Available online at www.sciencedirect.com
ScienceDirect
International Journal of Pharmaceutics 345 (2007) 116–124
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Effect of bee venom peptide–copolymer interactions on thermosensitive hydrogel delivery systems

Mingxi Qiao [a], Dawei Chen [a,*], Tangna Hao [a], Xiuli Zhao [a], Haiyang Hu [a], Xichen Ma [b]

[a] *School of Pharmacy, P.O. Box 42, Shenyang Pharmaceutical University, Wenhua Road 103, Shenyang, Liaoning Province 110016, PR China*
[b] *Dalian Institute of Light Industry, No. 1, Qinggongyuan, Ganjingzi District, Dalian, Liaoning Province 116034, PR China*

Received 11 February 2007; received in revised form 4 May 2007; accepted 25 May 2007
Available online 31 May 2007

Abstract

The objectives of this study were to investigate the potential interactions between the model protein drug (bee venom peptide, BVP) and thermosensitive poly(DL-lactide-co-glycolide-b-ethyleneglycol-b-DL-lactide-co-glycolide) (PLGA–PEG–PLGA) copolymers and to examine the drug–copolymer interactions on the in vitro drug release and hydrogel degradation. The PLGA–PEG–PLGA copolymers were synthesized by ring-opening copolymerization of DL-lactide and glycolide with PEG as an initiator. Drug–copolymer co-precipitate blends were prepared and analyzed by Fourier transform infrared (FTIR) spectroscopy and X-ray diffraction (XRD) to characterize the specific interactions between drug and copolymer. For the better understanding the drug–copolymer interactions on drug release, insulin was selected for comparison. The release of the two protein drugs from the copolymer-based hydrogels and hydrogel degradation was studied at 37 °C under agitation. The results of FTIR and XRD indicated that the hydrogen bonding interactions existed between the N–H group of BVP and C=O group of the copolymers. The insulin and BVP released from the copolymer hydrogel over 15 and 40 days, respectively. The BVP–copolymer interactions retarded the BVP release rate and degradation of hydrogel, but did not significantly affect the biological activity of BVP. These results indicate that the drug–copolymer interactions need to be considered when attempting to use PLGA–PEG–PLGA hydrogels as sustained delivery carriers of protein or peptide drugs.

Keywords: Drug–copolymer interactions; Bee venom peptide; Hydrogen bonding; PLGA–PEG–PLGA; Thermosensitive hydrogels

1. Introduction

In recent years, thermosensitive hydrogels have gained great interest in drug delivery, cell encapsulation, and tissue engineering due to the sol–gel transition of the hydrogels in response to temperature changes (Eve and Leroux, 2004; Hatefi and Amsden, 2002; Jeong et al., 2002). These thermosensitive hydrogels are injectable fluids that can be introduced into the body in a minimally invasive manner prior to forming a hydrogel at the injection site, which slowly degrade over a period of several days or weeks. Injectable thermosensitive hydrogels have many advantages over systems shaped into their final form before implantation. For example, the loading of such a system with a drug can be achieved by simple mixing of the drug with the copolymer solution and it can be easily injected with a syringe into a desired body site avoiding a surgical procedure. If the hydrogels are biodegradable, they obviate the need for removal of the materials after the drug depot exhausted. Furthermore, this kind of hydrogel is held together by non-covalent forces avoiding the use of organic solvent or chemical reactions, which may be potentially deleterious to the loaded protein- and peptide-drugs (Bromberg and Ron, 1998).

Homopolymers or copolymers of *N*-isopropylacrylamide (Schild, 1992; Lin and Cheng, 2001), poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (known as poloxamers) (Brown et al., 1991), and poloxamer-g-poly(acrylic acid) (Bromberg, 1998) are typical examples of thermosensitive polymers. However, such copolymers are generally not biodegradable, limiting their practicality for use in the clinic. The thermosensitive systems based upon chitosan were developed and could be used as sustained delivery systems of paclitaxel

* Corresponding author. Tel.: +86 24 23986308; fax: +86 24 23986250.
E-mail addresses: qiaomingxi@163.com, qmx_1@tom.com (D. Chen).

0378-5173/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2007.05.056

(Ruel-Gariepy et al., 2004). Recently, a new class of thermosensitive and biodegradable poly(organophosphazene) hydrogels were reported (Couffin-Hoarau and Leroux, 2004; Lee et al., 2002; Lee and Song, 2004). The hydrogel characteristics such as gelation temperature and biodegradability can be controlled by introducing various hydrophobic and hydrophilic constituents. Thermoreversible block copolymers composed of poly(ethylene glycol) (PEG) (A) and biodegradable polyesters (B), such as polylactide (PLA), poly(DL-lactide-co-glycolide) (PLGA) arranged as ABA or BAB were studied as sustained release drug carriers (Duvvuri et al., 2005; Zentner et al., 2001; Qiao et al., 2005).

Early research in the field focused on synthesis of the thermosensitive hydrogel materials and application of them in sustained drug delivery systems. The thermosensitive hydrogels were considered as the promising carriers for the protein and peptide drugs and extensively investigated to use as sustained delivery systems of these drugs (Zentner et al., 2001; Qiao et al., 2006; Kim et al., 2001). However, the potential drug–copolymer interactions on the drug release, biological activity and hydrogel properties are neither well investigated nor ignored. There are only limited numbers of studies that have suggested the presence of ionic interactions between the loaded drug and copolymers, therefore influencing the drug release from the hydrogels (amEnde et al., 1995; Peppas and Wright, 1998; Alvarez-Lorenzo and Concheiro, 2002). In the case of thermosensitive drug delivery systems, drug–copolymer interactions such as hydrogen bonding interactions, ionic interactions and hydrophobic bonding interactions may potentially affect the drug release patterns from the hydrogels. It is especially important that when the protein or peptide drugs loading into the thermosensitive hydrogel are discussed, interactions between the drugs and copolymers are to be considered. The nature and extent of the interactions may not only alter the drug release, but also determine the ability of hydrogels to provide a safe environment for the drugs (Dong et al., 1992).

Bee venom peptide (BVP), also be named melittin, is a small peptide with a molecular weight of 2.84 kDa and consists of 26 amino acid residues in the following sequence: Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gin-NH_2. BVP is the principal active component of bee venom, and it has potent anti-inflammatory and anti-microbial activity. BVP has been utilized to relieve pain and to treat inflammatory diseases such as rheumatoid arthritis (RA) in humans. BVP therapy is carried out by the long-term administration of a series of injections. Conventional regimen entails a course of three times a week over 8–10 weeks. In order to improve the therapy efficacy and patient compliance, an injectable sustained delivery system for BVP is needed.

In this article, the injectable sustained delivery systems of BVP based on thermosensitive PLGA–PEG–PLGA copolymers were developed. The presented BVP–copolymer interactions were characterized by powder X-ray diffraction analysis (PXRD) and FTIR, and the effects on drug release and hydrogel degradation were also studied.

