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Biodegradable and biocompatible thermosensitive polymer based injectable implant for controlled release of protein

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ABSTRACT

This study was aimed at developing a suitable controlled release system for proteins by modifying the structure of thermosensitive copolymer monomethoxy poly(ethylene-glycol)-*co*-poly(D,L-lactide-*co*-glycolide)-*co*-monomethoxy poly(ethylene-glycol) (mPEG–PLGA–mPEG). Eleven mPEG–PLGA–mPEG copolymers were synthesized and characterized by ^{1}H NMR and gel permeation chromatography (GPC). Thermosensitivity of the copolymers was tested using the tube inversion method. Four of the eleven synthesized copolymers were dissolved in water as injectable solutions at room temperature which turned into gels abruptly at body temperature (37 °C), indicating the potential use as *in vivo* drug delivery system. Lysozyme was used as a model protein to study *in vitro* release characteristics of the copolymer based delivery system. The copolymer based formulations released lysozyme (quantified by micro-BCA protein assay) over 10–30 days, depending on copolymer structure. The released lysozyme was confirmed to conserve its structural stability by differential scanning calorimetry (DSC) and circular dichroism (CD), and biological activity by specific enzyme activity assay. Furthermore, the copolymer based formulations showed excellent biocompatibility as tested by MTT assay and *in vivo* histological evaluation. Therefore, the copolymers controlled the *in vitro* release of lysozyme while conserving protein stability and biological activity, indicating that it is an appropriate delivery system for long term controlled release of proteins.

1. Introduction

There are several therapeutically important proteins that have been discovered in the past two decades since the completion of human genome sequencing and functional studies (Venter, 2001); meanwhile, the tremendous advancements in biotechnology made the large-scale production of these proteins a reality (Lee, 2002). Unfortunately, the clinical application of many of these therapeutic proteins is limited by the lack of suitable delivery systems. So far, delivery by subcutaneous injection is the most commonly used method of administration of protein. In order to maintain the therapeutic effect, frequent injection is required, due to the short half-life of proteins *in vivo* (Pitt, 1990), but low patient compliance makes this administration method undesirable. Thus, a suitable delivery system of protein is warranted in order to deliver protein continuously in its active form over a longer period after a single injection.

Recently, thermosensitive polymer-based injectable *in situ* gel forming drug delivery systems have attracted great research interest because of their advantages, such as ease of manufacturing, avoidance of organic solvents, convenient application, and sustained release of incorporated drug (Hoffman, 1987; Stile et al., 1999). Poly(*N*-isopropylacrylamide) (poly-NIPAAM) is the prototype of thermosensitive polymers. However, due to its toxicity, poly-NIPAAM is not used for drug delivery (Bae et al., 1987; Schild, 1992). The first thermosensitive polymer approved by the FDA was triblock poly(ethylene oxide)-*co*-poly(propylene oxide)-*co*-poly(ethylene oxide) (PEO–PPO–PEO) copolymer, also called "Pluronics®" or "Poloxamer" (Merril and Pekala, 1987; BASF, 1993). Nonetheless, except for its use as surfactant and thickening agent in pharmaceutical products, PEO–PPO–PEO failed to meet expectations for pharmaceutical implants, due to non-biodegradability and occurrence of side effects (Wang and Johnston, 1991; Muller et al., 1997; Wasan et al., 2003). In 1997, MacroMed Inc. developed a type of biodegradable thermosensitive triblock copolymer by replacing the hydrophobic non-degradable PPO block of poloxamer with a biodegradable poly(D,L-lactide) (PLA) block. The new PEO–PLA–PEO (M_w 5000–2040–5000 Da) triblock copolymer was found to control the release of dextran, a high molecular weight hydrophilic molecule, for 12 days (Jeong et al., 1997). Although the PEO–PLA–PEO/water system exhibits sol–gel transition at body temperature (37 °C), this system can only be loaded with

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therapeutic agents at an elevated temperature of 45 °C at which it exists as a Newtonian fluid. This physicochemical property limits its application in protein delivery since most proteins get denatured at high temperatures.

Later, MacroMed Inc. discovered that the thermosensitive sol–gel transition of the copolymer/water system is directly related to the block composition and arrangement of the copolymer (Jeong et al., 1999a,b). After replacing the PLA block with PLGA block, modifying the block length of PEO, and rearranging the block sequence, MacroMed reported that both mPEG–PLGA–mPEG and PLGA–PEG–PLGA (Regel®) are thermosensitive liquid drug carrier systems which can be loaded with therapeutic agents at a temperature lower than 30 °C with a sol–gel transition properties at 37 °C. PLGA–PEG–PLGA (Regel®) has been studied extensively, due to the ease of one-step synthesis. Unfortunately, Regel® system is usually required to be prepared at the temperature lower than room temperature for injectability and was reported to control the release of loaded protein for only ~7 days, further increasing the length of hydrophobic PLGA block caused protein aggregation in the formulations (Zentner et al., 2001; Chen et al., 2005a).

Due to the different gelation mechanism from Regel®, mPEG–PLGA–mPEG/water system was claimed to be able to load with therapeutic agents at room temperature with abrupt sol–gel transition at ~30 °C. Such a special sol–gel transition of the mPEG–PLGA–mPEG/water system not only avoids the high temperature denaturation of loaded protein but also provides convenience in administration because of the system's suitable injectability at room temperature. mPEG–PLGA–mPEG (M_w 550–2810–550 Da) was found to continuously release a hydrophilic drug, ketoprofen, for 3 days and another larger mPEG–PLGA–mPEG (M_w 12,798 by GPC) was reported to release loaded pDNA for ~14 days (Jeong et al., 2000; Li et al., 2003). So far, no further investigation has been published for the application of mPEG–PLGA–mPEG in the controlled release of proteins. According to the reported literature, the block length of biodegradable triblock copolymers has significant effects on the initial burst and the release duration of incorporated therapeutic agents, for two reasons: (1) proper ratio of the length of hydrophilic mPEG block and hydrophobic PLGA block induces less push-out effect by increasing gel's stability, and (2) larger PLGA block results in a controlled release for longer duration of loaded therapeutic agents by slower degradation (Jeong et al., 1999a,b; Chen et al., 2005a; Chen and Singh, 2005b). Thus, extension of the length of PLGA block while conserving the thermosensitivity of the copolymer and stability of formed gel is necessary to produce a suitable delivery system for controlled release of macromolecules over a long period.

The purpose of the present study was to further modify the block length of mPEG–PLGA–mPEG copolymer for extending the release of incorporated protein and to investigate the effects of the copolymers on protein stability. Eleven mPEG–PLGA–mPEG triblock copolymers were synthesized having serially increased length of both hydrophilic mPEG and hydrophobic PLGA block, in order to find a copolymer consisting of the longest hydrophobic PLGA block while retaining the system's injectability at room temperature, sol–gel transition property at 37 °C and satisfactory gel stability. The copolymers with appropriate thermosensitive sol–gel transition property were selected for controlled release formulation of a model protein, lysozyme, and for evaluating the effect of copolymers on stability and activity of lysozyme. Moreover, the copolymers which showed better controlled release of lysozyme were examined for their *in vitro* biocompatibility using MTT assay and *in vivo* biocompatibility by histological examination of the skin tissue at injection sites.

