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Sustained release of bee venom peptide from biodegradable thermosensitive PLGA-PEG-PLGA triblock copolymer-based hydrogels *in vitro*

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Biodegradable thermosensitive poly (DL-lactide-co-glycolide-b-ethylene glycol-b-DL-lactide-co-glycolide) (PLGA-PEG-PLGA) triblock copolymers with DL-lactide/glycolide molar ratio ranging from 6/1 to 15/l were synthesized from monomers of DL-lactide, glycolide and polyethylene glycol and were evaluated for sustained release of bee venom peptide *in vitro*. The resulting copolymers are soluble in water to form free flowing fluid at room temperature but become hydrogels at body temperature. The gelation temperature of the copolymer solutions can be influenced by the concentration and DL-lactide/glycolide molar ratio of the copolymers. The release of bee venom peptide from the copolymer-based hydrogel and hydrogel degradation in the phosphate buffer (pH 7.4) was studied at 37 °C under agitation. Bee venom peptide was released from the copolymer-based hydrogels over 40 days *in vitro* and the variation of DL-lactide/glycolide molar ratio in the PLGA block of the copolymer did not significantly affect the release rate of bee venom peptide ($P > 0.05$). The hydrogels undergo slower degradation and then faster degradation rate during the whole release stage. Accordingly, the mechanism of bee venom peptide was Fickian diffusion during initial stage and then may be a combination of diffusion and degradation. The synthesized copolymers have the advantage of gelation temperature over the ReGel system. These results indicate that the PLGA-PEG-PLGA copolymer-based hydrogel could be a promising platform for sustained delivery of bee venom peptide.

1. Introduction

Thermosensitive polymers known as "intelligent" polymers have gained considerable attention in the controlled delivery of protein- and peptide-drugs over the past decade (Bromberg and Ron 1998; Jeong et al. 2002). They are free-flowing liquid at or below room temperature and exhibit sol-gel transition at body temperature. In contrast to the chemically cross-linked hydrogels, this kind of hydrogel is held together by non-covalent forces avoiding the use of organic solvents or chemical reactions, which may be potentially deleterious to the loaded protein- and peptide-drugs (Bromberg and Ron 1998). Furthermore, the loading of such a system with a drug can be achieved by simple mixing of the drug with prepared copolymer solution and it can be easily injected with a syringe into a desired body site and the formed hydrogel is capable of sustained delivery of incorporated drugs.

Recent work have demonstrated that triblock copolymers PLGA-PEG-PLGA (poly(DL-lactide-co-glycolide-b-ethyleneglycol-b-DL-lactide-co-glycolide), ReGel) showed reversible thermal sol-gel transition in aqueous solution (Shim et al. 2002) and have been used as the carrier in injectable drug delivery systems (Zentner et al. 2001; Kim et al. 2001). ReGel is the trademark of the reported copolymers, which the PEG molecular weight was 1000 or 1450 and DL-lactide/glcolide molar ratio of the copolymers was confined to a certain range (from 1.5 to 4). These thermosensitive copolymers hold particular promise for the sustained delivery of protein- and peptide-drugs.

Bee venom comes from the stingers of honeybees who use it in defense of the bee colony. Bee venom peptide (BVP) has been used to relieve pain and to treat inflammatory diseases such as rheumatoid arthritis (RA) in humans (Billingham et al. 1973) and experimental animals (Eiseman et al. 1982; Hadjipetrou- Kourounakis and Yiangou 1984). 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. Previous studies suggested that local application of a low dose of BVP near the focus showed a more potent therapeutic effect (Eiseman et al. 1982). Thus, it is necessary to develop an injectable BVP sustained release system for the enhancement of the therapeutic efficacy and patient compliance. However, there is no report on the injectable sustained delivery of BVP until now.

In this study, thermosensitive PLGA-PEG-PLGA triblock copolymers with the DL-lactide/glycolide molar ratio ranging from 6 to 15 were firstly reported to possess the thermosensitive property. The factors influencing the gelation temperature and *in vitro* BVP release from the copolymer-based hydrogels and BVP release mechanism were investigated.