2. Materials and methods

2.1. Materials

Polyethylene glycol (PEG 1500) was purchased from Shanghai Pudong Gaonan Chemical Corporation. DL-Lactide and glycolide were purchased from CONAN Medical Supply Company and used without further purification. Bee venom peptide was supplied by Jilin Natural Pharmaceutical Co. Ltd. Stannous 2-ethylhexanoate and Lowry Reagent were purchased from Sigma (St. Louis, MO, USA). Zinc–insulin (27.5 I.U./mg, zinc content, 0.2%) was purchased from Wanbang Biopharmaceutical Company (China). All other chemicals were of reagent grade.

2.2. Synthesis and characterization of PLGA–PEG–PLGA triblock copolymers

The PLGA–PEG–PLGA triblock copolymer was synthesized by ring-opening polymerization as described in our previous paper (Qiao et al., 2005). Briefly, PEG was dried in a three-necked flask under vacuum and stirring at 120 °C for 2 h before copolymerization. The ring-opening polymerization in the bulk state was carried out with different molar ratio of DL-lactide/glycolide (6/1 and 15/1) and the weight ratio of PEG was adjusted to 30% (w/w). Stannous 2-ethylhexanoate (0.2%, w/w) was used as catalyst. The resulting PLGA–PEG–PLGA triblock copolymers were dried under vacuum. The copolymer nomenclature was designated PLGA–PEG–PLGA(*X*/*Y*), where *X*/*Y* is the molar ratio of DL-lactide/glycolide.

^{1}H NMR spectra of PLGA–PEG–PLGA copolymers were obtained in $CDCl_3$ using a NMR instrument (Bruker ARX-300) at 300 MHz. If x is the peak area at $\delta = 5.2$ ppm; y is the peak area at $\delta = 4.8$ ppm, the molar ratio of DL-lactide/glycolide can be calculated through the following equation: molar ratio of DL-lactide/glycolide = $2x/y$.

The molecular weights and molecular weight distribution indexes of the PLGA–PEG–PLGA copolymers were monitored by GPC system with a Shimadzu LC-10AD HPLC pump, Shimadzu RID-6A refractive index detector (Kyoto, Japan) and Hewlett-Packard Plgel columns. Tetrahydrofunan was served as solvent with a flow rate of 1 ml/min. The molecular weights of the copolymers were determined relative to polystyrene standards.

2.3. Measurement of gelation temperature

The distilled water was mixed with PLGA–PEG–PLGA copolymer and allowed to dissolve under magnetic stirring at room temperature. The copolymer solutions at concentrations of 15%, 17%, 20%, 23% and 25% (w/w) were prepared for the measurement of gelation temperature. A 20 ml transparent vial containing 2.6 g of magnetic bar (cylinder, 10 mm × 5 mm i.d.) and 10 g copolymer solution was placed in a water bath. Each sample at a given concentration was heated at a constant rate of 2 °C per minute with constant stirring (200 rpm). When the magnetic bar stopped stirring due to gelation of the solution, the

temperature read from the thermometer was determined as the gelation temperature (Qiao et al., 2005).

2.4. In vitro drug release studies

Various amounts of BVP and zinc–insulin were mixed with the copolymer solutions (20%, w/w). If any air bubbles produced, placed at room temperature until the air bubbles disappeared. The mixture (0.4 ml) containing 5 mg, 10 mg, and 20 mg of BVP and 1 mg of insulin were placed into the test tubes and incubated for 2 min at 37 °C. Three milliliters of phosphate buffer (pH 7.4) containing 0.02% (w/v) NaN_3 were added to the formed hydrogels and gently shaken at 20 rpm. At sampling times, the release medium was all removed for the measurement of BVP and insulin and replaced with fresh phosphate buffer.

The amount of BVP in the release medium was determined by the Lowry method. The samples of 0.6 ml were transferred to tubes followed by adding 2.5 ml of alkaline copper reagent (a freshly prepared solution by mixing 1 ml of 1% (w/v) $CuSO_4{\cdot}5H_2O$ and 1 ml of 2% (w/v) potassium tartrate in water with 100 ml of 2% (w/v) Na_2CO_3 in 0.1 M NaOH) and allowed to stand for 10 min at room temperature. Next, 0.1 ml of diluted Folin reagent (1:3 dilution in distilled water) was added and mixed well. After stored for 30 min in darkness, the assay mixture was measured at 500 nm using a UV-9100 spectrophotometer (Rayleigh Analytical Instrument Corp., Beijing, China). The BVP content in the release medium was determined by the standard curve of absorbance as a function of standard concentration.

Insulin was measured by a reverse phase HPLC method (SPD-10Avp, Shimadzu). The analysis was performed at 214 nm on a C_{18} column (5 μm particle, 150 mm × 4.6 mm i.d., Diamonsil™). The mobile phase was an isocratic mixture of 0.2 M sodium sulfate anhydrous solution adjusted to pH 2.5 with phosphoric acid and acetonitrile (72:28, v/v). The injection volume was 20 μl and the flow rate was 1.0 ml/min.

The release data of BVP were evaluated by model-dependent methods to elucidate the drug release mechanism (Jeong et al., 1999). Drugs diffusion from the polymer matrix can be described by Eq. (1). The equation can be simplified as Eqs. (2) or (3) depending on the value of M_t/M_∞.

$$\frac{M_t}{M_\infty} = 1 - \sum_{n=0}^{\infty} \frac{8}{(2n+1)^2\pi^2} \exp\left[\frac{-D(2n+1)^2\pi^2}{l^2}t\right] \quad (1)$$

$$\frac{M_t}{M_\infty} = 4\sqrt{\frac{Dt}{\pi l^2}} \quad \frac{M_t}{M_\infty} \leq 0.6 \quad (2)$$

$$\frac{M_t}{M_\infty} = 1 - \frac{8}{\pi^2} \exp\left[\frac{-\pi Dt}{l^2}\right] \quad \frac{M_t}{M_\infty} > 0.4 \quad (3)$$

where M_t is defined as the mass of the drug released at time t, M_∞ is the mass of drug released as time approaches infinity, and D and l are the diffusion coefficient of the drug and the thickness of the device, respectively.

2.5. BVP bioactivity assay

BVP is non-cell-selective anti-microbial peptide which possesses activity against both bacterial and mammalian cells and the interaction with mammalian cell membrane will cause hemolysis (Zhu et al., 2007). The anti-microbial and hemolytic activities of BVP were believed to be correlated to its binding to cell membranes and the anti-microbial activity of BVP is of a similar potency to its hemolytic activity (Blondelle and Houghten, 1991; Sun et al., 2005). In this study, the hemolytic activity of the BVP was tested by using human red blood cells (RBCs) to reflect the membrane binding activity. Fresh RBCs were isolated in vacutainers containing ethylene diaminetetraacetic acid (EDTA), centrifuged at 2000 rpm for 10 min and washed three times with phosphate buffer saline (PBS, phosphate buffer, 0.15 M NaCl, pH7.4). The RBCs were suspended in the PBS. The hemolytic assay was performed by adding the BVP samples in phosphate buffer to 0.5 ml of a 5% solution of the stock RBCs in PBS to reach a final volume of 1.0 ml. Following gentle shaking, the suspension was incubated in a 37 °C water bath for 60 min. The samples were then centrifuged at 2000 rpm for 10 min. The absorbance of the supernatant was then measured at 541 nm. Zero hemolysis (blank) and 100% hemolysis controls were determined by using RBCs suspended in PBS and distilled water, respectively. All hemolysis experiments were done in triplicate.

