Thermosensitive PCL-PEG-PCL Hydrogels: Synthesis, Characterization, and Delivery of Proteins

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ABSTRACT: In this work, a biodegradable and injectable *in situ* gel-forming controlled drug delivery system based on thermosensitive poly(ε-caprolactone)-poly(ethylene glycol)-poly(ε-caprolactone) (PCL-PEG-PCL) hydrogels was studied. A series of PCL-PEG-PCL triblock copolymers were synthesized and characterized by ^{1}H-NMR and gel permeation chromatography (GPC). Thermosensitivity of the PCL-PEG-PCL triblock copolymers was tested using the tube inversion method. The *in vitro* release behaviors of two model proteins, including bovine serum albumin (BSA) and horseradish peroxidase (HRP), from PCL-PEG-PCL hydrogels were studied in detail. The *in vivo* gel formation and degradation of the PCL-PEG-PCL triblock copolymers were also investigated in this study. The results showed that aqueous solutions of the synthesized PCL-PEG-PCL copolymers can form *in situ* gel rapidly after injection under physiological conditions. The PCL-PEG-PCL hydrogels showed the ability to control the release of incorporated BSA and HRP. The released HRP was confirmed to conserve its biological activity by specific enzymatic activity assay. The *in vivo* gel formation and degradation studies indicated that PCL-PEG-PCL copolymers hydrogels can sustain at least 45 days by subcutaneous injection. Therefore, owing to great thermosensitivity and biodegradability of these copolymers, PCL-PEG-PCL copolymers hydrogels show promise as an *in situ* gel-forming controlled drug delivery system for therapeutic proteins. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 116: 1985–1993, 2010

Key words: hydrogel; thermosensitive; injectable; controlled release; protein delivery

INTRODUCTION

Recently, thermosensitive polymers-based injectable *in situ* forming hydrogels have received increasing attention as controlled drug carriers because of their many advantages such as the convenience of application, high drug loadings, no organic solvents, sustained drug release behaviors, and less systemic toxicity.[1–3] These drug delivery systems are flowable aqueous solutions before administration, but once injected, rapidly form gel under physiological conditions. Among various sorts of thermosensitive hydrogels, physically thermogelling polymers consisting of hydrophilic poly(ethylene glycol)(PEG) and biodegradable hydrophobic polyester such as PEG/poly(L-lactide) (PEG/PLLA) diblock or triblock copolymers, PEG/poly(D,L-lactide-*co*-glycolide) (PEG/PLGA) diblock or triblock copolymers and PEG/Poly(ε-caprolactone) (PEG/PCL) triblock copolymers are especially attractive due to their excellent biodegradability.[4–10]

Among the physically thermogelling polymers mentioned earlier, PLGA-PEG-PLGA triblock copolymers were extensively studied as an attractive system for drug delivery.[1–3] Unfortunately, the PLGA-based thermosensitive copolymers have a sticky paste morphology and thus are usually difficult to transfer or weigh. In particular, PLGA-PEG-PLGA triblock copolymers required several hours to be dissolved in water and suffered from very slow redissolution/reconstitution. To solve the problem mentioned earlier, substitution of PLGA with PCL in the hydrophobic polyester backbone of the thermosensitive hydrogels was attempted.[9,10] According to Bae et al., PCL-PEG-PCL triblock copolymer has a powder morphology at room temperature, whereas the previous biodegradable thermogelling polymers such as PLGA-PEG-PLGA have a sticky paste morphology.[10] The PCL-PEG-PCL triblock copolymer is not only simple to transfer or weigh but also easily dissolved in water. The reconstitution/redissolution can be done in a couple of minutes by heating the polymer aqueous suspension above a melting point of the polymer (50°C), followed by quick cooling in an ice bath. Based on the advantages mentioned earlier, PCL-PEG-PCL triblock copolymer can be

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thought of as an important progress in thermogelling systems.

As we all know, the clinical application of many of therapeutic proteins is limited by the lack of suitable delivery systems. A suitable delivery system of protein is warranted in order to deliver protein continuously in its active form over a longer period after a single injection. On the basis of aforementioned consideration, injectable *in situ* forming hydrogels-based PCL-PEG-PCL triblock copolymer should be promising for controlled protein delivery. The mixing of proteins with the aqueous solutions of PCL-PEG-PCL triblock copolymer at low temperatures is beneficial for protecting protein away from denaturation, aggregation and any undesired chemical reaction. In this article, the injectable controlled delivery system of proteins based on thermosensitive PCL-PEG-PCL triblock copolymer is developed. Four PCL-PEG-PCL triblock copolymers were synthesized and characterized by ^{1}H-NMR and gel permeation chromatography (GPC). Thermosensitivity of the PCL-PEG-PCL triblock copolymers was tested using the tube inversion method. BSA as the model protein was incorporated into the delivery systems and the influences of composition and concentration of PCL-PEG-PCL copolymers on BSA release is discussed. Furthermore, the activity and stability of HRP released from the PCL-PEG-PCL hydrogels was studied for evaluating whether the released proteins maintain their activity and the active protein release could be achieved over a long-term period. The *in situ* gel formation and degradation of the PCL-PEG-PCL triblock copolymer in the body of mice were also investigated.

