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Thermosensitive polymers: Synthesis, characterization, and delivery of proteins

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Abstract

Three triblock copolymers based on the poly(lactide) or poly(lactide-co-glycolide) and poly(ethylene glycol) or poly(ethylene oxide) blocks were synthesized and characterized. The weight average molecular weight and number average molecular weight were determined by gel permeation chromatography and proton nuclear magnetic resonance spectroscopy, respectively. Fourier transform infrared spectroscopy was used to determine the completion of synthesis of polymers. Thermoreversible sol–gel transition temperature and concentration were determined by an inverted tube method. Two formulations each of three synthesized polymers containing 5% (w/v) of lysozyme or bromelain but differing in polymer concentrations (20–30%, w/v) were prepared and studied for *in vitro* release of the incorporated protein. *In vitro* biocompatibility of the delivery systems was studied by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay. Biological activities of lysozyme and bromelain were determined by enzyme activity assays. Critical gelling concentrations were found in the range of 20–30% (w/v). *In vitro* biocompatibility study showed that all the formulations were biocompatible. Increasing the polymer concentration led to a decrease in burst release and extended the *in vitro* release of proteins. Furthermore, biological activities of lysozyme and bromelain in released samples were found to be significantly ($p < 0.05$) greater in comparison to the control. Thus, the above thermosensitive polymers were able to deliver proteins in biologically active forms at a controlled rate for 2–8 weeks.

Keywords: Controlled release; Thermosensitive smart polymer; Lysozyme; Bromelain; *In vitro* biocompatibility; Biologically active form

1. Introduction

With the completion of human genome sequencing, it is possible to produce significant quantities of therapeutically active proteins (Chen et al., 2005). Several proteins are undergoing clinical trials. Proteins are generally metabolized by liver and have short plasma half-life. Therefore, proteins are delivered by frequent injections, although a wide variety of routes of administration and delivery systems exist. The available delivery methods can be critically evaluated by considering ease of manufacturing, impact on conformational stability and biological activity, bioavailability, and toxicity. All these obstacles have been overcome by recent developments in depot delivery systems except for complex and costly manufacturing (Stevenson and Tan, 2000; Fowler et al., 2000; Benoit et al., 2000). Pulmonary and transdermal routes have low protein bioavailability (Shen et al., 1999; Naik et al., 2000; Chen et al., 2005). Therefore, safe and efficient delivery of proteins is the key to commercial success of current and future biotechnology products (Cleland, 2001).

"Smart polymers" that display a physiochemical response in nonlinear fashion to external stimuli (*i.e.*, temperature, pH, solvent, magnetic field, electric field, ultrasound, *etc.*) are widely explored as potential drug-delivery systems (Chen and Singh, 2005; Stevenson and Tan, 2000; Fowler et al., 2000; Benoit et al., 2000; Shen et al., 1999; Naik et al., 2000; Chen et al., 2005). Several approaches have been reported for the delivery of bioactive molecules in controlled and pulsatile manner using polymeric carriers (Cleland, 2001). Thermosensitive polymers exhibit temperature-dependent reversible gel–sol transitions (Malmsten, 2002; Molinaro et al., 2002). The bioactive molecules can be incorporated into aqueous solution

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of thermosensitive polymers at an appropriate temperature at which formulations are injectable. On subcutaneous injection, the polymer forms a gel that can act as a sustained-release matrix for drugs (Jeong et al., 1997; Hoffman et al., 1997; Wu et al., 2005; Lee et al., 2001; Zentner et al., 2001).

A new poly(ethylene glycol)-based copolymer containing multiple thiol (–SH) groups was cross-linked *in situ* to form a polymer hydrogel under mild conditions without using any organic solvent, elevated temperature, or harsh pH. *In vitro* release of fluorescein-labeled bovine serum albumin and *in vivo* release of the model and therapeutic proteins demonstrated controlled release and prolonged biological activity of the proteins, making it particularly useful for delivery of fragile therapeutics (Qiu et al., 2003). Cross-linkable pluronic dimethyacrylate was found injectable at room temperature but forms a reversible gel at 37 °C (Sosnik et al., 2003).

Thermoreversible block copolymers composed of polyethylene glycol (PEG) and biodegradable polyesters, such as polylactic acid (PLA) and DL-lactide/glycolide copolymer (PLGA) were studied as controlled release drug carriers. PEG/PLGA hydrogels are particularly attractive systems for pharmaceutical applications because of their biodegradability and good safety profile. Compared to the widely utilized PLGA, protein delivery systems based on hydrophilic–hydrophobic block copolymers 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, 2003; Kim et al., 2001; Zentner et al., 2001).

In this study, three triblock copolymers were synthesized and characterized for their structure and composition, molecular weight and molecular weight distribution, phase separation temperature, and biocompatibility. Furthermore, two model proteins (*i.e.*, lysozyme and bromelain) were incorporated into the delivery systems and their *in vitro* release and biological activity were studied.

2. Materials and methods

2.1. Materials

DL-Polylactide (DL-PLA) (M_w ~16,000 Da) was purchased from Boehringer Ingelheim, Petersburg, VA. L-Polylactic acid (PLA) (M_w ~2500 Da) was purchased from Polysciences Inc., Warrington, PA. DL-Lactide/glycolide copolymer (PLGA) (molar ratio 75/25; M_w ~6000) was a gift sample from PURAC America, Petersburg, VA. Isophorone diisocyanate, stannous octoate, lysozyme (EC 3.2.1.17) from chicken egg white and Micrococcus Lysodeikticus (*Micrococcus luteus*) were purchased from Sigma Chemical Company, St. Louis, MO. Micro BCA protein assay reagent kit was purchased from Pierce Biotechnology, Inc., Rockford, IL. Bromelain and its substrate Nα-carboxybenzoyl-L-lysine *p*-nitrophenyl ester (Nα-CBZ-LNPE) were obtained from Sigma Chemical Company, St. Louis, MO. Leuprolide acetate was a gift sample from TAP Pharmaceuticals Inc. (Deerfield, IL). All other chemicals used were of analytical grade.

