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Synthesis and characterization of PEG-PCL-PEG thermosensitive hydrogel

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ABSTRACT

In this work, a series of biodegradable triblock poly(ethylene glycol)-poly(ε-caprolactone)-poly(ethylene glycol) (PEG-PCL-PEG, PECE) copolymers were successfully synthesized by ring-opening copolymerization, and were characterized by ^{1}H NMR, FT-IR, GPC, and DSC. Aqueous solutions of PECE copolymers underwent thermosensitive sol–gel–sol transition as temperature increases when the concentration was above corresponding critical gel concentration (CGC). Sol–gel–sol phase transition diagrams were recorded using test tube inverting method, which depended on hydrophilic/hydrophobic balance in macromolecular structure, as well as some other factors, including topology of triblock copolymers and solution composition of the hydrogel. As a result, the sol–gel–sol transition temperature range could be varied, which might be very useful for its application as injectable drug delivery systems. The *in vivo* gel formation and degradation behavior was conducted by injecting aqueous PECE solution into KunMing mice subcutaneously. *In vitro* degradation behavior, *in vitro* drug release behavior, and cytotoxicity were also investigated in this paper. Therefore, owing to great thermosensitivity and biodegradability of these copolymers, PECE hydrogel is believed to be promising for *in situ* gel-forming controlled drug delivery system.

1. Introduction

Hydrogels are a special class of materials that could absorb considerable amount of water while maintaining their integrity in water. In past decades, stimuli-sensitive copolymer hydrogels have gained increasing attention owing to their smart responsibility to the environmental stimuli and good biocompatibility. Especially, thermosensitive physically crosslinked hydrogels consisted of hydrophobic and hydrophilic blocks have been extensively studied because of their potential biomedical applications in *in situ* gel-forming controlled drug delivery, etc. (Kissel et al., 2002; Gong et al., 2007; Gariépy and Leroux, 2004; Choi et al., 1999; Jeong et al., 1999a,b, 2000, 1997; Lee et al., 2001a; Song et al., 2004; Kim et al., 2003; Li et al., 2003a, 2005; Liu et al., 2008; Malsmsten and Lindman, 1992; Nicolas et al., 1993; Zentner et al., 2001).

Poly(ethylene glycol)-poly(propylene glycol)-poly(ethylene glycol) triblock copolymer (PEG-PPG-PEG), known as Pluronic or Poloxamer, has been extensively studied as a potential drug delivery vehicle due to their excellent biodegradability and thermosensitivity (Xiong et al., 2003). These copolymers have been widely used as emulsifiers, wetting agents, and solubilizers (Rangelov et al., 2005). However, the critical micelle concentration (CMC) of Pluronic is high due to the weak hydrophobicity of PPG block. Pluronic copolymer forms a fast-eroding gel but cannot persist longer than a few hours. Furthermore, Pluronic was found to induce the toxic enhancement of plasma cholesterol and triglycerol because it is non-biodegradable and can be accumulated in the body (Choi et al., 1999; Lee et al., 2001b). Thus, the application of Pluronic in biomedical fields has been greatly restricted.

To solve the problems mentioned above, substituting PPG with poly(ε-caprolactone) (PCL) in the Pluronic copolymer backbone was attempted. Incorporation of biodegradable and more hydrophobic blocks into Pluronic copolymer backbone will result in a distinct decrease in macromolecular weight after degradation, faster elimination from the body, and an evident decrease in CMC (Kissel et al., 2002; Hwang et al., 2005). PCL and PEG are both well-known FDA-approved biodegradable and biocompatible materials, which have been widely used in the biomedical field (Chung et al., 2002; Chen et al., 2008; Bea et al., 2005; Jeong et al., 2002; Li et al., 2006, 2003b,c; Liu et al., 2007; Jeong et al., 1999c; Hatefi and Amsden, 2002; Iza et al., 1998). And PCL is non-toxic and has great permeability (Zhou et al., 2003). Due to the integration of respective advantages of PEG and PCL, PEG-PCL-PEG copolymer might have even wider applications in the biomedical field.

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In this study, we prepared a new kind of biodegradable and injectable poly(ethylene glycol)-poly(ε-caprolactone)-poly(ethylene glycol) (PEG-PCL-PEG, PECE) hydrogel for developing controlled drug delivery systems. Due to the difference in PEG/PCL ratio and total molecular weight, this new hydrogel undergoes sol–gel–sol transition, which is different from the gel–sol transition behavior of PECE reported previously by our laboratory (Gong et al., 2007). Aqueous solution of PECE triblock copolymers is free-flowing sol at either room temperature or below the corresponding critical gel temperature (CGT), and it becomes gel at body temperature. The structure–property relationship of the sol–gel–sol transition of this hydrogel was investigated in terms of PCL length, total molecular weight, and topological variation (Hwang et al., 2005; Bea et al., 2005). For comparison, PCL-PEG-PCL (PCEC) copolymer was synthesized and its sol–gel–sol transition behavior was investigated. Based on this unique sol–gel–sol transition property, one can formulate a drug delivery system by simply mixing a copolymer aqueous solution with pharmaceutical agents. A mice model was applied to evaluate the *in situ* gel formation and its subsequent degradation following subcutaneous injection of PECE hydrogel. *In vitro* degradation behavior and *in vitro* drug release behavior were also studied in this paper.

2. Materials and methods

2.1. Materials

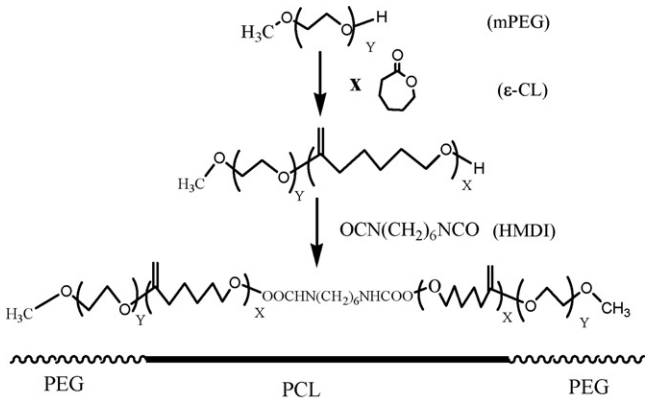
Poly(ethylene glycol) methyl ether (MPEG, M_n = 550 and 750, Aldrich, USA), poly(ethylene glycol) (PEG, M_n = 1000, Fluka, USA), ε-caprolactone (ε-CL, Alfa Aesar, USA), Pluronic F127 and F68 (Fluka, USA), hexamethylene diisocyanate (HMDI, Aldrich, USA), stannous octoate ($Sn(Oct)_2$, Sigma, USA), Dulbecco's modified Eagle's medium (DMEM, Sigma, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma, USA), bovine serum albumin (BSA, BR, BoAo Co. Ltd., China) and VB_{12} (Sigma, USA) were used without further purification. Honokiol was isolated and purified in our lab. All the materials used in this article were analytic reagent (AR) grade and used as received, expect for honokiol and BSA.