MATERIALS AND METHODS

Materials

ε-Caprolactone, poly(ethylene glycol) (PEG) (M_w = 1000 and 1500), stannous octoate, bovine serum albumin (BSA), and horseradish peroxidase (HRP, M_w 40 kDa) were used as received from Sigma-Aldrich. BCA protein assay Kit was supplied by Beyotime Institute of Biotechnology (Shanghai, China). 3, 20, 5, 50-tetramethylbenzidine (TMB) substrate kits was purchased from Tiangen Biotechnology (Peking, China). All other chemicals were reagent grade and used as purchased without further purification.

Synthesis of PCL-PEG-PCL triblock copolymers

The PCL-PEG-PCL triblock copolymer was prepared by ring-opening polymerization of caprolactone in the presence of PEG as an initiator and stannous octoate as a catalyst.[9,11] For example, to synthesize the PCL-PEG-PCL (800-1000-800) triblock copolymer, PEG (15.0 g) was dissolved in anhydrous toluene (80 mL), and the solvent was distilled off to a final volume of 30 mL to remove the residual water adsorbed to the polymer. ε-Caprolactone (22.2 g) and stannous octoate (40 μL) were added to the reaction mixtures and stirred at 120°C for 24 h. The product was isolated by precipitation into diethyl ether. The copolymer was redissolved in methylene chloride and fractionally precipitated by slowly adding diethyl ether. The residual solvent was removed under vacuum.

^{1}H-NMR analysis

A 500-MHz proton NMR spectrometer (Bruker, DMX500 spectrometer) was used for ^{1}H-NMR in $CDCl_3$ to study the molecular structure and composition of the triblock copolymers.

Gel permeation chromatography

Molecular weights and molecular weight distributions of copolymers were determined using a Waters 515 gel permeation chromatography (GPC) apparatus with a refractive index detector (Waters 410). Tetrahydrofuran was used as an eluting solvent at a flow rate of 1.0 mL/min at 35°C, and polystyrene standards were used as the calibration sample.

Critical micellization concentration determination

The critical micellization concentration (CMC) of PCL-PEG-PCL triblock copolymers in distilled water was determined with a fluorescence spectrophotometer (F-4500, Hitachi, and Tokyo, Japan).[12,13] Pyrene was used as the fluorescence probe. The fluorescence excitation spectra of pyrene were measured at various concentrations of PCL-PEG-PCL copolymers as previously described.[14] The concentration of PCL-PEG-PCL ranged from 5.0×10^{-5} to 1.0 mg/mL and the pyrene concentration was fixed at 6.0×10^{-7} mol/L. The fluorescence excitation spectra were measured at emission wavelength of 390 nm. The slit width for both excitation and emission was 1.5 nm. The ratio of fluorescence intensity at 334 and 337 nm (I_{337}/I_{334}) was calculated and plotted against the logarithm of the copolymer concentrations.

Transmission electronic microscopy

The morphology of the micelle of PCL-PEG-PCL triblock copolymers was observed using a transmission electron microscope (TEM; Tecnai-F20, FEI, The Netherlands). A drop of polymeric micelle suspension, which had passed through a 450 nm filter, was placed onto copper grids coated with a thin carbon film and allowed to dry under IR light.

Determination of sol-to-gel transition

Sol-to-gel transition

The sol-to-gel transition was determined by the test tube inverting method.[15,16] The sample solution was prepared according to the literature[9] and briefly described as follows: the 10 mL vials containing 1 mL solutions of PCL-PEG-PCL triblock copolymers in distilled water were immersed in a water bath at 50°C to dissolve quickly the triblock copolymer above the melting point of PCL; the solutions were then quenched in an ice bath for 30 s. The phase transitions of the polymer solutions were investigated by heating from 10 to 60°C in increments of 2°C. The vials were kept at each temperature for 5 min, and they were removed from the water bath and then tilted. The sample was regarded as a "gel" in the case of no flow within 30 s by inverting the vial. The phase transition temperature was determined from the results of these tilting tests.

In situ gel formation

In situ gel formation of the PCL-PEG-PCL triblock copolymer was confirmed as follows: the PCL-PEG-PCL triblock copolymer aqueous solutions (20 wt %; 0.5 mL) at room temperature were injected over 5 s into a vial containing 37°C water. Red dye (0.01 mg of methyl red) was added to the sample solutions to see the gel clearly. The gel formation was photographed during the injection. For comparison, the aqueous solutions of red dye were also injected into 37°C waters in the same procedures mentioned earlier.

In vitro BSA release

For the protein release experiment, different amounts of BSA were added to the 10 mL vials containing 1 mL solutions of 15–30 wt % PCL-PEG-PCL triblock copolymers at room temperature, and the mixtures were gently mixed until completely dissolved. The vials were incubated at 37°C for 10 min to form a gel. Then, 5 mL of 0.01*M* phosphate buffer (PBS, pH 7.4) at 37°C was added to each gel, and the vials were shaken at 60 rpm and 37°C. At predetermined time, 3 mL of PBS was removed from the vial and the same volume of fresh phosphate buffer at 37°C was added to the vial. The amount of BSA in the released solutions was determined using a BCA Protein Assay according to the kit instructions. The results are expressed as mean ± standard error. Statistical significance of differences was assessed by Student's *t* test. A probability value of <0.05 was considered significant.

