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Triblock copolymers: synthesis, characterization, and delivery of a model protein

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Abstract

The purpose of this study was to synthesize and characterize biodegradable and thermosensitive triblock copolymers for delivering protein at controlled rate in biologically active form for longer duration of time. A series of thermosensitive triblock copolymers with different block lengths (PLGA–PEG–PLGA) were synthesized by ring-opening polymerization of D,L-lactide and glycolide with polyethylene glycol (PEG) in the presence of stannous octoate. Compositions and molecular weight of triblock copolymers were characterized by ^{1}H NMR spectrometry and gel permeation chromatography, respectively. A single test-tube inverting method was employed to determine the sol–gel transition temperature. Lysozyme was used as a model protein. Lysozyme solution formulation was prepared with different triblock copolymers for in vitro release. Lysozyme concentration and its biological activity in the released sample were determined using a standard MicroBCA method and bacterial cell lysis method, respectively. The effects of varying block lengths and concentrations of copolymers on the in vitro release of lysozyme were evaluated. The release profiles from formulations showed a higher initial release followed by slower release up to 4 weeks. Increasing the block lengths of copolymers decreased burst release of lysozyme from $41.2 \pm 5.4\%$ to $16.1 \pm 3.9\%$. Increasing copolymer concentrations decreased the drug release. Lysozyme in the 4 weeks released samples retained most of its biological activity (>80%). It is feasible to deliver protein in biologically active form for longer duration by varying block lengths and concentrations of triblock copolymers.

Keywords: Controlled release; Protein; Thermosensitive; Biodegradable polymer; Lysozyme; Biological activity

1. Introduction

The tremendous growth in biotechnology and the completion of human genome sequencing have made large-scale production of therapeutic proteins a reality (Lee, 2002). The need to develop delivery systems

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that can control the release of proteins in biologically active form is now well acknowledged. Entrapment of proteins in biodegradable poly(lactide-co-glycolide) (PLGA) microspheres has been widely investigated as a technique to produce sustained release formulations for protein administration (Cleland and Jones, 1996; Chen et al., 1997; Shendrova et al., 1997). However, obtaining suitable protein release kinetics and preservation of protein integrity are still a problem in the PLGA system because most proteins do not maintain full activity when exposed to an organic solvent/water interface during preparation of microspheres (Bezemer et al., 2000a; Van de Weert et al., 2000a). Moreover, there are significant manufacturing challenges involved in production of microspheres which requires five to six major processing steps. Once injected, microsphere formulations may cause an acute tissue reaction (e.g., nodule) and, possibly transient irritation resulting in the presence of particles. In contrast, "smart" polymer based injectable aqueous solution is simple to prepare and forms an implant upon injection (Eeckman et al., 2004). Smart polymer solutions typically form an implant with a low surface area to volume ratio resulting in less total protein released from the surface. In addition, these formulations tend to spread into the tissue space to minimize tissue irritation.

Thermosensitive polymers, also called "intelligent" or "smart" polymers, have met with increasing interest during the past two decades, particularly in the field of controlled drug release (Eeckman et al., 2004). With these polymers, it is possible to administer the formulation as a solution, which then gels at body temperature. These injectable formulations allow ease of manufacture, single injections of high doses with small volumes, and enhanced protein stability (Cleland et al., 2001). Many commercially available block copolymers, such as Pluronics or poloxamers can undergo a temperature-induced reversible gel–sol transition upon heating or cooling of the aqueous solution. Poloxamer hydrogels perhaps represent the most extensively studied systems since they are commercially available in a wide range of molecular weights and block ratios (Ruel-Gariepy and Leroux, 2004). However, despite the clinical acceptance of poloxamers as solubilizer and thickening agents, these polymers have not met initial expectations as pharmaceutical and biomedical implant, mainly due to their non-biodegradability and inability to provide sustained drug delivery over more than just a few days (Katakam et al., 1997; Wenzel et al., 2002).

Thermoreversible block copolymers composed of poly(ethylene glycol) (PEG) (A) and biodegradable polyesters (B), such as polylactide (PLA), polyglycolide (PGA), PLGA, poly(ε-caprolactone) (PCL) and poly(ethylene terephthalate) (PET) were studied as controlled release drug carriers (Gilding and Reed, 1979; Casey et al., 1987; Cohn and Younes, 1988; Cerrai et al., 1989; Li and Kissel, 1993). PEG/PLGA hydrogels are particularly attractive systems for pharmaceutical applications since they are biodegradable and generally have a good safety profile. Compared to the widely utilized PLGA, protein delivery systems based on hydrophilic–hydrophobic block copolymers may have some important advantages. Incorporation of hydrophilic blocks in a hydrophobic polymer can be utilized to modify the degradation rate as well as the permeability of the matrix, leading to release kinetics which can be readily modulated by adjusting the copolymer compositions. Their compositions can be tailored to provide drug delivery over weeks or months after single injection (Jeong et al., 2000; Kim et al., 2001; Zentner et al., 2001; Jeong et al., 2002).

