Preparation and Drug-Release Behavior of 5-Fluorouracil-Loaded Poly(lactic acid–4-hydroxyproline–polyethylene glycol) Amphipathic Copolymer Nanoparticles

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ABSTRACT: Poly(lactic acid–4-hydroxyproline–polyethylene glycol) (PLA–Hpr–PEG) was synthesized via melt copolymerization with stannous chloride as a catalyst activated by a proton acid. Copolymers with different poly(ethylene glycol) (PEG) concentrations (0.1, 0.5, 1, and 5 wt %) were synthesized and exhibited moderate molecular weights (weight-average molecular weight = 9705–13,600 g/mol) and reasonable molecular weight distributions (weight-average molecular weight/number-average molecular weight = 1.35–1.66). The structure of the polymers was verified with infrared spectroscopy and proton nuclear magnetic resonance spectroscopy. The nanoparticles were made by the nanoprecipitation method with PLA–Hpr–PEG. The size and size distribution of the nanoparticles were investigated with laser light scattering, and the surface morphology of the nanoparticles was investigated with transmission electron microscopy. The drug encapsulation efficiency and drug loading content were measured with ultraviolet absorption spectroscopy. The effects of various formulation parameters were evaluated. The prepared nanoparticles were spherical and greater than 100 nm in size. The drug loading content and encapsulation efficiency were greatly influenced by the amount of the copolymer and the volume of the solvent. The PEG content in the polymer could affect the release of drugs from the PLA–Hpr–PEG nanoparticles. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 103: 2654–2659, 2007

Key words: microencapsulation; polyesters

INTRODUCTION

A number of different polymers, both synthetic and natural, have been used in formulating biodegradable nanoparticles.[1] The polymers used for the formulation of nanoparticles include synthetic polymers such as poly(D,L-lactide-*co*-glycolide) (PLGA) copolymers and polycaprolactones and natural polymers such as albumin, gelatin, alginate, collagen, and chitosan.[1] Of these polymers, polylactides (PLAs) and PLGAs have been the most extensively investigated for drug delivery.[2,3]

A wide variety of PLAs are commercially available at present, but it often happens that we need PLA specimens with physical or chemical properties different from those of commercially available ones. In such cases, we should synthesize the polymer specimens through the polymerization of lactides or lactic acids. The introduction of functional groups into PLA for the modification of its properties has been investigated.[4] In our research, we have chosen 4-hydroxyproline to introduce functional groups because 4-hydroxyproline is an amino acid needed by the human body and has the advantage of being nontoxic, biodegradable, biocompatible, and fitted with pendant functional groups on the backbone;[5] after copolymerization with other polymers, it still retains some active groups.[6–10]

We have designed a novel amphipathic polymer, poly(lactic acid–4-hydroxyproline–polyethylene glycol) (PLA–Hpr–PEG) (Scheme 1), which has a free amino group in the 4-hydroxyproline residue that can be further used to chemically attach a biologically active peptide, by melt copolymerization. PLA–Hpr–PEG has been used to prepare nanoparticles by the nanoprecipitation method. Nanoparticles have been found that can penetrate the submucosal layers, whereas large microparticles are predominantly localized in the epithelial lining.[11] Others have shown that nanoparticles can cross the blood–brain barrier after the opening of tight junctions by hyperosmotic mannitol. Such a strategy could provide a sustained delivery of therapeutic agents for difficult-to-treat diseases such as brain tumors.[12] The size and size distribution of the prepared nanoparticles have been measured with laser light scattering (LLS). The surface morphology has been investigated with transmission electron microscopy (TEM). Ultraviolet (UV) has been used to measure the drug loading content (DLC) and encapsulation efficiency (EE) of the nanoparticles and the *in vitro* release profile. The effects of formulation variables, such as the amount of the copolymer, the oil/water phase ratio, and drug loading, have been evaluated.

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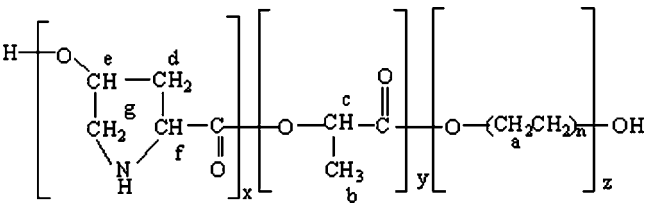

Scheme 1 Structure of PLA–Hpr–PEG.