2.2. Synthesis of thermosensitive triblock copolymer

The following three types of thermosensitive polymers were synthesized:

TP1 = (PEO:DL-PLA:IPDI:DL-PLA-PEO (5000:16,000:168:16,000:5000);
TP2 = PLGA:PEG:PLGA (6000:3350:6000);
TP3 = PLA:PEG:PLA (2500:1000:2500).

2.2.1. Synthesis of thermosensitive triblock copolymer (TP1)

The triblock copolymer (TP1) was synthesized following the method of Jeong et al. (1997). Fig. 1 shows the synthetic scheme of TP1. Briefly, monomethoxy poly(ethylene oxide) (PEO; M_w 5000) and DL-poly(lactide) (PLA, IV 0.19 dl/g) in equal molar ratio, along with stannous octoate (0.05%, w/w) were dissolved in toluene under reflux for 8 h. This step resulted in the synthesis of diblock copolymer. The coupling reaction of diblock copolymers was performed by dissolving with isophosphodiisocyanate (IPDI) in toluene at 60 °C for 12 h, followed by refluxing for 6 h. The triblock polymer obtained was purified by fractional precipitation of polymer from methylene chloride using diethyl ether.

2.2.2. Thermosensitive triblock copolymers (TP2 and TP3)

The triblock copolymers, TP2 and TP3, were synthesized following the method of Zentner et al. (2001). The synthetic schemes of triblock copolymers, TP2 and TP3, are shown in Fig. 2. Briefly, under dry nitrogen atmosphere, polyethylene glycol (M_w 3350 or 1000) was dried in a 3-necked flask by stirring at 150 °C for 3 h. DL-PLGA (PLA:GA 3:1, IV 0.16 dl/g, M_w 6000) or L-PLA (M_w 2500) was added, and the reaction mixture was heated for 30 min. After all the polymer was melted, 0.03% (w/w) of stannous 2-ethylhexanoate was added, and the reaction mixture was further heated at 155 °C for 8 h. Crude polymer obtained was dissolved in ice-cold water (5–8 °C). After complete dissolution, the polymer solution was heated to 80 °C to effect precipitation of the polymer and to remove water-soluble, low-molecular weight polymer and unreacted monomers. Precipitated polymer was isolated by decanting the supernatant and re-dissolving in ice-cold water. Heating, precipitation, and decantation were repeated once again, and purified polymer was obtained. Residual water was removed by freeze drying.

2.2.3. Polymer characterization

The phase transition from sol to gel of the triblock copolymers in aqueous solution was determined by an inverted test tube method at a heating interval of 2 °C. The polymer was dissolved in water and the sol–gel transition was determined by inverting the tube horizontally after it had been kept for 10 min at

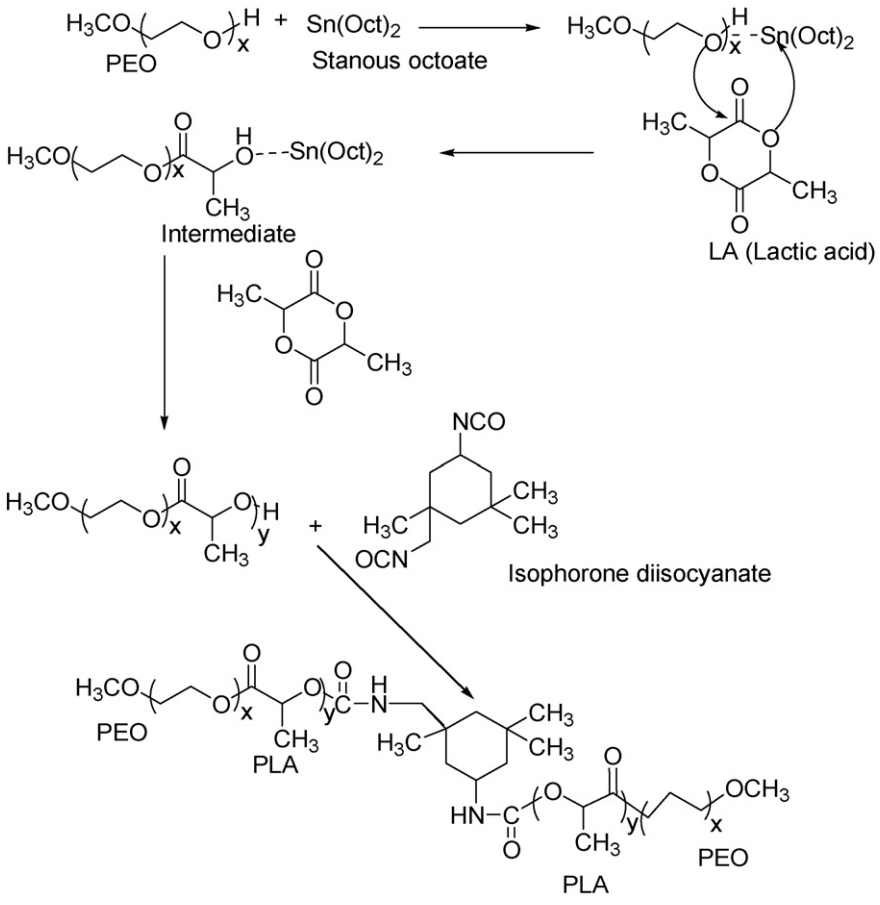

Fig. 1. Schematic presentation of the synthesis of thermosensitive polymer TP1 (PEO:DL-PLA:IPDI:DL-PLA-PEO; 5000:16,000:168:16,000:5000).

constant temperature to achieve an equilibrium state (Lee et al., 2001).

FTIR spectra of polymers were performed on a Nicolet 210 FTIR (Nicolet Instrument Corporation, Madison, WI) to characterize their molecular structure. Spectra were obtained in the frequency range 4000–1000 cm^{-1} in absorbance mode. All spectra analyzed represent an average of 32 scans with resolution of 4 cm^{-1}, Happ-Genzel apodization, and zero filling factor of none. OMNIC® FTIR software (Nicolet Instrument Corporation, Madison, WI) was used to analyze the spectra.

The weight average molecular weight (M_w), the number average molecular weight (M_n), and molecular weight distribution (MD) were determined using gel permeation chromatography (GPC) on a Waters Model 510 equipped with two styragel HR 4E and styragel HR 5E columns in series at a flow rate of 1.2 ml/min (eluent:terahydrofuran, room temperature, and calibrated by polystyrene standard samples). The detector used was a Waters 2410 refractive index detector. Millennium 3.2 Software was used to analyze the GPC chromatogram.