In vitro HRP release and enzymatic activity assay

In vitro HRP release from PCL-PEG-PCL hydrogels was also studied using the same procedures as *in vitro* BSA release study mentioned earlier. HRP (1.0 %, w/w) was added to the 10 mL vials containing 1 mL solutions of 20 wt % PCL-PEG-PCL aqueous solutions. Enzymatic activity of HRP was calculated by quantification of oxidized TMB substrate in a peroxide solution using a TMB Substrate Kit.[17,18] TMB (3, 20, 5, 50-tetramethylbenzidine) is a chromogenic substrate for HRP. Once oxidized by the enzyme, this substrate yields a blue product that absorbs at 370 nm and 652 nm. The assay was carried out according to the kit instructions.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed to experimentally confirm the electrophoretic properties and integrity of released HRP. An aliquot of release solutions containing released HRP and an aliquot of stock HRP solutions were analyzed. The protein molecular weight standard used was the low molecular weight calibration kit for SDS electrophoresis: phophorylase b (97.0 kDa), albumin (66.0 kDa), egg albumin (40.0 kDa) and carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.0 kDa), lactalbumin (14.0 kDa).

In vivo gel formation and degradation

The *in vivo* gel formation and degradation were confirmed by subcutaneous injection of the PCL-PEG-PCL triblock copolymers aqueous solutions into Kunming experimental mice (20 ± 2 g). The aqueous solution of PCL-PEG-PCL copolymer (0.3 mL, 20 wt %) was injected into dorsal areas of mice through a 25-G needle. At appropriate intervals, the animals were sacrificed by cervical dislocation. And then the injection site was carefully cut open and the *in situ* formed gel was taken photo.

RESULTS AND DISCUSSION

Characterization of synthesized triblock copolymers

The molecular structure and molecular weight of PCL-PEG-PCL copolymer was characterized by ^{1}H-NMR and GPC. In the ^{1}H-NMR spectra, the sharp peak at 3.64 ppm is attributed to methylene protons of —CH_2—CH_2O— in PEG units in block copolymer. Peaks at 1.38, 1.64, 2.32, and 4.06 ppm are assigned to in that order to the a, b, c, and d methylene protons of the oxycarbonyl-1, 5-pentamethylene unit homosequence derived from ε-CL ring opening. The very weak peak at 4.23 and 3.78 ppm are attributed to methylene protons of —O—CH_2—CH_2— in PEG end block that are linked with PCL blocks (Fig. 1).

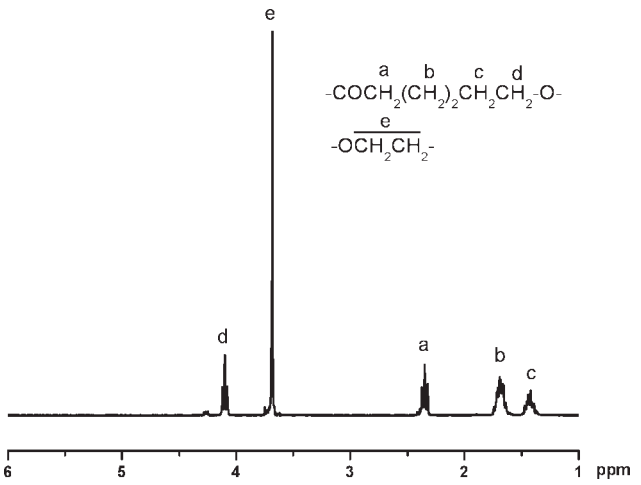

Figure 1 ^{1}H-NMR of PCL-PEG-PCL triblock copolymer.

The ethylene peak of the ethylene glycol (CH_2CH_2O) unit at 3.64 ppm, the methylene peak of the caprolactone ($COCH_2CH_2CH_2CH_2CH_2O$) unit at 4.06 ppm and the methylene peak of the ethylene glycol end unit at 4.23 ppm in the ^{1}H-NMR spectra were used for the determination of number average molecular weight (M_n) of the PCL-PEG-PCL triblock copolymer[19] The molecular weight and molecular weight distribution determined by GPC were in a range of 2650–4350 and 1.1–1.3, respectively. Table I summarizes the polymers investigated in this study.

The aggregation of micelles has been suggested as a sol-to-gel transition mechanism of the copolymers consisting of PEG and hydrophobic polyesters such as PLGA-PEG-PLGA copolymers.[20] In this study, the micelle formation of synthesized PCL-PEG-PCL copolymers in water was monitored with the fluorescence spectra of the pyrene probe.[12–14] It has been reported that fluorescence spectra of pyrene probe solutions contain a vibrational band exhibiting high sensitivity to the polarity of the pyrene environment. When polymeric micelles formed, the pyrene probe was preferentially distributed in the hydrophobic micelle core instead of in the hydrophilic outer shell, thus the environment of the pyrene probe was changed from polar to nonpolar. Accordingly, a red shift was observed with increasing concentration of PCL-PEG-PCL copolymers in the pyrene excitation spectra. The critical micellization concentration (CMC) can be obtained from the plot of the fluorescence intensity ratio I_{337}/I_{334} from pyrene excitation spectra versus the logarithm of the copolymers concentration: a major change in the slope indicates the onset of micellization and this concentration is defined as the CMC. From Figure 2, the CMC of PCL-PEG-PCL (P_2) copolymer was determined to be 0.0002 g/L. Table I summarizes the CMC values of a series of PCL-PEG-PCL copolymers. A typical TEM image of micelles was visualized in Figure 3.