KunMing mice, at weight of 20 ± 2 g, were used for *in vivo* gel formation and degradation test. The animals were purchased from the Laboratory Animal Center of Sichuan University (Chengdu, China). The animals were housed at temperature of 20–22 °C, relative humidity of 50–60% and 12 h light-dark cycles. Free access to food and water was allowed. All the animals would be in quarantine for a week before treatment. All animal care and experimental procedures were conducted according to Institutional Animal Care and Use guidelines.

2.2. Synthesis and purification of PECE copolymers

PEG-PCL diblock copolymers were prepared by ring-opening copolymerization of ε-CL initiated by MPEG using stannous octoate as catalyst; PECE triblock copolymers were synthesized by coupling PEG-PCL diblock copolymers using HMDI according to Scheme 1, which was similar to the protocol reported previously (Gong et al., 2007).

A typical PECE (E_{550}-C_{2200}-E_{550}) copolymer (S1) was synthesized as follows: 11 g (0.01 mol) of ε-CL, 5.5 g (0.01 mol) of MPEG (M_n = 550), and 0.08 g of $Sn(Oct)_2$ (0.5 wt.% of total reactants) were added into a reaction vessel under dry nitrogen atmosphere, and the reaction system was kept at 130 °C for 12 h. Then, 1.68 g of HMDI (0.01 mol) was added to the reaction mixture, and the mixture solution was stirred at 80 °C for 6 h. After the mixture was degassed under vacuum for 1 h, the resultant copolymer was then cooled to room temperature. In this paper, the synthesized copolymers were denoted as E_Y-C_{2X}-E_Y, where 2*X* and *Y* represented the number average molecular weight (M_n) of PCL and PEG block, respectively.



Scheme 1. Synthesis scheme of PECE copolymers.

The just-obtained PECE block copolymers were first dissolved in dichloromethane, and reprecipitated from the filtrate using excess cold petroleum ether. Then the mixture was filtered and vacuum dried to constant weight at room temperature. The purified copolymers were kept in air-tight bags prior to use.

2.3. Characterization of PECE and PCEC copolymers

2.3.1. Fourier transform infrared spectroscopy (FT-IR)

The copolymer samples were dissolved in chloroform and cast on KBr plates. FT-IR spectra were recorded on a NICOLET 200SXV Infrared Spectrophotometer (Nicolet, USA).

2.3.2. Nuclear magnetic resonance analysis (^{1}H NMR)

^{1}H NMR spectra (in $CDCl_3$) were recorded on a Varian 400 spectrometer (Varian, USA) at 400 MHz to characterize chemical composition and macromolecular weight of the copolymers.

2.3.3. Gel permeation chromatography (GPC)

GPC (Agilent 110 HPLC, USA) was used to determine the macromolecular weight and macromolecular weight distribution of PECE or PCEC copolymers. The samples were dissolved in freshly distilled tetrahydrofuran (THF) at a concentration of 1–2 mg/mL. THF was eluted at a rate of 1.0 mL/min through two Waters Styragel HT columns and a linear column. The external and column temperature were kept at 35 °C. The molecular weights of samples were calculated based on polystyrene standard samples with a known narrow molecular weight distribution.

2.3.4. Differential scanning calorimetry (DSC)

The thermal properties of copolymers were characterized by using a differential scanning calorimeter (NETSCZ 204, NETSCZ, Germany). Copolymer specimens were first heated from −40 to 90 °C under nitrogen atmosphere at a heating rate of 10 °C/min, then cooled to −40 °C, and then reheated to 90 °C at the same rate.

2.4. Sol–gel–sol phase transition behavior study

In this study, each sample at given concentration was prepared by dissolving a sample of known amount in deionized water at a designated temperature. The volume of the solution was kept at 1 mL in total regardless of the concentration. After being incubated in a water bath at 0 °C for 20 min, the hydrated samples were slowly

heated at a rate of 0.5 °C/min, from 0 °C to the temperature when precipitation occurred.

Sol–gel–sol phase transition diagram of triblock copolymer hydrogels was recorded using test tube-inverting method with a 10-mL tightly screw-capped vial with inner diameter of 13 mm (Gong et al., 2007; Liu et al., 2008; Liu et al., 2007). The sol–gel–sol transition was visually observed by inverting the vials, and conditions of sol and gel were defined as "flow liquid sol" and "no flow solid gel" in 1 min, respectively.

2.5. In vitro hydrolytic degradation

In vitro hydrolytic degradation tests were carried out as follows: the copolymers were placed in a bottle filled with 30 mL of PBS solution at 37 and 4 °C, respectively. The degradation media were refreshed every 2 days. The samples were partly removed from the bottles at a predetermined time, rinsed thoroughly with distilled water, and dried in vacuum at 25 °C to achieve a constant weight. The degree of degradation was characterized by decrease in macromolecular weight, which was calculated according to ^{1}H NMR results.

2.6. In vivo gel formation and degradation

In vivo gel formation and degradation tests were performed on normal KunMing experimental mice (20 ± 2 g). The aqueous solutions of PECE triblock copolymer (S1, 30 wt.%, 0.5 mL) were prepared and subcutaneously injected into mice by a syringe with a 25-gauge needle. For comparison, Pluronic (F127) hydrogel (30 wt.%) was also evaluated under the same condition. At predetermined time, 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.

2.7. In vitro drug release

2.7.1. Release of hydrophilic small-molecular-weight drugs

In vitro release of hydrophilic small-molecular-weight drug VB_{12} from PECE (S1) hydrogel was determined. Briefly, 200 μL of VB_{12} loaded PECE hydrogel complexes (30 wt.% of hydrogel with 0.8 mg of VB_{12}, 30 wt.% of hydrogel with 2 mg of VB_{12}, and 20 wt.% of hydrogel with 0.8 mg of VB12, respectively) were placed into 5 mL EP tubes and allowed to form gels in an incubator at 37 °C for 12 h. Then, the gels were immersed in 2 mL of PBS (pH 7.4) and were shaken at 100 rpm at 37 °C. At specific time intervals (T = 0.5, 1, 2, 4, 8, 12, 24, 48, 72, 96, 120, 144 and 168 h), 1 mL of release media were removed and replaced with fresh release media of a equal volume. After centrifugation at 13,000 rpm for 10 min, the supernatant of the removed release media were collected and stored at −20 °C prior to analysis. The collected supernatants were detected on a UV–vis spectrophotometer at 362 nm to determine the concentration of VB_{12}. The accumulative release of VB_{12} was calculated according to the following equation (Jia et al., 2007):

$$Q = C_n V_t + V_s \sum C_{n-1} \quad (1)$$

where Q was accumulative release weight, and C_n was the VB_{12} concentration at time t. V_t was the volume of medium (V_t = 2 mL), and V_s was the volume of solution removed from supernatant (V_s = 1 mL).

2.7.2. Release of hydrophobic small-molecular-weight drugs

Freshly prepared honokiol loaded PECE hydrogel complexes were used to assay *in vitro* release behavior of hydrophobic drugs. In detail, 200 μL of prepared honokiol loaded PECE hydrogel complexes containing 1 and 2 mg honokiol, respectively were transferred into 5 mL EP tubes and allowed to form gels in an incubator at 37 °C for 12 h. Then, the gels were immersed in 2 mL of PBS (pH 7.4) and were shaken at 100 rpm at 37 °C. At specific time intervals, 1 mL of release media were removed and replaced with fresh release media of a equal volume. After centrifugation at 13,000 rpm for 10 min, the supernatant of the removed release media were collected and stored at −20 °C prior to analysis.