2. Materials and methods

2.1. Materials

Monomethoxy poly(ethylene glycol) (mPEG550 and mPEG750) was purchased from Sigma (St. Louis, MO, USA). D,L-Lactide was obtained from TCI (Tokyo, Japan). Glycolide was bought from Maybridge (Cornwall, UK). Lysozyme (EC 3.2.1.17) from chicken egg white and *Micrococcus Lysodeikticus* (*Micrococcus luteus*) were purchased from Sigma (St. Louis, MO, USA). Micro-BCA protein assay reagent kit was purchased from Pierce (Rockford, IL, USA). Human embryonic kidney (HEK 293) cell line was obtained from American Type Culture Collection (Rockville, MD, USA). All other chemicals used were of analytical grade.

2.2. Copolymer synthesis

The triblock copolymer (mPEG–PLGA–mPEG) was synthesized by ring-opening polymerization following diblock condensation (Jeong et al., 1997; Singh et al., 2007a). Briefly, lactide and glycolide were polymerized onto mPEG chain to produce mPEG–PLGA diblocks which were then connected by the coupling agent isophorone diisocyanate (IPDI) to generate triblock mPEG–PLGA–mPEG copolymer. Fig. 1 shows the modified synthetic scheme of mPEG–PLGA–mPEG triblock copolymer. According to previously reported investigations, larger hydrophobic block sustained copolymer degradation over a longer duration, thereby resulting in the controlled release of incorporated therapeutic agents over a longer period (Chen et al., 2005a). Moreover, the ratio of the length of mPEG and PLGA blocks affected the thermosensitive sol–gel transition in a critical way (Packhaeuser et al., 2004). Thus, the lengths of both mPEG and PLGA blocks were elongated carefully, in order to find the copolymer with longest hydrophobic PLGA block length while conserving the copolymer/water system's injectability at room temperature, sol–gel transition and gel stability at 37 °C. The different block lengths of triblock copolymers were achieved by varying the reaction initiator mPEG (mPEG550 or mPEG 750) and adjusting the feeding ratio of mPEG to monomers (lactide and glycolide).

2.3. Copolymer characterization

2.3.1. ^1H NMR analysis

^1H NMR (Varian Unity, 300 MHz) was used to determine the structural composition of synthesized copolymers. All spectra were recorded at 25 °C in deuterated chloroform ($CDCl_3$) and tetramethylsilane (TMS) signal was taken as the zero chemical shift. Number average molecular weight (M_n) and the ratio of lactic acid (LA) to glycolic acid (GA) portions were calculated by integrating the signals pertaining to each monomer, such as the peaks from CH_3 of LA (at 1.55 ppm), CH_3 of mPEG end group (at 3.38 ppm), CH_2 of mPEG (at 3.65 ppm), CH_2 of GA (at 4.80 ppm), and CH of LA (at 5.20 ppm) (Jeong et al., 1999a).

2.3.2. Gel permeation chromatography (GPC)

GPC was used to determine molecular weight distribution of synthesized copolymers. The measurement was carried out on a Waters 515 (Milford, MA) apparatus equipped with a refractive index detector and two Styragel® HR4E and HR5E columns (Milford, MA). The analyses were performed at 30 °C, using tetrahydrofuran (THF) as an eluant at a flow rate of 1 ml/min. Polystyrenes having molecular weight of 162–6,035,000 Da were used as standards for molecular weight calibration.

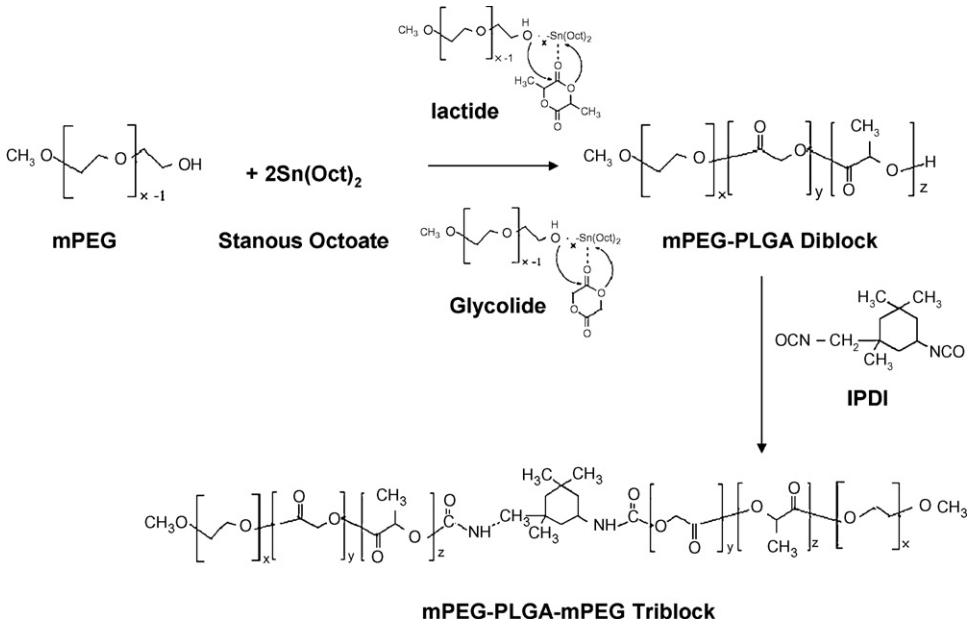

Fig. 1. The synthetic scheme of mPEG–PLGA–mPEG triblock copolymers following the Jeong's procedure with modifications (Jeong et al., 1997).

2.3.3. Thermosensitive sol–gel transition of copolymer solutions

mPEG–PLGA–mPEG triblock copolymers were dissolved in distilled water at a series of concentrations of 40%, 30%, 20%, 10% and 5% (w/v). After equilibration at 4 °C for 12 h, vials containing copolymer solutions were immersed in a water bath equilibrated at designated temperatures. After equilibration at a constant temperature for 10 min, the sol–gel transition was determined by inverting the tube for 1 min until no fluidity was observed (Jeong et al., 1999b). The testing temperatures ranged from 10 to 90 °C with the temperature increments of 2 °C/step. The determined critical gelation temperatures (CGT) and critical gelation concentrations (CGC) of triblock copolymers were depicted in phase diagrams.

2.4. Preparation of thermosensitive in situ gel forming formulations for lysozyme

Copolymer was dissolved in distilled water at a concentration of 40% (w/v). Model protein lysozyme (2%, w/v) was added to the copolymer solution and homogenized at a speed of 8000 rpm for 3 s at room temperature. The solution formulation of lysozyme was pushed through a 25-gauge needle to test its injectability.

2.5. In vitro release of lysozyme

An aliquot of 0.5 ml solution formulation was injected into a 10 ml tube which was then incubated at 37 °C for gel formation. Phosphate buffer saline (PBS, 0.01 M, pH 7.4) containing NaN_3 (0.02%, w/v) was added into the tube as a release medium. The tube was incubated in a reciprocal shaking water bath at 37 °C and 35 rpm during the release studies. Samples were withdrawn periodically and replaced with the same amount of fresh release medium. The amount of released protein was determined by micro-BCA protein assay. An aliquot of 150 μl suitably diluted sample was mixed with 150 μl of working reagent (micro-BCA reagent A, B and C in a volume ratio of 50:48:2). The mixture was incubated at 37 °C for 2 h and then cooled down to room temperature. Absorbance at 570 nm was measured by Dynex MRX Revelation TC model 96-well microplate reader (Vienna, VA). The standard sample of lysozyme was prepared in PBS within the concentration range of 3–80 μg/ml for standard calibration curve.