In this study, we investigated the suitability of PLGA–PEG–PLGA triblock copolymer as a matrix material to control the release of a model protein, lysozyme. For this reason, a series of ABA triblock copolymers (PLGA–PEG–PLGA) were synthesized in which the D,L-lactide/glycolide (D,L-LA/GA) ratios were varied. The structure and composition were characterized by ^{1}H NMR; molecular weight and molecular weight distribution were determined by gel permeation chromatography (GPC). Phase separation temperature in aqueous solution of the copolymer was investigated using an inverted test-tube method. Furthermore, in vitro release and biological activity of the model protein, lysozyme, was investigated from triblock copolymer formulations.

2. Materials and methods

2.1. Materials

Polyethylene glycol (PEG 1000) was purchased from Sigma (St. Louis, MO). D,L-Lactide and glycolide

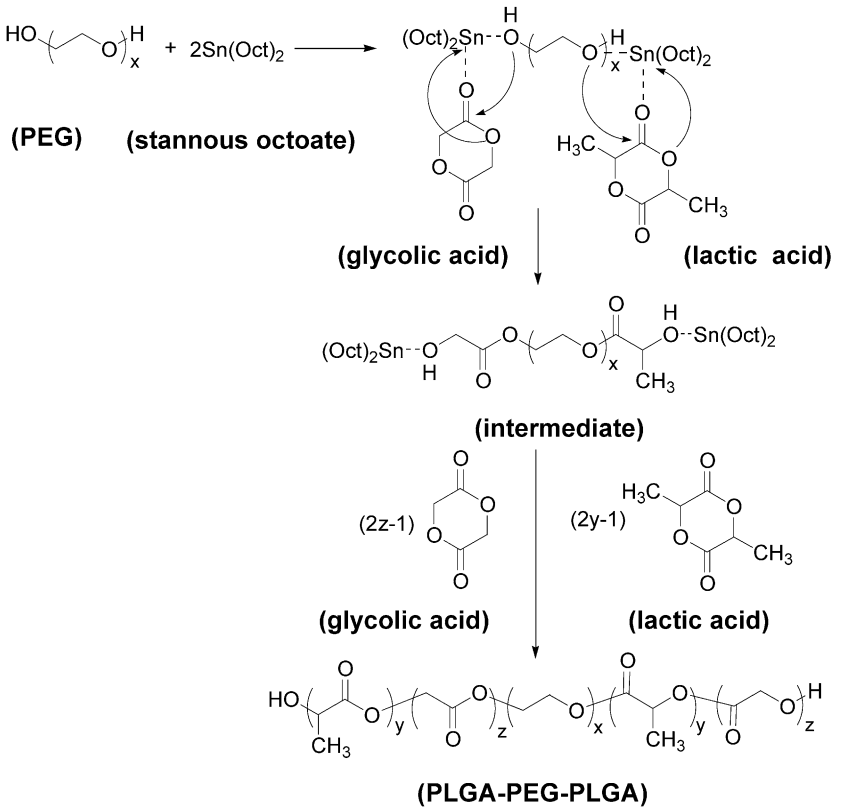

Fig. 1. The synthetic scheme of PLGA–PEG–PLGA triblock copolymer.

were purchased from Alfa Aesar (Ward Hill, MA) and used without further purification. Stannous 2-ethylhexanoate (Stannous Octoate) was obtained from Sigma and was used as received. Lysozyme (EC 3.2.1.17) from chicken egg white and Micrococcus Lysodeikticus (*Micrococcus luteus*) were purchased from Sigma. Micro BCA protein assay reagent kit was purchased from Pierce (Rockford, IL). All other chemicals used were of analytical grade.

2.2. Polymerizations

Triblock copolymers (PLGA–PEG–PLGA) were synthesized following the method described by Zentner et al. (2001). Fig. 1 shows the synthetic scheme of PLGA–PEG–PLGA triblock copolymers. A series of four triblock copolymers with different LA/GA ratios (2:1, 2.5:1, 3:1, 3.5:1) were synthesized and purified.

2.3. Characterization of the copolymers

2.3.1. ^{1}H NMR analysis

^{1}H NMR was used to determine the structure and composition of triblock copolymers. Spectra were recorded at 300 MHz on a Varian spectrometer at 25 °C. The solvent used was deuterated chloroform ($CDCl_3$). A tetramethylsilane (TMS) signal was taken as the zero chemical shift. Number average molecular weight and lactide to glycolide ratios were determined by ^{1}H NMR by integrating the signals pertaining to each monomer such as the peaks from CH and CH_3 of LA, CH_2 of ethylene glycol and CH_2 of GA.

2.3.2. Molecular weight evaluation

The molecular weight and molecular weight distribution of copolymers were determined by GPC. A Waters 515 (Milford, MA) apparatus equipped with a refractive index detector and two Styragel® HR4E and HR5E columns (Milford, MA) was used. The analyses

were performed at 20 °C, using tetrahydrofuran (THF) as an eluent, at a flow rate of 1 mL/min. GPC was calibrated with polystyrene standards.