EXPERIMENTAL

Materials

Lactic acid was purchased as a 90% aqueous solution from Shanghai Chemical Industries (Shanghai, China). Tin(II) chloride dihydrate and *p*-tolulenesulfonic acid monohydrate were purchased from Guangzhou Chemical Industries (Guangzhou, China), and all these materials were used without purification. All other chemicals were analytical-grade and were purchased in China.

Synthesis of the PLA–Hpr–PEG copolymer

Lactic acid (33.33 g, 0.333 mol) was dehydrated at 150°C for a determined time to make the oligomer. The oligomer (20 g), 4-hydroxy-1-(1-oxoethyl)-L-proline[13] (0.8 g, 0.0061 mol), and poly(ethylene glycol) (PEG; weight-average molecular weight = 2000; 1 g, 0.0005 mol) were placed in a 250-mL flask and mixed with 3 wt % tin(II) chloride dihydrate and a proton acid. Then, the mixture was heated at 180°C in a vacuum of 5 mmHg under mechanical stirring at 5 h, and a 9-h reaction followed at the same pressure with the reaction temperature changed from 180 to 150°C. At the end of the reaction, the flask was cooled, and the product was dissolved in acetone and subsequently precipitated into distilled water. The resulting solids were filtered and dried *in vacuo*. Then, catalytic hydrogenation in a reduction catalyst (5 wt % palladium on charcoal) was used to reduce the protected amino group of proline (yield = 64%).

Preparation of the 5-fluorouracil (5-Fu)-loaded PLA–Hpr–PEG nanoparticles

5-Fu-loaded nanoparticles were prepared with the nanoprecipitation method. Briefly, 10 mg of 5-Fu and 100 mg of PLA–Hpr–PEG (5 wt % PEG) were dissolved in 10 mL of acetone. This organic solution was dropwise added to 50 mL of deionized water under magnetic stirring. The nanoparticles were formed immediately, and acetone was removed through overnight evaporation at room temperature. The suspension was filtered by a microfilter with a pore size of 5 μm to remove polymer aggregates. The obtained micellar solutions were frozen and lyophilized by a freeze-dryer system to obtain the dried nanoparticle product.

PLA–Hpr–PEG characterization

Infrared (IR) spectra were measured on a Nicolet 20DXB IR spectrophotometer. Samples were pressed into KBr pellets. Proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) spectra were measured on a Varian Inova 400-MHz spectrometer in dimethyl sulfoxide containing 1 vol % tetramethylsilane as the internal reference. The contact angles of the polymer films were measured statically on a JY-82 contact-angle meter (Harke Ltd., Beijing, China). The water sorption was evaluated by immersing polymer films in distilled water for 24 h at room temperature.

Nanoparticle yield, DLC, and entrapment efficiency

5-Fu had a strong absorption band at a wavelength of 265 nm. This could be used for quantitative analysis. A solution of 5-Fu was measured with an HP8453 UV spectrophotometer (Agilent Technologies, Palo Alto, CA) at a wavelength of 265 nm, and the weight of the drug entrapped in the nanoparticles was calculated with a calibration curve. The nanoparticle yield, DLC, and drug entrapment efficiency were calculated with eqs. (1)–(3), respectively:

$$\text{Nanoparticle yield (\%)} = \frac{\text{Weight of the nanoparticles}}{\text{Weight of the polymer and drug fed initially}} \times 100\% \quad (1)$$

$$\text{DLC (\%)} = \frac{\text{Weight of the drug in the nanoparticles}}{\text{Weight of the nanoparticles}} \times 100\% \quad (2)$$

$$\text{Entrapment efficiency (\%)} = \frac{\text{Weight of the drug in the nanoparticles}}{\text{Weight of the drug fed initially}} \times 100\% \quad (3)$$

Size and size distribution

The particles size and size distribution were determined by LLS with a Zetasizer 1000 particle size analyzer (Malvern Instruments Ltd., Worcestershire, UK). Each suspension sample was diluted to the appropriate concentration with filtered and distilled water before the measurements.

Morphology

The morphological examination of 5-Fu-loaded nanoparticles was performed with a Tecnai G^2 transmission electron microscope (FEI, Eindhoven, The Netherlands).