2.6. In vitro degradation of the hydrogels

The copolymer solutions (20% w/w, 0.4 ml) alone or the copolymer solutions loaded with various amount of BVP (5 mg, 10 mg, and 20 mg) were used for degradation study and manipulated according to Section 2.4. At sampling times, the release medium of each test tube was transferred to the pre-weighed vial and evaporated until constant weight. The amount of copolymer degradation was calculated as: $W = W_d - W_o - W_p$, or $W = W_d - W_o - W_p - W_{BVP}$, where W is the amount of degradation, W_d is the weight of vial after drying, W_o is the original weight of the vial, W_p is the weight of phosphate salt, W_{BVP} is the amount of released BVP in the release medium.

2.7. Preparation of copolymer/drug co-precipitate blends

A solvent casting method (Nair et al., 2001) was used to prepare the blends of copolymer and BVP. Copolymer solutions (20%, w/w, 1 ml) containing the appropriate amounts of BVP (12.5 mg, 25 mg, and 50 mg) were prepared in water. The solution was poured into a Petri dish and dried under vacuum at room temperature.

2.8. Powder X-ray diffraction study

Powder X-ray diffraction patterns of the pure BVP, copolymer, and copolymer/drug co-precipitate blends were obtained using a D/Max-2400 diffractometer (Rigaku Corporation, Japan). Measurement conditions included target Cu Kα, voltage

30 kV, and current 40 mA. Patterns were obtained using a step width of 0.04° 2θ between 3 and 45° at ambient temperature.

2.9. Fourier transform infrared spectroscopy (FTIR)

Infrared spectra of BVP or copolymer alone, physical mixture and copolymer/drug co-precipitate blends were measured by using a Flourier transform infrared spectrophotometer (Bruker IFS-55, Switzerland). For analysis, the pellets were prepared by mixing the samples with 200 mg of KBr and compressing the mixture under high pressure. Each FTIR spectrum was obtained by averaging 16 scans at a resolution of 1 cm^{-1}.

3. Results and discussion

3.1. Thermosensitive PLGA–PEG–PLGA triblock copolymers

Thermosensitive PLGA–PEG–PLGA copolymers with the trade name of ReGel were reported (Zentner et al., 2001), which the DL-lactide/glycolide molar ratio was 3/1 and the molecular weight of PEG used for copolymerization was 1000. However, the gelation temperature was below the room temperature, and will bring troubles in formulation preparation and administration. By changing the molecular weight of PEG and molar ratio of DL-lactide/glycolide, we successfully synthesized the thermosensitive copolymer with more appropriate gelation temperature (30–37 °C). The aqueous solution (25%, w/w) of the PLGA–PEG–PLGA(6/1) copolymer flowed freely at room temperature, but became hydrogel at evaluated temperature such as 37 °C (Fig. 1). The typical phase diagram illustrating the gelation behavior of aqueous solutions of the copolymer is shown in Fig. 2. The copolymer solution exhibited phase transition from sol to gel (lower critical temperature) and from gel to sol (higher critical temperature) as the temperature monotonically increased. As shown in our previous article (Qiao et al., 2005), the characteristic signals in ^{1}H NMR spectrum appearing at 5.2 ppm, 4.8 ppm, 3.6 ppm, and 1.5 ppm are assigned to the methine hydrogen of the DL-lactide units, methylene hydrogen of the glycolide units, the methylene hydrogen of the PEG and the methyl hydrogen of the DL-lactide units, respectively. The molecular weights, DL-lactide/glycolide molar ratio and polydispersity indexes of the copolymers are shown in Table 1. As shown in Table 1, the obtained composition was in good agreement with the copolymer composition expected from the feed ratio.

Table 1
The molecular weights, compositions and polydispersity indexes of the copolymers

Copolymer	M_w of copolymer		DL-Lactide/glycolide ratio[a]	Polydispesity index
	M_w[b]	M_n[b]		
PLGA–PEG–PLGA(6/1)	4842	3824	5.7	1.27
PLGA–PEG–PLGA(15/1)	5084	4067	14.8	1.25

[a] Determined by ^{1}H NMR.
[b] Measured by GPC, relative to polystyrene standards.

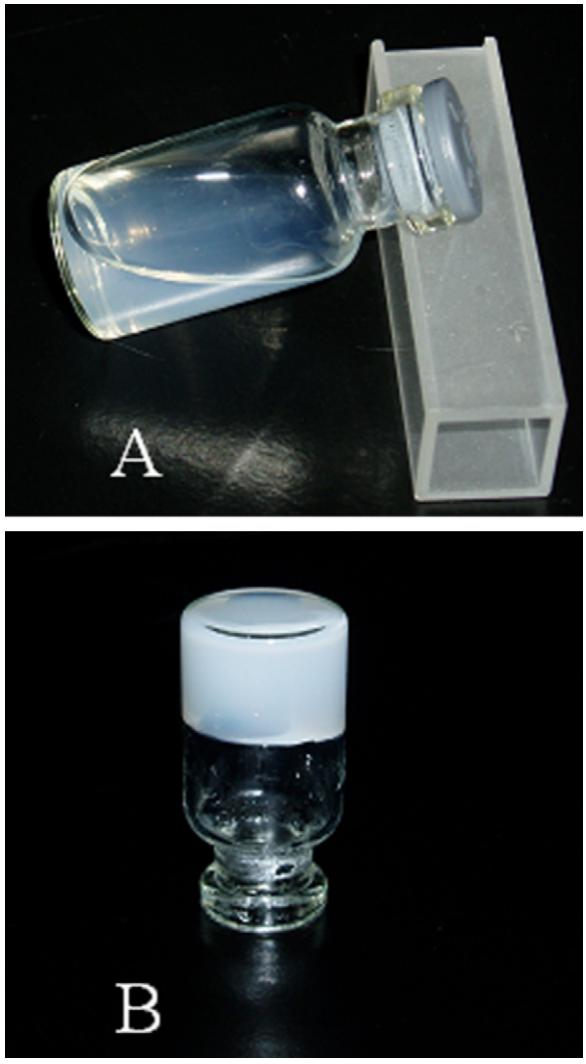

Fig. 1. The photographs of aqueous solution of PLGA–PEG–PLGA(6/1) copolymer at 27 °C (A) and 37 °C (B).

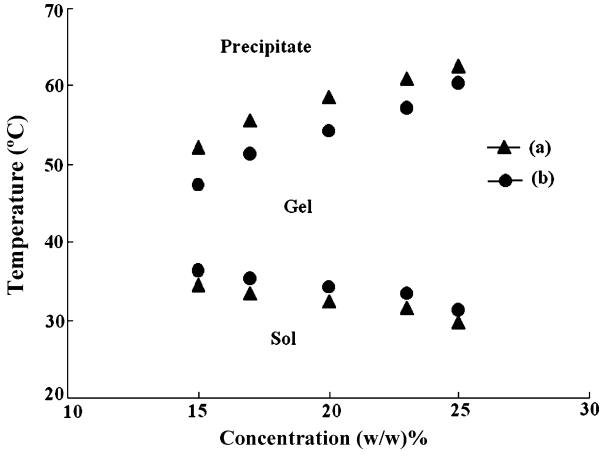

Fig. 2. Phase diagram of PLGA–PEG–PLGA(15/1) (a) and PLGA–PEG–-PLGA(6/1) (b) copolymer aqueous solutions.