2.3.3. Phase diagram of the copolymers

PLGA–PEG–PLGA copolymers having concentrations of 15, 20, 25, 30 wt%, respectively, were dissolved into distilled water in 4 ml vials. After equilibration at 4 °C overnight, copolymer solutions were immersed in a water bath equilibrated at given temperatures, ranging from 10 to 60 °C. The sol–gel transition was determined by inverting the vial horizontally after keeping the sample at a constant temperature for 10 min to allow the establishment of equilibrium. Sol to gel transition temperature was measured by test tube inverting method by increasing 2 °C/step (Jeong et al., 1999a).

2.4. Preparation of thermosensitive in situ gel depot forming formulations

Lysozyme (2.5%, w/v) was added to 30% (w/v) copolymer aqueous solution and homogenized at 8000 rpm for 30 s to form a homogeneous clear solution at room temperature or below. The polymer formulation was loaded into 1 ml syringe and pushed through 25-gauge needle to investigate its injectability.

2.5. In vitro release of lysozyme

A 1 ml polymer solution formulation of lysozyme was injected into a 20 ml test tube and transferred to 37 °C water bath for 10 min to form a gel, 15 ml of phosphate buffer saline (pH 7.4) containing NaN_3 (0.025%, w/v) was added to the tube as release medium. The tube containing gel was kept in reciprocal shaking water bath at 37 °C and 35 rpm for the entire period of study. A 5 ml aliquot was withdrawn at intervals from releasing media and replaced with the same amount of fresh releasing media. The amount of lysozyme in the released samples was determined by MicroBCA protein assay method using microplate (Steadman et al., 1992). Briefly, sample was centrifuged at 3000 rpm for 30 min. A 150 μl aliquot of suitably diluted supernatant/standard was mixed with 150 μl of working reagent solution made by mixing Micro BCA Reagent A, B, and C in the ratio of 50, 48, and 2, respectively. This mixture was incubated at 37 °C for 120 min, then cooled to room temperature, and used for measurement of absorbance at 570 nm by Dynex MRX & MRX Revelation TC model 96 well microplate reader (Vienna, VA). Sample from formulation without lysozyme was used as blank control for absorbance. Amount of lysozyme in the released sample was obtained from the standard curve and corrected for sample removal. The effect of different block lengths of copolymers on the lysozyme release was investigated. Also, the effect of copolymer concentrations on the lysozyme release was studied.

2.6. Biological activity of lysozyme by enzyme activity assay

For enzyme activity assay, a portion of vigorously shaken *M. luteus* stock suspension (0.01%, w/v) into phosphate buffer (66 mM, pH 6.15) was diluted so that it had an A_{450} between 0.2 and 0.6. A 2.5 ml of this diluted *M. luteus* solution was taken into a spectrophotometer cell and 0.1 ml of an appropriately diluted lysozyme sample was added to it. The rate of decrease of absorbance at A_{450} was monitored by UV spectrophotometer during a total incubation period of 2 min at 25 °C. The slope of the linear portion of the plot (between absorbance and time) in absorbance units per min gave the amount of lysozyme in enzyme unit (EU) (Shugar, 1952). The detailed calculation of unit of biologically active lysozyme was described by Singh and Singh (2004). Unit of biologically active lysozyme was determined by using the following formula:

$$\text{Units of lysozyme/ml sample} = \frac{(\Delta A_{450\text{nm}}/\text{minTest} - \Delta A_{450\text{nm}}/\text{minBlank})(\text{df})}{(0.001)(0.1)}$$

where df is the dilution factor, 0.001 the change in absorbance at A as per the unit definition, and 0.1 is the volume (in ml) of sample/standard used.

2.7. Data analysis

The results were expressed as a mean ± S.D. ($n = 4$). Statistical comparisons were made using Student's t-test. The level of significance was used as $p < 0.05$.

Table 1
Characterization of triblock copolymer PLGA–PEG–PLGA

Triblock copolymer (PLGA–PEG–PLGA)	NMR		GPC		
	Mn[a]	LA/GA[b]	Mn[c]	Mw[d]	Mw/Mn[e]
Copolymer 1	995–1000æ995	2.08	1187	1602	1.35
Copolymer 2	1125–1000æ1125	2.45	3002	3771	1.26
Copolymer 3	1350–1000æ1350	3.18	4622	5805	1.26
Copolymer 4	1400–1000æ1400	3.21	6072	7859	1.29

[a] Number average molecular weight determined by NMR.
[b] Molar ratio of lactic acid to glycolic acid determined by NMR.
[c] Number average molecular weight determined by GPC.
[d] Weight average molecular weight determined by GPC.
[e] Polydispersity determined by GPC.

3. Results and discussion

3.1. Characterization of triblock copolymers

3.1.1. ^1H NMR analysis

Various PLGA–PEG–PLGA triblock copolymers were prepared by the ring-opening polymerization of D,L-lactide and glycolide in the presence of α,ω-dihydroxy-terminated PEG and a small amount of stannous octoate. The characteristics of the triblock copolymers are listed in Table 1.