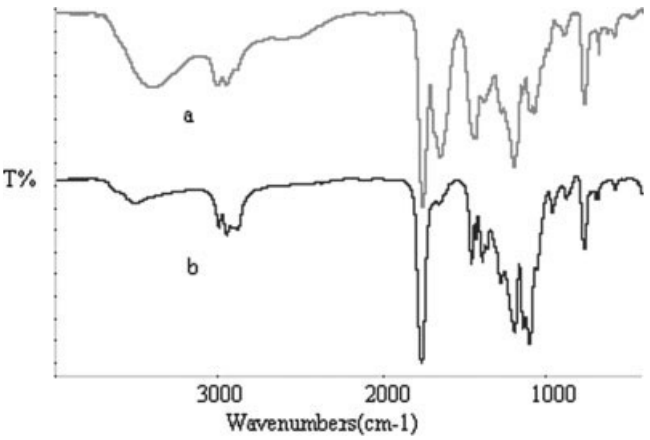

Figure 1 IR spectra of (a) PLA–Hpr and (b) PLA–Hpr–PEG (T% = transmittance percentage).

In vitro release

Nanoparticles (3 mg) were placed in a dialysis membrane bag with a molecular weight cutoff of 6000 g/mol, and the bag was tied and dropped into 200 mL of a phosphate buffer solution (pH 7.4, sink conditions). The entire system was kept at 37°C with continuous stirring. At selected time intervals, 5 mL of the aqueous solution was withdrawn from the release medium. The absorbance of the phosphate buffer solution could be negligible at 265 nm in comparison with 5-Fu in this release medium, so the solution was assayed at 265 nm, a stronger absorption band, with a UV spectrophotometer. Each sample was measured three times, and the release of the drug was determined by a calibration curve; the reported values are the mean values for two replicate samples.

RESULTS AND DISCUSSION

Synthesis of PLA–Hpr–PEG

Representative IR spectra of the PLA–Hpr–PEG (1 wt % PEG) copolymers are shown in Figure 1. The major peaks assigned to the structure of PLA–Hpr–PEG are as follows: 2900–3000 (C—H stretching) and 1760 cm^{-1} (ester C=O stretching). Characteristic absorption bands related to CH_3—,CH—, and CH_2— can be observed at 2996.12, 2945.34, and 2878.81 cm^{-1}. The most distinctive feature of PEG in PLA–Hpr–PEG is the presence of a strong peak in 2878.81 cm^{-1} in comparison with the same absorption peaks of poly(lactic acid–4-hydroxyproline) (PLA–Hpr).

To gain insight into their chemical structure, the PLA–Hpr–PEG (1 wt % PEG) and PLA–Hpr copolymers were subjected to ^{1}H-NMR measurements. A typical spectrum of the PLA–Hpr–PEG copolymers is shown in Figure 2. The ^{1}H-NMR spectra of the copolymers are consistent with the IR data. The characteristic absorption peaks at $\delta = 4.85$ ppm [due to the methine proton of the (—OC—CH*$_2$—C—O—) methylene protons], $\delta = 5.25$ ppm [due to the methane (C_4—H) of the proline], $\delta = 5.15$ ppm (due to the methine proton of the lactic acid), $\delta = 2.01$ ppm (due to the methyl proton of the acetyl protecting group), and $\delta = 1.45$–1.53 ppm (due to the methyl proton of the lactic acid) are exhibited. The methine proton of PEG appears at $\delta = 3.66$ ppm. The ^{13}C-NMR spectrum of the PLA–Hpr–PEG copolymers is shown in Figure 3. The characteristic absorption peaks are at $\delta = 15$ ppm [due to the carbon of the (C*H_3) of lactic acid], $\delta = 69$ ppm [due to the carbon (C*H_2) of the PEG], $\delta = 77$ ppm [due to the carbon (C*H) of the proline], and $\delta = 172$ ppm [due to the carbon (C*=O) of the ester].

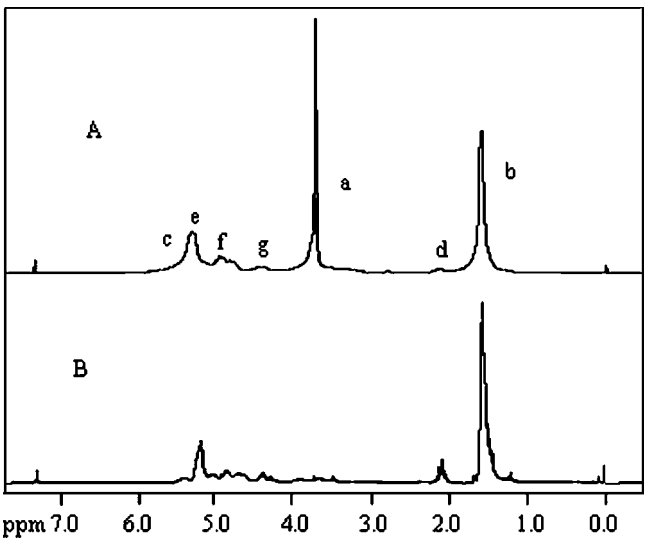

Figure 2 ^{1}H-NMR spectra of (A) PLA–Hpr–PEG and (B) PLA–Hpr.

