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Research Paper

Thermoresponsive Gelatin/Monomethoxy Poly(Ethylene Glycol)–Poly(D,L-lactide) Hydrogels: Formulation, Characterization, and Antibacterial Drug Delivery

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Purpose. The primary objective of this study was to prepare novel thermoresponsive binary component hydrogels composed of gelatin and monomethoxy poly(ethylene glycol)–poly(D,L-lactide) (MPEG–PDLLA) diblock copolymer and to obtain optimal formulations capable of forming gels upon a narrow temperature range between body temperature and room temperature.
Methods. MPEG–PDLLA diblock copolymers with a lower critical solution temperature (LCST) feature were synthesized by using a ring-opening polymerization method. The starting weight ratio of MPEG/DLLA was varied to obtain a series of copolymers with a wide range of molecular weight and hydrophilicity. The copolymers were characterized by ^{1}H nuclear magnetic resonance (^{1}H NMR) and thermogravimetric analysis. MPEG (2K)–PDLLA (1:4) was chosen to construct hydrogels with gelatin. To obtain optimal thermoresponsive formulation, various hydrogels were formulated and quantified in terms of sol–gel phase transition kinetics and rheological properties. Selected hydrogels were studied as drug carrier for gentamicin sulfate.
Results. Gelatin/MPEG–PDLLA hydrogels underwent gelation in less than 15 min when 30 wt.% MPEG (2K)–PDLLA (1:4) was mixed with 10, 50, or 100 mg/mL gelatin. Hydrogels showed rapid gelation when 100 mg/mL gelatin was mixed with 15, 20, or 25 wt.% MPEG–PDLLA as temperature fell from 37°C to room temperature. The viscosity of hydrogels depended on the frequency applied in the rheological tests, the environment temperature, and the concentration of both polymer components. The time needed for 50% gentamicin sulfate release was 5 days or longer at room temperature, and the release lasted up to 40 days. ^{1}H NMR confirmed that MPEG–PDLLA hydrolyzed under *in vitro* situations.
Conclusions. The incorporation of a second polymer component MPEG–PDLLA into the gelatin hydrogel could modify the thermal characteristic of gelatin and the resulting binary component hydrogels obtained different thermal characteristics from the individual polymer components. Formulation of gelatin/MPEG–PDLLA hydrogels could be varied for obtaining such gels that can undergo gelation promptly upon a narrow temperature change.

KEY WORDS: gentamicin sulfate; *in vitro* degradation; rheology; thermoresponsive; tissue engineering.

INTRODUCTION

Hydrogels, composed of either chemically or physically cross-linked polymeric backbone, are capable of absorbing large amounts of aqueous solution and undergoing degradation via erosion, hydrolysis, solubilization, and other biodegradation mechanisms, and have been extensively investigated and widely used in tissue engineering (1,2) and drug delivery (3,4). Modulating hydrogel properties such as swelling/degradation, mechanical strength, drug release kinetics, and environment-oriented sensitivity to pH, light, and temperature may provide significant advantages for a specific biomedical application. In particular, hydrogels that can undergo gelation *in situ* upon mild external stimuli are of great interest to be explored as potential drug delivery scaffolds (5,6). One type of promising environmentally responsive hydrogels is thermoresponsive hydrogels, which form physical cross-links upon temperature change (7,8). Consequently, the use of cross-linking reagents can be avoided in the preparation of thermoresponsive hydrogels and the potential side effects such as toxicity caused by cross-linking agents can be minimized.

One of thermoresponsive hydrogels is gelatin-based hydrogels. Gelatin has biomedically favorable properties including biodegradability, low immunogenicity, low cytotox-

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Table I. Feeding Compositions of MPEG–PDLLA Diblock Copolymers and Respective Solvation Capability in Water at RT

Feeding composition			Solvation in water			
MPEG–PDLLA	MW of MPEG	MPEG–DLLA (w/w)	15 wt.%	20 wt.%	25 wt.%	30 wt.%
MPEG (5K)–PDLLA (1:4)	5 kDa	1:4	+	+	+−	+−
MPEG (5K)–PDLLA (1:1)	5 kDa	1:1	+	+−	+−	+−
MPEG (5K)–PDLLA (4:1)	5 kDa	4:1	++	++	++	++
MPEG (2K)–PDLLA (1:4)	2 kDa	1:4	+	+	+	+
MPEG (2K)–PDLLA (1:1)	2 kDa	1:1	+	+	+	+
MPEG (2K)–PDLLA (4:1)	2 kDa	4:1	++	++	++	++

++: Good solubility, clear solution; +: fully solvated, viscous solution; +−: partly solvated.