In order to gain insight into their chemical structure, the copolymers were analyzed using ^{1}H NMR spectrometry. A typical spectrum of PLGA–PEG–PLGA copolymer with its chemical structure is presented in Fig. 2. This spectrum was very similar to the reported spectrum and all the signals were assigned on the spectrum (Jeong et al., 1999b). The signals pertaining to PLGA–PEG–PLGA are found in $\delta = 5.20$ ppm (CH of LA, a), 1.55 ppm (CH_3 of LA, b), 4.80 ppm (CH_2 of GA, c), 3.65 ppm (CH_2 of ethylene glycol, d), 4.20 ppm (CH_2 of ethylene glycol, e), and 2.75 ppm (OH of LA, f). The complicated split in these peaks was due to the random copolymerization of glycolide and lactide. To obtain the number average molecular weight, the peaks at 5.20 ppm (CH of LA), 4.80 ppm (CH_2 of GA), 3.65 ppm (CH_2 of ethylene glycol), and 1.55 ppm (CH_3 of LA) were used. The detailed calculation can be obtained in the work of Jeong et al. (1999b).

3.1.2. Molecular weight evaluation

GPC was used to obtain the molecular weight and molecular weight distribution.

Fig. 3 represents the results of GPC analysis. The retention time of the triblock copolymer is about 18 min, the other two peaks in the chromatogram (retention time about 21 min) are from the solvent. The polydispersity of all copolymers was found to be about 1.3, which was shown a symmetric peak and had a relative narrow molecular weight distribution. Unimodal GPC trace with a low polydispersity value confirms the formation of triblock copolymers. All the quantitative data on weight average molecular weight (Mw) and polydispersity of the copolymers are listed in Table 1.

The molecular weight was controlled by fixing monomer (GA) to initiator (PEG) ratio and changing LA/GA ratio in the ring opening polymerization step. Molecular weight increased with the increased LA/GA ratio. Unimodal GPC trace with a low polydispersity of triblock copolymers suggested that purity was sufficient to study their physical properties.

3.1.3. Phase diagram of PLGA–PEG–PLGA triblock copolymers

The phase diagram of PLGA–PEG–PLGA triblock copolymers with different D,L-LA/GA ratios is shown in Fig. 4. PLGA–PEG–PLGA triblock copolymers demonstrated a thermoreversible sol–gel transition. It was reported that triblock copolymers based on a central PEG block (Mn 1000) and PLGA blocks (Mn 900–1600) show a temperature-dependent gel–sol transition in water. Beyond this range, the copolymers are either too hydrophobic or too hydrophilic to show thermoreversible gelation (Lee et al., 2001). All these four copolymers are within this range.

The sol–gel transition temperature was found to be a function of both the concentration and composition