Thermosensitivity characterization of PCL-PEG-PCL copolymers

All synthesized PCL-PEG-PCL triblock copolymers in this study exhibited a temperature-dependent reversible sol-to-gel transition in water: a sol-to-gel transition (at the lower transition temperature) and a gel-to-precipitate transition (at the upper transition temperature). The phase diagram (Fig. 4) revealed that the reversible sol-to-gel transition behavior of PCL-PEG-PCL triblock copolymers in aqueous solutions was highly dependent on their chemical composition and copolymer concentration. As shown in Figure 4, increasing the length of hydrophobic PCL block with the same PEG block length resulted in a lower sol-to-gel transition temperature at a given copolymer concentration, which might be attributed to the enhanced hydrophobicity of the copolymer macromolecular backbone. This indicated that the thermodynamic driving force of such a physical sol-to-gel transition was the hydrophobic interaction.[20,21] From Figure 4, increasing the length of hydrophobic PCL block with the same PEG block length resulted in a higher precipitate temperature at a given copolymer concentration. Precipitates will occur when the micellar structure is broken, indicating that the stability of micellar structure plays a relatively important role on precipitates formation.[20] For PCL-PEG-

TABLE I
List of PCL-PEG-PCL Triblock Copolymers Studied

	Triblock copolymer	PCL/PEG[a] (Theoretical)	Total M_n[a] (Theoretical)	PCL/PEG[b] (Calculated)	Total M_n[b] (Calculated)	Total M_n (GPC)[c]	PDI[c]	CMC[d] (g/L)
P1	PCL_{1100}-PEG_{1500}- PCL_{1100}	1.47:1	3700	1.67:1	4000	4100	1.2	0.0004
P2	PCL_{1250}-PEG_{1500}- PCL_{1250}	1.67:1	4000	1.8:1	4200	4350	1.3	0.0002
P3	PCL_{1500}-PEG_{1500}- PCL_{1500}	2.0:1	4500	2.04:1	4560	4680	1.3	0.0001
P4	PCL_{750}-PEG_{1000}- PCL_{750}	1.5:1	2500	1.6:1	2600	2650	1.1	0.0005

[a] Theoretical value, calculated according to the feed ratio.
[b] Calculated from ^{1}H-NMR results.
[c] Determined by GPC analysis.
[d] Determined by a fluorescence probe technique using pyrene as a hydrophobic probe.

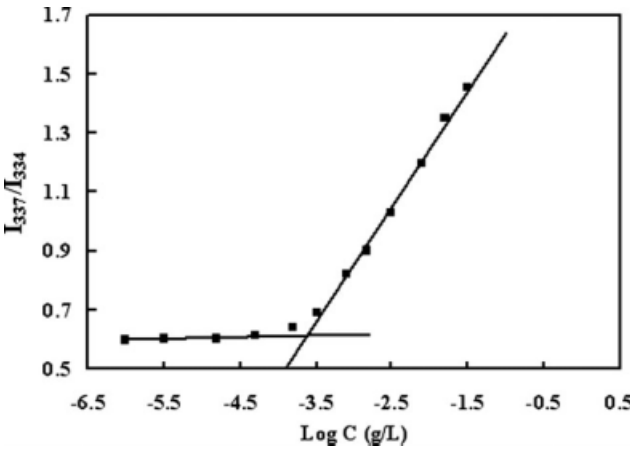

Figure 2 Plot of the fluorescence intensity ratio of I_{337}/I_{334} from pyrene excitation spectra versus log C (g/L) of the PCL-PEG-PCL copolymer in distilled water ($\lambda_{em} = 390$ nm, concentration of pyrene $= 6 \times 10^{-7}$ mol /L).

PCL micelles, the increase in the length of a PCL hydrophobic block at a given length of a PEG hydrophilic block causes a decrease in CMC and an increase in micelle stability. Therefore, it is believed that the micellar structure with a longer PCL block is more stable and, thus, will be broken at higher temperatures.

Besides the effects of chemical composition, copolymer concentration of the triblock copolymers also showed significant influence on the sol-to-gel transitions. An increase in the copolymer concentration from 15 to 30 wt % dramatically shifted sol-to-gel transitions to the lower temperature. Furthermore,

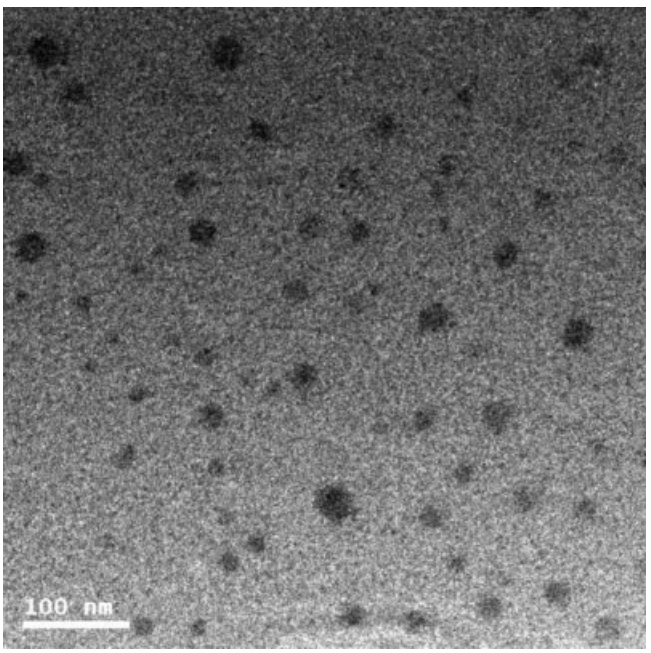

Figure 3 A TEM image showing typical micelles of PCL-PEG-PCL copolymers (P2) formed in an aqueous suspension at a concentration of 0.05 wt %.