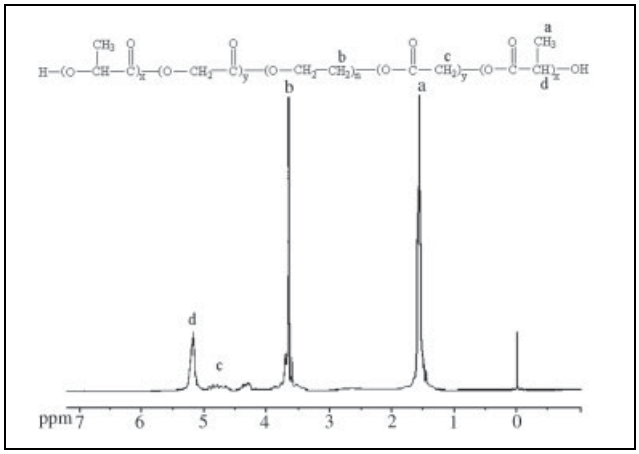

Fig. 1: ^{1}H NMR spectrum of PLGA-PEG-PLGA(10/1) copolymer

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

Copolymer	MW of copolymer		DL-lactide/glycolide ratio[b]	Polydispersity index
	M_w[a]	M_n[a]		
PLGA-PEG-PLGA(6/1)	4842	3824	5.7	1.27
PLGA-PEG-PLGA(10/1)	4584	3555	9.0	1.29
PLGA-PEG-PLGA(15/1)	5084	4067	14.8	1.25

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

2. Investigations, results and discussion

2.1. Characterization of PLGA-PEG-PLGA triblock copolymers

A typical ^{1}H NMR-spectrum of PLGA-PEG-PLGA copolymer is shown in Fig. 1. The characteristic signals 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. Because the ^{1}H NMR signals of each monomer residue are distinguished and well separated, the molar ratio of DL-lactide/glycolide in the copolymer can readily be determined by calculation of the corresponding peak areas. 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:

$$\text{Molar ratio of DL-lactide/glycolide} = 2x/y \qquad (1)$$

The molecular weights, DL-lactide/glycolide molar ratio and polydispersity indexes of the copolymers are shown in Table 1. The obtained composition was in good agreement with the copolymer composition expected from the feed composition. The characterization results demonstrated that the PLGA-PEG-PLGA triblock copolymer with narrow molecular weight distribution of $M_w/M_n < 1.3$ have been synthesized.

2.2. Gelation temperatures of PLGA-PEG-PLGA copolymer solutions

A typical phase diagram illustrating the gelation behavior of aqueous solutions of PLGA-PEG-PLGA(6/1) copolymer is shown in Fig. 2. The hydrogel formed by the copolymer existed between two critical temperatures. The copolymer solution exhibited phase transition from sol to gel