Proton nuclear magnetic resonance (^{1}H NMR) was used to determine the structure and composition of triblock copolymers. ^{1}H NMR measurements were performed by a Varian 300 MHz NMR spectrometer operating at 300 MHz. All spectra were obtained at room temperature from 15 mg polymer/ml dimethyl sufoxide (DMSO) solution. The integration values of peaks due to PLA (CH_3), EG (CH_2), GA (CH_2), and PLA (CH) were used to get an empirical idea of the molecular weight of synthesized polymers.

2.3. Preparation of drug-delivery systems

The solutions of TP1, TP2, and TP3 were prepared in deionized water in the concentration range of 20–30% (w/v). The polymer mixture in water was kept at the required temperature for 24 h to allow complete penetration/equilibration with water. The specified amount of TP1 was added to deionized water maintained at 40 °C. However, in case of TP2 and TP3, the required amount of polymers was added to deionized water maintained at room temperature. Lysozyme or bromelain (5%, w/v) was added to the polymer solution and homogenized (8000 rpm, 2 min) (Silverson laboratory mixer, model L4RT, Silverson Machines Inc., East Longmeadow, MA). Two formulations were prepared with each of the three polymers differing in polymer concentration. Thus, six formulations were prepared. Injectability of the formulations was observed through a 25-gauge needle.

2.4. In vitro release of proteins

Five hundred microliter of the formulation containing lysozyme or bromelain was injected into 15 ml of releasing media (isotonic phosphate buffer, pH 7.4) contained in a glass vial. The vials containing *in situ* formed gel depot were kept

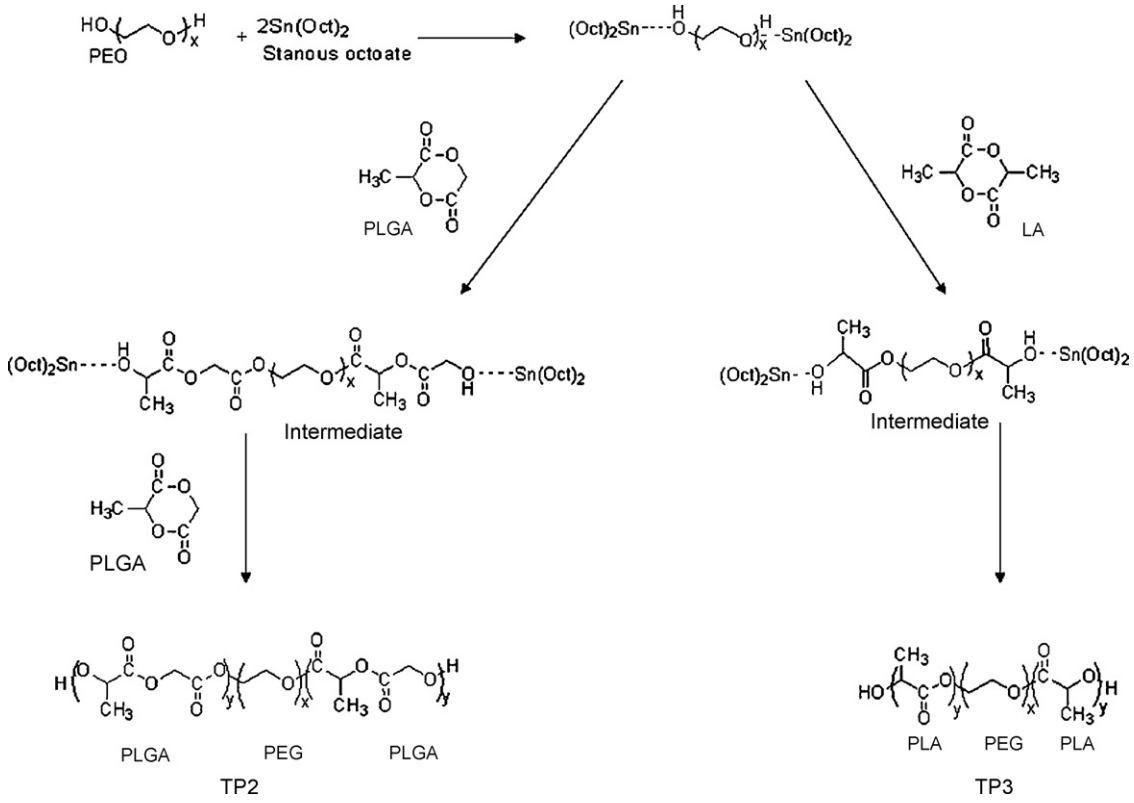

Fig. 2. Schematic presentation of the synthesis of thermosensitive polymer TP2 (PLGA:PEG:PLGA; 6000:3350:6000) and TP3 (L-PLA:PEG:L-PLA; 2500:1000:2500).

in reciprocal shaking water bath (Precision Scientific, Winchester, VA) at 37 °C and 35 rpm. Five milliliter of aliquot was withdrawn from releasing media at specified time points. The volume withdrawn was replaced with fresh releasing media.

The amount of lysozyme or bromelain in the released samples was determined by MicroBCA protein assay method (Smith et al., 1985). Briefly, samples were centrifuged at 4229 × *g* for 30 min. One milliliter of suitably diluted supernatant/standard was mixed with 1 ml of working reagent solution obtained by mixing Micro BCA Reagent A, B, and C in the ratio of 50, 48, and 2, respectively. This mixture was incubated at 60 °C for 60 min, cooled to room temperature, and used for measurement of absorbance at 562 nm by UV–visible spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan). The linearity was observed in the range of 6.25–100 μg/ml for lysozyme and 12.5–100 μg/ml for bromelain. The correlation coefficient (*R* value) between concentration and absorbance were 0.99 for both lysozyme and bromelain with *y*-intercept value equal to 0.075 for lysozyme and 0.014 for bromelain. Samples from formulation without lysozyme were used as blank control for absorbance. Concentrations were corrected for sample removal (Hayton and Chen, 1982).