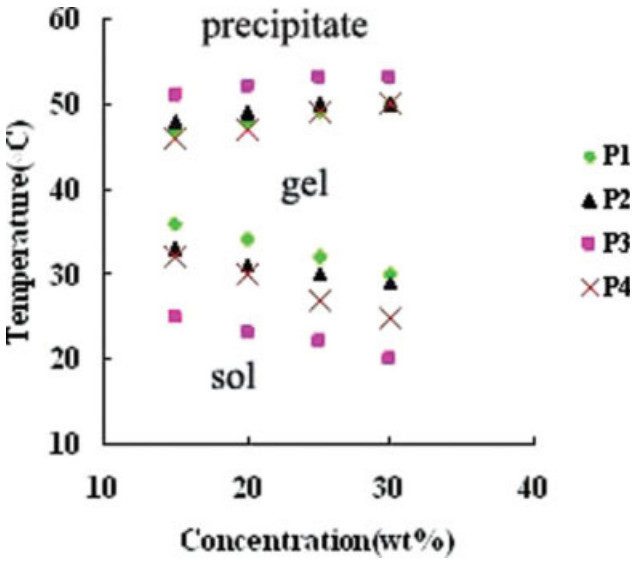

Figure 4 Phase diagrams of PCL-PEG-PCL aqueous solutions. The legends represented copolymer samples listed in Table I. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

copolymer P3 spontaneously gelled under the room temperature and thus led to difficulty in handling and injecting. The sol-to-gel transition temperatures of other copolymers synthesized in this study were between room temperature and physiological temperature, and thus suitable for drug delivery as an injectable biomaterial.

The physical gelation process of PCL-PEG-PCL triblock copolymers in water was shown in Figure 5, which was in agreement with the earlier reports about PLGA-PEG-PLGA copolymers hydrogels. Yu et al.[20] summarized the hierarchy mechanism to interpret the physical gelation process of these kinds of amphiphilic triblock copolymers in water: first, amphiphilic block copolymers were self assembled into micelles [Fig. 5(A)]; second, the micelles were aggregated into macroscopic gel with mesoscopically inhomogeneous micelle network (transparent gel) [Fig. 5(B)]; third, the micelle-network was coarsened until the mesh size was in the order of wavelength of visible light, and the gel was thus opaque [Fig. 5(C)]; finally, the micelle structure was destroyed due to the over-hydrophobicity of the copolymers at higher temperature, eventually leading to macroscopic precipitate [Fig. 5(D)].

Besides the capability of gelation at physiological conditions, another prerequisite of PCL-PEG-PCL copolymers hydrogels as an injectable biomaterial drug delivery system is to form gel within an appropriate gelation time. The gelation time should be as short as possible, which enable the PCL-PEG-PCL copolymer aqueous solutions to form *in situ* gel instantaneously after injection. To confirm the *in situ* gel formation, the PCL-PEG-PCL copolymer aqueous

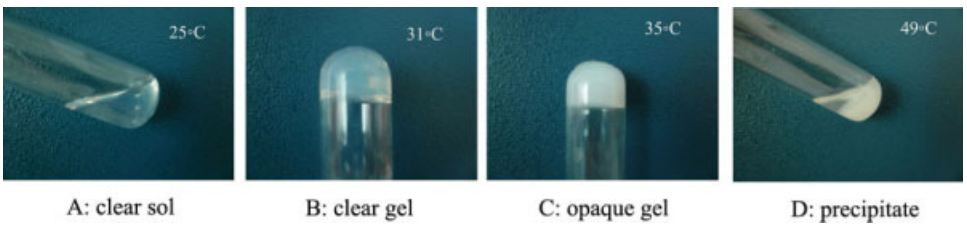

Figure 5 Optical images of PCL-PEG-PCL copolymer solutions (P2: 20 wt %) in the test tube at the indicated temperatures leading to the sol (A), transparent gel (B), opaque gel (C), and precipitate (D). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

solutions were injected into water at 37°C using a syringe with a 21-gage needle. To see the gel formation clearly, red dye was added to the sample solutions. As shown in Figure 6, the polymer P2 aqueous solutions formed instantaneously into a gel after injection whereas red dye aqueous solutions diffused into waters spontaneously.