The concentration of honokiol was determined by HPLC (Waters Alliance 2695). The solvent delivery system was equipped with a column heater and a plus autosampler. Detection was made on a Waters 2996 detector. Chromatographic separations were performed on a reversed phase C18 column (4.6 mm × 150 mm-5 μm, Sunfire Analysis column). The column was maintained at 28 °C. Acetonitrile/water (60/40, v/v) was used as eluent at a flow rate of 1 mL/min. The standard curve equation is: H = 105,000X + 4680 (H: the area of peak; X: the concentration of honokiol) and the correlation coefficient is 0.999994.

2.7.3. Release of hydrophilic macromolecular protein drugs

BSA was used as a model protein drug for determining the *in vitro* release behavior of protein or peptide from PECE hydrogels. The procedure was similar to that detailed in Section 2.7.2. The initial drug loading amounts were 4 and 10 mg, respectively. The amount of BSA released was determined by bicinchoninic acid (BCA) assay, in which BCA™ Protein Assay Kit (PIERCE, USA) was used. The SDS-polyacrylamide gel electrophoretic (PAGE) analysis was used to assess the stability of BSA in the supernatant.

2.8. Cytotoxicity assay of PECE copolymer

Cytotoxicity of PECE triblock copolymer to human HEK 293 cells was evaluated by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. All media were supplemented with 10% of FBS and 1% of PS. The cells were cultured in a 5–95% CO_2–O_2 atmosphere at 37 °C. Cells were seeded on 96-well plates at a density of 10^4 cells/well in their usual culture media supplemented with 10% FBS and 1% PS. After 24 h, the media without FBS were used to replace the usual ones, and different amounts of PECE copolymer or Pluronic F68 were added from 5 to 200 μg each well, respectively. After 12 h, the media with PECE copolymer or Pluronic F68 were replaced with fresh media containing 10% FBS. The normal saline was used as the control groups. Cytotoxicity studies were performed using MTT assay after 24 h. Cells cultured in 100 μL culture medium per well received 10 μL of MTT (5 mg/mL), followed by incubation at 37 °C for 4 h. Then MTT-containing media were replaced with 200 μL of isopropanol–HCl (0.1 mol/L). Finally, the absorbance of the samples was measured at 570 nm. The cell cytotoxicity of PECE copolymer is defined as the relative viability, which is the ratio of the number of live cells to that of the control cells (100%).

3. Results

3.1. Synthesis and characterization of PECE copolymers

A series of PEG-PCL diblock copolymers were prepared through ring-opening copolymerization of ε-CL initiated by MPEG using stannous octoate as catalyst, and biodegradable PECE triblock copolymers were synthesized from PEG-PCL diblock copolymers using HMDI as coupling agent (Gong et al., 2007). FT-IR, ^{1}H NMR and GPC were used to characterize the chemical structure of PECE copolymers.

In FT-IR spectra of PEG-PCL diblock copolymer, a strong C=O stretching band appeared at 1721 cm^{-1}, which was attributed to the

Table 1
The PECE copolymers and PCEC copolymer prepared in this work

Code	Copolymer	PEG/PCL[a] (theoretical)	Total Mn[a] (theoretical)	PEG/PCL (^{1}H NMR)	Total Mn (^{1}H NMR)	Total Mn (GPC)	PDI (GPC)
S1	E_{550}-C_{2200}-E_{550}	1100/2200	3300	960/2448	3408	4391	1.30
S2	E_{550}-C_{2000}-E_{550}	1100/2000	3100	1055/2181	3236	2992	1.26
S3	E_{750}-C_{3000}-E_{750}	1500/3000	4500	1518/3116	4634	5046	1.18
S4	C_{1100}-E_{1000}-C_{1100}	1000/2200	3200	1022/2092	3114	3619	1.21

[a] Theoretical value, calculated according to the feed ratio.

ester bond. And in FT-IR spectra of PECE triblock copolymer, there is no absorption in 2250–2270 cm^{-1}, which indicates that the –NCO groups of hexamethylene diisocyanate disappeared completely due to coupling reaction of –NCO with –OH group. The absorption bands at 1528 cm^{-1} were attributed to the N–H bending vibrations, which confirmed the formation of PECE triblock copolymers. In the ^{1}H NMR spectra, the methylene peak of the caprolactone (–$COCH_2CH_2CH_2CH_2\boldsymbol{H_2}O$–) unit at 4.06 ppm and the ethylene peak of the ethylene glycol (–$C\boldsymbol{H_2}C\boldsymbol{H_2}O$–) unit at 3.60 ppm were used for the determination of number average molecular weight (M_n) of the PECE triblock copolymer. Macromolecular weight and macromolecular weight distribution (polydispersity, PDI, M_w/M_n) of PECE triblock copolymers determined by GPC were in the range of 3000–5000 and 1.18–1.30, respectively. The PECE copolymers prepared in this study were summarized in Table 1.

According to Table 1, the macromolecular weight (M_n) and PEG/PCL block ratios estimated from ^{1}H NMR spectrum were consistent with theoretical value calculated from feed ratio. So, for simplicity, the feed ratio was used in the following text instead of experimental composition ratio calculated from ^{1}H NMR spectra. FT-IR and ^{1}H NMR results indicated that the PECE triblock copolymer designed by controlling the feed composition was synthesized successfully.

3.2. Temperature-dependent sol–gel–sol transition

Owing to the combination of hydrophilic PEG block and hydrophobic PCL block, the PECE triblock copolymer is amphiphilic in nature. As listed in Table 1, PECE copolymers based on central PCL blocks (total M_n = 2000, 2200, 3000) and end PEG blocks (M_n = 550, 750) from S1 to S3 showed the temperature-dependent sol–gel transition (lower transition) and gel–sol transition (upper transition) in water. The aqueous solution of S1 (30 wt.%) flowed freely at lower temperature, but became opaque at elevated body temperatures about 37 °C (Fig. 1).

Fig. 2 presented the sol–gel–sol transition phase diagram of PECE triblock copolymers in aqueous solutions. These PECE copolymer aqueous solutions all had concentration-dependent critical gelation concentration (CGC), lower critical gelation temperature (LCGT), and upper critical gelation temperature (UCGT).

Aqueous solutions of PECE copolymers changed from "sol" phase to "gel" phase with increase in temperature when the copolymer concentrations are above the CGC, which seems to be driven by the micelle packing and aggregation (Hwang et al., 2005; Bea et al., 2005). Further increase in temperature can cause the molecular motion of PCL block to increase, thus leading to the transition from "gel" phase to "sol" phase (Choi et al., 1999; Bea et al., 2005). The sol–gel–sol transition behavior of PECE triblock copolymers in aqueous solutions was highly dependent on their chemical composition and solution composition, which are discussed in the following sections.

3.2.1. Effect of PCL block length on sol–gel–sol transition

Fig. 2A shows the effect of PCL block length on sol–gel–sol transition behavior while the macromolecular weight of PEG block was kept at 550. As the length of PCL blocks increased from 2000 to 2200, the LCGT of PECE copolymers decreased from 35 °C for S2 (30 wt.%) to 31 °C for S1 (30 wt.%); the CGC decreased from 20 wt.% for S2 to 15 wt.% for S1; and the UCGT increased slightly. In total, when the PCL block length increased, the gel region became wider.