2.6. Conformational stability and biological activity studies of lysozyme

2.6.1. Conformational stability study by differential scanning calorimetry (DSC)

DSC measurements were carried out on an ultra-sensitive scanning calorimeter (VP-DSC, MicroCal, Northampton, MA). All the samples and references were degassed by stirring under vacuum before loading into the sample cell and reference cell. The heat flow required for thermally balancing the sample cell and reference cell was recorded from 25 to 95 °C. A scan rate of 1.5 °C/min was used for all samples. The release medium background was subtracted from lysozyme sample scan during data analysis. The transition temperature (T_m) and the calorimetric enthalpy change (ΔH) of thermal denaturation were used for evaluating conformational stability of lysozyme. All thermodynamic values were averaged over four measurements. Data analysis was performed using Origin5.0® software (OriginLab, Northampton, MA).

2.6.2. Secondary structure stability study by circular dichroism (CD)

CD measurement was performed in a quartz 0.1 cm cell, using a Jasco J-815 CD spectrophotometer (Jasco, Tokyo, Japan). CD signals were recorded from 260 to 200 nm at 25 °C using a band width of 1 nm and a scanning rate of 100 nm/min. All spectra were the average of five consecutive scans. Fresh release medium (PBS) was scanned in the same wavelength range for obtaining a baseline to eliminate the background interference. The molar ellipticity was calculated using the following equation:

$$\text{molar ellipticity } [\theta] = \frac{\theta}{Cl}$$

where θ was the ellipticity in mdeg; l was the pathlength (0.1 cm) of the cell and C was the concentration of protein sample in mmol/l.

2.6.3. Biological activity of lysozyme by enzymatic method

Micrococcus luteus (0.01%, w/v) was suspended into phosphate buffer (66 mM, pH 6.15) and diluted to obtain an A_{450} between 0.2 and 0.6. A 0.1 ml aliquot of appropriately diluted lysozyme sample was mixed with 2.5 ml of prepared *Micrococcus luteus* suspension

in a quartz cell (path length 1 cm), which was then immediately placed into a spectrophotometer. The rate of decrease of absorbance at 450 nm was monitored by the UV spectrophotometer during a total period of 2 min at 25 °C. The slope of the linear portion in the plot of absorbance against time provided the amount of lysozyme in enzyme unit (EU) (Shugar, 1952; Al-Tahami et al., 2006; Singh et al., 2007a). Units of biological active lysozyme were calculated based on EU, using the following equations:

$$\text{Units of lysozyme/ml sample} = \frac{(\Delta A_{450\text{nm}}/\text{min Test} - \Delta A_{450\text{nm}}/\text{min Blank}) \times (\text{df})}{0.001 \times 0.1}$$

$$\text{Units of lysozyme/mg} = \frac{\text{Units of lysozyme/ml sample}}{\text{mg lysozyme/ml sample}}$$

In the equation, df was the dilution factor and 0.001 was from the definition of lysozyme unit as one unit will produce a $\Delta A_{450\,\text{nm}}$ of 0.001 per minute at pH 6.15 and 25 °C using a suspension of *Micrococcus luteus* as substrate in a 2.6 ml reaction mixture. The volume in milliliters of lysozyme sample used was 0.1 ml. The biological activity of released lysozyme sample was compared with native lysozyme sample (freshly prepared lysozyme solution in PBS) to evaluate the effect of polymeric formulation on lysozyme's biological activity.

2.7. Biocompatibility of copolymer based delivery system

2.7.1. In vitro biocompatibility

The *in vitro* biocompatibility of triblock copolymers was investigated by MTT assay using human embryonic kidney cells (HEK 293). Because living cells can reduce a water soluble yellow dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)) to a water insoluble purple formazan product by mitochondrial succinate dehydrogenases, the absorbance of the formazan product at 570 nm can be used to measure the cell viability (Ignatius and Claes, 1996; Singh and Singh, 2007b). In this study, copolymer extracts were prepared by incubating the copolymer formulation in phosphate buffer saline (PBS, pH 7.4) for degradation at 37 and 70 °C over 10 days and then the pH was adjusted to 7.4 using 1 M NaOH. Copolymer extracts and fresh PBS were filtered through a 0.2 μM membrane and diluted serially with growth medium (modified Eagle's minimum essential medium supplemented with 10% of fetal bovine serum) at various dilution ratios. Then copolymer extracts, fresh PBS as well as their diluents were added into 96-well plates containing HEK293 cells. Cells treated with the same amount of growth medium were used as a control group. The plates were incubated at 37 °C in a humidified 5% CO_2 atmosphere for 24, 48, and 72 h. At indicated time points, 20 μl of MTT solution (5 mg/ml in PBS) was added into each well followed by 4 h incubation. After culture medium was suctioned completely, 100 μl per well of dimethyl sulfoxide (DMSO) was added to dissolve the formed formazan crystals. The absorbance that correlated with the number of viable cells in each well was measured by an MRX-Microplate Reader at 570 nm. Cell viability of the control group was considered as 100%. The absorbance of groups treated with copolymer extract, PBS as well as the diluents was compared with that of the control group to obtain the percentage of cell viability.

2.7.2. In vivo biocompatibility

In vivo bicompatibility study was carried out using female Wistar rats (Harlan, Indianapolis, IN). The skin tissue from the injection site was evaluated for the biocompatibility of the polymeric delivery system. In this study, 300 μl of blank polymeric delivery system was injected subcutaneously at the back of the neck of rats. Rats were euthanized at 1 day, 1 week, 2 weeks, 1 month, 2 months, and 3 months after treatments. Skin tissue from the injection site was surgically removed, fixed in 10% neutral buffered formalin for 48 h, dehydrated in a graded series of alcohol and embedded in paraffin. Transverse sections (5-μm thick) were prepared using rotatory microtome and mounted on glass slides followed by counter staining with hematoxylin and eosin. The slides were examined under a light microscope for any signs of acute and chronic inflammations, such as tissue granulation, fibrous capsule formation, and fibrosis.

2.8. Data analysis and statistics

Analysis of variance (ANOVA) and student's *t*-test were used for statistical comparison. A probability value less than 0.05 was considered significant.

3. Results

3.1. Copolymer characterization

3.1.1. Structure and molecular weight analysis using ^1H NMR

Eleven copolymers were synthesized following the scheme shown in Fig. 1. A typical ^1H NMR spectrum of synthesized copolymers was presented in Fig. 2, which was similar to the reported spectrum of mPEG–PLGA–mPEG triblock copolymer (Jeong et al., 1999a). The characteristic peaks pertaining to mPEG–PLGA–mPEG triblock copolymer were found at 1.55 ppm (CH_3 of LA), 3.38 ppm (CH_3 of mPEG end group), 3.65 ppm (CH_2 of mPEG), 4.30 ppm (connecting CH_2 group between PLGA and mPEG), 4.80 ppm (CH_2 of GA), and 5.20 ppm (CH of LA). Integrations of the peaks at 1.55 ppm (CH_3 of LA), 3.38 ppm (CH_3 of mPEG end group), 3.65 ppm (CH_2 of mPEG), and 4.80 ppm (CH_2 of GA) were used to calculate the number average molecular weight (M_n) (Table 1). Copolymers from P1 to P5 had mPEG block in the same length of 550 Da with gradually elongated PLGA block from 2741 to 4112 Da. Copolymers from P6 to P11 comprised of larger mPEG block of 750 Da with serially enlarged PLGA block from 3839 to 5066 Da. The ratio of LA to GA moieties was fixed at ~3 for all copolymers.