2.5. Biological activity of lysozyme

A portion of vigorously shaken *Micrococcus luteus* stock suspension (0.01%, w/v) into phosphate buffer (0.1 M, pH 7.0) was diluted to obtain an absorbance between 0.2 and 0.6 at 450 nm. One hundred microliter of an appropriately diluted lysozyme sample was added to 3 ml of the diluted *Micrococcus luteus* solution into a spectrophotometer cell. The rate of decrease of absorbance was monitored by UV spectrophotometer during a total incubation period of 2 min at 25 °C. Slope of the linear portion of the plot (between absorbance and time) in absorbance units per min provided the amount of lysozyme in enzyme unit (EU) (Shugar, 1952). Unit of biologically active lysozyme was determined by using the following formula:

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

where d*f* 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.

2.6. Biological activity of bromelain

Biological activity of bromelain in the released samples was determined by using Nα-CBZ-LNPE as a substrate (Heinrikson and Kezdy, 1976). Reagent A (30 mM sodium acetate buffer with 100 mM potassium chloride and 1.0 mM L-cysteine, pH 4.6) and reagent B (50 mM LNPE in 1:4 acetonitrile and deionized water) were prepared. In a spectrophotometric cuvette, 2.6 ml of reagent A, 0.1 ml of sample/standard (centrifuged and filtered), and 0.1 ml of reagent B were added, and a rate of increase in absorbance per min at 340 nm was measured.

Samples were suitably diluted so that the increase in absorbance may not be greater than 0.3 absorbance units. A mixture of reagent A (2.7 ml) and reagent B (0.1 ml) served as a blank. Units of bromelain in the released sample were calculated by using the following equation:

$$\text{Units of bromelain (ml sample)} = \frac{(\Delta A_{340\,\text{nm}}\,(\min\text{Test}) - \Delta A_{340\,\text{nm}}\,(\min\text{Blank}))(2.8)(\text{d}f)}{(6.28)(0.1)},$$

where df is the dilution factor, 6.32 the millimolar extinction coefficient of p-nitrophenol at 340 nm, 0.1 the volume (in ml) of enzyme/standard used and 2.8 is the volume (in ml) of assay.

2.7. In vitro biocompatibility of the delivery systems

The *in vitro* biocompatibility of the delivery systems was evaluated by MTT assay (Ignatius and Claes, 1996). The polymer-based delivery systems (without protein) were extracted in PBS (pH 7.4) for 10 days at 70 °C. The polymers degrade faster at elevated temperatures, which simulate the long-term effects of *in situ* gel formed implant (artificial ageing extract) (USP, **27**). After 10 days of incubation, the pH of the extract was adjusted to 7.4 by adding 1 M NaOH. The extract was filtered through 0.2 μm filter and handled aseptically. The total concentration of the degradation products from TP1, TP2, and TP3 in polymer extracts PE1, PE2, and PE3 were 200, 250, and 250 mg/ml, respectively.

The extracts were diluted with double concentrated growth medium to varying ratios of 1:1 to 1:16. The diluted extract (200 μl) was added to Crandell Feline Kidney Cell (CRFK cells) cultures. The growth medium without polymer extract was used as a negative control (PBS, pH 7.4), and 2% dimethyl sulfoxide (DMSO) in growth medium served as a positive control.

The MTT assay is based on the ability of living cells to reduce a water-soluble yellow MTT dye to a purple formazan product by mitochondrial succinate dehydrogenase. After 5 h of plating 8×10^3 cells per well of 96-well microtiter plates, the growth medium was decanted and replaced with 200 μl per well of a freshly prepared dilution series of the extracts. Eight replicates were prepared for each dilution and controls. The extracts were exposed to the cells for 24, 48 and 72 h at 37 °C in a humidified 5% CO_2 atmosphere. The MTT solution was prepared at a concentration of 5 mg/ml in phosphate-buffered saline (pH 7.4). Ten microliter per well of an MTT solution was added and incubated for 4 h. One hundred microliter per well of a freshly prepared acidified isopropanol was added to dissolve the formazan crystals. The colorimetric staining of the plates was evaluated on a multi-well plate reader at 570 nm. In order to ensure that the polymers themselves do not contribute to the reduction of the dye, the polymer solutions alone were also assayed in a microtiter plate by the MTT assay (Ignatius and Claes, 1996).

2.8. Data analysis

The results were expressed as a mean ± S.D. ($n = 3$). Statistical comparisons were made using Student's t-test. The level of significance was used as $p < 0.05$.

3. Results and discussion

3.1. Synthesis and characterization

Completion of the polymerization reaction was confirmed by the disappearance of a peak from 1700 to 1725 cm^{-1} due to the carboxylic acid group of PLA and the appearance of peak in the region of 1730–1750 cm^{-1} due to ester groups in the FTIR spectra. The peak of ester group appeared at 1151 cm^{-1} in case of TP3. The peak at 2175 cm^{-1} was characteristic of isophorone diisocyanate (IPDI), which was used as a coupling agent. We used IPDI because it gives a sharp characteristic peak in the FTIR spectrum. The disappearance of peak at 2175 cm^{-1} was used as a diagnostic of completion of coupling reaction in TP1. Stannous octoate was used as an initiator of polymerization. TP1 was stored in a desiccator under vacuum to expel any organic solvent entrapped during the purification step.

Fig. 2 shows the schematic illustrations of steps involved in the synthesis of TP2 and TP3, which were carried out by condensation polymerization without using any toxic solvent. The progression of the reaction was monitored by the appearance of peaks characteristic of both PEG and PLA in the FTIR spectra of the product. Peaks at 1129 cm^{-1} and 1751 cm^{-1} were due to C–O–C stretching vibrations of the repeated OCH_2CH_2 units of PEG and C=O stretching vibrations of the ester carbonyl from PLA, respectively (Fig. 3).