3.2. Drug–copolymer interactions characterization

3.2.1. X-ray analysis

Powder X-ray diffraction analysis is often used to evaluate drug–polymer interactions and the state of a drug in a polymer matrix (Liu et al., 2004). The drug–polymer interactions result in a change in the drug molecular mobility of the drug, ultimately leading to an inhibitory effect on crystallization of drug molecules and an amorphous dispersion in the polymer (Yoshioka et al., 1995). X-ray diffraction patterns for BVP alone, copolymer alone, and BVP–copolymer blends are shown in Fig. 3. The presence of one broad peak in the X-ray powder diffraction patterns for the copolymer confirms that it is a semi-crystalline polymer. There are two peaks in the diffraction graph for BVP alone. Although one of the peak of BVP alone is overlapped by the broad peak of copolymer, the peak at a 2-theta-scale of 9.46 degree disappeared in the BVP–copolymer blends suggests that BVP is either in an amorphous state or in the molecular dispersion. The decrease of BVP in the crystalline state in the BVP–copolymer blends indicates that the interactions may exist between drug and copolymer in the hydrogels. Because the copolymer and BVP is semisolid and powder under room temperature respectively, the X-ray diffraction analysis of physical mixture was not performed due to the difficulty in preparing the physical mixture samples.

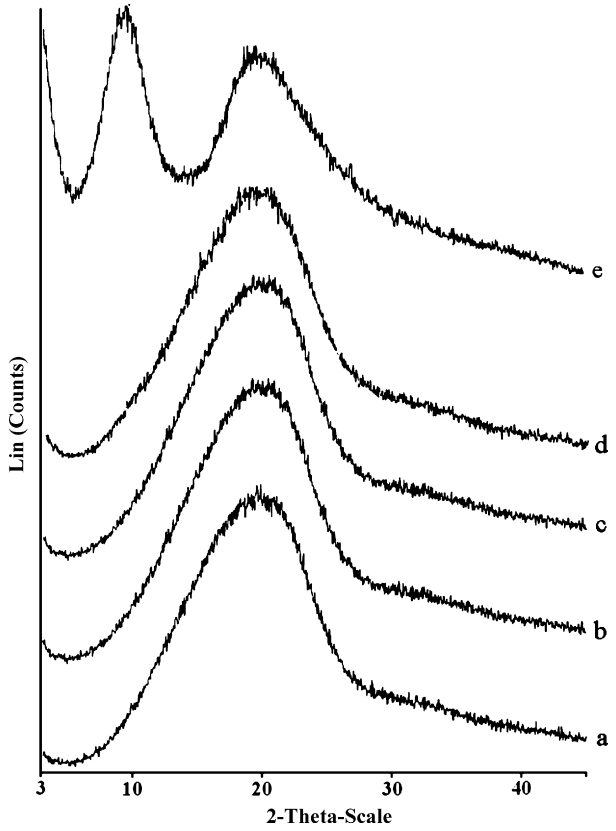

Fig. 3. Powder X-ray diffraction patterns for BVP and PLGA–PEG–PLGA copolymer alone and the various BVP–copolymer blends. (a) Pure PLGA–PEG–PLGA copolymer; (b) BVP(5 mg)–copolymer blends; (c) BVP(10 mg)–copolymer blend; (d) BVP(20 mg)–copolymer blend; (e) pure BVP.

3.2.2. FTIR analysis

In order to elucidate the drug–copolymer interactions, the samples were further tested by FTIR. Many studies have used FTIR to probe the nature and extent of interactions in drug–polymer blends (Nair et al., 2001; Puttipipatkhachorn et al., 2001; Kuo et al., 2002). The premise of using an IR to study drug–polymer blends is that the mixing of two components at the molecular level which will cause changes in the oscillating dipole of the molecules. This will manifest itself as changes in the frequency and bandwidth of interacting groups in the spectrum compared to the spectrum of the pure drug and polymer.

The FTIR spectra of pure BVP, PLGA–PEG–PLGA copolymer alone, physical mixture of BVP and copolymer, and BVP–copolymer blends are shown in Fig. 4. Fig. 4 shows the hydroxyl stretching region 3200–3600 cm^{-1} of the pure PLGA–PEG–PLGA copolymer, N–H stretching vibrations region 3100–3600 $cm^{-1}$of the pure BVP, and the overtone of hydroxyl and N–H stretching vibrations of the BVP–copolymer blends. The peak observed at 3422 cm^{-1} indicates the absorbance of free N–H stretching vibrations in the pure BVP spectrum. From the peak (∼1654 cm^{-1}) presented

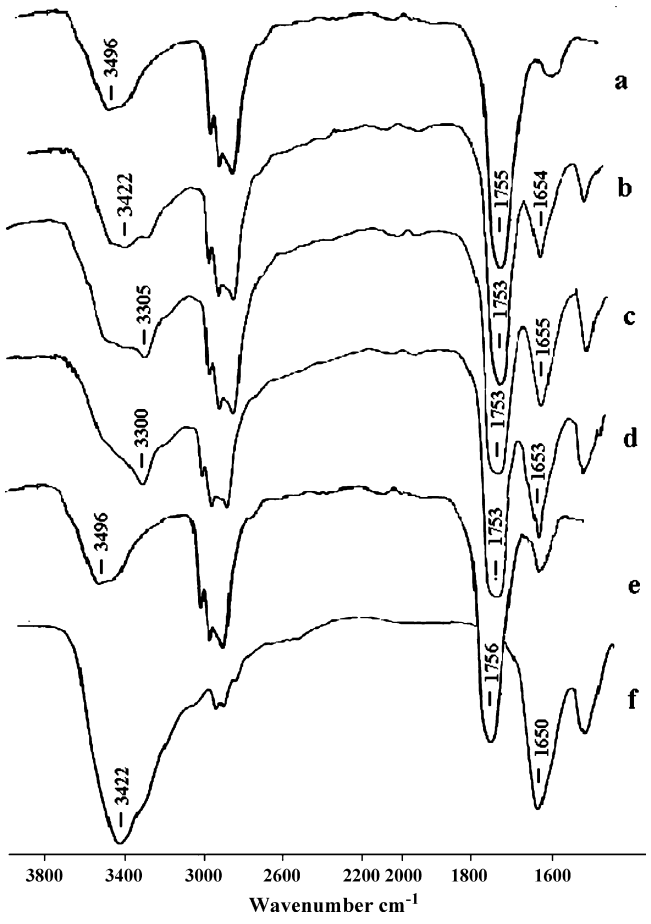

Fig. 4. The FTIR spectra of BVP, PLGA–PEG–PLGA copolymer, and the various BVP–copolymer blends. (a) Pure PLGA–PEG–PLGA copolymer; (b) BVP(5 mg)–copolymer blends; (c) BVP(10 mg)–copolymer blends; (d) BVP(20 mg)–copolymer blends; (e) the physical mixture of BVP and copolymer; (f) pure BVP.