3.1.2. Molecular weight distribution analysis by gel permeation chromatography (GPC)

A unimodal GPC trace was found for all synthesized copolymers (graphs not shown). The progressively increased molecular

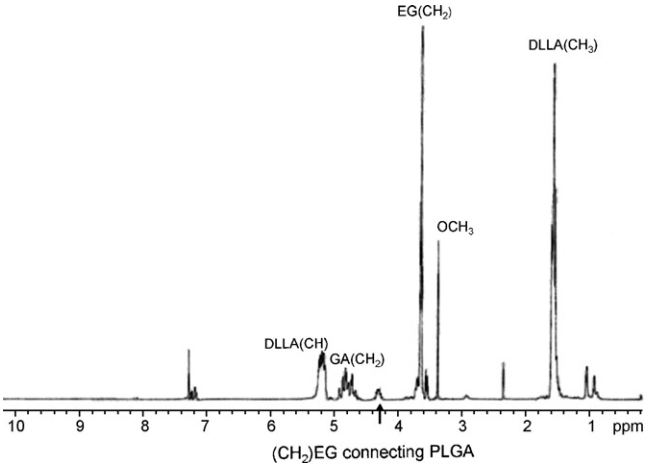

Fig. 2. ^1H NMR spectrum of synthesized mPEG–PLGA–mPEG triblock copolymer.

Table 1
^{1}H NMR and GPC characterization of synthesized copolymers

Triblock copolymer mEPG–PLGA–mPEG	^{1}H NMR	GPC			
	Structure	M_n[a]	M_n[b]	M_w[c]	PDI[d]
P1	EG_{12}–$L_{30}G_{10}$–EG_{12}	3841	3561	4163	1.17
P2	EG_{12}–$L_{35}G_{12}$–EG_{12}	4318	3761	4392	1.17
P3	EG_{12}–$L_{39}G_{13}$–EG_{12}	4715	4057	4728	1.16
P4	EG_{12}–$L_{41}G_{14}$–EG_{12}	4866	4524	5312	1.17
P5	EG_{12}–$L_{45}G_{15}$–EG_{12}	5212	4973	5994	1.21
P6	EG_{17}–$L_{42}G_{14}$–EG_{17}	5339	5648	6655	1.18
P7	EG_{17}–$L_{45}G_{15}$–EG_{17}	5613	5985	7144	1.19
P8	EG_{17}–$L_{48}G_{16}$–EG_{17}	5887	6427	7770	1.21
P9	EG_{17}–$L_{50}G_{17}$–EG_{17}	6089	6761	8376	1.24
P10	EG_{17}–$L_{52}G_{18}$–EG_{17}	6291	6807	8238	1.21
P11	EG_{17}–$L_{55}G_{19}$–EG_{17}	6566	7054	8590	1.22

Note: EG: mPEG block of mPEG–PLGA–mPEG triblock copolymer. The subscript indicates the number of repeat units of ethylene glycol in the copolymer, LG: PLGA block of mPEG–PLGA–mPEG triblock copolymer. The subscript indicates the number of repeat units of lactic acid (L) and glycolic acid (G) in the copolymer.

[a] Number average molecular weight obtained from ^{1}H NMR analysis.
[b] Number average molecular weight obtained from GPC measurements.
[c] Weight average molecular weight obtained from GPC measurements.
[d] Polydispersity index obtained from GPC measurements.

weights [both number average molecular weight (M_n) and weight average molecular weight (M_w)] were observed by GPC, which were in accordance with the ^{1}H NMR analysis (Table 1). The molecular weight distribution of copolymers was expressed as polydispersity index (PDI = M_w/M_n). All synthesized copolymers were observed to have a low polydispersity index (close to 1). A unimodal GPC trace and low polydispersity index further confirmed the formation of triblock copolymers as well as sufficient purity for further study of copolymer thermosensitivity.

3.1.3. Thermosensitivity characterization of mPEG–PLGA–mPEG copolymers

The thermosensitive sol–gel transition of mPEG–PLGA–mPEG copolymer solutions was summarized by phase diagrams (Fig. 3). Copolymer P1 (EG_{12}–$L_{30}G_{10}$–EG_{12}), P2 (EG_{12}–$L_{35}G_{12}$–EG_{12}), P6 (EG_{17}–$L_{42}G_{14}$–EG_{17}) and P7 (EG_{17}–$L_{45}G_{15}$–EG_{17}) were completely dissolved in distilled water as clear solutions up to polymer concentration of 40% (w/v) at 4 °C. These polymeric solutions showed a fast sol–gel–sol transition in response to the elevated temperature from 10 to 90 °C. Other copolymers were soluble at a concentration below 15% (w/v) but failed to exhibit the thermosensitive sol–gel transition. Thus, phase diagrams were used to depict the sol–gel transitions of copolymer P1, P2, P6 and P7 solutions only. The transition temperature was found to be a function of both copolymer concentration and structural composition. In the temperature range of 10–90 °C, copolymer solutions at concentrations higher than 15% (w/v) existed in three physical states: solution, gel, and precipitate. At a given copolymer concentration, the minimal temperature required for the sol–gel transition of copolymer solution was defined as lower critical gelation temperature (LCGT), and the temperature required for gel to precipitate was referred to as upper critical gelation temperature (UCGT). As shown in Fig. 3, an increase in the copolymer concentration resulted in a decrease in the LCGT with a concurrent increase in the UCGT. Besides the effects of copolymer concentration, structural composition of the triblock copolymers also showed significant influence on the sol–gel–precipitate transitions. An increase in the mPEG block length from 550 Da (EG_{12}) to 750 Da (EG_{17}) dramatically shifted both sol–gel and gel–precipitate transitions to higher temperature ($\Delta T = \sim 10$ to 12 °C). An increase in the PLGA block length from 2741 Da ($L_{30}G_{10}$, copolymer P1) to 3218 Da ($L_{35}G_{12}$, copolymer P2), keeping the mPEG block length constant (550 Da), resulted in the shift of sol–gel transition to lower temperature and the gel–precipitate transition to a higher temperature. The same phenomenon was also observed for copolymers P6 and P7. Furthermore, the P1, P2, P6 and P7 copolymers that had a concentration in the range of 35–40% (w/v) existed as clear solutions at room temperature (~22–25 °C) and turned into a white gel immediately at body temperature. This physicochemical property makes these copolymers suitable for drug delivery applications.

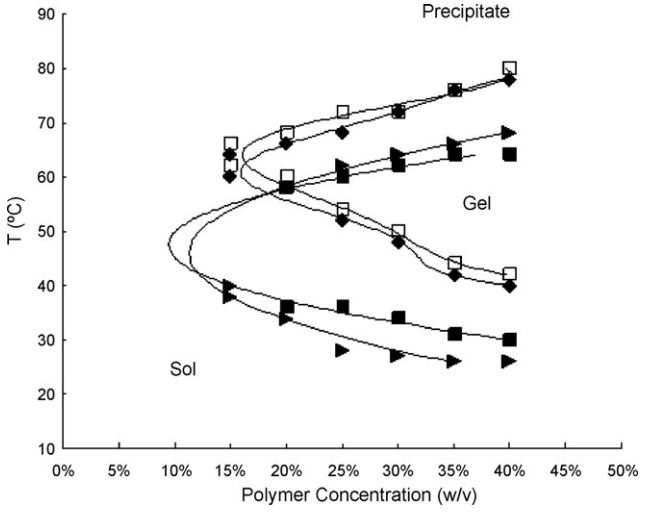

Fig. 3. Phase diagrams of mPEG–PLGA–mPEG triblock copolymers. Keys: (■) copolymer P1 (EG_{12}–$L_{30}G_{10}$–EG_{12}); (►) copolymer P2 (EG_{12}–$L_{35}G_{12}$–EG_{12}); (♦) copolymer P6 (EG_{17}–$L_{42}G_{14}$–EG_{17}); (□) copolymer P7 (EG_{17}–$L_{45}G_{15}$–EG_{17}).