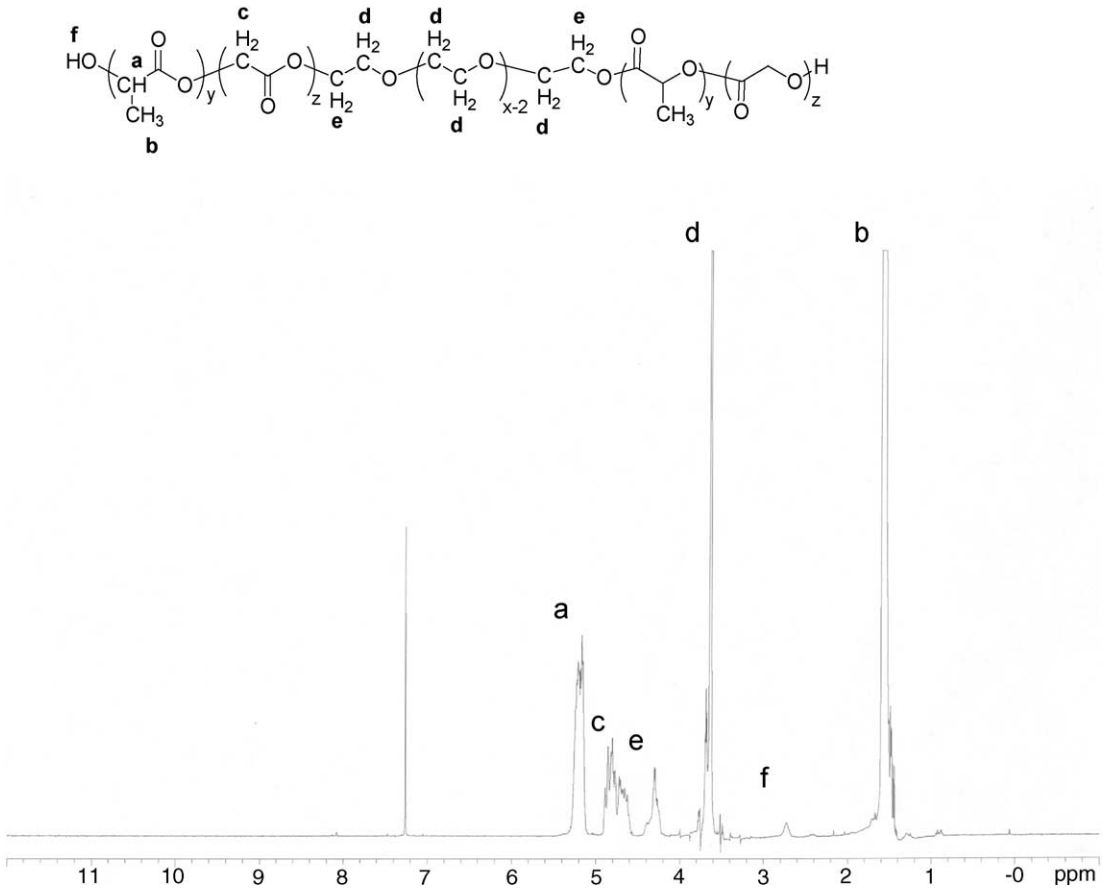

Fig. 2. The typical ^{1}H NMR spectra of PLGA–PEG–PLGA triblock copolymer (a) CH of LA, (b) CH_3 of LA, (c) CH_2 of GA, (d and e) CH_2 of ethylene glycol, and (f) OH of LA.

of the block copolymers. From 10 to 60 °C, all hydrogels presented three physical states: solution, gel, precipitate. In this study, transition between solution and gel is defined as sol–gel transition; transition between gel and precipitate is defined as gel–sol transition. Table 2 shows the sol–gel and gel–sol transition temperatures of triblock copolymers at different concentrations. When the copolymer concentration increased from 15% to 30%, sol–gel transition temperature decreased ∼2–8 °C and gel–sol transition

Table 2
Characterization of sol–gel transition and gel–sol transition temperature of triblock copolymer PLGA–PEG–PLGA

Triblock copolymer (PLGA–PEG–PLGA)	15%		20%		25%		30%	
	T_{lower}[a]	T_{upper}[b]	T_{lower}	T_{upper}	T_{lower}	T_{upper}	T_{lower}	T_{upper}
Copolymer 1	30	32	30	32	28	32	28	38
Copolymer 2	26	32	26	34	24	34	24	38
Copolymer 3	20	32	16	34	16	36	16	42
Copolymer 4	20	38	14	40	12	50	12	54

[a] T_{lower}, lower transition temperature from sol to gel.
[b] T_{upper}, upper transition temperature from gel to sol.

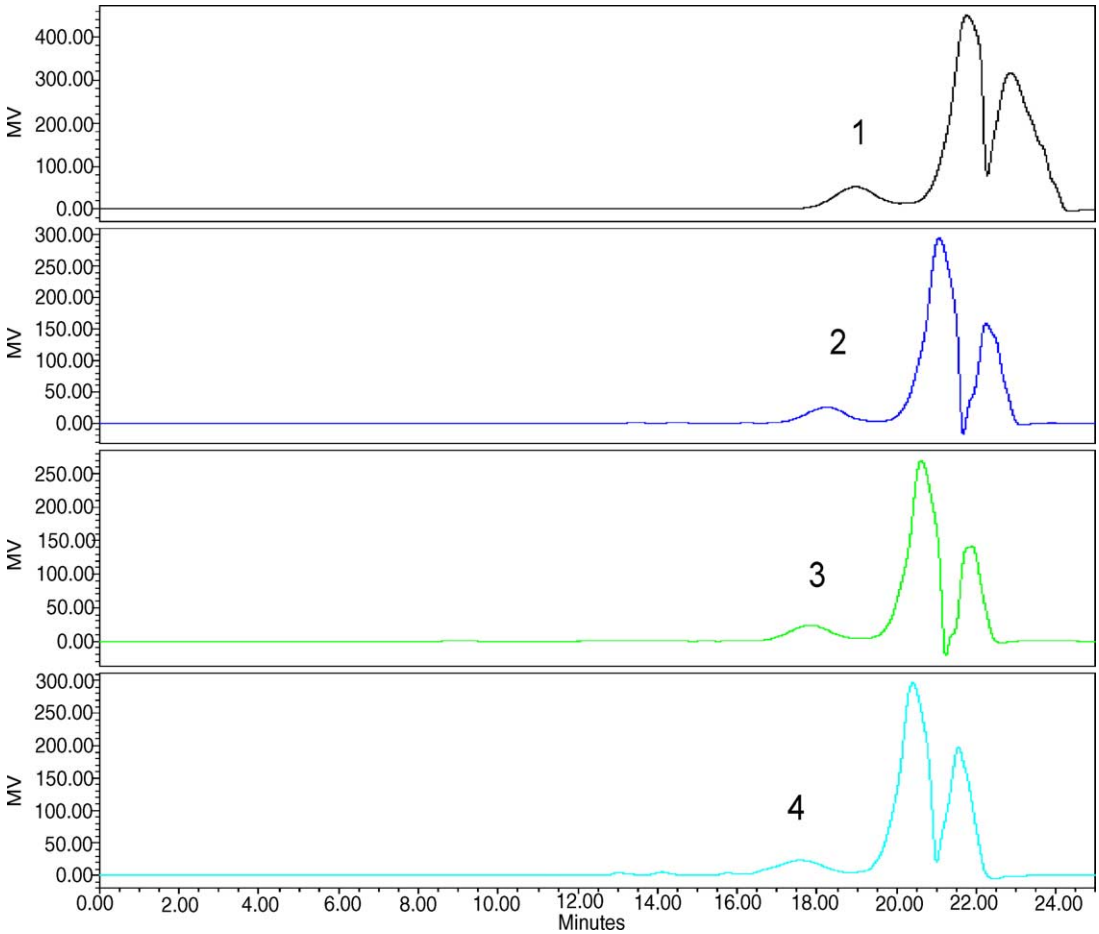

Fig. 3. The GPC spectrum of PLGA–PEG–PLGA triblock copolymer. (1) Copolymer 1, (2) copolymer 2, (3) copolymer 3, and (4) copolymer 4.

temperature increased ∼2–16 °C for all four copolymers.

