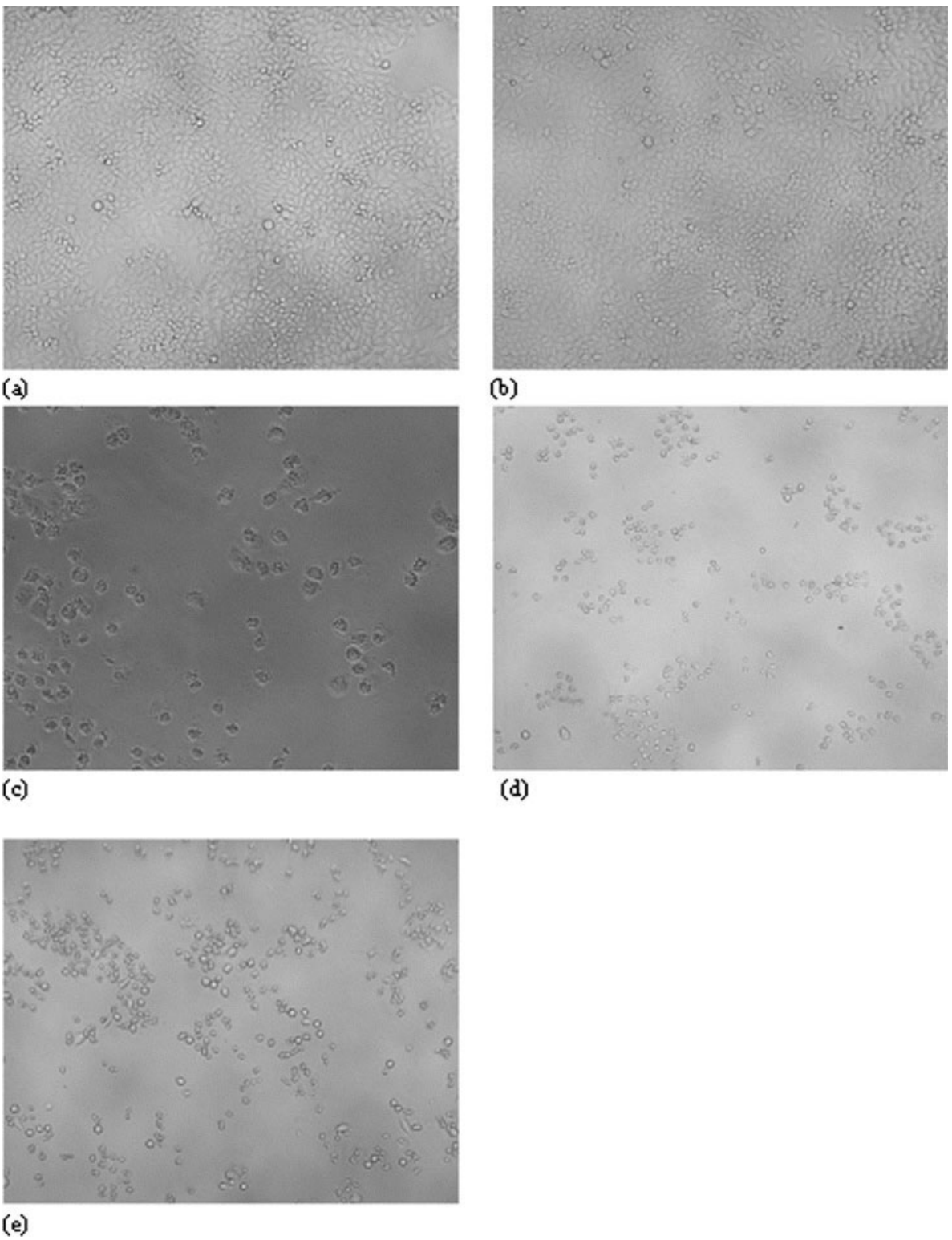


Figure 11 Typical micrographs of the cells after incubation for 48 h in the presence of (a) the copolymer, (b) the copolymer micelle, (c) PTX, (d) the conjugate, and (e) the conjugate micelles.

whole field. However, almost all Hela cancer cells died in the case of pure PTX or the PLA–PEG–PLA/PTX conjugate. From these results, it can be concluded that the PLA–PEG–PLA/PTX conjugates show high cytotoxicity against Hela cancer cells and can be used furthermore as clinical medicine.

CONCLUSIONS

The main conclusions from this work can be summarized as follows:

1. Triblock PLA–PEG–PLA/PTX conjugates were synthesized in three steps: (1) the ring-opening polymerization of LA was conducted in the presence of PEG with two terminal hydroxyl end groups, (2) the terminal hydroxyl end groups of PLA–PEG–PLA were converted into terminal carboxyl end groups by a reaction with succinic anhydride in the presence of DMAP and TEA, and (3) the carboxyl end groups were reacted with PTX with the help of DCC and DMAP. The structure of the copolymer obtained at each step was confirmed by ^{1}H-NMR and GPC.
2. The self-assembly behavior of the PLA–PEG–PLA/PTX conjugate in water was studied by fluorescence spectroscopy, DLS, and ESEM. PLA–PEG–PLA/PTX micelles showed a spherical structure with smooth surfaces. The amphiphilic property could be used in developing new drug delivery vehicles.
3. The antitumor activity of the conjugate against woman Hela cancer cells evaluated by the MTT method showed that the conjugate had antitumor activity similar to that of pure PTX and could be used furthermore in clinical medicine.

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