3.2. In vitro release of lysozyme from polymeric formulation

The copolymer P1, P2, P6 and P7 based solution formulations of lysozyme were injectable solutions through 25 G needle at room temperature and turned into gels rapidly at 37 °C. In the *in vitro* release study (Fig. 4), the copolymer P1 based formulation showed an initial burst release of 26.3 ± 1.1% and controlled the release of lysozyme for ~21 days. Copolymer P2 based formulation suppressed the initial burst release to 22.1 ± 0.5% and prolonged the release of the protein over 28 days. A high initial burst release of 67.9 ± 1.8% and 66.2 ± 1.3% was observed for copolymer P6 and P7 based formulations, respectively, followed by a slow release for only 10 days.

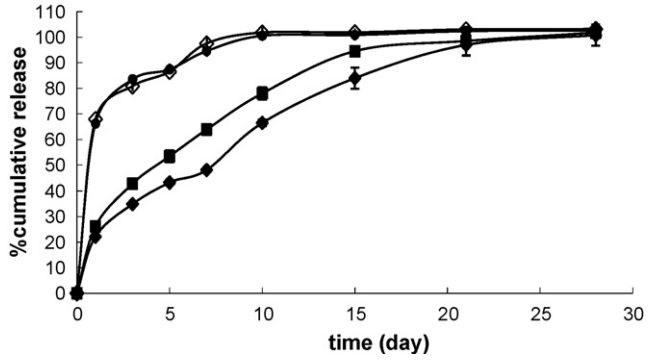

Fig. 4. Effect of copolymer structural composition on *in vitro* release of lysozyme. Keys: (■) formulation of 40% (w/v) of copolymer P1; (♦) formulation of 40% (w/v) P2; (◊) formulation of 40% (w/v) P6; (●) formulation of 40% (w/v) P7.

Table 2
Thermal parameters of lysozyme released from thermosensitive formulations (mean ± S.D., $n = 4$)

Sample	Calorimetric enthalpy ΔH (kcal/mol)			Transition temperature T_m (°C)		
	0 days	7 days	15 days	0 days	7 days	15 days
Native lysozyme	102.2 ± 4.1	–	–	75.7 ± 0.5	–	–
Lysozyme control	–	74.9 ± 5.8*	67.0 ± 8.8*	–	72.7 ± 0.2*	72.6 ± 0.3*
Released lysozyme						
P1 formulation	–	77.1 ± 4.1*	74.5 ± 8.0*	–	75.7 ± 0.2[a]	76.0 ± 0.3[a]
P2 formulation	–	98.5 ± 9.1[a]	93.8 ± 5.9[a]	–	75.1 ± 0.3[a]	76.1 ± 0.2[a]

* $p < 0.05$ compared to native lysozyme.
[a] $p < 0.05$ compared to lysozyme control at corresponding time points.

3.3. Stability studies of released proteins

3.3.1. Conformational stability by differential scanning calorimetry (DSC)

DSC was used to investigate the conformational stability of protein against thermal denaturation. The DSC parameters (T_m and ΔH) of released lysozyme were compared to native lysozyme (freshly prepared lysozyme solution in PBS) and lysozyme control (lysozyme solution in PBS incubated at 37 °C and sampled at the same time points as released lysozyme) to evaluate the effect of polymeric formulation on the protein stability (Table 2). Native lysozyme showed a transition peak at 75.7 ± 0.5 °C (T_m) with a calorimetric enthalpy change (ΔH) of 102.2 ± 4.1 kcal/mol during the thermal denaturation. After 7 days incubation at 37 °C, the T_m and ΔH of the control lysozyme were significantly ($p < 0.05$) decreased to 72.7 ± 0.2 °C and 74.9 ± 5.8 kcal/mol, respectively, while extension of the incubation time to 15 days resulted in further decrease in ΔH (67.0 ± 8.8 kcal/mol). Released lysozyme from copolymer P1 formulation demonstrated the fold → unfold transition at a similar temperature (75.7 ± 0.2 °C of sample withdrawn at day 7, and 76.0 ± 0.3 °C of sample withdrawn at day 15) as native lysozyme. However, the ΔH of the transition decreased to 77.1 ± 4.1 kcal/mol and 74.5 ± 8.0 kcal/mol for sample withdrawn at day 7, and day 15, respectively, which were slightly higher than control lysozyme but significantly ($p < 0.05$) lower than native lysozyme. The T_m of released lysozyme from copolymer P2 formulation was also observed at a similar temperature as native lysozyme (75.1 ± 0.3 °C at day 7, and 76.1 ± 0.2 °C at day 15) during thermal denaturation, and the ΔH (98.5 ± 9.1 kcal/mol at 7 day, and 93.8 ± 5.9 kcal/mol at day 15) were significantly ($p < 0.05$) higher than control lysozyme and similar to native lysozyme.

3.3.2. Secondary structure stability by circular dichroism (CD)

Secondary structure of released lysozyme was probed by CD to further examine the possible effects of copolymer formulations on the structural stability of lysozyme. Fig. 5 shows the CD spectra of native lysozyme and released lysozyme from copolymer P1 and P2 based formulations. All the released lysozyme displayed a strong negative band in the range of 200–240 nm with two minima at 208 nm and 222 nm, which was similar to native lysozyme.

3.3.3. Biological activity of lysozyme

Released lysozyme from P1 and P2 formulations withdrawn at day 28 were tested for specific enzyme activity and compared with native lysozyme. The enzyme activity of released lysozyme was 35.3 ± 1.6 (×10^4 EU/mg) and 37.4 ± 1.5 (×10^4 EU/mg) for P1 and P2 formulations, respectively. No significant difference was found between native lysozyme (37.2 ± 1.6 (×10^4 EU/mg)) and released lysozyme from P2 formulation. Released lysozyme from P1 formulation showed a slightly but insignificantly ($p > 0.05$) lower specific enzyme activity compared to native lysozyme.