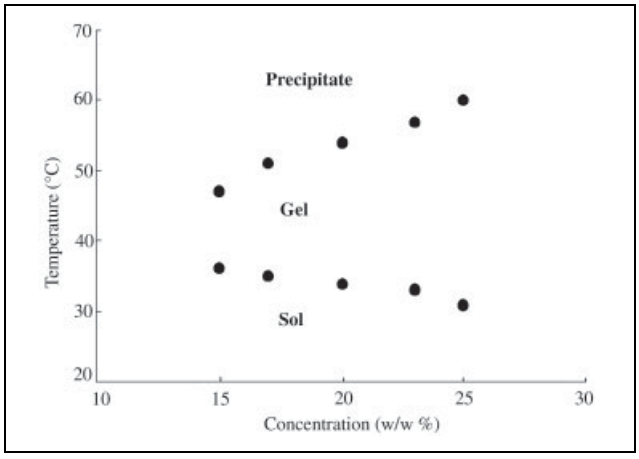

Fig. 2: Phase diagram of PLGA-PEG-PLGA(6/1) copolymer aqueous solutions

(lower critical temperature) and from gel to sol (higher critical temperature) as the temperature monotonically increased. The gelation temperature of the copolymers at various concentrations is shown in Fig. 3. Increasing the copolymer (6/1, 10/1, 15/1) concentration from 15% (w/w) to 25% (w/w), the gelation temperatures decrease from 36.2 °C to 31.2 °C, 35.4 °C to 30.2 °C and 34.5 °C to 29.5 °C, respectively. The gelation temperature also decreased with the increasing DL-lactide/glycolide molar ratio from 6/1 to 15/1. At all concentration levels, there was a significant difference ($P < 0.05$) in gelation temperatures among the three copolymers.

Thermosensitive PLGA-PEG-PLGA copolymers are a kind of block copolymers which are composed of hydrophobic PLGA segment function as formation of associative crosslinks and a hydrophilic PEG segments which will permit the copolymer molecules stay in the solution. At lower temperatures, hydrogen bonding between hydrophilic PEG segments of the copolymer chain and water molecules dominates in the aqueous solution, resulting in their dissolution in water. As the temperature increases, the hydrogen bonding becomes weaker, while hydrophobic forces among the hydrophobic PLGA segments strengthened leading to sol-gel transition. The hydrophobicity of the copolymer increases in the order PLGA-PEG-PLGA(6/1), PLGA-PEG-PLGA(10/1) and PLGA-PEG-

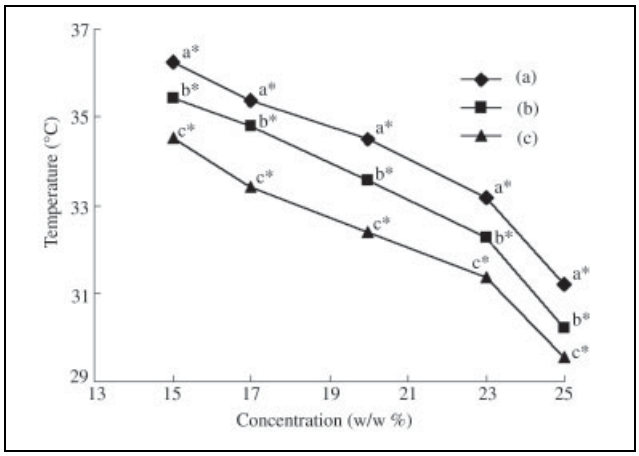

Fig. 3: Effect of DL-lactide/glycolide molar ratio on gelation temperature of the copolymers. (a) PLGA-PEG-PLGA(6/1), (b) PLGA-PEG-PLGA(10/1), (c) PLGA-PEG-PLGA(15/1). a* $P < 0.05$ PLGA-PEG-PLGA(6/1) vs. PLGA-PEG-PLGA(10/1). b* $P < 0.05$ PLGA-PEG-PLGA(10/1) vs. PLGA-PEG-PLGA(15/1). c* $P < 0.05$ PLGA-PEG-PLGA(15/1) vs. PLGA-PEG-PLGA(6/1)

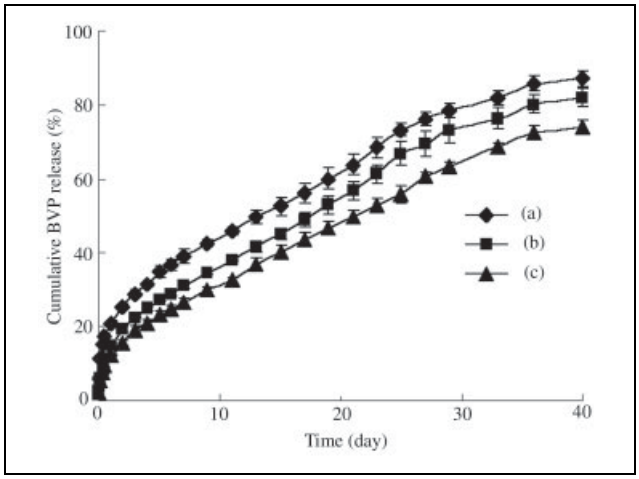

Fig. 4: Effect of PLGA-PEG-PLGA(6/1) concentration on BVP release at 37 °C. (a) 15% (w/w), (b) 20% (w/w), (c) 25% (w/w). Each point represents the mean ± S.D.; n = 3