3.2.2. Effect of total molecular weight on sol–gel–sol transition

Fig. 2B presents the effect of total molecular weight on sol–gel–sol transition diagram of PECE copolymers when the PEG/PCL ratio was kept constant. Obviously, with the increase in the total molecular weight from 3300 for S1 to 4500 for S3, the LCGT and UCGT increased from 31 °C for S1 to 38 °C for S3 and from 45 °C for S1 to 51 °C for S3, respectively, and the CGC of the copolymers decreased by approximately 3 wt.%. Thus, the phase transition behavior was affected by the hydrophilic/hydrophobic balance in the macromolecular structure as well as by the total macromolecular weight of PECE copolymers.

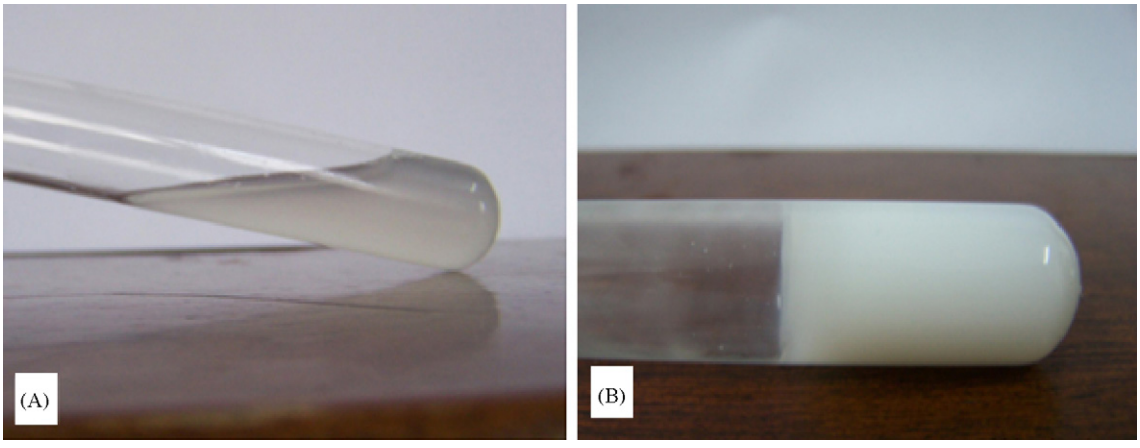


Fig. 1. Photograph of PECE hydrogel at different temperatures: (A) at room temperature and (B) at 37 °C.

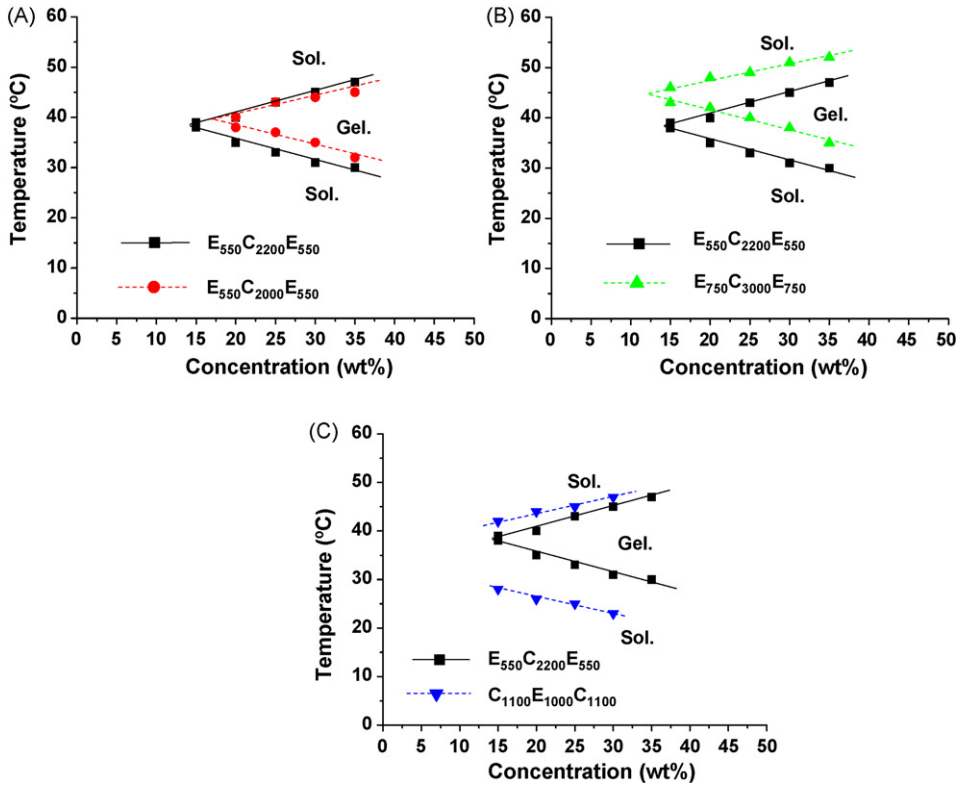


Fig. 2. Sol–gel–sol transition phase diagram of PECE hydrogel: (A) effect of PCL block length, (B) effect of total molecular weight and (C) effect of topology.

3.2.3. Effect of macromolecular topology structure on the sol–gel–sol transition of copolymer aqueous solutions

To investigate the effect of topology on sol–gel–sol transition of copolymer aqueous solutions, PCL_{1100}-PEG_{1000}-PCL_{1100} (S4) was synthesized through ring-opening copolymerization of ε-CL initiated by PEG (Liu et al., 2008; Bea et al., 2005). S4 prepared in this work undergoes sol–gel–sol transition, which is different from the gel–sol transition behavior of PCEC reported previously by our laboratory. The might be due to the difference in PEG/PCL ratio, total molecular weight and administration procedure (Liu et al., 2008). The BAB-type E_{550}-C_{2200}-E_{550} (S1) and ABA-type C_{1100}-E_{1000}-C_{1100} (S4) have a similar total molecular weight and a similar PEG/PCL ratio, and their aqueous solutions both underwent sol–gel–sol transition. According to Fig. 2C, ABA-type copolymer has a lower sol–gel transition temperature and a higher gel–sol transition temperature compared with BAB-type copolymer, which means a wider gelation window. But the CGC of two copolymer types was nearly the same.

3.2.4. Effect of glucose solution on the phase diagram of copolymer aqueous solutions

The effect of glucose solution on sol–gel–sol transition phase diagram of copolymer aqueous solution for sample S1 is shown in Fig. 3A. Samples at the same concentration in deionized water, 5% glucose solution, and 10% glucose solution were prepared and then processed by the same treatment procedure. As shown in Fig. 3A, with the increase of glucose solution concentration of copolymer from 0% to 10%, both LCGT and UCGT decreased and the gelation curve shifted to the lower temperature region, while the CGC kept constant.