Increasing polymer concentration caused higher micelle concentrations, gelation was induced by the packing of aggregated micelles, and the fast increase in the numbers of aggregated micelles induced the sol–gel transition at lower temperature. Thus, one can modify polymer concentration to meet specific requirements in drug delivery systems, for example, subcutaneous implantation or injection through catheter.

The phase diagram of these triblock copolymers in water demonstrates a critical gel concentration (CGC), a lower transition temperature curve from sol to gel and an upper transition temperature curve from gel to sol. An increase in the LA content in PLGA increases the hydrophobicity of PLGA blocks. A longer PLGA chain in the triblock copolymers induces a stronger hydrophobic interaction, leading to an increase in the association tendency. Hydrophobic PLGA blocks in copolymers can participate in different micelles, causing bridging which facilitates aggregation. More bridging connections between micelles can be produced when the two end blocks are located in different micelles, leading to a lower CGC (Lee et al., 2001). It is obvious that the strong interaction between hydrophobic PLGA blocks and the high bridging possibility between micelles lead to lower sol–gel transition temperatures, and higher gel–sol transition temperatures. Therefore, the gel zone in the phase diagram becomes larger with

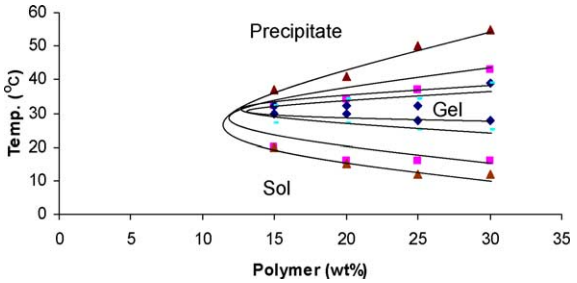

Fig. 4. The phase diagram of PLGA–PEG–PLGA triblock copolymer. Key: (♦) Copolymer 1, (▬) copolymer 2, (■) copolymer 3, and (▲) copolymer 4.

increasing PLGA block lengths in the triblock copolymers.

Micelle formation and micellar behavior are dependent on the polymeric molecular structure and its hydrophobic–hydrophilic balance in water. They play an important role in the gelation of ABA- or BAB-type copolymers. Lee et al. (2001) used dynamic light scattering (DLS) to determine the size distribution of micelles in the aqueous copolymer system, the results provided good evidence that the PLGA–PEG–PLGA (ABA) copolymer solution forms a gel via micelles but by a different mechanism, as described for BAB-type copolymer such as pluronic by Jeong et al. (1999a).

3.2. Preparation and characterization of polymer formulation

Injectability was first examined because it is a critical factor for drug delivery systems, as compared with implants that require surgery (Molina et al., 2001). All formulations easily passed through the 25-gauge needle at room temperature or below. Lysozyme (2.5%, w/v) was mixed with PLGA–PEG–PLGA triblock copolymers solutions in a 20 ml test tube to form a homogeneous clear solution at room temperature or below and formed gel at 37 °C. Fig. 5 shows the sol–gel transition.

3.3. In vitro release of lysozyme

Fig. 6 shows the in vitro release profile of lysozyme from different thermosensitive polymer gel formulations. The amount of lysozyme in day 1 released samples was treated as burst release. We found 41.2 ± 5.4, 38.4 ± 3.8, 27.3 ± 6.8, and $16.1 \pm 3.9\%$ burst release of lysozyme, respectively, from formulations with different block lengths of compolymers 1, 2, 3, and 4. Higher burst release was found with formulations containing shorter PLGA block lengths. Fig. 7 shows the in vitro release profile of lysozyme