The critical gelling concentrations at 37 °C were 20% (w/v) for TP1 and 25% (w/v) for both TP2 and TP3 polymers. TP1 was solution at temperatures higher than body temperature (37 °C) but a gel at the body temperature. On the other hand, both TP2 and TP3 were sol at room temperature but gel at 37 °C. Several mechanisms have been proposed as driving force for such sol–gel interconversions (Song et al., 2004). Sol–gel transition has been related with intrinsic changes in micelle properties (Jeong et al., 2002a,b) leading to formation of an ordered three-dimensional structure or network (Wanka et al., 1994). Structural neutron scattering studies have shown that the observed change in viscosity during sol–gel conversion is due to a hard sphere crystallization as the micelle concentration approaches the critical volume fraction of 0.53 (Mortensen and Pedersen, 1993). Sol–gel conversion of the synthesized polymers might be due to the presence of a domain (core–shell micelle) structure in aqueous environment (Jeong et al., 1999). The hydrophilic PEG occupies the outer shell region, and the hydrophobic PLA/PLGA constitutes the inner core in order to decrease free energy of hydration. At low bulk concentration, these triblock copolymers form micelles in water. At a particular concentration termed as critical gelling concentration, association of micelles and phase separation take place resulting into gel formation.

Fig. 4 shows a typical NMR spectrum of TP3. The integration values of different peaks in the NMR spectrum of synthesized

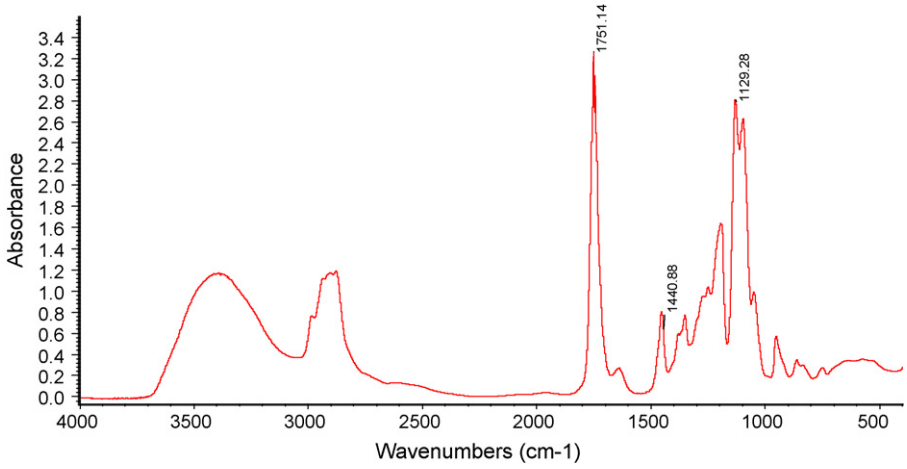

Fig. 3. FTIR spectrum of triblock thermosensitive polymer TP3.

polymers were used to determine M_n which were found equal to 38,700, 5400, and 6200 for TP1, TP2, and TP3, respectively. GPC was used to determine the M_w which were found to be 42,300, 14,200, and 6100 for TP1, TP2, and TP3, respectively (data not shown). Thus, polydispersities (PD) were 1.09, 1.46, and 1.12 for TP1, TP2, and TP3, respectively. The polydispersity of all the three polymers was in the range of 1.09–1.46 with symmetric peak and a relative narrow molecular weight distribution. Unimodal GPC trace with low polydispersity values confirmed the formation of triblock polymers and suggested that polymers had sufficient purity for use in drug delivery.

3.2. In vitro release

The molecular weights of lysozyme and bromelain are 14,200 and 33,800 Da, respectively. Proteins differ widely in their structural organization as their size varies. Therefore, lysozyme and bromelain were used in this study to investigate the effect of protein size on their *in vitro* release from the thermosensitive gel formulations. Fig. 5 shows the *in vitro* release of lysozyme from 20% and 25% (w/v) TP1-based formulations. We found a 20–22% burst release, followed by a controlled rate of release of incorporated lysozyme up to 8 weeks. Increasing the concentration of TP1 to 25% (w/v) significantly ($p = 0.0059$) decreased the rate of release of lysozyme. Fig. 5 shows the *in vitro* release of bromelain. We found a 15–17% burst release and thereafter a controlled rate of release of incorporated bromelain through 6 weeks. Increasing the concentration of TP1 to 25% (w/v) significantly ($p = 0.0262$) extended the release of bromelain.

Fig. 6 shows the release of lysozyme from 25% and 30% (w/v) TP2-based formulations. There was 10–12% burst release followed by a controlled release of incorporated lysozyme through 15 days. Increasing the concentration of TP2 (30%, w/v) significantly ($p = 0.0102$) controlled the release of lysozyme for a longer duration. In case of bromelain, we observed an 11–13% burst release. Increasing the concentration of TP2 (30%, w/v) significantly ($p = 0.0321$) extended the release of bromelain for a longer duration. Fig. 7 shows the release of lysozyme and

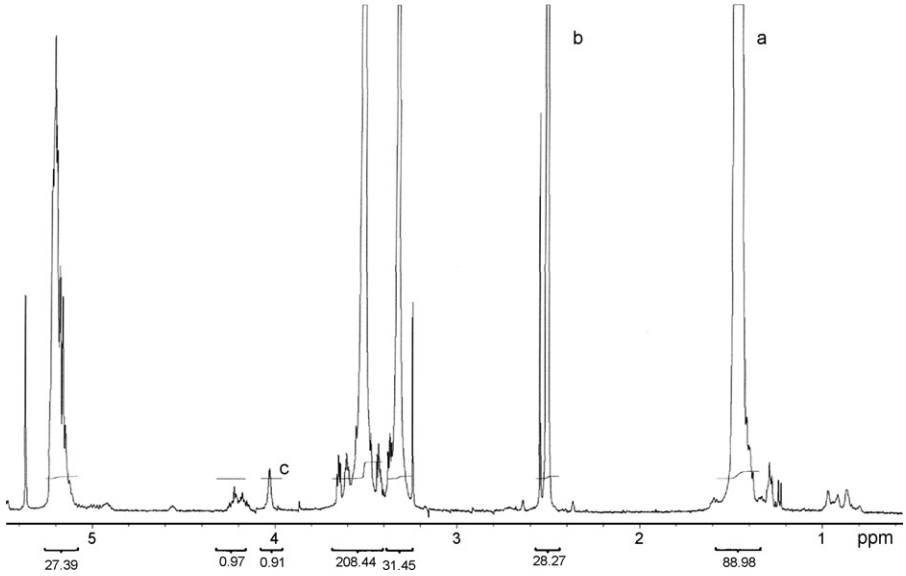

Fig. 4. ^{1}H NMR spectrum of thermosensitive polymer TP3 (a) CH_3 of LA, (b) CH_2 of ethylene glycol, and (c) CH of LA.