in the BVP–copolymer blends, we can postulate N–H peak at 3422 cm^{-1} will also contribute the adsorption at corresponding frequency. But we cannot observe obvious increase in absorption intensity at 3422 cm^{-1} in the spectra of BVP–copolymer blends. In the spectra of BVP–copolymer blends, the peak of free N–H stretching vibrations at 3422 cm^{-1} is reduced and shifts to around 3305 cm^{-1} (Fig. 4b–d). The peak at 1755 cm^{-1} and 1753 cm^{-1} denotes the carbonyl stretching of the pure copolymer and drug–copolymer blends (Fig. 4b–d). Pure PLGA–PEG–PLGA copolymer shows a peak at 1755 cm^{-1} indicative of C=O stretching vibrations of the ester group. By comparing C=O stretching vibrations of pure copolymer with drug–copolymer blends, as the proportion of BVP in the blends increased from 5 to 10 mg or 20 mg, the peak at 1755 cm^{-1} shifted to 1753 cm^{-1} and the C=O stretching peak become broader than the pure copolymer. Previous studies on the hydrogen bonding between carbonyl group and N–H group proved that the carbonyl band in the spectrum will show band shifts to lower wave numbers and broadening compared to the band of free carbonyl (Nair et al., 2001; Silverstein et al., 1991) and N–H group often showed a lower band region between 3283 and 3340 cm^{-1} compared to the free N–H band at 3440 cm^{-1} (Coleman et al., 1986; Coleman et al., 1988; Brunette et al., 1982). The copolymer C=O stretch and BVP N–H stretch shift to lower wave numbers with the broadening and reduction in intensity respectively, indicating the formation of hydrogen bonding between the N–H group of BVP and the C=O group of the copolymer.

The peak around 1650 cm^{-1} in the Fig. 4 b–d and f indicate the amide I band of the drug–copolymer blends and pure BVP. For the drug–copolymer blends, the amide I band shifts to higher wave numbers (1655 cm^{-1}) compared with the pure BVP (1650 cm^{-1}). As shown in Fig. 4(f), the shoulder peak observed at around 3300 cm^{-1} suggests that a part of N–H form hydrogen bonding with C=O group in the pure BVP. In the drug–copolymer blends, the presence of the copolymer interfered the hydrogen bonding between the N–H group and C=O group due to the interactions of BVP with copolymer and resulted in the shifts of the amide I to higher wave numbers.

3.3. The effect of drug–copolymer interactions on hydrogel degradation and drug release

The in vitro degradation profiles of the blank hydrogels containing 20% (w/w) copolymers are presented in Fig. 5. It can be seen from the Fig. 5 that the blank hydrogels almost completely degraded within about 30 days and the degradation rate decreased with increasing the DL-lactide/glycolide molar ratio in the copolymers from 6/1 to 15/1. Fig. 6 shows the effect of BVP loading on the degradation of the hydrogels. As shown in Fig. 6, the hydrogels loaded with 5 mg or 10 mg BVP shows similar degradation rate and both are slower than that of the hydrogel loaded with 20 mg BVP. At 40 days, about 28%, 30% and 19% of the initial amount of the copolymers remained in the BVP loaded (5 mg, 10 mg, and 20 mg) hydrogels, respectively. Compared with the blank hydrogel, the BVP (5 mg, 10 mg, and 20 mg) loaded hydrogels underwent a slower degradation and showed an apparent half-life of degradation about 30 days and 26 days, respectively.

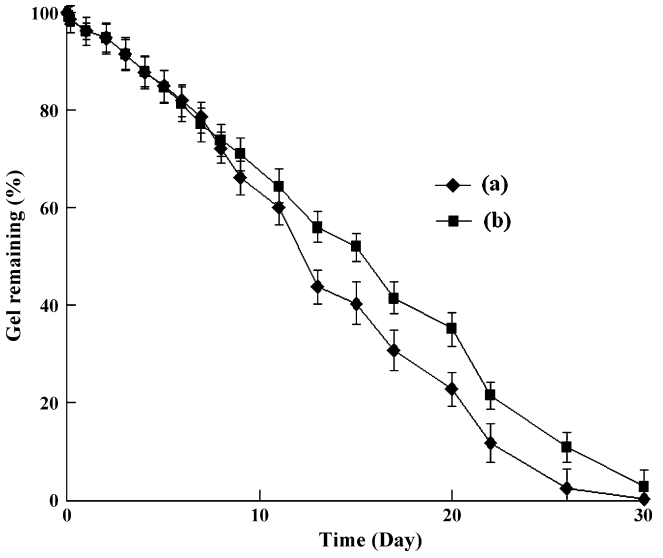

Fig. 5. Degradation profiles of blank copolymer based hydrogels at 37 °C. (a) PLGA–PEG–PLGA(15/1), (b) PLGA–PEG–PLGA(6/1). Both the copolymer concentration is 20% (w/w). Each point represents the mean ± S.D.; $n = 3$.

The thermosensitive hydrogel maintains its network structure through hydrophobic interactions, which exist among the hydrophobic PLGA block of the copolymer chains. The copolymer hydrophobicity increases with increasing the DL-lactide/glycolide molar ratio because the DL-lactide moiety is more hydrophobic than glycolide. As a result, the hydrogel formed by copolymer with higher DL-lactide/glycolide molar ratio shows slower degradation rate (See Fig. 5). The investigation of the degradation of BAB-triblock copolymers from hydrophobic poly(L(+)lactic acid) (PLLA) B-blocks and hydrophilic PEG A-blocks indicated that a preferential cleavage

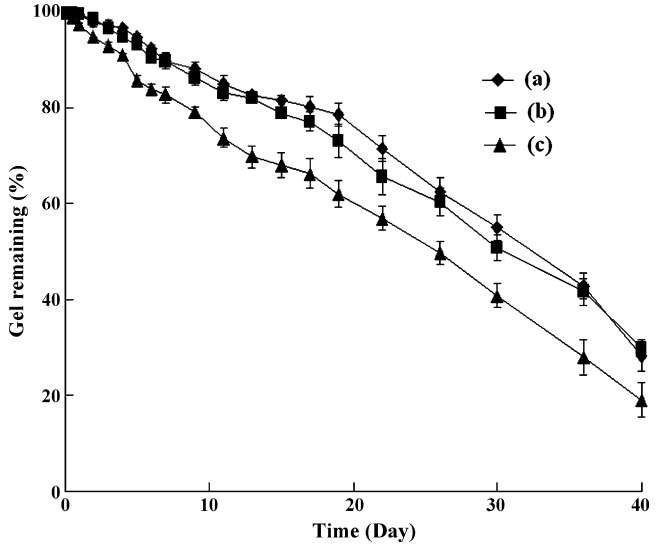

Fig. 6. Degradation profiles of the BVP loaded copolymer-based (PLGA–PEG–PLGA, 6/1) hydrogels at 37 °C. (a) 5 mg, (b)10 mg, (c) 20 mg. The copolymer concentration was 20% (w/w). Each point represents the mean ± S.D.; $n = 3$.