In vitro BSA release from PCL-PEG-PCL hydrogels

BSA was used as a model protein to examine whether the PCL-PEG-PCL hydrogels were suitable for controlled drug delivery system. *In vitro* release behaviors of BSA from PCL-PEG-PCL hydrogels in PBS was shown in Figure 7. BSA release profiles were affected to some extent by chemical composition and concentrations of PCL-PEG-PCL copolymers hydrogels. In Figure 7(A), a high initial burst release of 40.06% was observed for copolymer P4-based formulations, followed by a controlled release for only 11 days. Copolymer P1-, P2-, and P3-based formulation, respectively, suppressed the initial burst release to less than 20% and prolonged the release of the protein over 30 days. Moreover, the formulation of copolymer P4 which had a less PEG block ($M_w = 1000$) achieved the cumulative BSA release of almost 100%, higher than copolymer P1, P2 and P3 ($M_w = 1500$; $P < 0.05$). The partitioning of protein between the hydrophilic domain and hydrophobic domain in the hydrogels was a critical factor for the initial burst release.[22,23] During the sol-to-gel transition, the system's volume contraction led to the expulsion of the aqueous phase. Protein located in the hydrophilic domain of the hydrogels was subjected to the push-out effect and resulted in the initial burst release. Furthermore, the copolymer hydrogels with less PEG block might have less gel stability and more volume contraction during sol-to-gel transition; as a result, copolymer P4 ($M_w = 1000$) showed higher initial burst release compared to copolymers P1, P2, and P3 ($M_w = 1500$), although further studies would be needed to substantiate this.

Moreover, it was found that the length of hydrophobic PCL block also played an important role in the BSA release from PCL-PEG-PCL copolymers. As shown in Figure 7(A), the larger PCL block showed the slower BSA release behaviors. Drug release behaviors from copolymer hydrogels are driven by two forces: drug diffusion and matrix degradation.[24,25] A larger PCL block leads to a stronger

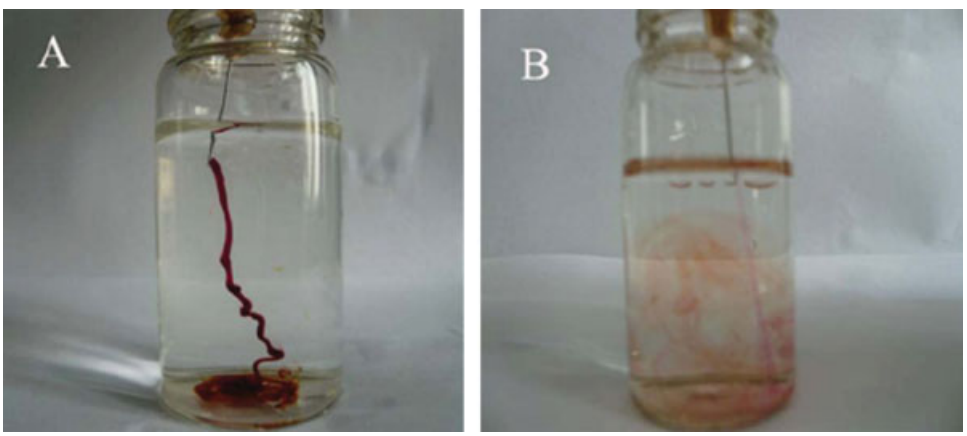

Figure 6 For *in situ* gel formation study, the PCL-PEG-PCL copolymer aqueous solutions and waters at room temperature were injected into an excess amount of water at 37°C, respectively. Methyl red was added to observe clearly. (A) *In situ* gel formation of the PCL-PEG-PCL triblock copolymers aqueous solution (P2: 20 wt %). (B) Diffusion of water after injection. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

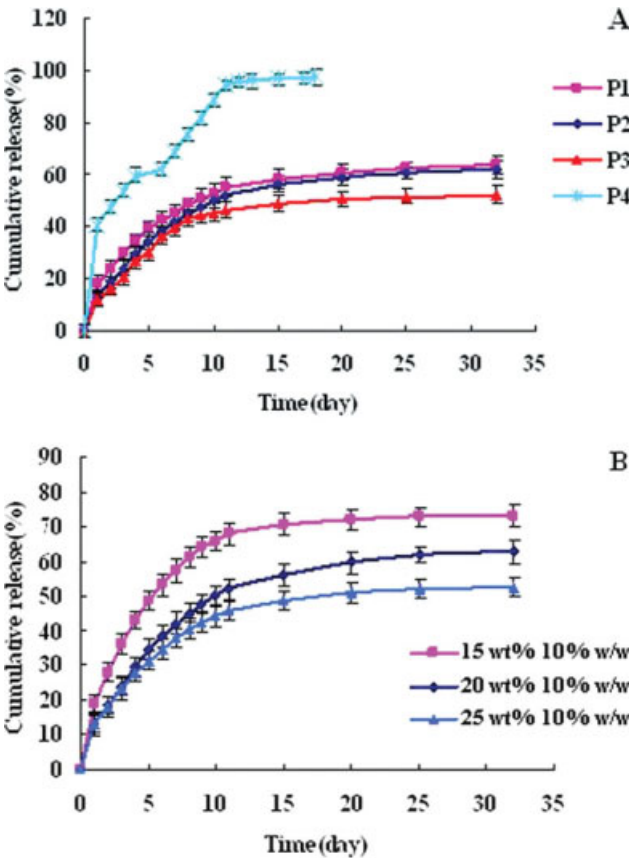

Figure 7 *In vitro* release of BSA from PCL-PEG-PCL hydrogels in PBS at 37°C with marked copolymer and drug loadings. (A) Effect of different compositions on the BSA release. The legends represent copolymer samples listed in Table I. The copolymer concentration was 20 wt %, and the drug loading was 10.0% (w/w). (B) Effect of varying polymer concentrations on the BSA release from copolymer P2 formulations. Each point represents the mean ± SD, $n = 3$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley. com.]

hydrophobicity of the copolymer hydrogels and thus a lower degradation ratio, which presumably resulted in a slower protein release.