icity, and great capacity for potential modification at the level of amino acids, and it has already been approved as a clotting agent and as an exudate absorbing construct by FDA. Gelatin alone can form physical hydrogels by responding to temperature change; however, such hydrogels have poor mechanical and chemical stability (9,10). Gelatin's mechanical and chemical stability can be improved with the introduction of irreversible chemical cross-links into the gelatin matrix (11). For the incorporation of chemical cross-links into the matrix, gelatin can be either modified with photosensitive cross-linking groups or mixed with photosensitive group-containing polymers, then photocured to trigger cross-linking to form interpenetrating networks (11–17). Although thermal gelation is particularly preferred over photocuring strategy when light-sensitive bioactive agents are involved (18–24), no studies have been reported to use thermal gelation strategy to prepare gelatin-based binary component or multicomponent hydrogels of physical cross-links. Therefore, our primary goal was to use thermal gelation strategy to formulate gelatin-based binary component hydrogels *in situ*. We hypothesized that a second polymer with opposite thermal response would modify the thermal characteristic of gelatin, and the combination of these two polymers could provide a unique effect to produce hydrogels with thermal characteristics different from the individual polymer components. We designed a new family of hydrogels that are composed of gelatin and monomethoxy poly(ethylene glycol)–poly(D,L-lactide) (MPEG–PDLLA) diblock copolymer. An aqueous solution of gelatin flows at elevated temperatures (35°C or higher) and undergoes gelation as temperature decreases. In contrast to gelatin, monomethoxy poly(ethylene glycol)–poly(D,L-lactide) (MPEG–PDLLA) diblock copolymers have a lower critical solution temperature (LCST) feature, allowing the polymers to flow at temperatures below the LCST and to form a gel as temperature increases above the LCST (7,8). In addition, MPEG–PDLLA has desired biodegradability, nonimmunogenicity, and noncytotoxicity, and its hydrophilicity and molecular weight can also be modified. We prepared a series of formulations of gelatin/MPEG–PDLLA hydrogels in an attempt to determine the optimal formulations that can make gelatin/MPEG–PDLLA binary solutions undergo gelation upon mild temperature changes, that is, from room temperature to body temperature, during a reasonable period of time. The thermal property of the hydrogels was quantified in terms of sol–gel phase transition kinetics and rheological behaviors. Gentamicin sulfate, a broad-spectrum antibiotic commonly used against infections, was chosen to be incorporated into the hydrogel matrix to provide a bacteria-free environment favorable to cell growth, proliferation, and differentiation, and the drug release kinetics and the stability of hydrogels were determined under *in vitro* conditions.

MATERIALS AND METHODS

Materials

Monomethoxy poly(ethylene glycol) (MPEG, MW = 2 or 5 kDa), 3,6-dimethyl-1,4-dioxane-2,5-dione(D,L-lactide) (DLLA), tin ethyl hexanoate, gelatin (type A, derived from porcine skin, 300 bloom, 50–100 kDa), toluene, dichloromethane (DCM), diethyl ether, gentamicin sulfate (potency: ~600 μg gentamicin/mg), *o*-phthaldialdehyde (OPA), phosphate-buffered saline (PBS, 10×), and chloroform-d ($CDCl_3$, 99.8 at.% D) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Milli-Q deionized water was used throughout the experiment.

Synthesis of MPEG–PDLLA Diblock Copolymers

MPEG–PDLLA diblock copolymers with various molecular weights of MPEG (2 or 5 kDa), and with various feeding weight ratios of MPEG/DLLA (4:1, 1:1, or 1:4) were synthesized based on a previous work (25). The synthesis of MPEG (2K)–PDLLA (1:4) was as follows: 1 g of MPEG with MW = 2 kDa and 4 g of lactide were dissolved in 20 mL toluene, which was azeotropically distilled prior to use. The catalyst, 20 μL of tin ethyl hexanoate, was added to the solution. The solution was flushed with argon for 10 min and then placed in a tightly capped vial under argon. The reaction was allowed to proceed for 2 h in a silicone oil bath at 95–100°C, during which MPEG–PDLLA diblock copolymers were created in the ring-opening polymerization. Upon completion of the reaction, toluene was evaporated using rotary evaporation. The resulting polymer was dissolved in a small volume of DCM, and then the polymer was precipitated in cold diethyl ether, filtered, and vacuum-dried. The final product, MPEG (2K)–PDLLA (1:4), was subsequently lyophilized into a white powder. The other copolymers with varying feed ratios were synthesized following a similar method (Table I).

Characterization of MPEG–PDLLA Diblock Copolymers

The copolymers were characterized by ^{1}H nuclear magnetic resonance (^{1}H NMR) spectroscopy on a Varian

Table II. Time Scales of Sol–Gel Phase Transition of Gelatin/MPEG (2K)–PDLLA (1:4) Binary Hydrogels at RT and 37°C

	MPEG (2K)–PDLLA (1:4) (wt.%)									
	0		15		20		25		30	
Gelatin (mg/mL)	37°C	RT	37°C	RT	37°C	RT	37°C	RT	37°C	RT
0	/	/	‡	‡	‡	‡	+	‡	†	‡
10	‡	‡	‡	‡	‡	‡	‡	‡	#	−
50	‡	†	‡	‡	‡	‡	+	−	#	−
100	‡	#	‡	#	‡	#	‡	#	#	−

−: no flow; /: no material tested; ‡: flow up to 20 h and beyond; #: flow up to 15 min, then no flow thereafter; †: flow up to 5 h, then no flow thereafter; +: flow up to 10–20 h, then no flow thereafter.