3.4. Biocompatibility of copolymer based delivery system

3.4.1. In vitro biocompatibility

The biocompatibility of P1 and P2 copolymer based on formulations were evaluated by the viability assay of HEK293 cells. The value of cell viability was expressed as a percentage of control cells (which were treated with growth medium only and considered to have 100% cell viability). Gradual increase in the cell viability was observed with increasing dilution of copolymer extracts and PBS

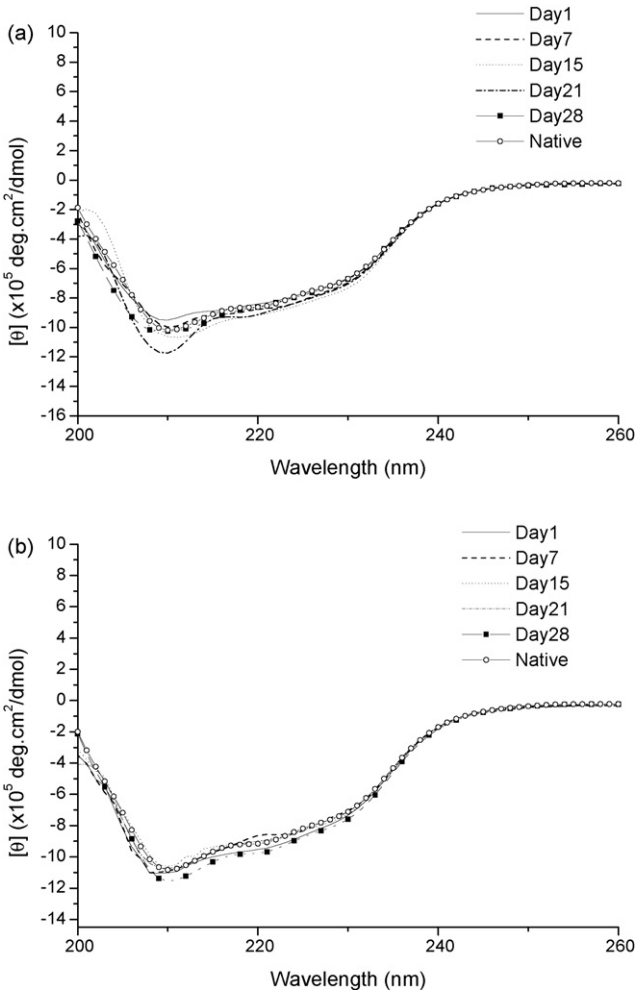

Fig. 5. CD spectra of released lysozyme samples from thermosensitive formulations. (a) Lysozyme (2%, w/v) released from 40% (w/v) P1 formulation; (b) lysozyme (2%, w/v) released from 40% (w/v) P2 formulation. Keys: CD spectra of released lysozyme sample withdrawn at day 1, day 7, day 15 and day 21. (Native) is freshly prepared lysozyme solution in PBS.

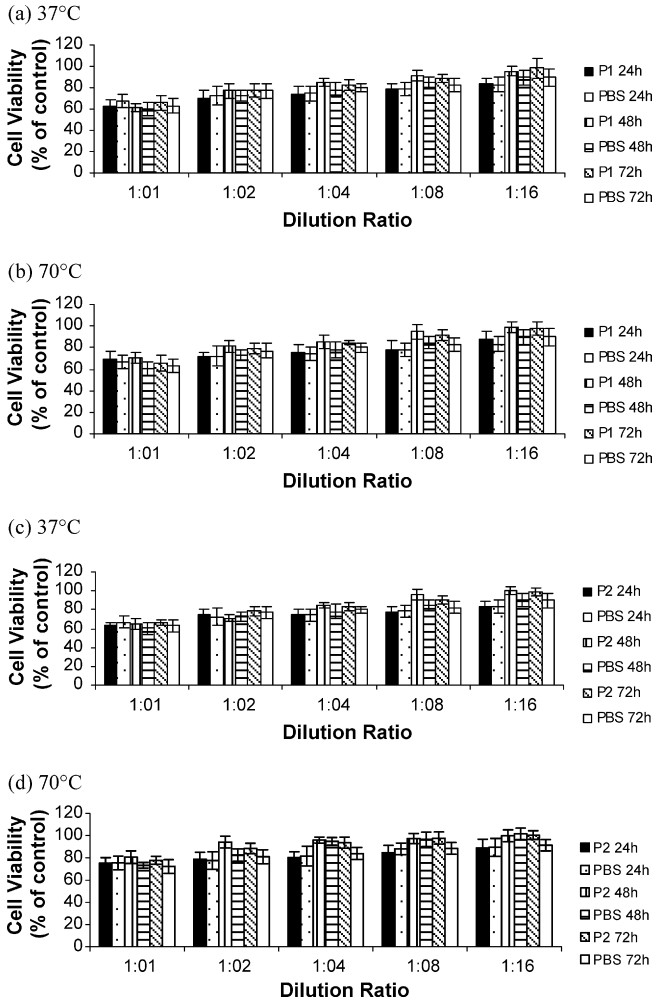

Fig. 6. Biocompatibility of mEPG–PLGA–mPEG triblock copolymer P1 and P2. The biocompatibility was expressed as cell viability in terms of percentage of control group. (a and b) Comparison of cytotoxicity between copolymer P1 extracts (37 and 70 °C) and PBS. (c and d) Comparison of cytotoxicity between copolymer P2 extracts (37 and 70 °C) and PBS.

with growth medium (Fig. 6). HEK293 cells treated with P1 and P2 copolymer extracts prepared at 37 °C showed comparable cell viability to PBS at all dilution ratios (Fig. 6a and c). Although an increase in viability was observed for cells treated with copolymer extracts prepared at 70 °C, the increase was statistically insignificant compared to PBS treatment (Fig. 6b and d).

3.4.2. *In vivo* biocompatibility

In vivo biocompatibility of the polymeric delivery system was evaluated in the rat after administration of blank polymeric delivery system. Fig. 7a showed the image of normal subcutaneous histology of rat skin where no formulation was injected. The subcutaneous histologies of rat skin following administration of the copolymer P2 based polymeric delivery system were presented in Fig. 7b–g. One day after administration of the polymeric delivery system, an acute inflammatory response occurred at the injection site characterized by increased permeability of the capillaries and infusion of abundant neutrophiles and lymphocytes. 1–2 weeks later, the number of lymphocytes and neutrophiles dropped but the count of macrophages became predominant. The presence of macrophages was seen for more than a month after subcutaneous administration of the polymeric delivery system. After 3 months, the histology of the skin sample from polymer delivery site was similar to normal skin. No signs of fibrosis, muscle damage, or necrosis were observed during the study, indicating the acceptable biocompatibility of the polymeric delivery system.

4. Discussion

The synthesized copolymers were proven to have mPEG–PLGA–mPEG triblock structure, but not all of them displayed suitable characteristics for the application to *in situ* gel forming implant delivery systems. The hydrophobic PLGA block was carefully extended from $L_{30}G_{10}$ (L: lactic acid moiety; G: glycolic acid moiety) to $L_{45}G_{15}$ while fixing the LA/GA ratio (~3:1) and keeping mPEG block length constant (EG_{12}, 550 Da). Copolymer P1 (EG_{12}–$L_{30}G_{10}$–EG_{12}, 550–2741–550 Da) and P2 (EG_{12}–$L_{35}G_{12}$–EG_{12}, 550–3218–550 Da) showed satisfactory solubility in water at equilibrium temperature of 4 °C. However, further extension of the hydrophobic PLGA block from $L_{39}G_{13}$ (3615 Da) to $L_{45}G_{15}$ (4112 Da) resulted in the marked decrease in copolymer solubility. Increase in the hydrophilic mPEG block was known to increase the solubility of copolymers. Therefore, copolymers P6 (EG_{17}–$L_{42}G_{14}$–EG_{17}) and P7 (EG_{17}–$L_{45}G_{15}$–EG_{17}) were synthesized by replacing the hydrophilic mPEG (EG_{12}, 550 Da) of copolymer P4 (EG_{12}–$L_{41}G_{14}$–EG_{12}) and P5 (EG_{12}–$L_{45}G_{15}$–EG_{12}) with a more hydrophilic mPEG (EG_{17}, 750 Da). Consequently, the copolymers P6 and P7 regained a satisfactory solubility for sol–gel transition. Unfortunately, further increase in the length of hydrophobic PLGA block from $L_{48}G_{16}$ (copolymer P8) to $L_{55}G_{19}$ (copolymer P11) decreased the solubility of the copolymer and failed to achieve the critical gelation concentration (CGC) (Jeong et al., 1999b). Thus, copolymers P1, P2, P6 and P7 were selected for delivery and stability studies of the model protein, lysozyme.