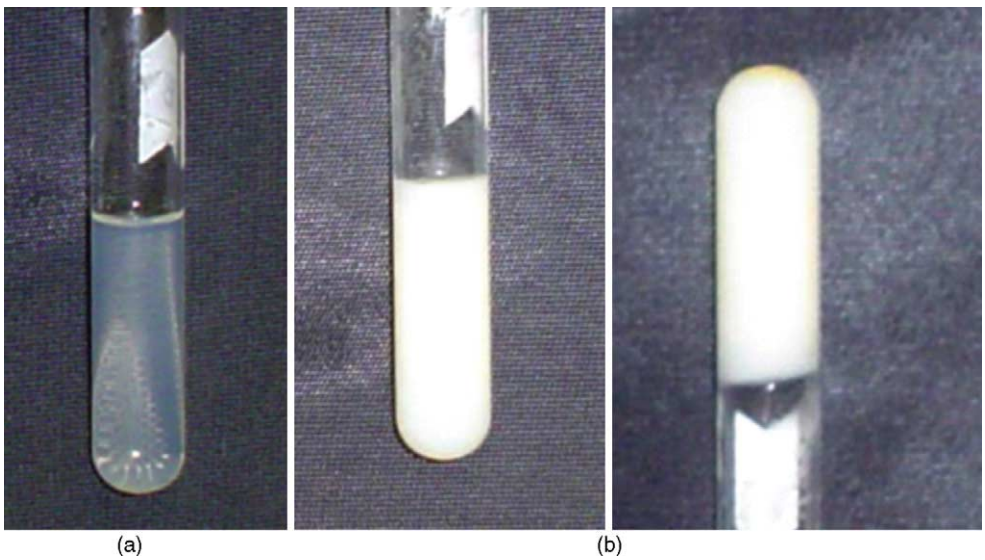

Fig. 5. Sol–gel transition of PLGA–PEG–PLGA triblock copolymers at different temperatures. (a) At room temperature or below (≤25 °C) and (b) at body temperature (37 °C).

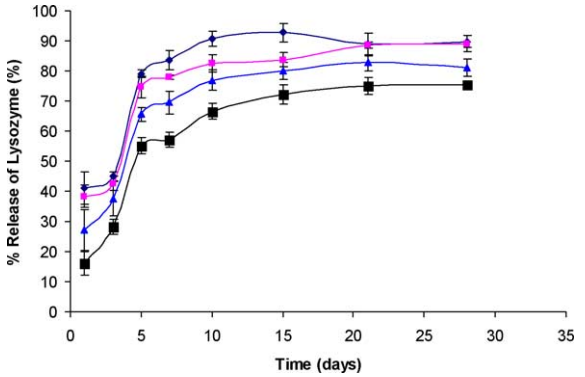

Fig. 6. Effect of varying block lengths of copolymers on the in vitro release of lysozyme from polymer formulations. Key: (♦) 30% (w/v) copolymer 1, (■) 30% (w/v) copolymer 2, (▲) 30% (w/v) copolymer 3, and (■) 30% (w/v) copolymer 4.

from gel formulations with different concentrations of copolymer 4. We found 22.0 ± 4.0, 19.1 ± 2.3, 17.5 ± 0.6, and 16.1 ± 3.9% burst release of lysozyme, respectively, from formulations containing 15, 20, 25, 30% (w/v) polymer concentration. Higher polymer concentration decreased burst release.

The release profile of lysozyme from the gel presents three phases: an initial burst release phase in which 16–42% of the entrapped lysozyme was released during 24 h, a sequential high release phase in which more than 50% of the drug was released in a continuous way during 15 days, and a plateau region in which very little lysozyme was released. Compared to the large burst release (50%) from microsphere formulation (Van de Weert et al., 2000b), this copolymer can decrease burst release to a relatively low level. The burst release of drugs was normally considered to be due to surface located drug and sequential drug release may be due to diffusion of drugs from polymers as well as due to erosion of polymers.

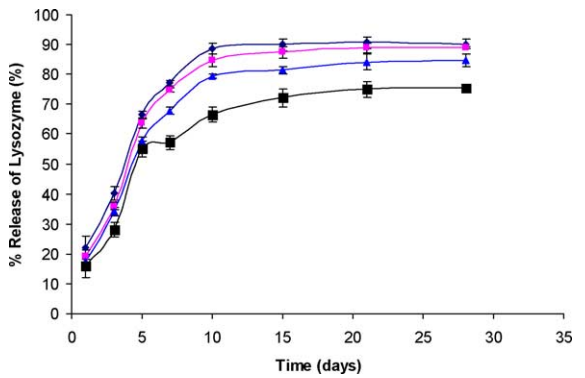

Fig. 7. Effect of varying polymer concentrations on the in vitro release of lysozyme from copolymer 4 formulations. Key: (♦) 15% (w/v), (■) 20% (w/v), (▲) 25% (w/v), and (■) 30% (w/v).

This hydrogel system is thought to have a core-shell structure in an aqueous environment. It has a flower-type micellar shape, in which the middle hydrophilic PEG chain makes a loop on the surface and hydrophobic PLGA resides in the core in order to decrease surface free energy (Jeong et al., 2004). Assuming a domain structure of the hydrogel, the partitioning of drug between the hydrophilic domain and the hydrophobic domain was considered to be a critical factor for drug release. At the sol–gel transition state, the system's volume will contract leading to the expulsion of the aqueous phase in which proteins are dissolved. This effect causes some initial drug burst and only those proteins associated with or dissolved in the hydrophobic core do not experience this push-out effect (Packhaeuser et al., 2004).