We also examined *in vitro* release behaviors of BSA from the hydrogels with different polymer concentrations. As shown in Figure 7(B), increasing the PCL-PEG-PCL hydrogel concentration significantly slowed down the release of BSA. 50% of the BSA was released from the 25 wt % gel after 30 days, whereas 70% of the BSA was released from the 15 wt % gel in the same time period ($P < 0.05$). Therefore, it is quite flexible to select the copolymer concentration within a certain range as release rate is concerned.

In vitro HRP release and enzymatic activity assay

A suitable controlled delivery system should be able to release protein in its biologically active form. In this study, we investigated the biological activity of the released HRP from the PCL-PEG-PCL copolymers hydrogels. HRP is a 43 kDa protein, which is positively charged at a neutral pH (pI = 9.0). HRP detection in the release medium was achieved by enzymatic reaction, which required maintenance of protein activity during and after release. The controlled release behavior of HRP from copolymer P2 was observed in Figure 8. The release profile was characterized by an initial sharp release phase spanning the first 5 days followed by a much slower and sustained release for 15 days. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to evaluate the stability of HRP during the *in vitro* release study. According to Figure 9, the single band for HRP in the release solutions

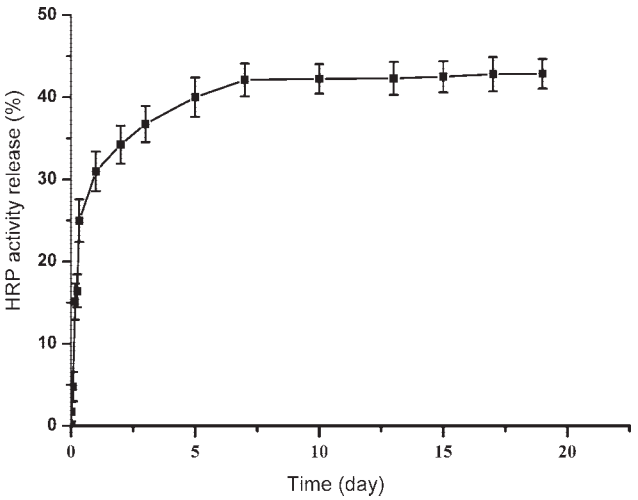

Figure 8 Enzymatic activity of HRP released from the copolymer P2 hydrogels at different time intervals (15 wt %, 1% w/w).

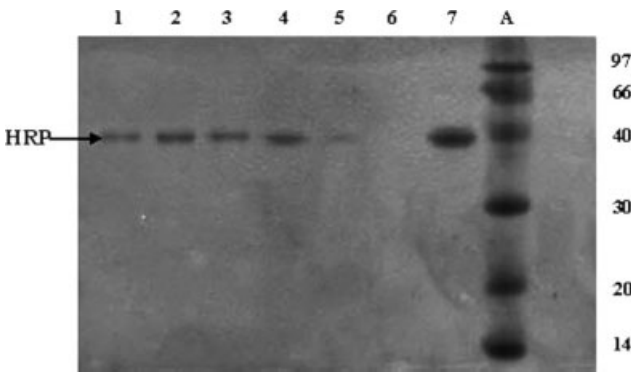

Figure 9 SDS-PAGE gel of the HRP release solutions from PCL-PEG-PCL copolymers hydrogels at different time intervals: Lane 1, 2h; Lane 2, 1d; Lane 3, 3d; Lane 4, 7d; Lane 5, 15d; Lane 6, release solutions from the copolymer hydrogels without HRP; Lane 7, native HRP solutions as supplied by the manufacturer; lane A, protein molecular weight markers. Ten microliters of the release solutions were loaded in each lane.