Table 1
Release kinetics of lysozyme and bromelain from thermosensitive controlled release formulations

Formulations	Rate Constants (μg/day)			
	Lysozyme		Bromelain	
	Zero-order	Higuchi Model	Zero-order	Higuchi Model
TP1 (20%, w/v)	$1.41 \pm 2.46 \times 10^3$; $R = 0.71$	$1.85 \pm 2.19 \times 10^3$; $R = 0.79$	$1.42 \pm 1.87 \times 10^3$; $R = 0.78$	$2.12 \pm 1.46 \times 10^3$; $R = 0.92$
TP1 (25%, w/v)	$1.26 \pm 2.42 \times 10^3$; $R = 0.71$	$1.69 \pm 0.65 \times 10^3$; $R = 0.87$	$1.59 \pm 2.27 \times 10^3$; $R = 0.77$	$1.90 \pm 1.24 \times 10^3$; $R = 0.93$
TP2 (25%, w/v)	$1.83 \pm 0.61 \times 10^3$; $R = 0.86$	$1.83 \pm 2.19 \times 10^3$; $R = 0.79$	$2.64 \pm 0.68 \times 10^3$; $R = 0.91$	$2.53 \pm 0.82 \times 10^3$; $R = 0.97$
TP2 (30%, w/v)	$1.60 \pm 0.46 \times 10^3$; $R = 0.80$	$1.63 \pm 0.51 \times 10^3$; $R = 0.90$	$2.21 \pm 0.36 \times 10^3$; $R = 0.76$	$2.12 \pm 0.50 \times 10^3$; $R = 0.91$
TP3 (25%, w/v)	$1.40 \pm 0.54 \times 10^3$; $R = 0.75$	$1.40 \pm 0.44 \times 10^3$; $R = 0.93$	$1.94 \pm 0.68 \times 10^3$; $R = 0.92$	$1.90 \pm 0.64 \times 10^3$; $R = 0.91$
TP3 (30%, w/v)	$1.19 \pm 0.25 \times 10^3$; $R = 0.76$	$1.35 \pm 0.21 \times 10^3$; $R = 0.92$	$1.58 \pm 0.24 \times 10^3$; $R = 0.76$	$1.62 \pm 0.28 \times 10^3$; $R = 0.89$

Note: Thermosensitive controlled release formulations contained either 5% lysozyme or 5% bromelain. Zero-order and Higuchi square root rate constants were calculated from the release data from day 1 to end of the study. R is the correlation coefficient.

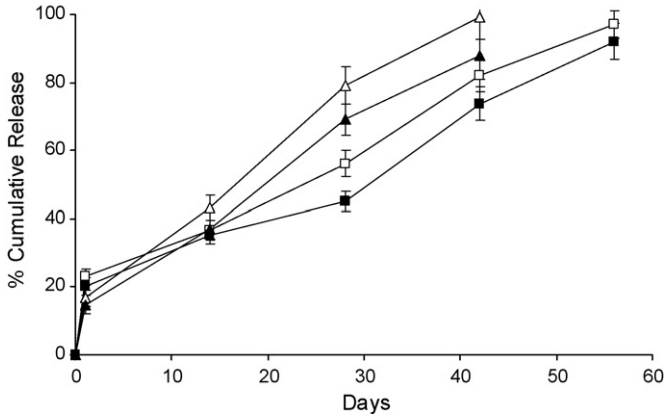

Fig. 5. *In vitro* release profiles of lysozyme and bromelain from thermosensitive polymer TP1-based formulations. Each data point is expressed as a mean ± S.D., $n = 3$ [Keys: (□) lysozyme, 20% (w/v) TP1; (■) lysozyme, 25% (w/v) TP1; (△) bromelain, 20% (w/v) TP1; (▲) bromelain, 25% (w/v) TP1].

bromelain from 20% and 25% (w/v) TP3-based formulations. The release of lysozyme and bromelain was reduced as the polymer concentration increased from 20 to 25% (w/v) in the formulations.

Bromelain is more hydrophobic than lysozyme due to the presence of hydrophobic amino acid residues such as Trp, Phe, Ile, and Leu. Therefore, hydrophobic interaction between bromelain and PLA/PLGA block of polymers is greater than lysozyme. As a result, bromelain should be released at a slower rate than lysozyme due to its lower diffusivity provided by relatively stronger hydrophobic interaction. However, release rate of bromelain was greater than lysozyme, which can be explained on the basis of predominant role of degradation of the polymer. The protein was added to polymer solution, which changed to gel at body temperature. Bromelain (M_W 33,000 Da) after initial diffusion from gel possibly led to larger pore pathways followed by faster degradation than the gel containing smaller molecule, lysozyme (M_W 14,200 Da). Therefore, bromelain was released at a relatively faster rate than lysozyme.

The burst release of proteins from formulations can be explained assuming a domain structure of the hydrogels. The partitioning of protein between the hydrophilic domain and hydrophobic domain is a critical factor for protein release. At the sol–gel transition state, the system's volume contracts leading to the expulsion of the aqueous phase in which proteins are dissolved. This effect causes initial protein burst release which is greater with faster rate of gelation (Packhaeuser et al., 2004).

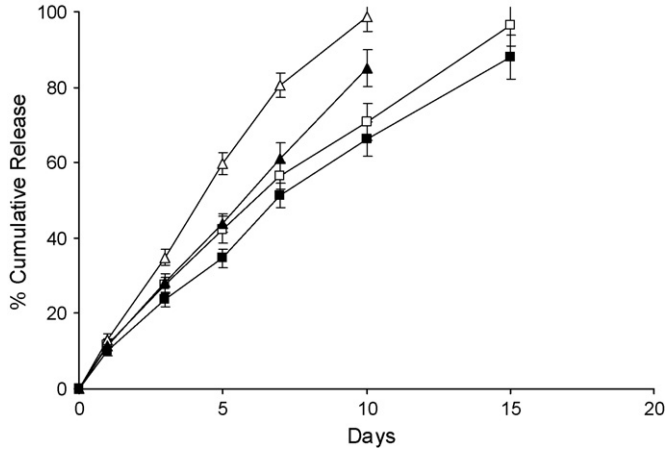

Fig. 6. *In vitro* release profiles of lysozyme and bromelain from thermosensitive polymer, TP2-based formulations [Keys: (□) lysozyme, 25% (w/v) TP2; (■) lysozyme, 30% (w/v) TP2; (△) bromelain, 25% (w/v) TP2; (▲) bromelain, 30% (w/v) TP2].