At the early stage, lysozyme released from gel formulations in a way mainly depending on the diffusion process. We expect to observe a consistent and complete release due to the progressive structural erosion of the copolymer. However, almost all the formulations showed incomplete release of lysozyme. The formation of protein aggregates and formation of acid degradation products from PLGA may be responsible for an incomplete and very slow protein release at the final stage (Lu and Park, 1995a,b; Bittner et al., 1998; Morlock et al., 1998). Ionic interaction and non-specific adsorption are also believed to be possible causes of the incomplete release (Park et al., 1998). We found that medium pH value dropped only about 1 unit (from 7.4 to 6.5) for the fourth week samples since we replaced the buffer solution frequently, but the internal pH within the degrading gels might be lower than the observed bulk medium pH. In order to elucidate the mechanism, more techniques such as gel electrophoresis (SDS-PAGE) and size-exclusion chromatography (SEC) need to be used to determine the formation of protein aggregates (Van de Weert et al., 2000b).

Tables 3 and 4 provide data on the specific enzyme activity of lysozyme in the fourth week released samples. We found significantly greater ($p < 0.05$) enzyme activity for lysozyme in all of the released samples in comparison to the control sample. Singh and

Table 3
Specific enzyme activity of lysozyme in the 28th day released sample from thermosensitive polymer formulations with different block lengths

Formulations	Specific enzyme activity (EU/mg) × 10^3 (mean ± S.D., $n = 4$)
Lysozyme (freshly prepared)	47.97 ± 3.32
Control	5.91 ± 1.40
Copolymer 1	40.00 ± 3.29[a]
Copolymer 2	40.21 ± 1.94[a]
Copolymer 3	43.37 ± 1.14[a]
Copolymer 4	44.49 ± 1.87[a]

Note: The formulation contained 2.5% (w/v) lysozyme and 30% (w/v) copolymers. Control = lysozyme (25 mg in releasing buffer) kept at 37 °C in oscillating water bath for 4 weeks.
[a] Significantly different from control ($p < 0.05$).

Singh (2004) found that increasing the hydrophilic content (Benzyl Alcohol) in a phase sensitive smart polymer system can increase the biological activity of lysozyme in the 2 weeks released sample. Ghaderi and Carlfors (1997) reported that the retained biological activity of the entrapped lysozyme was increased from 59 to 83% when the PLGA concentration was increased from 4.5 to 37% and a high concentration of lysozyme (30%) was used. The improvement was probably caused by the higher rate of solidification of the microspheres as a result of the higher PLGA concentration to protect the lysozyme from the contact of organic phase for the shorter duration during the preparation of microspheres. However, we didn't see any significant difference in enzyme activity between copolymers with different block lengths as well as with different concentrations, this might be due to the fact that we did not use any organic solvents in the formulations. PLGA–PEG–PLGA can maintain most of the enzyme activity of lysozyme, this may due to the lysozyme being protected in an amorphous core of the PLGA. Determination of protein conformation inside polymeric matrices has long been difficult. For most analytical methods, the protein must first be extracted from the matrix, a process that may induce conformational changes itself. Kang et al. (2002) developed a novel technique, using differential scanning calorimetry (DSC), to assess the conformational stability of lysozyme in the primary emulsion phase of the PLGA microspheres preparation process. Compared to these techniques, the enzyme activity of lysozyme is relatively simple to measure. Lysozyme is a relatively small enzyme (14.6 kDa), it preferentially hydrolyses the β-1,4 glucosidic linkages between *N*-acetylemuramic acid and *N*-acetylglucosamine which occur in the mucopeptide cell wall structure of certain micro-organisms. The rate of lysis of this specific linkage in the cell walls of micro-organisms can be measured and is known as the specific biological activity of lysozyme (Ghaderi and Carlfors, 1997). It was reported that the PLGA matrix can negatively affect the stability of the incorporated protein drug (Crotts and Park, 1997; Crotts et al., 1997). The hydrophobic nature of PLGA is responsible for protein adsorption onto the polymer surface, denaturation and aggregation. Using amphiphilic block copolymers as matrix material for a protein delivery system probably has prevented these problems (Bezemer et al., 2000b).

Table 4
Specific enzyme activity of lysozyme in the 28th day released sample from thermosensitive polymer formulations with different polymer concentrations

Formulations	Specific enzyme activity (EU/mg) × 10^3 (mean ± S.D., $n = 4$)
Lysozyme (freshly prepared)	50.41 ± 6.20
Control	7.83 ± 2.60
Copolymer 4 (15%)	41.03 ± 3.75[a]
Copolymer 4 (20%)	43.92 ± 5.74[a]
Copolymer 4 (25%)	46.52 ± 0.80[a]
Copolymer 4 (30%)	44.49 ± 1.87[a]

Note: The formulation contained 2.5% (w/v) lysozyme. Control = lysozyme (25 mg in releasing buffer) kept at 37 °C in oscillating water bath for 4 weeks.
[a] Significantly different from control ($p < 0.05$).

4. Conclusions

This study describes the self-association behavior of a novel PEG-based amphiphilic triblock copolymer, which consists of LA/GA as hydrophobic moieties joined at the two ends of the chain. Depending on the block length and the copolymer concentration, different phase diagrams and protein release profiles were achieved. Thermosensitive polymer delivery systems may be used for controlled delivery of proteins in a biologically active form for an extended period of time by adjusting the PLGA–PEG–PLGA triblock copolymer compositions and concentrations.

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