The mPEG–PLGA–mPEG copolymer has both hydrophobic PLGA block and hydrophilic mPEG block. Extent of hydrophobicity and hydrophilicity determines the sol–gel transition of the copolymer. As shown in the phase diagrams, increasing the length of hydrophobic PLGA block at fixed length of mPEG resulted in a lower sol–gel transition temperature and wider temperature region for gel phase at a given copolymer concentration. Increased in the PLGA block resulted in larger micelles, which had higher tendency to form a gel and improve gel stability at lower concentration and lower temperature (Jeong et al., 1999b). Besides the effect of PLGA, the hydrophilic mPEG block also remarkably affected the thermosensitive sol–gel transition of mPEG–PLGA–mPEG copolymer solution. Copolymer with longer mPEG block (EG_{17}, 750 Da) showed a higher gelation temperature than copolymer having a smaller mPEG block (EG_{12}, 550 Da), which was in agreement with the earlier reports (Jeong et al., 1999b; Chen et al., 2005a). The intermixing of hydrophobic PLGA core with hydrophilic mPEG corona was responsible for the thermosensitive sol–gel transition of the copolymer solution and also for the stability of formed gel. Longer mPEG usually had less tendency for phase mixing, thus mPEG–PLGA–mPEG copolymer having larger mPEG block required a higher temperature for enough phase mixing as well as sol–gel transition (Jeong et al., 1999b). Increasing the length of PLGA block was reported to compensate the effect of longer mPEG block on sol–gel transition temperature (Li et al., 2003). However, our study found that increasing the PLGA block length from $L_{48}G_{16}$ (4387 Da) to $L_{55}G_{19}$ (5066 Da) did not reverse the elevated sol–gel transition temperature but decreased water solubility of the copolymer.

Lysozyme was used as a model protein to examine whether the mPEG–PLGA–mPEG copolymers (P1, P2, P6 and P7) were suitable for controlled delivery system. Copolymer P1 and P2 based thermosensitive *in situ* gel forming formulations demonstrated significantly lower ($p < 0.05$) initial burst release of lysozyme than copolymer P6 and P7 formulations (Fig. 4). The initial burst release

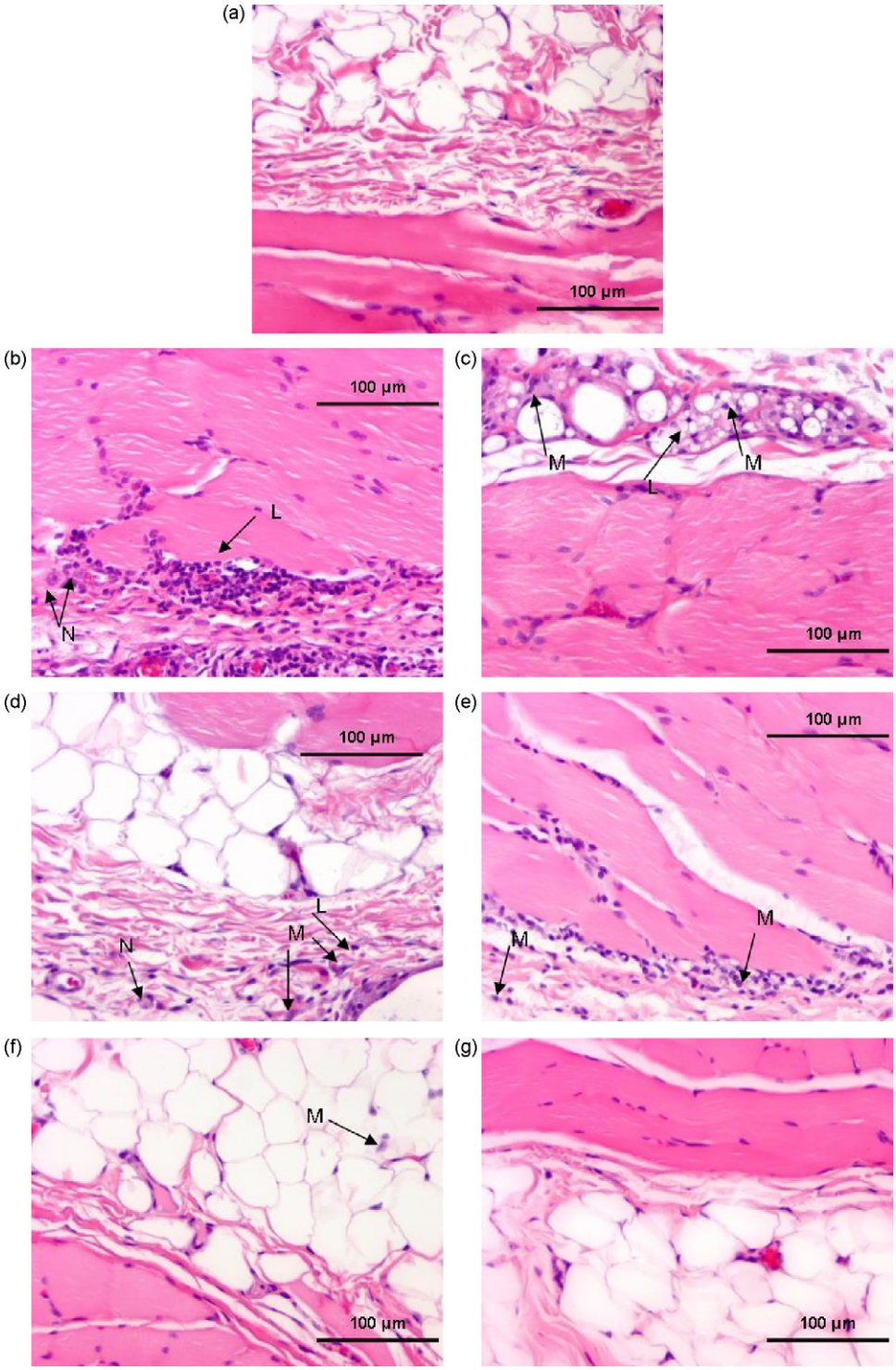

Fig. 7. Light micrographs of rat skin showing subcutaneous histology. Magnification 20×. (a) Normal subcutaneous histology, (b) rat skin sampled from the injection site after 1 day subcutaneous administration of the blank polymeric delivery system (copolymer P2), (c) rat skin sampled from the injection site after 1 week subcutaneous administration of the blank polymeric delivery system (copolymer P2), (d) rat skin sampled from the injection site after 2 weeks subcutaneous administration of the blank polymeric delivery system (copolymer P2), (e) rat skin sampled from the injection site after 1 month subcutaneous administration of the blank polymeric delivery system (copolymer P2), (f) rat skin sampled from the injection site after 2 months subcutaneous administration of the blank polymeric delivery system (copolymer P2), (g) rat skin sampled from the injection site after 3 months subcutaneous administration of the blank polymeric delivery system (copolymer P2) (L: lymphocytes; M: macrophage; N: neutrophils).

was normally considered to be due to the surface located protein. However, in the case of thermosensitive *in situ* forming hydrogel, the expulsion of the aqueous phase resulting from contraction of system volume during sol–gel transition was also responsible for the initial burst release. Protein located in the hydrophilic domain of the gel was subjected to the push-out effect and resulted in the initial burst release (Packhaeuser et al., 2004). Furthermore, gel with higher stability had less volume contraction than the gel hav-

ing lower stability; as a result, copolymers P6 and P7 having larger mPEG block and less gel stability showed higher initial burst release compared to copolymers P1 and P2.