3.2.5. Effect of normal saline solution on the sol–gel–sol transition

Fig. 3B presents the comparison between sol–gel–sol transition diagrams of aqueous solution and normal saline solution for sample S1. In this figure, copolymer solutions undergoing the same treatment procedure were compared. In a normal saline solution, the

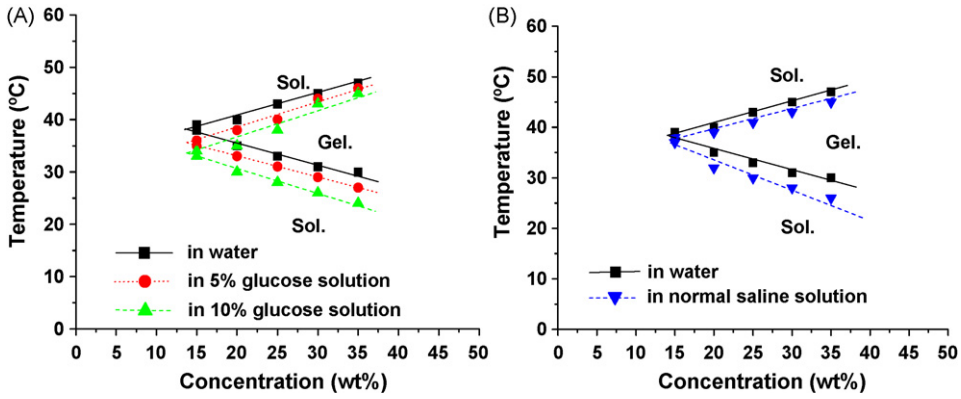


Fig. 3. Sol–gel–sol transition phase diagram of PECE hydrogel in different solutions: (A) effect of glucose solution and (B) effect of normal saline solution.

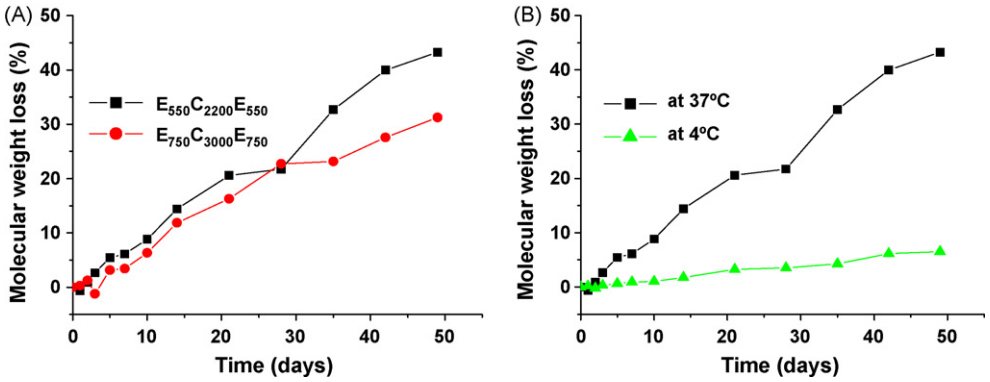


Fig. 4. Degradation behavior of PECE copolymer: (A) effect of molecular weight and (B) effect of temperature.

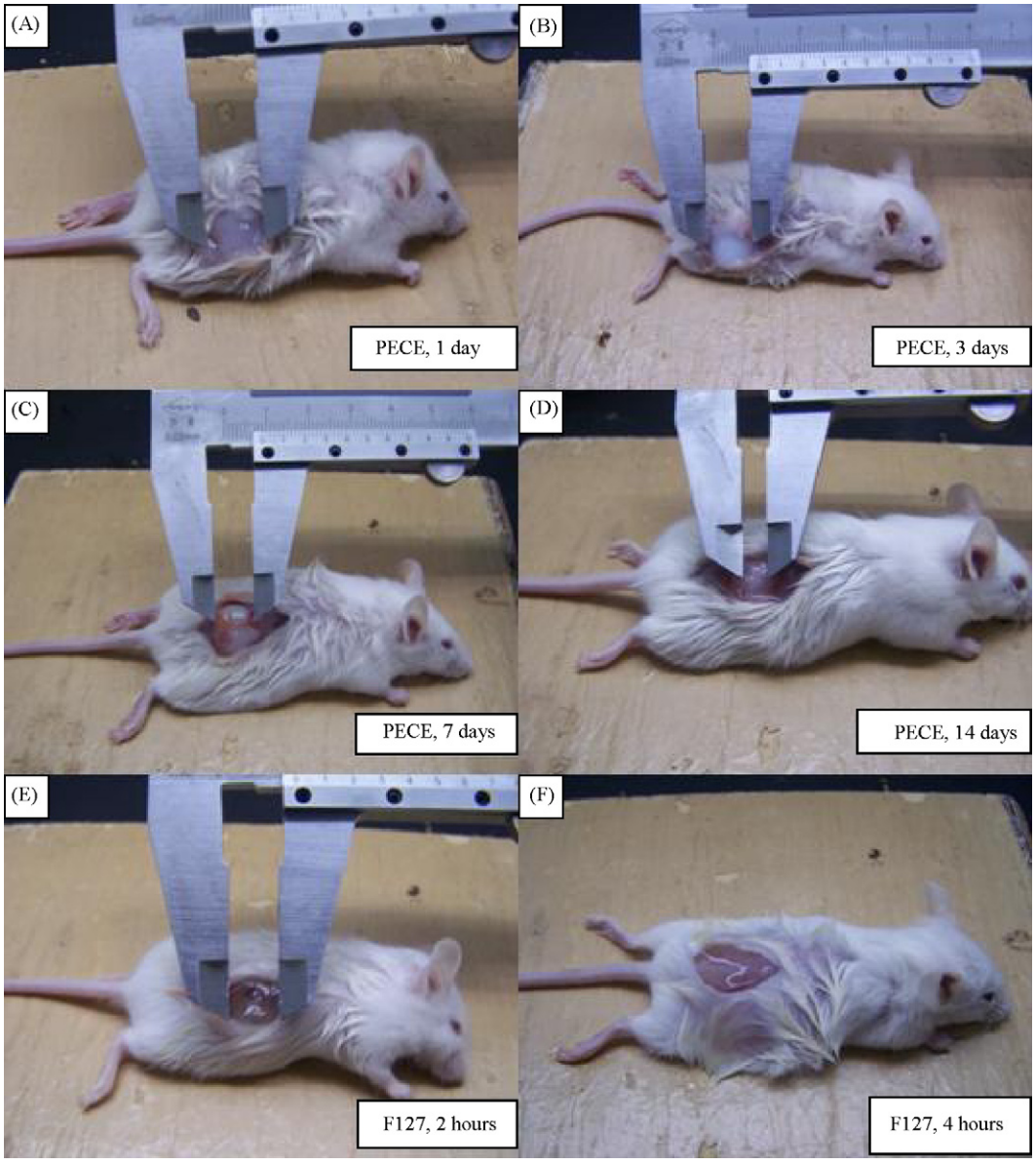


Fig. 5. *In situ* gel formation and degradation behavior of PECE hydrogel.

LCGT decreased by about 4 °C compared to aqueous solution, and the UCGT also decreased about 3 °C, which means that the gelation window shifted to lower temperature, whereas the CGC of copolymer in aqueous solution and the normal saline solution kept constant.

3.3. *In vitro hydrolytic degradation behavior of PECE copolymers*

In vitro hydrolytic degradation of these biodegradable PECE copolymers was affected by many factors, such as degradation temperature, molecular weight of triblock copolymers, etc.