PLGA(15/1) by increasing the molar ratio of DL-lactide/glycolide in the PLGA segment because DL-lactide moiety is more hydrophobic than glycolide. The copolymer with higher hydrophobicity facilitated the sol-gel transition due to the stronger hydrophobic forces among the copolymer molecules, leading to a decrease in gelation temperature.

2.3. In vitro BVP release and hydrogel degradation studies

The cumulative percent of BVP released from the copolymer-based hydrogel as a function of time is shown in Fig. 4. It can be observed that about 85% BVP were released from the hydrogel formed by 15% (w/w) copolymer over a 40-day release period. Higher copolymer concentration results in a shorter intermicellar distance, leading to greater numbers of cross-links and a greater number of micelles per unit volume in the hydrogel. The decrease in the release rate of BVP with an increase in the copolymer concentration is probably related to the increase in the number of micelles within the hydrogel structure. The effect of DL-lactide/glycolide molar ratio on the BVP release is shown in Fig. 5. Changing the DL-lactide/glycolide molar ratio did not significantly change the release rate of BVP ($P > 0.05$).

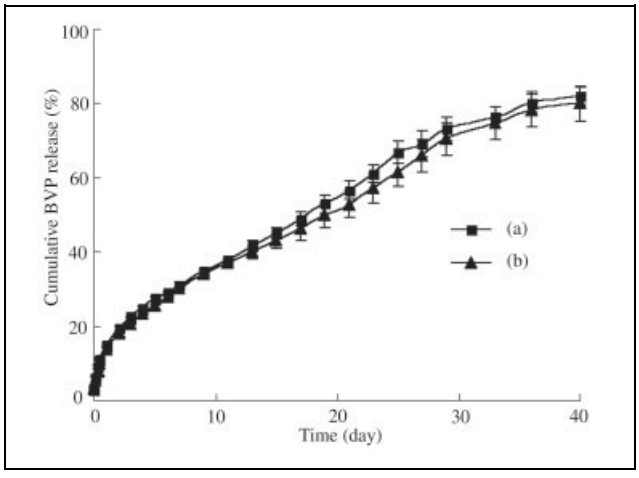

Fig. 5: Effect of DL-lactide/glycolide ratio on the release rate of BVP at 37 °C. The copolymer concentration fixed was at 20% (w/w). (a) PLGA-PEG-PLGA(6/1), (b) PLGA-PEG-PLGA(15/1). For the clarity, the release data of PLGA-PEG-PLGA(10/1) are not shown. Each point represents the mean ± S.D.; n = 3

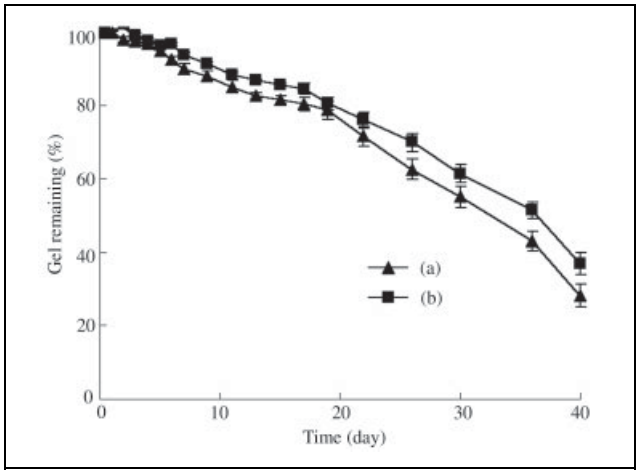

Fig. 6: *In vitro* degradation profiles of the copolymer-based hydrogels. (a) PLGA-PEG-PLGA(6/1), (b) PLGA-PEG-PLGA(15/1). For the clarity, the release data of PLGA-PEG-PLGA(10/1) is not shown. Each point represents the mean ± S.D.; n = 3