The size of PLGA block also played an important role in the controlled release of protein. The formulation of copolymer P2 which has a larger PLGA block ($L_{35}G_{12}$, 3218 Da) controlled the release of lysozyme for a longer duration than copolymer P1 (PLGA block: $L_{30}G_{10}$, 2741 Da). The release of large molecules from degradable polymeric formulation was known to be controlled by both diffusion of the molecules and degradation of copolymer matrix (Jeong et al., 2000; Chen et al., 2005a). Larger PLGA block caused the copolymer to be more hydrophobic, which consequently slowed down the degradation of copolymer matrix and resulted in the longer release period of loaded protein.

A suitable controlled delivery system should be able to release protein in its biologically active form, as proteins, being fragile, can be easily destabilized during formulation processes. Structural deformation is a major reason for the loss of protein's activity, because certain tertiary, secondary, and primary structures are required for eliciting the physiological function of protein. Therefore, it is important to examine the stability of proteins released from the mPEG–PLGA–mPEG copolymer based thermosensitive *in situ* gel forming formulations.

The most stable conformation of a protein is usually the native state (Wang, 1999). When a protein folds, most of the nonpolar chains are buried in the interior of protein molecules out of contact with water. This folded structure is stabilized by intramolecular interactions such as hydrophobic interactions, electrostatic interactions, hydrogen bonding, intrinsic propensities, and van der Waals forces. However, when intermolecular protein–solvent (or environmental) interactions dominate over the intramolecular interactions, proteins may denature (or unfold) into an inactive form through a process of conformational changes. Such conformational changes result in changes of excess apparent specific heat capacity of protein ($\Delta C_{p,ex}$) during thermal denaturation which can be measured by DSC. DSC transforms the structural information of protein into macroscopic scale thermodynamic parameters, such as ΔH and T_m. Thus, DSC measurement is now widely accepted for studying protein conformational (tertiary structure) stability. In this study, incubation of lysozyme in PBS at 37 °C led to decrease in both T_m and ΔH. The significant decrease of T_m might be as a result of the protein misfolding after unfolding and the reduction of ΔH was attributed to protein unfolding. These results indicated that lysozyme was gradually unfolded, or in other words destabilized, in PBS with increasing incubation time at 37 °C. Compared to lysozyme control, released lysozyme from the two polymeric formulations demonstrated a similar T_m and ΔH as native lysozyme suggesting that the polymeric formulation P2 continuously released conformationally stable lysozyme into the release medium (PBS).

CD is a sensitive technique to probe secondary structure of protein, particularly for diluted protein solution. According to reported literature, the secondary structure of lysozyme could be destabilized by the presence of glycolic acid and lactic acid (Determan et al., 2006). Since the lactic acid and glycolic acid could be produced by mPEG–PLGA–mPEG copolymer degradation, it was important to examine the secondary structure of released lysozyme from formulations. The CD spectra of released lysozyme did not display significant difference from native lysozyme, which means the copolymer formulation degradation had no effect on the secondary structure of loaded lysozyme (Fig. 5).

In order to correlate the conformational stability with the biological activity of lysozyme, the specific enzyme activity was tested for released lysozyme and native lysozyme. In agreement with the conformational stability studies, the released lysozyme retained its specific enzyme activity compared to native lysozyme. Hence, the copolymer P1 and P2 based formulations were confirmed to continuously release lysozyme in biologically active form.

Good biocompatibility is an important criterion for selection of implanted materials. The non-biocompatible materials could cause irreversible tissue damages, such as permanent tissue destruction, necrosis, significant fibrosis, and dystrophic calcification. mPEG–PLGA–mPEG copolymers is finally degraded into lactic acid, glycolic acid and mPEG. Both lactic acid and glycolic acids can be metabolized into carbon dioxide and water through the tricarboxylic acid cycle (Tice and Cowsar, 1984). Meanwhile, mPEG can be cleared from body via kidney. However, the good biodegradability does not insure good biocompatibility. Moreover, the reported biocompatibility of polymer containing PLGA is still controversial. It is important to investigate the biocompatibility of the synthesized copolymers. Therefore, *in vitro* and *in vivo* biocompatibility studies were carried out in this study.

The *in vitro* biocompatibility of the mPEG–PLGA–mPEG copolymer was tested by an MTT assay. This assay has proven to be a sensitive and reliable method for examining biocompatibility of biodegradable polymers (Singh and Singh, 2007b; Chen and Singh, 2008). In the present study, no significant difference in cell viability was observed between cells treated with PBS and copolymer extracts. Since PBS was non-cytotoxic, the copolymer-based formulation was considered to have good *in vitro* biocompatibility. The cell viability was found to gradually decrease with the increased concentration of copolymer extracts and PBS in growth medium, which was attributed to the depletion of nutrition for cell growth and proliferation.

MTT assay proved that the neutralized copolymer extracts have no cytotoxicity, but could not reveal the effects of acidity resulted from the copolymer degradation and the tissue response to the polymeric formulation. In order to address this issue, *in vivo* biocompatibility was evaluated by the extent and duration of tissue inflammatory responses. An acceptable tissue reaction to polymeric implants should be a temporary inflammatory response to injury caused by injection with minimal fibrosis from wound healing process (Ziats et al., 1987). Such a short process of inflammatory response is characterized by acute inflammatory responses for the first day to 2 weeks followed by a longer duration of chronic inflammation depending on the existence of stimuli (Anderson and Shive, 1997). In this study, histological examination of skin samples removed from the injection site showed a typical acute inflammatory response 1 day after subcutaneous administration of the polymeric formulation, distinguished by the localized hemorrhage resulting from increased permeability of capillaries and abundant infiltration of neutrophils and lymphocytes in the interstitial spaces. An acute inflammatory response was gradually replaced by chronic inflammation in the following 1–2 weeks with increased number of macrophages. The chronic inflammatory response lasted for more than 1 month and less inflammatory cells were observed 2 months after the administration of polymeric formulation. The skin tissue was restored to normal status after 3 months. No persistent tissue damage was observed in this study and the recovery of temporary tissue damage satisfies the regulation of International Organization of Standardization (ISO, 2007). Thus, the biocompatibility studies confirmed that the polymeric formulation has acceptable biocompatibility for use in drug delivery system.

5. Conclusion

A series of mPEG–PLGA–mPEG triblock copolymers were synthesized and characterized for their use in thermosensitive system for protein delivery. Four of the eleven synthesized copolymers showed optimal aqueous solubility, sol–gel transition at body

temperature, and ability to control the *in vitro* release of incorporated protein. The copolymer (P2) controlled burst release as well as extended the release of incorporated lysozyme in its conformationally stable and biologically active form. The release of lysozyme from the polymeric formulations was dependent on the structural composition of copolymers. Furthermore, the copolymer based formulation also showed good biocompatibility. Thus, the mPEG–PLGA–mPEG copolymer (P2) based *in situ* gel forming implant might be an appropriate delivery system for controlled release of therapeutic proteins.

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