3.3.1. *Effect of molecular weight of triblock copolymers*

From Fig. 4A, we could find that in PBS solution at 37 °C, the degradation rate decreased with the increase in the molecular weight of PECE copolymers, which was similar to the previous reports (Liu et al., 2005; Jia et al., 2006). With increase in the molecular weight from 3300 to 4500, the molecular weight loss rate (degradation rate) decreased from 43.23% to 31.24% after 49 days.

3.3.2. *Effect of degradation temperature*

Fig. 4B presents the effect of temperature on the degradation of PECE copolymer (S1). After 49 days, the total molecular weight of the copolymer decreased slightly by 6.53% of at 4 °C, while the total molecular weight of the same sample decreased by 43.23% at 37 °C. Therefore, for the PECE copolymer, the degradation rate decreased dramatically with decrease in temperature.

3.4. *In vivo gel formation and degradation of PECE hydrogel*

The application of PECE hydrogel as an *in situ* gel-forming system was tested. A hydrogel solution (S-1 sample, 30 wt.%, 0.5 mL) was subcutaneously injected into KunMing mice. The injected solution formed gel within seconds and the formed gel was spherical. Fig. 5A–D are the pictures taken at day 1, day 3, day 7, and day 14 following subcutaneous injection, and the opaque gel maintained its integrity in the period of observation. The size of gel decreased during degradation, and at day 14, the gel almost disappeared. In contrast, Pluronic (F127) gel disappeared in just a few hours (Fig. 5E and F).

3.5. *In vitro drug release from PECE hydrogel*

3.5.1. *Release of hydrophilic small-molecular-weight drug*

In vitro release of VB_{12} from VB_{12}/PECE hydrogel in PBS is shown in Fig. 6. According to Fig. 6, VB_{12} was released from VB_{12}/PECE hydrogel for a sustained period of time. The hydrogel concentration impacts VB_{12} release kinetics greatly as shown in Fig. 6A. In lower concentration hydrogel (20 wt.%), VB_{12} released faster and reached higher cumulative release rate (98.2%) as compared to higher concentration hydrogel (94.6%). In 20 wt.% hydrogel, an initial burst release of 51.2% of loaded VB_{12} occurred in the first 1 h, followed by release of 92.1% in one day, while in 30 wt.% hydrogel, the cumulative release rates for 1 h and 1 day were 28.3% and 83.2%, respectively. The effect of initial drug loading amount on the release profile was also investigated. As shown in Fig. 6B, PECE hydrogel with more than twice amount of VB_{12} only resulted in a slight decrease in the cumulative release rate, thus leading us to conclude that the effect of initial drug loading amount on drug release profile was limited.

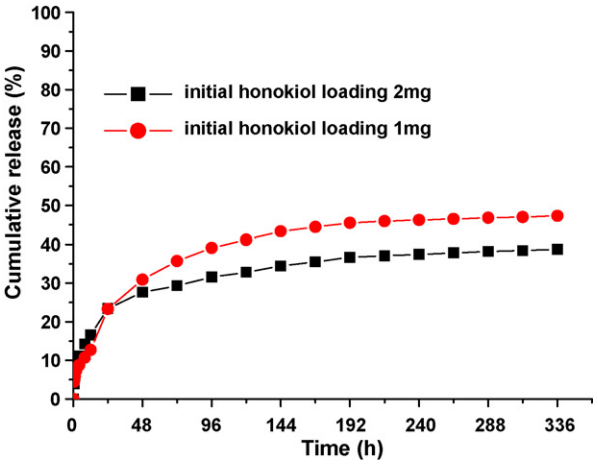


Fig. 7. *In vitro* release behavior of honokiol from honoliol/PECE hydrogel complexes.

3.5.2. *Release of hydrophobic small-molecular-weight drug*

Honokiol, a multi-functional drug, has a great potential application in human disease therapy especially in cancer therapy. An approach to rapidly isolate and purify honokiol has been developed using high-capacity high-speed counter-current chromatography (high-capacity HSCCC) by Chen et al. in our laboratory (Chen et al., 2007). Honokiol was chosen as a hydrophobic model drug in this *in vitro* drug release study.

The release of honokiol from PECE hydrogel was performed and its cumulative release profile is presented in Fig. 7. From Fig. 7, we could find that initial drug loading amount has a great effect on the release behavior of honokiol from PECE hydrogel. With increase of initial drug loading amount from 5 to 10 mg, the cumulative release rate of honokiol decreased dramatically from 47.3% to 38.8% in a 14-day period. The burst release rates of honokiol were both

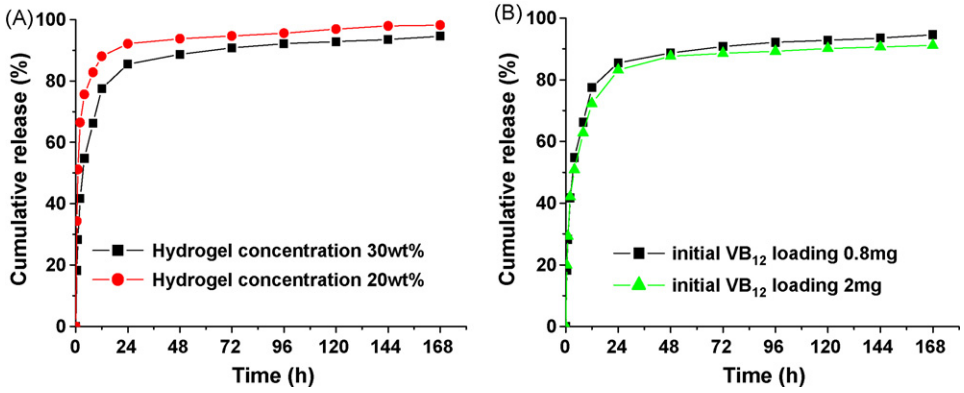


Fig. 6. *In vitro* release behavior of VB_{12} from VB_{12}/PECE hydrogel complexes. (A) Effect of hydrogel concentration and (B) effect of initial VB_{12} loading amount.