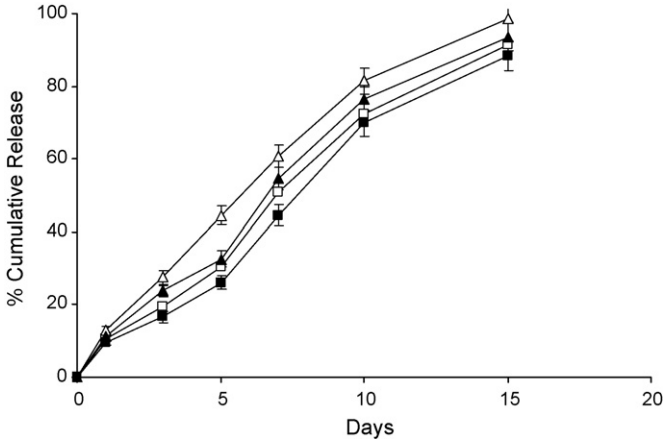

Fig. 7. *In vitro* release profiles of lysozyme and bromelain from thermosensitive polymer, TP3-based formulations [Keys: (□) lysozyme, 25% (w/v) TP3; (■) lysozyme, 30% (w/v) TP3; (△) bromelain, 25% (w/v) TP3; (▲) bromelain, 30% (w/v) TP3].

Table 2
Biological activity of lysozyme released from thermosensitive polymer TP1-based formulations

Days	Specific enzyme activity (EU/mg) × 10^3		
	Control	20% (w/v)	25% (w/v)
1	48.0 ± 1.8	48.5 ± 1.2	48.7 ± 1.7
14	41.2 ± 0.9	44.2 ± 1.5	44.5 ± 1.0
28	31.0 ± 1.1	41.0 ± 1.4	41.8 ± 1.1
42	22.5 ± 0.8	34.6 ± 0.9	35.2 ± 0.7
56	12.4 ± 0.7	26.4 ± 0.6	28.9 ± 0.5

TP1 = PEO:DL-PLA:IPDI:DL-PLA-PEO (5000:16,000:168:16,000:5000).

Table 3
Biological activity of bromelain released from thermosensitive polymer TP1-based formulations

Days	Specific enzyme activity (EU/mg)		
	Control	20% (w/v)	25% (w/v)
1	3.59 ± 0.45	3.65 ± 0.78	3.81 ± 0.44
14	1.56 ± 0.49	3.15 ± 0.49	3.47 ± 0.51
28	0.98 ± 0.15	2.56 ± 0.71	2.98 ± 0.46
42	0.15 ± 0.08	1.98 ± 0.19	2.24 ± 0.31

TP1 = PEO:DL-PLA:IPDI:DL-PLA-PEO (5000:16,000:168:16,000:5000).

Table 1 shows the release kinetics of lysozyme and bromelain from thermosensitive controlled release formulations. Release rate constants and correlation coefficients were calculated for zero-order and Higuchi's equations. The best fit of release kinetics with higher correlation coefficients was achieved with Higuchi's equation followed by zero-order release kinetics (Table 1).

In this study, we have not investigated the stability of the released lysozyme and bromelain from the delivery system. A protein must be stable to elicit the expected biological activity; therefore our future study will explore the chemical and conformational stability of the released lysozyme and bromelain.

3.3. Biological activity

Tables 2–7 show the biological activity of lysozyme and bromelain in released samples from the formulations. We found significantly ($p < 0.05$) greater specific enzyme activity in samples taken at various time points in comparison to the controls, except in day 1 released samples, for both lysozyme and bromelain.

Table 4
Biological activity of lysozyme released from thermosensitive polymer TP2-based formulations

Days	Specific enzyme activity (EU/mg) × 10^3		
	Control	20% (w/v)	25% (w/v)
1	48.0 ± 1.6	49.0 ± 1.3	49.7 ± 1.4
5	46.2 ± 1.0	48.1 ± 1.1	48.8 ± 1.5
10	44.1 ± 1.4	46.8 ± 1.7	47.1 ± 1.7
15	41.5 ± 0.7	45.0 ± 1.1	45.7 ± 1.2

TP2 = PLGA:PEG:PLGA (6000:3350:6000).

Table 5
Biological activity of bromelain released from thermosensitive polymer TP2-based formulations

Days	Specific enzyme activity (EU/mg)		
	Control	25% (w/v)	30% (w/v)
1	3.61 ± 0.57	3.75 ± 0.81	3.84 ± 0.47
5	3.19 ± 0.61	3.55 ± 0.59	3.62 ± 0.77
10	2.31 ± 0.71	3.12 ± 0.89	3.25 ± 0.69
15	1.26 ± 0.49	2.75 ± 0.49	2.85 ± 0.54

TP2 = PLGA:PEG:PLGA (6000:3350:6000).

Table 6
Biological activity of lysozyme released from thermosensitive polymer TP3-based formulations

Days	Specific enzyme activity (EU/mg) × 10^3		
	Control	25% (w/v)	30% (w/v)
1	48.4 ± 1.8	49.7 ± 1.3	50.0 ± 1.2
5	45.6 ± 2.5	47.4 ± 4.1	48.5 ± 2.3
10	41.5 ± 0.7	45.5 ± 1.3	45.6 ± 1.4
15	37.5 ± 0.8	40.7 ± 1.2	41.4 ± 1.5

TP3 = PLA:PEG:PLA (2500:1000:2500).

Organic solvents or any harsh treatments were not used in the preparation of thermosensitive formulations which might have entrapped protein in the gel structure preventing access of external aqueous media to the peptide backbone structure. Therefore, we found greater biological activity for lysozyme and bromelain from formulations in comparison to control samples.

Hydrophobic polymer matrix can lead to protein adsorption onto the polymer surface, denaturation and aggregation leading to instability and loss of biological activity of the incorporated protein (Crotts and Park, 1997; Crotts et al., 1997). The use of amphiphilic block copolymers in this study for proteins delivery has prevented the above problems (Bezemer et al., 2000).