The copolymer-based hydrogels undergo a slower degradation during the initial stage (0 to 21 days) which is independent of the DL-lactide/glycolide molar ratio and then faster degradation rate during the later stage (after 21 days) which decreases with the increasing of the DL-lactide/glycolide molar ratio (Fig. 6).

2.4. Drug release mechanism

In order to determine the release mechanism of BVP from the copolymer-based hydrogel, the release data were evaluated by model-dependent methods. For model-dependent analysis, two theoretical models describing drug release from polymeric systems according to Higuchi (2) (Higuchi 1963) and Korsmeyer-Peppas (3) were used (Korsmeyer et al. 1983; Peppas 1985).

$$M_t/M_\infty = k_H t^{1/2} \quad (2)$$

$$M_t/M_\infty = kt^n \quad (3)$$

Where M_t is the amount of drug released at time t, M_∞ is the quantity of drug released at infinite time, k_H is the Higuchi dissolution constant, k is the kinetic constant and n is the release exponent. The Korsemeyer-Peppas model takes into account the drug release mechanism, which deviates from Fick's law and follows an anomalous behavior. For cylindrical devices, n is equal to 0.45 for diffusion-controlled release, whereas n between 0.45 and 1.0 indicates an anomalous non-Fickian transport. Drug release from a porous system may lead to $n < 0.45$ due to the combination of diffusion through the matrix and partial diffusion through water-filled pores. For evaluation of the release data by the described models, the portion of the release curve where $M_t/M_\infty < 0.6$ (0 to 21 days) was used as described in the literature. In both cases, good correlation coefficients were obtained indicating that the BVP release from the hydrogels was consistent with a Fickian diffusion mechanism (Table 2). Drug release from degradable hydrogel following Fickian diffusion explains that the effect of hydrogel degradation on drug release is not pronounced. This is in accordance with the results of *in vitro* degradation study of the hydrogels, which the hydrogels maintain their structure integrity and undergo relative slow degradation during the initial 21 days. After 21 days, the mechanism of BVP release may be a combination of diffusion and degradation due to the faster degradation of hydrogels (see Fig. 6).

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

DL-lactide/glycolide ratio	Higuchi		Korsmeyer-Peppas Equation[a]	
	Slope	R^2	Slope	R^2
6/1[b]	2.08	0.9939	2.62	0.9932
10/1[b]	1.48	0.9966	2.60	0.9981
15/1[b]	1.32	0.9965	2.56	0.9976

[a] Release exponent n = 0.45
[b] The concentration of the copolymer is 20% (w/w)

2.5. The comparison of new synthesized copolymer with ReGel

For the thermosensitive copolymers, it is important to precisely control the gelation temperature in designing a drug delivery system. Gelation temperature means the temperature below which the copolymer is soluble in water and above which the copolymer undergoes phase transition to form a water-insoluble hydrogel. If the gelation temperature is lower than room temperature, gelation will occur at room temperature leading to difficulty in manufacturing, handling and administering. If the gelation temperature is higher than the body temperature, the formulation will maintain liquid at injection site resulting in drug leakage. The gelation temperature of the ReGel system is lower than the room temperature which needs to be improved. In contrast to the ReGel, the gelation temperature of copolymers synthesized by us ranges from 29.5 °C to 36 °C which is more suitable for drug delivery than ReGel (see Fig. 3). There is no significance of difference in the release of bee venom peptide *in vitro* between the new synthesized copolymers and ReGel (data not shown).