The surface and bulk hydrophilicity of various PLA–Hpr–PEG copolymers with different feed ratios of PEG have been determined from the contact angles and water uptake, and the data are presented in Table I. The contact angles of the copolymers are

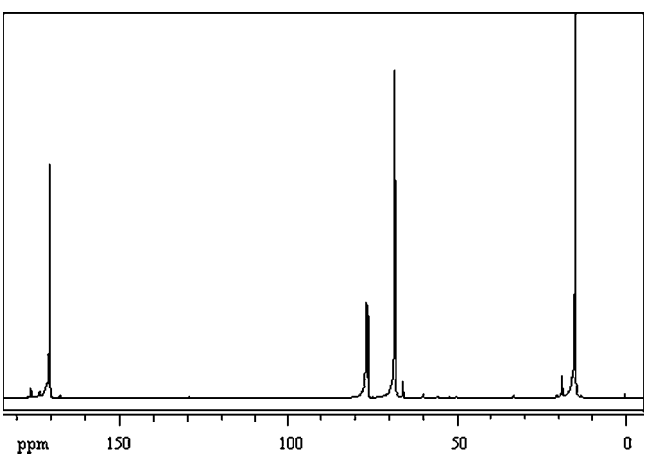

Figure 3 ^{13}C-NMR spectrum of PLA–Hpr–PEG.

TABLE I
Copolymerization Results for Lactic Acid and 4-Hydroxyproline with PEG

Sample	PEG (wt %)	M_w[a]	M_w/M_n[b]	Contact angle (°)	Water absorption (%)	Yield (%)
1	0.1	13,610	1.70	36.3	11.5	73
2	0.5	13,446	1.63	34.0	14.8	71
3	1	12,932	1.53	33.8	43.7	68
4	5	9,705	1.32	33.6	46.1	67

[a] Weight-average molecular weight.
[b] Weight-average molecular weight/number-average molecular weight.

smaller when the feed of PEG is higher. This means that the introduction of PEG segments enhances the surface hydrophilicity of the copolymers.[14,15] Samples 1–4 were enhanced as the concentration of PEG segments in the copolymer increased because lactic acid is more hydrophobic than PEG and hence copolymers are less hydrophilic and absorb less water.

5-Fu-loaded nanoparticles were prepared with the nanoprecipitation method. The surface and size of the particles are important for the delivery of targeted drugs to specific tissue sites.[16] PEG coatings have been used to minimize the nonspecific fouling of the surfaces of materials with biocomponents, particularly plasma proteins. For example, a PEGylated surface, that is, one whose surface is covered with tethered chains of PEG with the functionality of PEG end groups, extremely reduces protein adsorption[17,18] and results in high blood compatibility.[19,20]

Effects of the formulation variables on the nanoparticle properties

PEG-coated nanoparticles were prepared with amphiphilic PEG–R copolymers, where R is hydrophobic and biodegradable PLA–Hpr. Amphiphilic copolymers have been the focus of research especially because of the nanocharacter of self-assembling systems. This type of copolymer can form a spherical micelle structure in an aqueous medium. The hydrophobic blocks of the copolymer form the core of the micelle, whereas the hydrophilic blocks form the corona or outer shell. The hydrophobic micelle core serves as a microenvironment for incorporating hydrophobic drugs such as anticancer drugs, whereas the outer shell serves as a stabilizing interface between the hydrophobic drug and the external medium. Because most drugs have a hydrophobic character, the drugs can be easily incorporated into the micelle by simple physical entrapment through dialysis or by an oil/water emulsion method. Thus, incorporating a drug into the micelle is an effective method of preparing an efficient drug delivery system.