Tables 2–7 show a decrease in the protein activity in the *in vitro* released samples with time. This is probably due to the presence of protein in releasing media for a specific time period. The protein activity in samples from formulations was significantly ($p < 0.05$) greater than the control at each time point. Control samples were taken from a 5% (w/v) lysozyme or bromelain (*i.e.*, same concentration as used in formulations) solution prepared in releasing media and added to releasing medium similarly as formulations. Therefore, decrease in activity could be attributed to storage conditions which will not happen in *in vivo* situation

Table 7
Biological activity of bromelain released from thermosensitive polymer TP3-based formulations

Days	Specific enzyme activity (EU/mg)		
	Control	25% (w/v)	30% (w/v)
1	3.61 ± 0.57	3.78 ± 0.74	3.88 ± 0.57
5	3.1 ± 0.43	3.51 ± 0.67	3.72 ± 0.71
10	1.96 ± 0.49	3.38 ± 0.54	3.65 ± 0.57
15	1.05 ± 0.49	3.25 ± 0.49	3.45 ± 0.44

TP3 = PLA:PEG:PLA (2500:1000:2500).

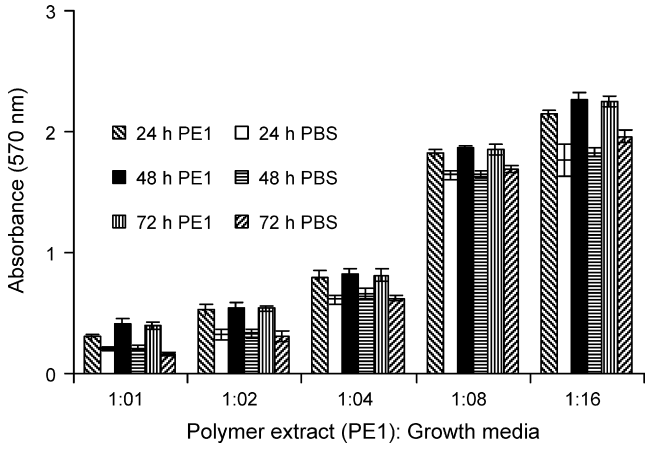

Fig. 8. *In vitro* biocompatibility polymer extract PE1 by MTT cell viability assay [PBS, phosphate-buffered saline, pH 7.4; PE1, polymer extract made with TP1].

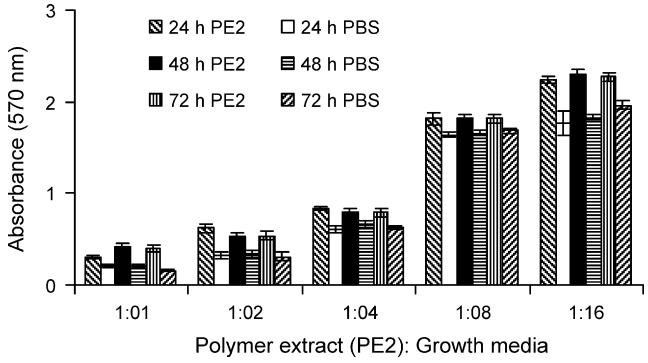

Fig. 9. *In vitro* biocompatibility of extract PE2 by MTT cell viability assay [PBS, phosphate-buffered saline, pH 7.4; PE2, polymer extract made with TP2].

where protein would be absorbed immediately after release from the gel formulation. The gel delays the easy access of water to its core and protects the incorporated protein from the destabilizing effect of external releasing medium.

3.4. Biocompatibility of the delivery systems

Figs. 8–10 show absorbances measured in the MTT cell viability assay. Higher absorbance readings mean greater cell viability (Ignatius and Claes, 1996). We found significantly greater ($p < 0.05$) cell viabilities in growth media diluted with polymer extracts than diluted with control for all dilutions and exposure periods.

Polymer extracts provided significantly ($p < 0.05$) higher cell viability than the control. Polymer extracts have lactic acid generated out of polymer degradation, which can stimulate mitochondrial activity by acting as a metabolic substrate. An increased in the mitochondrial activity may be the reason for significantly ($p < 0.05$) greater absorbance found for cells containing media diluted with polymer extract than the control.

Free urethane, which is known as ethyl carbamate, is reported to be toxic and carcinogenic. In our study, TP1 was synthesized using IPDI (isophorone diisocyanate) to link two mPEG–PLGA diblocks. Thus, TP1 is neither synthesized using free urethane nor contains free urethane. Our biocompatibility studies confirmed that TP1 is biocompatible based on cell viability studies.

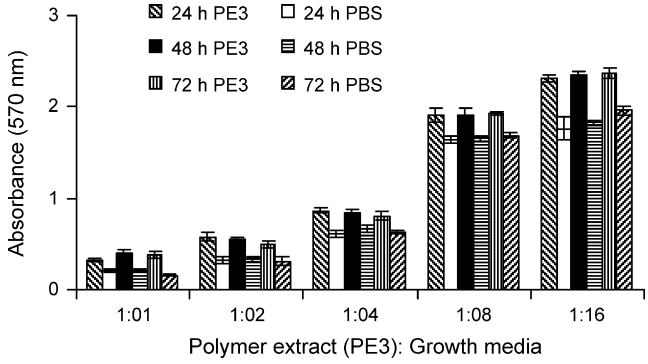

Fig. 10. *In vitro* biocompatibility of polymer extract PE3 by MTT cell viability assay [PBS, phosphate-buffered saline, pH 7.4; PE3, polymer extract made with TP3].

4. Conclusions

Three types of thermosensitive triblock copolymers were synthesized and characterized. Varying concentrations of polymers were used in the delivery systems to control the release rate of incorporated model proteins, lysozyme and bromelain. *In vitro* biocompatibility study using MTT assay showed that all the three polymers were biocompatible. The polymers were able to deliver the proteins at controlled rates in biologically active form up to 2–8 weeks. However, synthesis and characterization of different chain length of block polymers for further prolonging the duration of release, *in vivo* biocompatibility of polymers in an animal model, and *in vivo* absorption of proteins (especially therapeutic proteins) are major goals of our future studies.

Acknowledgement

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