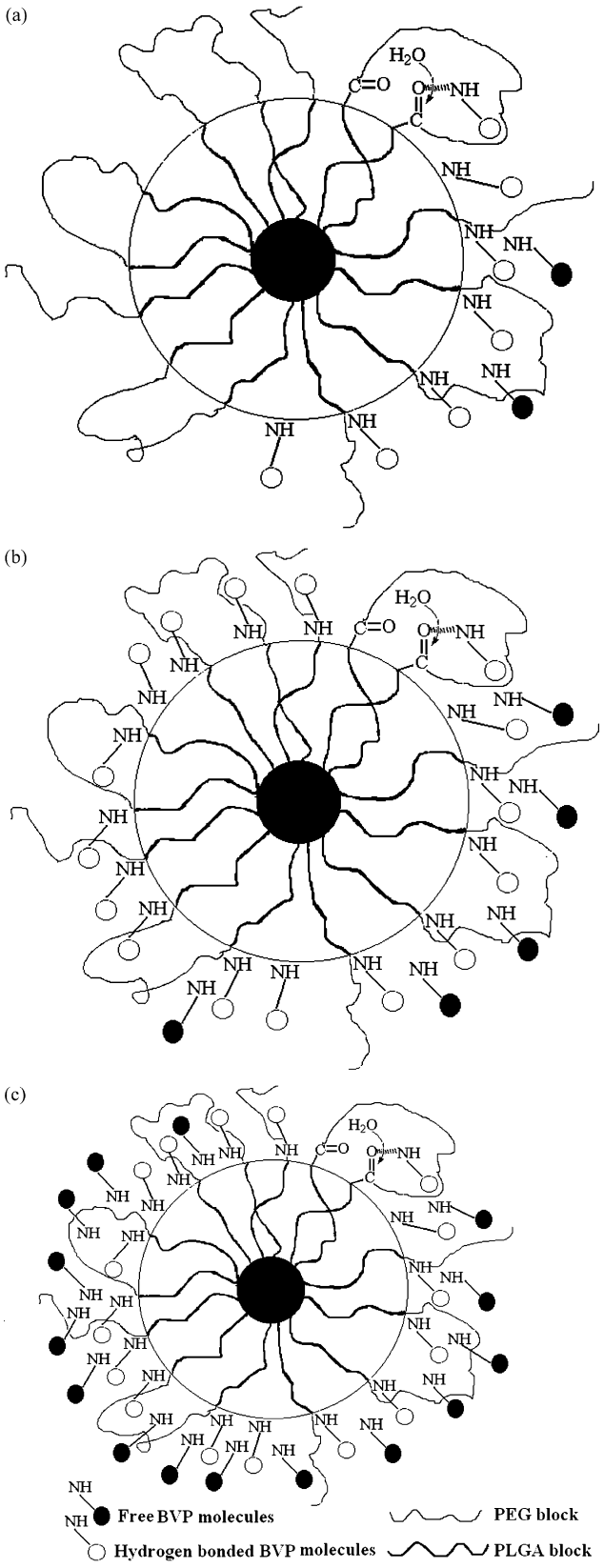

Fig. 7. Schematic diagram of the possible mechanism of drug–copolymer interactions on the drug release and hydrogel degradation. The "a" state shows the hydrogen bonding between the BVP and copolymers when the drug loading is low. The "b" and "c" shows the binding comes to saturated state and the free BVP percentage increases with the increasing of drug loading.

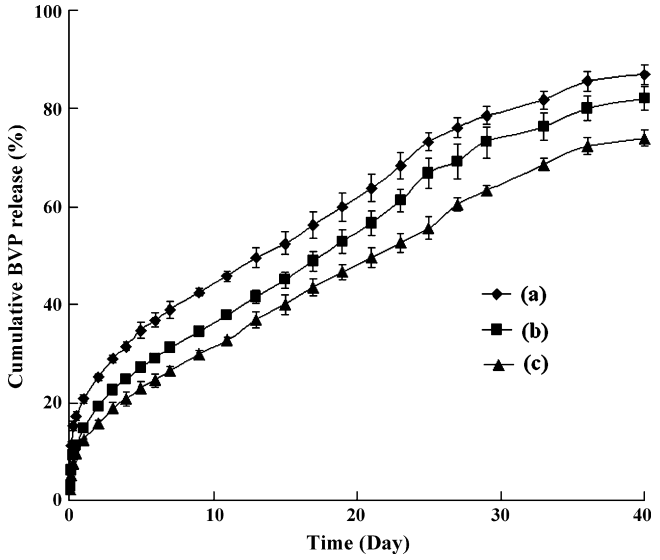

Fig. 8. Cumulative release of BVP from the copolymer-based hydrogels with various BVP loading at 37 °C. (a) 5 mg, (b) 10 mg, (c) 20 mg. The copolymer concentration is 20% (w/w). Each point represents the mean ± S.D.; $n = 3$.

of the copolymers occurred in the vicinity of the PLLA/PEG interface by using ^{1}H NMR analysis (Li et al., 1994). The initial degradation of the copolymers began with the cleavage of C=O bond which linked hydrophilic PEG segment and hydrophobic polyester segment. This postulation is reasonable by analyzing the structure of the micelles. The PLGA–PEG–PLGA copolymers simultaneously possess hydrophobic and hydrophilic blocks and form spherical micelles in water, which have hydrophobic PLGA core and hydrophilic PEG shell. Water molecules easily closed to the outermost C=O group which linked hydrophilic PEG segment and hydrophobic PLGA segment due to the hydrophobicity of the micelle cores. As the water soluble drug, BVP molecules would like to distribute in the hydrophilic PEG domain and formed hydrogen bonding with the outermost C=O group (See Fig. 7b). The hydrogen bonding interactions may shield the ester bonds from hydrolysis by water, resulting in slower degradation rate than the blank hydrogel. Unlike the blank hydrogels, the degradation of the BVP loaded hydrogels was independent of DL-lactide/glycolide molar ratio (data not shown), indicating that the effect of drug–copolymer interactions on degradation is more pronounced than the DL-lactide/glycolide molar ratio. The hydrogel loaded with 20 mg BVP showed faster degradation than lower BVP loaded hydrogels (See Fig. 6). The reason for this behavior is not known at present and further investigations are required to clarify this. However, this has little effect on the main topics of the current investigation.