Nanoparticles of the copolymer with a mean particle size around 100 nm were prepared. The size and size distribution play important roles in determining the drug release behavior of 5-Fu-loaded nanoparticles as well as their fate after administration. It has been reported that smaller particles tend to accumulate in tumor sites because of facilitated extravasation,[21] and greater internalization has also been observed.[22] Less than 200 nm particles can prevent spleen filtering.[23] In addition, smaller particles make intravenous injection easier, and their sterilization may be simply performed by filtration.[24,25]

The drug EE is another factor to be considered. Therefore, in this work, the effects of the formulation variables on the prepared nanoparticles were evaluated in terms of the size and the drug EE, which are summarized in Table II.

No significant effect of the drug loading was observed on the particle size. However, the DLC and EE were greatly affected by the drug loading. With an increase in the drug loading from 10 to 30 mg, the DLC and EE declined drastically from 3.8 to 2.03% and from 34.75 to 11.19%, respectively. It seems that the higher the drug loading is, the lower the DLC and EE can be. A possible explanation is that a higher drug loading results in an increased drug concentration gradient between the polymer matrix and the outer aqueous phase, which in turn leads to more drug loss in the fabrication process. The polymer itself may have a limited capacity to encapsulate a specific drug. Beyond its maximum capacity, more drugs might be wasted during the fabrication process, and the EE thus decreases correspondingly. This phenomenon is in accordance with the literature.[26,27]

The particle size increased from 101.2 to 134.6 nm when the amount of the polymer in the organic solvent was increased from 100 to 300 mg but the drug loading was kept at 10 mg. Also, the DLC declined from 3.8 to 1.8%, and the EE showed a drastic enhancement from 34.75 to 47.68%. This demonstrates that the particle size, DLC, and EE can be significantly affected by the polymer amount when other formulation variables are kept constant. It is well accepted that the size of nanoparticles is directly dependent on the rate of diffusion of the organic solvent to the outer aqueous environment. The reduction of the organic phase viscosity or the interfacial tension can facilitate solvent diffusion and thus tends to produce nanoparticles of a smaller size.[28,29]

TABLE II
Effects of the Fabrication Variables on the Size and Drug EE of the PLA–Hpr–PEG Nanoparticles

Fabrication variable	Size (nm)	Polydispersity	DLC (%)	EE (%)	Yield (%)
Drug (mg)					
10	101.2	0.13	3.80	34.75	33.45
20	104.5	0.09	2.03	18.69	36.42
30	103.2	0.09	2.03	11.19	34.77
Polymer (mg)					
100	101.2	0.13	3.80	34.75	33.45
200	108.5	0.08	2.19	42.40	30.89
300	134.6	0.04	1.80	47.68	40.50
Solvent (mL)					
5	109.7	0.09	1.32	12.90	37.09
10	101.2	0.13	3.80	34.75	33.45
15	97.5	0.10	4.92	46.00	38.73

The data were obtained from the reactions described in the Preparation of the 5-Fu-Loaded PLA–Hpr–PEG Nanoparticles section; one fabrication variable was changed while the others were kept constant during the fabrication process.

The lower the diffusion rate is, the larger the particles will be. The increasing organic phase viscosity will make the solvent diffusion rate slow down and thus tend to produce nanoparticles of a larger size. In this work, more polymer in the solvent resulted in a higher viscosity, and this may have produced greater contact opportunity for the drug and polymer, thus leading to a higher EE value. When the amount of the polymer was increased from 100 to 300 mg, while the loading of 5-Fu was kept constant, the upswing in the denominator of DLC correspondingly resulted in a DLC reduction from 3.8 to 1.8%.

The solvent volume increase from 5 to 15 mL reduced the particle size from 109.7 to 97.5 nm. This size decrease may still be ascribed to the reduced viscosity of the organic phase, which facilitated solvent diffusion into water. More solvent resulted in higher DLC and EE values. This may be due to the decreased drug concentration gradient between the polymer matrix and the outer aqueous phase, which could lead to less drug loss in the fabrication process.

Morphology

The particles under TEM investigation, as shown in Figure 4, were spherical and 100 nm or so in size; this is similar to the results obtained by the LLS technique. An improved safety profile of PLA–Hpr–PEG nanoparticles was observed in comparison with the PLA nanoparticles, and this was attributed to the presence of PEG on the particle surface, which prevented a coagulation cascade. Amphipathic PLA–Hpr–PEG block copolymers in a selective solvent have a tendency to self-assemble at surfaces and into micelles.[30–33] The nanometer ranges of these delivery systems offer certain distinct advantages for drug delivery. Because of their subcellular and submicrometer size, nanoparticles can penetrate deep into tissues through fine capillaries, can cross the fenestration present in the epithelial lining (e.g., liver), and are generally taken up efficiently by the cells.[34] This allows efficient delivery of therapeutic agents to target sites in the body.