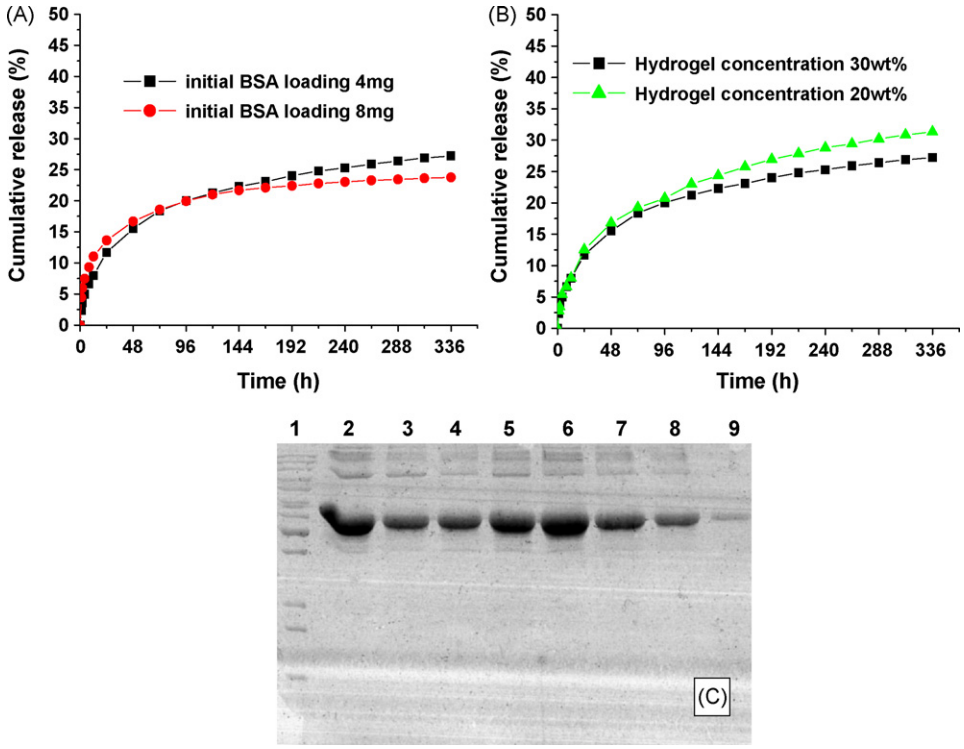


Fig. 8. *In vitro* release behavior of BSA from BSA/PECE hydrogel complexes: (A) effect of initial BSA loading amount, (B) effect of hydrogel concentration and (C) SDS-PAGE results of BSA *in vitro* release profile: Lane 1, marker; Lane 2, BSA standard; Lane 3, 1 h; Lane 4, 4 h; Lane 5, 12 h; Lane 6, 24 h; Lane 7, 72 h; Lane 8, 168 h; Lane 9, 336 h.

approximately 6% in the first 24 h, and after that, honokiol could be released steadily from the hydrogels, which finally reached 47.3% and 38.8% of cumulative drug release rates in the next 13 days, for the 1 and 2 mg honokiol loaded hydrogels, respectively.

3.5.3. Release of hydrophilic macromolecular protein drugs

To investigate *in vitro* release behavior of protein or peptide drugs from PECE hydrogel, BSA was used as a model macromolecular drug. As presented in Fig. 8A and B, initial BSA loading content and hydrogel concentration substantially affected the BSA release behavior from PECE hydrogel, where higher hydrogel concentration or higher initial drug loading amount resulted in slower release of BSA from PECE hydrogel. SDS-PAGE was performed to evaluate the stability of BSA during the *in vitro* release study. According to Fig. 8C, the major band for BSA appeared at about 67 kDa (lanes 3–9) according to the protein markers, which means that BSA remained stable within the observation period.

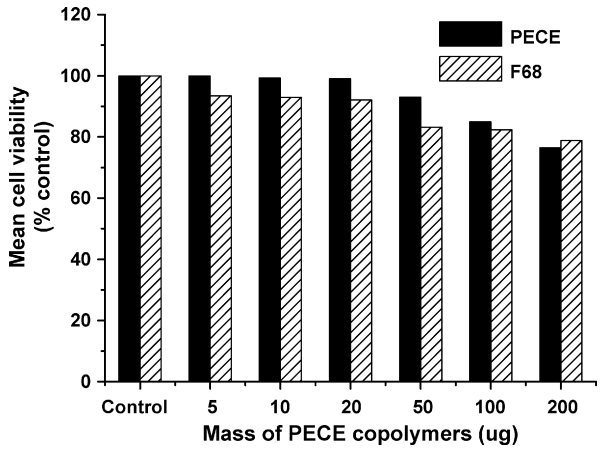


Fig. 9. The 293 cell viability assay. Cell containing 1×10^4 cells in DMEM containing 10% FBS was incubated with PECE copolymers in 96-well in a humidified atmosphere containing 5% CO_2 at 37 °C for 12 h.

3.6. Cell cytotoxicity of PECE copolymers

The cytotoxicity of the prepared PECE copolymer was evaluated. Pluronic F68 was used as a positive control group. Fig. 9 exhibits the 293 cell viability at the presence of PECE copolymer and Pluronic F68 at different concentrations. The cell viability decreased with increase of PECE copolymer or Pluronic F68 amount. But the 293 cell viability was yet higher than 76% even when the input PECE copolymers were 200 μg per well. According to Fig. 9, the 293 cell viability of PECE copolymer was higher compared to Pluronic F68 group. Thus, we could say that the PECE copolymers prepared in this paper were biocompatible with low cell cytotoxicity.

4. Discussion

Over the past decades, *in situ* gel-forming controlled drug delivery systems have received considerable attention due to the simplicity of pharmaceutical formulation. An *in situ* gel-forming controlled drug delivery system enables drugs to be easily mixed and form a depot through a syringe injection at a target location, where the depot works as a sustained drug delivery system (Kissel et al., 2002; Gong et al., 2007; Gariépy and Leroux, 2004; Choi et al., 1999; Jeong et al., 1999a,b, 2000; Lee et al., 2001a; Song et al., 2004; Kim et al., 2003). In particular, thermosensitive hydrogels based on synthetic block copolymers have been extensively studied since their potential biomedical applications in the *in situ* gel-forming system, including controlled drug delivery, cell encapsulation and tissue repair. Since, Perret and Skoulios (1972) for the first time prepared a series of block copolymers consisting of PEG and PCL, these triblock copolymers containing PCL blocks and PEG blocks have been widely studied (Gong et al., 2007; Liu et al., 2008;

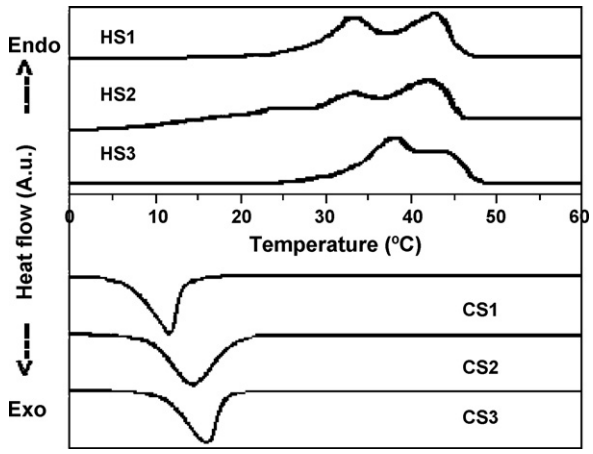


Fig. 10. DSC curves of PECE triblock copolymers. HS1, HS2, and HS3 are heating curves of S1, S2, and S3, respectively. CS1, CS2, and CS3 are cooling curves of S1, S2, and S3, respectively. Heating and cooling rate were 10 °C/min.

Hwang et al., 2005; Bea et al., 2005). In this paper, by ring-opening copolymerization of ε-CL on MPEG and coupled with HMDI, PECE copolymer was obtained. ^{1}H NMR, FT-IR and GPC results showed that PECE copolymers were successfully synthesized. Compared to Pluronic, PECE copolymer is biodegradable, and its formed hydrogel could sustain a longer time, which are very important and useful for its application in biomedical field.