Fig. 8 shows the effect of BVP loading levels on the release patterns as a function of time. The amount of the BVP released from the hydrogel loaded with 5 mg, 10 mg, and 20 mg of BVP during 40 days reached 73%, 82%, and 87%, respectively. It can be observed from Fig. 8 that the drug release rate at higher drug loading level was faster than that at lower drug loading level in the initial time period. After that, the drug release rate was similar independent of drug loading. As discussed above, the

Table 2
Kinetic assessments of in vitro release data from the hydrogels

Drug loading (mg)	Eq. (1)		Eq. (2)	
	Slope	R^2	Slope	R^2
5	7.52	0.9937	3.59	0.9889
10	2.08	0.9939	3.13	0.9846
20	0.48	0.9923	3.61	0.9996

BVP molecules formed hydrogen bonding with the copolymer molecules in the hydrogel. When the drug loading was very low, almost all the drug molecules formed hydrogen bonding with the copolymer (Fig. 7a). As the drug loading increased, the hydrogen bonding interaction sites should be close to a saturated state, due to the limited hydrogen bonding interaction sites in the hydrogel (Fig. 7b). When almost all the interaction sites were bonded, further increasing the drug loading would increase the percentage of free drug molecules in the hydrogels (See Fig. 7c). The free drug molecules were easily diffused from the hydrophilic channel of the hydrogel due to the well water solubility of the BVP molecules. Therefore, the higher drug loading hydrogel showed faster drug release rate than that of lower drug loading hydrogels in the initial time. After most of the free BVP molecules released, the remaining bonded drug molecules were released from the hydrogel at similar rate.

The release data of BVP were fitted to both the Eqs. (2) and (3). According to the models, a straight line is expected for each plot if drug release from the matrix is based on a diffusion mechanism. In both cases, good correlation coefficients were obtained indicating that BVP release from the hydrogels was diffusion dominant (Table 2). Drug release from degradable hydrogel following Fickian diffusion explained that the effect of hydrogel degradation on drug release was not pronounced. This can be attributed to the hydrogen bonding interactions, which altered the drug release pattern and counteract the effect of hydrogel degradation on the drug release. This also explains the phenomenon that higher drug loading (20 mg) hydrogel showed faster degradation rate, but the drug release rate at later period is not faster than that of the other two lower drug loading hydrogels.

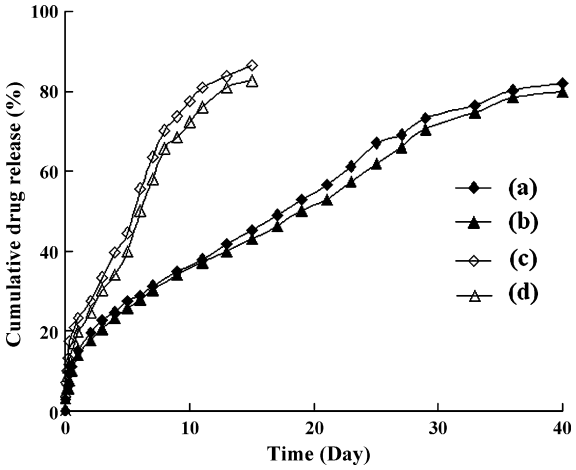

Fig. 9. Cumulative release of BVP and insulin from the copolymer-based hydrogels at 37 °C. (a) BVP, PLGA–PEG–PLGA(6/1), (b) BVP, PLGA–PEG–PLGA(15/1), (c) insulin, PLGA–PEG–PLGA(6/1), (d) insulin, PLGA–PEG–PLGA(15/1). The copolymer concentration is 20% (w/w). Each point represents the mean of triplicates, and error bars were omitted for clarity.

Table 3
Hemolysis activity of BVP released from the copolymer-based hydrogels at different times

Copolymer	Day	Hemolysis activity (%)[a]
PLGA–PEG–PLGA(6/1)	1	95
	5	90
	10	89
	20	84
	36	80

[a] The hemolysis activity is relative to freshly reconstituted BVP in the release medium. The values are the mean of three measurements deviated 5–10%.

Both the copolymer (PLGA–PEG–PLGA(6/1) and PLGA–-PEG–PLGA(15/1)) solutions (20%, w/w) were used to examine the effect of DL-lactide/glycolide molar ratio on the BVP release. The release profiles of BVP from the two different copolymer-based hydrogels loaded with 10 mg BVP are shown in Fig. 9. The BVP release from the hydrogels was independent of the DL-lactide/glycolide molar ratio in the copolymer. For the better understanding the drug–copolymer interactions on the BVP release, insulin was selected as another model protein drug. The release profile of insulin from the copolymer hydrogels was shown in Fig. 9. In contrast to BVP, insulin was released with relatively faster rate over 15 days. The comparison clearly showed that the BVP–copolymer interactions retarded the BVP release.

For the thermosensitive hydrogel drug delivery systems, potential incorporated drug–copolymer interactions would have effects on the drug release rate below the lower critical solution temperature (LCST), the magnitude of drug pulse during the sol–gel transition process, and drug release rate from the copolymer formed hydrogel. Drug–copolymer interactions between a drug and a hydrogel can potentially alter the drug release from the hydrogel in two ways. First, binding to the hydrogel could directly slow the release rate of the drug due to the interactions with the copolymer chains, and therefore depressed or eliminated "burst effect". Secondly, the binding may affect the hydrogel characteristic such as swelling or degradation, which could affect the concomitant drug release from the hydrogel. As for the proteins or peptides sustained delivery systems, the drug–copolymer interactions do not simply influence the drug release behavior. The potential drug–copolymer interactions may possibly affect their biological activity because the interactions will likely change the protein structure. Therefore, it is important to investigate any drug–copolymer interactions when the thermosensitive hydrogels were used as delivery systems for protein or peptide drugs. As shown in Table 3, BVP released from the hydrogels remained 80–95% of its original activity. The results indicated the hydrogen bonding interactions did not significantly influence the biological activity of BVP in the hydrogels.

4. Conclusion

The potential interactions between BVP and thermosensitive PLGA–PEG–PLGA copolymers were examined. The nature of binding was characterized by analysis of the FTIR and XRD of BVP–copolymer co-precipitate blends. The binding was shown to be of the hydrogen bonding between the N–H group of BVP and C=O group of the copolymers. The hydrogen bonding interactions decreased the BVP release and degradation of the hydrogels, but did not influence the biological activity of the BVP. The importance of investigating drug–copolymer interactions was shown when attempting to use the thermosensitive copolymers as protein or peptide drug delivery systems.

Acknowledgement

This study was supported financially by the Educational Department of Liaoning Province (No. 05L432).

References

Alvarez-Lorenzo, C., Concheiro, A., 2002. Reversible adsorption by a pH and temperature sensitive acrylic hydrogel. J. Controlled Release 80, 247–257.

amEnde, M.T., Hariharan, D., Peppas, N.A., 1995. Factors influencing drug and protein transport and release from ionic hydrogels. React. Polym. 25, 127–137.

Blondelle, S.E., Houghten, R.A., 1991. Hemolytic and antimicrobial cctivities of the twenty-four individual omission analogues of melittin. Biochemistry 30, 4671–4678.

Bromberg, L.E., 1998. Novel family of thermogelling materials via C–C bonding between poly(acrylic acid) and poly(ethylene oxide)-b-poly(propylene oxide)-b-poly(ethylene oxide). J. Phys. Chem. B 102, 1956–1963.

Bromberg, L.E., Ron, E.S., 1998. Temprature-reponsive gels and thermo gelling polymer matrices for protein and peptide delivery. Adv. Drug Deliv. Rev. 31, 197–221.