In vitro release

The release behaviors of 5-Fu from PLA–Hpr–PEG nanoparticles in a phosphate buffer are exhibited in Figure 5. The release behavior of the nanoparticles exhibited an early rapid release phase, followed by a slower phase. The initial stage led to rapid release of about 40% of the drug during the first 8 h. The faster release phase was probably due to a pore diffusion mechanism through an interconnecting network and the higher solubility of 5-Fu in water. At a later stage, the drug was released more slowly. That rate of re-

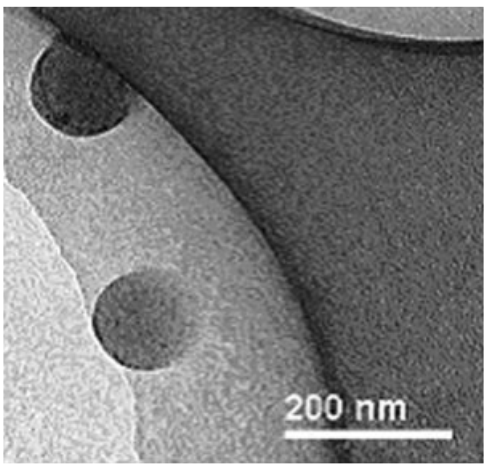

Figure 4 TEM image of 5-Fu-loaded PLA–Hpr–PEG nanoparticles.

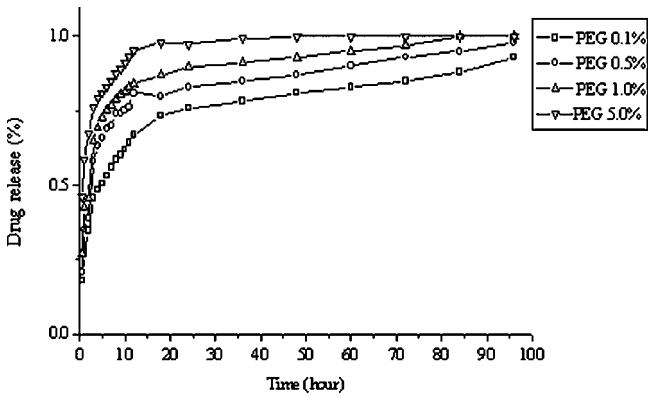

Figure 5 *In vitro* release profile of entrapped 5-Fu from samples 1–4 of the PLA–Hpr–PEG nanoparticles.

lease might have been controlled by the 5-Fu fraction that was entrapped within the internal globules. After 10 h, the sample reached a maximum in the drug release curves, showing a significant burst effect. Usually, a faster release rate proportional to increasing amounts of a drug in nanoparticles is typical of crystalline dispersions of a drug in a polymer matrix, and this can be explained by an assisted-diffusion model.[35] The release behavior of 5-Fu from the PLA–Hpr–PEG nanoparticle is illustrated in Figure 5, which indicates that 60% of 5-Fu was released from the microspheres within 24 h. The introduction of PEG segments was responsible for this significant increase in the drug release. Compared with the release profile of 5-Fu from PLA–Hpr–PEG nanoparticles, the release profile of sample 4 of the PLA–Hpr–PEG nanoparticles exhibited a much faster release rate. This may have been caused by the presence of PEG in the polymer matrix, which reduced the hydrophobic interaction between the polymer and drug. Moreover, PEG could induce easier penetration of the water and promote the erosion of the polymer matrix. Another possible explanation is the weight-average molecular weight reduction of the four samples. All these factors could accelerate the release of the drug from the particles.

CONCLUSIONS

A novel amphipathic copolymer from lactic acid, 4-hydroxyproline, and PEG was synthesized by melt copolymerization. This work demonstrated that stealthy PLA–Hpr–PEG nanoparticles loaded with 5-Fu could be readily prepared by the nanoprecipitation method with good reproducibility. The prepared particles appeared spherical on a nanometer scale under TEM. The particle size depended mainly on the organic phase viscosity: a less viscous organic phase tended to produce smaller particles. Therefore, increasing the solvent volume or reducing the amount of the polymer tended to produce smaller particles. The DLC and EE could be adjusted by the formulation variables. The drug was released from the nanoparticles in an aqueous medium. More PEG in the polymer accelerated the release of the drug from the particles.

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