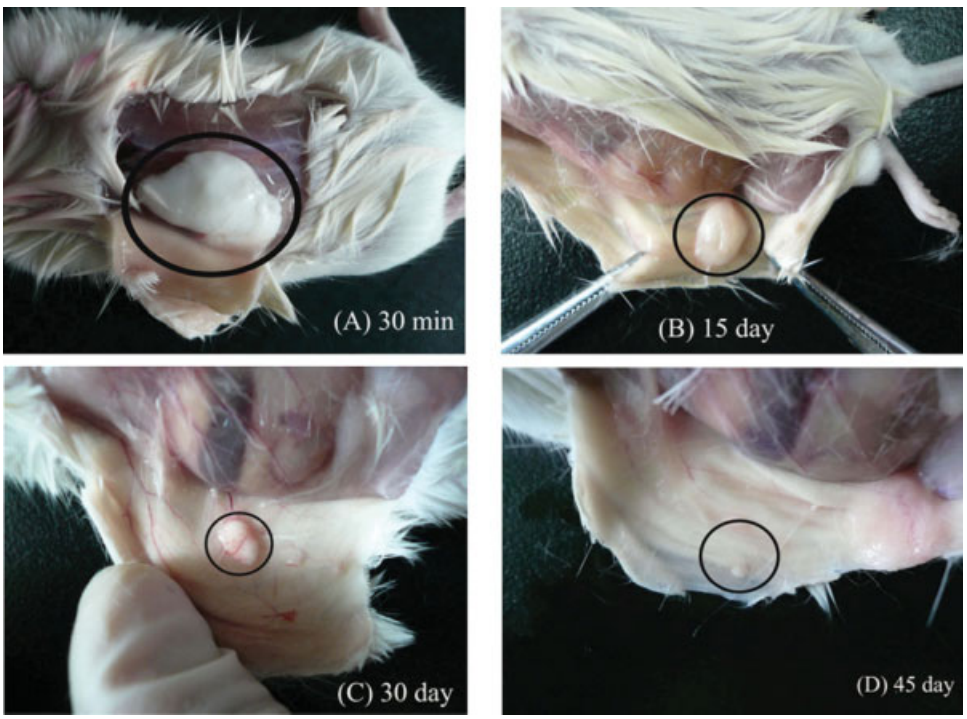

Figure 10 *In situ* gel formation and degradation behaviors of PCL-PEG-PCL hydrogels after subcutaneous injection of 0.3 mL 20 wt % copolymers P2 aqueous solutions. After injections of the copolymer solutions, polymeric gel depots were observed at 30 min, days 15, 30, and 45. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

appeared at the same position as the native HRP solution, suggesting that the protein was being released in its native form from PCL-PEG-PCL copolymer hydrogels.

In vivo gel formation and degradation of PCL-PEG-PCL copolymers hydrogels

In vivo gel formation and degradation of PCL-PEG-PCL copolymers hydrogels was observed in Kunming experimental mice. The hydrogel solution (copolymer P2, 20 wt %, 0.3 mL) was subcutaneously injected into mice. As shown in Figure 10, the polymer solutions immediately formed a gel at the injection site. The size of gel decreased in the period of observation, and at Day 45, the gel almost disappeared. Considering the *in vivo* results, PCL-PEG-PCL copolymers hydrogels can be useful for the application as *in situ* gel-forming drug delivery system for long term controlled release of therapeutic proteins.

In this study, we prepared PCL-PEG-PCL hydrogels that undergo sol-gel transition as the temperature increased from 20°C to 60°C. The thermosensitive behavior of the present PCL-PEG-PCL hydrogels is consistent with Bae's results but contrary to Liu's results that the special gel-sol transition behavior of PCL-PEG-PCL hydrogels was reported.[9,10,19] In fact, the gel-sol phase transition behavior of PCL-PEG-PCL copolymers was greatly dependent not only on the hydrophilic/hydrophobic balance in macromolecular structure, but also on the heating history of copolymer aqueous solutions. The aqueous solution of the present PCL-PEG-PCL triblock copolymer in this study is a free-flowing sol at ambient temperature and becomes a gel at body temperature, and thus PCL-PEG-PCL hydrogels are suitable for drug delivery as an injectable biomaterial. An injectable PCL-PEG-PCL/DMSO system was evaluated for its acute toxicity, and the results indicate that the PCL-PEG-PCL might be a safe candidate for an *in situ* forming controlled drug delivery system.[26]

Recently, the PEG-PCL-PEG-based thermosensitive hydrogel has been synthesized and applied for controlled drug delivery.[27,28] Compared with the PEG-PCL-PEG triblock copolymer, the PCL-PEG-PCL triblock copolymer has several advantages: first, the PCL-PEG-PCL triblock copolymer can be synthesized in one step without using any coupling agent; second, PCL-PEG-PCL hydrogels have a wider gel window; third, PCL-PEG-PCL hydrogels can persist for a longer period *in vivo* (about 6 weeks) compared with PEG-PCL-PEG hydrogels (about 2 weeks). Both PCL-PEG-PCL hydrogels and PEG-PCL-PEG hydrogels can play important roles in the biodegradable *in situ* gel-forming controlled drug delivery system because they show sol-gel-sol transition, are compatible and nontoxic, can persist at least 2 weeks *in*

vivo, and can sustained release drugs in a extended period. We could choose to use PCL-PEG-PCL or PEG-PCL-PEG hydrogels according to the practical needs of different applications.

CONCLUSIONS

A series of thermosensitive PCL-PEG-PCL copolymers with different compositions were synthesized and characterized for their application in injectable *in situ* forming hydrogels as controlled protein delivery systems. The synthesized PCL-PEG-PCL copolymers aqueous solutions can form *in situ* gel rapidly after injection under physiological conditions. The sol-to-gel transition behavior of the copolymers depended on the chemical composition and the copolymer concentration. As a result, the temperature range of phase transition could be varied, which might be useful for its application in many fields, such as drug delivery systems. The PCL-PEG-PCL hydrogels showed the ability to control the release of incorporated BSA and HRP. The released HRP was confirmed to conserve its biological activity by specific enzymatic activity assay. The *in vivo* gel formation and degradation studies indicated that PCL-PEG-PCL copolymers hydrogels can sustain at least 45 days by subcutaneous injection, which is very useful for its application as *in situ* gel-forming drug delivery systems. Therefore, owing to great thermosensitivity and biodegradability of these copolymers, PCL-PEG-PCL copolymers hydrogels show promise as an *in situ* gel-forming controlled drug delivery system for therapeutic proteins.

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