3. Experimental

3.1. Materials

Polyethylene glycol (PEG 1500) was purchased from Shanghai Pudong Gaonan Chemical Corporation. DL-Lactide and glycolide were purchased from China Rehabilitation Research Center CONAN Polymer R & D Center and used without further purification. Stannous 2-ethylhexanoate and Lowry Reagent were purchased from sigma (St. Louis, MO, USA). All other chemicals were of reagent grade.

3.2. Synthesis of PLGA-PEG-PLGA triblock copolymers

Copolymerization in the bulk state was carried out with various molar ratios of DL-lactide/glycolide (6/1, 10/1, 15/1) and the weight ratio of PEG was adjusted to 30% (w/w). A total of 50 g of DL-lactide, glycolide plus PEG were used for the polymerization. Stannous 2-ethylhexanoate (0.2% (w/w)) was added into a vigorously dried polymerization tube followed by the addition of DL-lactide, glycolide and PEG. Then the tube was sealed under vacuum. The sealed tube was immersed and kept in an oil bath thermostated at 150 °C for 8 h. The tube was subsequently broken and the product was dissolved in water. After completely dissolved, the copolymer solution was heated to 80 °C to precipitate the copolymer and to remove the water-soluble low molecular weight copolymer and unreacted monomers. The supernatant was decanted to obtain the precipitated copolymer. The process was repeated three times to purify the copolymer. The resulting copolymer was dried under vacuum at room temperature until constant weight. The copolymer nomenclature was designated PLGA-PEG-PLGA(X/Y), where X/Y is the molar ratio of DL-lactide/glycolide.

3.3. Gel permeation chromatography (GPC)

The molecule weights of the PLGA-PEG-PLGA copolymers were determined using a Shimadzu LC-10AD HPLC pump and Shimadzu RID-6A refractive index detector (Kyoto, Japan) coupled to a Hewlett Packard Plgel column. Tetrahydrofuran served as solvent with a flow rate of 1 ml/min. The molecular weights of the copolymers were determined relative to polystyrene standards.

3.4. ^{1}H-Nuclear magnetic resonance (NMR)

^{1}H NMR spectra of PLGA-PEG-PLGA copolymers were obtained in $CDCl_3$ using a NMR instrument (Bruker ARX-300) at 300 MHz. The DL-lactide to glycolide ratio of the copolymer was determined by ^{1}H NMR.

3.5. Measurement of gelation temperature (Yong et al. 2001)

A 20 ml transparent vial containing a 2.6 g of magnetic bar (cylinder, 10 × 5 mm i.d.) and 10 g water solution of PLGA-PEG-PLGA copolymer was placed in a water bath. The solution was heated at a constant rate of 2 °C per min 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.

3.6. In vitro drug release and hydrogel degradation studies

The PLGA-PEG-PLGA copolymers were dissolved in phosphate buffer (pH 7.4) to form various concentrations of copolymer solution (15%, 20%, 25%, (w/w)). The copolymer solution (0.4 ml) was placed into the test tube and mixed with BVP (6 mg). The tubes were incubated for 5 min at 37 °C. Three milliliter of phosphate buffer (pH 7.4) containing 0.02% (w/v) NaN_3 were added to the formed hydrogel and gently shaken at 20 rpm. At sampling times, the release medium was removed for the measurement of the amount of BVP or transferred to the pre-weighed vial and evaporated until constant weight. The amount of BVP in the release medium was determined by the Lowry method, and compared with a standard curve of data obtained by assaying known concentrations of BVP solution (Xing et al. 2003). The amount of degradation was calculated as:

$$W = W_d - W_o - W_p \quad (4)$$

where W is the amount of hydrogel 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.

3.7. Statistics

Statistical analysis of the effects of increasing DL-lactide/glycolide ratio (6/1, 10/1, 15/1) on the gelation temperature and drug release was performed using a one-way ANOVA at all different pints. In all cases, post-hoc comparisons of the means of individual groups were performed using Tukey's Honestly Significant Difference test. A significance level of $P < 0.05$ denoted significance in all cases.

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