To investigate the thermal property of PECE copolymers, DSC was performed. According to Fig. 10, the heating process displayed two melting peaks at 33–38 and 40–45 °C. And during the cooling process, one exothermic transition at 10–20 °C was observed. Two endothermic peaks at 33–38 and 40–45 °C on the heating trace and one exothermic peak at 10–20 °C on the cooling trace were attributed to the melting of PCL segment at 33–38 °C followed by melting of re-crystallized PCL domain during the heating process, which was similar to the two endothermic peaks of multiblock PEG-PCL copolymer composed of low molecular weight PEG and PCL during the heating process (Ferruti et al., 2003).

In our previous study (Gong et al., 2007), we reported the gel–sol phase transition of PECE triblock copolymers and identified the factors that affected the phase transition, including chemical composition and treatment procedure of the copolymers. But it is very interesting to note that the phase transition diagrams of such triblock copolymers studied in this work were quite different from what we observed previously (Gong et al., 2007). This difference may be due to the variation of chemical composition and treatment procedure of the copolymers as compared to our previous work. All the factors including chemical composition (PEG/PCL ratio, total molecular weight), and treatment procedure of the copolymers greatly influence the phase transition of the aqueous copolymer solution. Therefore, the sol–gel phase transition behavior could be varied by altering the treatment manner of hydrous copolymers or chemical composition of copolymers.

In this study, sol–gel–sol phase transition and factors affecting the transition diagram were investigated in detail. With increase in the length of hydrophobic PCL block, gelation occurred at lower temperatures and lower concentrations (Jeong et al., 1999c), which might be attributed to the enhanced hydrophobicity of the copolymer macromolecular backbone. This indicated that the hydrophobic interactions induced sol–gel transition of an aqueous PECE system (Choi et al., 1999). As the total molecular weight increased, the sol–gel–sol transition curves shifted toward higher temperature even though the molar ratio of PCL to PEG was kept constant. Therefore, PEG block length has a significant influence on the sol–gel–sol transition curve of PECE triblock copolymer aqueous solution. Furthermore, the two kinds of triblock copolymers, BAB-type PECE and ABA-type PCEC copolymers, tended to form a hydrophobic PCL micelle core and a hydrophilic PEG shell. The BAB-type PECE copolymers formed a regular hydrophobic core and a hydrophilic shell, whereas the ABA-type PCEC copolymers formed a micelle core with inter-micellar bridges which caused ABA-type copolymers more easily to form gelation in low temperature than BAB-type copolymers. Our results were consistent with the work reported by Jeong's group (Bea et al., 2005).

Glucose solution and normal saline solution were commonly used to dissolve the drugs for injection in clinic. It is clinically relevant to investigate the influence of glucose solution and normal saline solution on the phase transition behavior of the PECE hydrogel system. Our results suggested that glucose in water might interact with micelles to enhance micelle aggregation rather than disturb the micelle interactions. The possible reason might be that glucose in water interacts with the PCL core which is mixed with the PEG shell. The enhanced inter-micellar interactions act as bridges between the micelles, hence leading to gelation at lower temperatures. NaCl is known as the water structure making salt. By adding NaCl to water, ion–water interactions exclude the copolymers around the ion, resulting in a salt-out effect on water, which was the cause of the phenomenon mentioned above. It was obvious that the sol–gel–sol transition behavior of PECE triblock copolymers aqueous solution depended on the solutions mentioned above besides chemical composition of copolymers. And altering the solutions of copolymers broadened the temperature range of sol–gel–sol phase transition, which might be useful for developing injectable drug delivery systems.

The *in vitro* degradation of PECE copolymers was studied as well as the factors affecting *in vitro* degradation behavior. From Fig. 4, we could find that both of the factors investigated here have significant influence on *in vitro* degradation behavior of PECE copolymers, including degradation temperature and molecular weight of triblock copolymers. With increase in macromolecular weight of PECE copolymers or decrease in degradation temperature, the degradation rate of PECE copolymer decreased dramatically, which is consistent with the degradation behavior of polyester or polyester copolymers reported previously (Liu et al., 2006; Qian et al., 2004a,b; Chao et al., 2007). In the *in vivo* gel formation and degradation test in KunMing mice, PECE hydrogel can sustain at least 14 days by subcutaneous injection, which is much longer than that of Pluronic F-127 hydrogel. And this feature is very useful for its application as *in situ* gel-forming drug delivery system.

In vitro drug release behavior of PECE hydrogel was studied and factors affecting *in vitro* drug release behavior were also investigated. According to Figs. 6–8, drugs concluding hydrophilic drug, hydrophobic drug, and protein drug could released slowly from PECE hydrogel in a sustained period, and drug release profiles were affected in some extent by initial drug loading and hydrogel concentration. Hydrophilic drug were almost completely released from PECE hydrogel in a week with high release rate (>85% in 24 h) and high initial burst rate (about 30% in 1 h), whereas hydrophobic drug and protein drug could be released slowly in a longer period with lower cumulative release rate (38.8% for honokiol and 27.2% for BSA in 14 days, respectively). Drug release behavior from hydrogel was driven by two forces: diffusion effect and degradation or erosion of the hydrogel (Bromberg and Ron, 1998). Because of the great solubility, hydrophilic drug could diffuse through the pores of hydrogel in a short time and almost all the drug in hydrogel was released. But for hydrophobic and protein drugs, low diffusion rate in water and strong intermolecular interactions with hydrogel dominated the drug release profile, which resulted in the low release rate and high residual drug in hydrogel.

The cytotoxicity of PECE copolymer was evaluated by MTT assay in human HEK 293 cells, which depended on the dose of PECE copolymer. As shown in Fig. 9, with increase in amount of PECE copolymer, the cytotoxicity increased accordingly. Compared with the control group, the cytotoxicity of PECE copolymer at 20 μg/well or lower were almost the same (>99%) as that of normal saline, and when the amount of PECE copolymer were 50 μg/well or higher, the cytotoxicity increased accordingly. But even at amount of 200 μg/well, HEK293 cell viability was higher than 76.4%. Compared to FDA-approved Pluronic F68 copolymers, HEK293 cell viability of PECE administration group was higher, especially at lower amount. Cell viability study implied that the cytotoxicity of PECE copolymer is low, but it was dose dependent. Therefore, PECE hydrogel could be regarded as safe drug delivery carrier and is very promising for *in situ* gel-forming controlled drug delivery system.

5. Conclusions

A series of biodegradable PECE triblock copolymers were successfully synthesized and characterized by FT-IR, ^{1}H NMR, GPC, and DSC. The PECE copolymers aqueous solution undergoes sol–gel–sol transition as the temperature increases. And the sol–gel–sol phase transition behavior of the copolymers aqueous solutions was determined using the test tube inverting method. The sol–gel–sol transition behavior of the copolymers depended on a number of factors, such as hydrophilic/hydrophobic balance (PEG/PCL ratio) in the molecular structure, topology of the triblock copolymers, and the solution composition of the hydrogel. 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. Furthermore, the *in situ* gel formation and degradation test were studied by injecting subcutaneously PECE copolymer aqueous solution in the mice model. *In vitro* degradation behavior, *in vitro* drug release behavior, and cytotoxicity were also investigated. Owing to the great thermosensitivity and biodegradability of these copolymers, a solvent-free (no organic solvent) injectable system could be designed as an *in situ* gel-forming controlled drug release system (Kissel et al., 2002).

Acknowledgements

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