Brown, W., Schillen, K., Almgren, M., Bahadur, P., 1991. Micelle and gel formation in a poly (ethylene oxide)/poly (propylene oxide)/poly (ethylene oxide) triblock co-polymer in water solution. Dynamic and static light scattering and oscillatory shear measurements. J. Phys. Chem. 95, 1850–1858.

Brunette, C.M., Hsu, S.L., MacKnight, W.J., 1982. Hydrogen-bonding properties of hard-segment model compounds in polyurethane block copolymers. Macromolecules 15, 71–77.

Coleman, M.M., Lee, H.K., Skrovanek, D.J., Painter, P.C., 1986. Hydrogen bonding in polymers. 4. Infrared temperature studies of simple polyurethane. Macromolecules 19, 2149–2157.

Coleman, M.M., Skrovanek, D.J., Hu, J.B., Painter, P.C., 1988. Hydrogen bonding in polymer blends. 1. FTIR studies of urethane-ether blends. Macromolecules 21, 59–65.

Couffin-Hoarau, A.C., Leroux, J.C., 2004. Report on the use of poly(organophosphazenes) for the design of stimuli-responsive vesicles. Biomacromolecules 5, 2082–2087.

Dong, L.C., Yan, Q., Hoffman, A.S., 1992. Controlled release of amylase from a thermal and pH-sensitive, macroporous hydrogel. J. Controlled Release 19, 171–177.

Duvvuri, S., Janoria, K.G., Mitra, A.K., 2005. Development of a novel formulation containing poly (D,L-lactide-co-glycolide) microspheres dispersed in PLGA–PEG–PLGA gel for sustained delivery of ganciclovir. J. Controlled Release 108, 282–293.

Eve, R.G., Leroux, J.C., 2004. In situ-forming hydrogels-review of temperature-sensitive systems. Eur. J. Pharm. Biopharm. 58, 409–426.

Hatefi, A., Amsden, B., 2002. Biodegradable injectable in situ forming drug delivery systems. J. Controlled Release 80, 9–28.

Jeong, B., Choi, Y.K., Bae, Y.H., Zentner, G., Kim, S.W., 1999. New biodegradable polymers for injectable drug delivery systems. J. Controlled Release 62, 109–114.

Jeong, B., Kim, S.W., Bae, Y.H., 2002. Thermosensitive sol-gel reversible hydrogels. Adv. Drug Deliv. Rev. 54, 37–51.

Kim, Y.J., Choi, S., Koh, J.J., Lee, M., Ko, K.S., Kim, S.W., 2001. Controlled release of insulin from injectable biodegradable triblock copolymer. Pharm. Res. 18, 548–550.

Kuo, S.W., Lin, C.L., Chang, F.C., 2002. The study of hydrogen bonding and miscibility in poly(vinylpyridines) with phenolic resin. Polymer 43, 3943–3949.

Lee, B.H., Song, S.C., 2004. Synthesis and characterization of biodegradable thermosensitive poly (organophosphazene) gels. Macromolecules 37, 4533–4537.

Lee, B.H., Lee, Y.M., Sohn, Y.S., Song, S.C., 2002. A thermosensitive poly(organophosphazene) gel. Macromolecules 35, 3876–3879.

Li, Y., Volland, C., Kissel, T., 1994. In-vitro degradation and bovine serum albumin release of the ABA triblock copolymers consisting of poly(L(+)lactic acid), or poly(L(+) lactic acid-co-glycolic acid) A-blocks attached to central polyoxyethylene B-blocks. J. Controlled Release 32, 121–128.

Lin, H.H., Cheng, Y.L., 2001. In situ thermoreversible gelation of block and star copolymers of poly (ethylene glycol) and poly(*N*-isopropylacrylamide) of varying architectures. Macromolecules 34, 3710–3715.

Liu, J.B., Xiao, Y.H., Allen, C.J., 2004. Polymer-drug compatibility: a guide to the development of delivery systems for the anticancer agent, ellipticine. J. Pharm. Sci. 93, 132–143.

Nair, R., Nyamweya, N., Martínz-Miranda, L.J., Hoag, S.W., 2001. Influence of various drugs on the glass transition temperature of poly(vinylpyrrolidone): a thermodynamic and spectroscopic investigation. Int. J. Pharm. 225, 83–96.

Peppas, N.A., Wright, S.L., 1998. Drug diffusion and binding in ionizable interpenetrating networks from poly(vinyl alcohol) and poly(acrylic acid). Eur. J. Pharm. Biopharm. 46, 15–29.

Puttipipatkhachorn, S., Nunthanid, J., Yanamoto, K., Peck, G.E., 2001. Drug physical state and drug-polymer interaction on drug release from chitosan films. J. Controlled Release 75, 143–153.

Qiao, M.X., Chen, D.W., Ma, X.C., Liu, Y.J., 2005. Injectable biodegradable temperature-responsive PLGA–PEG–PLGA copolymers: synthesis and effect of copolymer composition on the drug release from the copolymer-based hydrogels. Int. J. Pharm. 294, 103–112.

Qiao, M.X., Chen, D.W., Ma, X.C., Hu, H.Y., 2006. Sustained release of bee venom peptide from biodegradable thermosensitive PLGA–PEG–PLGA triblock copolymer-based hydrogels in vitro. Pharmazie 61, 199–202.

Ruel-Gariepy, E., Shive, M., Bichara, A., Berrada, M., Le Garrec, D., Chenite, A., Leroux, J.C., 2004. A thermosensitive chitosan-based hydrogel for the local delivery of paclitaxel. Eur. J. Pharm. Biopharm. 57, 53–63.

Schild, H.G., 1992. Poly(*N*-isopropylacrylamide): experiment, theory and application. Prog. Polym. Sci. 17, 63–249.

Silverstein, R.M., Bassler, G.G., Morril, T.C., 1991. Spectrometric Identification of Organic Compounds. Wiley, New York, pp. 91–131.

Sun, X.J., Chen, S.X., Li, S.Z., Yan, H.H., Fan, Y.G., Mi, H.F., 2005. Deletion of two C-terminal Gln residues of 12–26-residue fragment of melittin improves its antimicrobial activity. Peptides 26, 369–375.

Yoshioka, M., Hancock, B.C., Zografi, G., 1995. Inhibition of indomethacin crystallization in poly(vinylpyrrolidone) coprecipitates. J. Pharm. Sci. 84, 983–986.

Zentner, G., Rathi, R., Shih, C., Mcrea, J.C., Seo, M.H., Oh, H., Rhee, B.G., Mestechy, J., Moldoveanu, Z., Morgan, M., Weitman, S., 2001. Biodegradable block copolymers for delivery of proteins and water-insoluble drugs. J. Controlled Release 72, 203–215.

Zhu, W.L., Song, Y.M., Park, Y., Park, K.H., Yang, S.T., Kim, J.I., Prak, I.S., Hahm, K.S., Shin, S.Y., 2007. Substitution of the leucine zipper sequence in melittin with peptoid residues affects self-association, cell selectivity, and mode of action. Biochim. Biophys. Acta 1768, 1506–1517.