^{UNITY}INOVA spectrometer (400 MHz) (Palo Alto, CA, USA) using $CDCl_3$ as solvent.

The water solubility of the synthesized copolymers was examined with solvation tests. Each dry polymer sample was mixed with water with the weight ratios of 15, 20, 25, or 30 wt.% (w/w, polymer/water). The tests were carried out at room temperature (RT) and observed after 5 h during which the samples were completely equilibrated with water. The extent of solubility of samples are listed in Table I.

Thermogravimetric analysis (TGA) tests were carried out on a Q600 SDT thermogravimetric analyzer (TA Instruments, New Castle, DE, USA). Samples (3–5 mg) were heated at a heating rate of 10°C/min under nitrogen flow of 100 mL/min. Weight and temperature differences as a function of time were recorded.

Formulation of Gelatin/MPEG–PDLLA Hydrogels

MPEG (2K)–PDLLA (1:4) was chosen to formulate hydrogels with gelatin for the study of sol–gel phase transition kinetics, because of the good solvation in water and reasonable required concentration for gelation. Solutions of 10, 50, and 100 mg/mL gelatin were prepared by dissolving appropriate amounts of gelatin in deionized water at 80°C and maintained at 37°C. Dry powder of MPEG (2K)–PDLLA (1:4) was added to each gelatin solution and vortexed to obtain 15, 20, 25, and 30 wt.% solutions (MPEG–PDLLA/water, w/w). Sol–gel phase transition kinetics of hydrogel samples was measured at 37°C for up to 24 h, and then the samples were cooled to RT for another 24 h measurement.

Characterization of Gelatin/MPEG–PDLLA Hydrogels

Sol–gel phase transition was estimated by "flow" or "no-flow" over 30 s in capped vials that were inverted at predetermined time intervals (26). The kinetics of phase transition is listed in Table II.

Rheological measurements were performed at 21 and 37°C on a Bohlin VOR rheometer equipped with a parallel-plate geometry. Each gel solution was placed between parallel plates (diameter: 25 mm) and a gap of 1 mm and tested under frequency ranging from 6.28×10^{-2} to 6.28 rad/s. Viscosity of gels as a function of frequency was measured, and two replicate runs were performed for each condition. For clarity, each data point is presented as an average of viscosity data points.

Antibacterial Drug Delivery

Gentamicin sulfate delivery using gelatin/MPEG–PDLLA hydrogels was investigated in PBS buffer (pH 7.4) at RT and 37°C. A stock solution of gentamicin sulfate (50 mg/mL) was prepared first. Gelatin of appropriate amount was dissolved in 1 mL of gentamicin stock solution in a 4-mL scintillation vial, and the solution was heated to 80°C for 5 min after which the solution became clear. The solution was maintained at 37°C thereafter. Gentamicin-loaded matrices were prepared by vortexing the dry powder of MPEG (2K)–PDLLA (1:4) with a predetermined amount in a gelatin solution, then allowing the formulated matrices to stand at RT or 37°C for 24 h during which gelation occurred. The formulations of matrices for the drug release study are listed in Table III.

Table III. Formulations of the Gentamicin-Loaded Matrices

	Composition of gentamicin-loaded matrices		
Matrix no.	MPEG (2K)–PDLLA (1:4) (mg)	Gelatin (mg)	50 mg/mL of gentamicin solution (mL)
M1	428	0	1.0
M2	428	10	1.0
M3	428	50	1.0
M4	428	100	1.0
M5	0	100	1.0
M6	0	50	1.0
M7	150	100	1.0
M8	200	100	1.0
M9	250	100	1.0

For the drug release study, 1.5 mL of PBS buffer (pH 7.4) was added to each vial containing the matrix and incubated at RT or 37°C. At each time point, the remaining hydrogels were weighed after carefully transferring the buffer to a test tube. After each measurement, 1.5 mL of fresh buffer was added. Each test tube of collected buffer was centrifuged at 8000 rpm, and the supernatant was collected and then spectroscopically measured to determine the amounts of the released gentamicin. A modified Sampath and Robinson method (27) was used to determine gentamicin concentrations. A stock solution of OPA was prepared by mixing 2.5 g of OPA, 62.5 mL of methanol, and 3 mL of 2-mercaptalethanol with 560 mL of 0.04 M sodium borate solution in an aluminum-foil-wrapped bottle, then left for 24 h prior to measurement. The reagent was stored at 4°C and kept for no longer than 1 month (28). For calibration, a series of gentamicin solutions at known concentrations were vortexed with a mixture solution of 0.7 mL of isopropyl alcohol, 0.45 mL of OPA solution, and 0.35 mL of deionized water, and the reaction was allowed to proceed at RT for 45 min. The final concentrations of gentamicin for calibration were between 0 and 50 μg/mL. A correlation coefficient of 0.90 was obtained from the calibration curve: absorbance (Abs) = 0.0015 × concentration (μg/mL) + 0.0042. The absorbance of the aliquots of the removed buffer relative to a blank reagent solution was measured at a wavelength of 332 nm on a Genesys™ 8 UV–Vis spectrophotometer (Thermospectronic, Rochester, NY, USA). The mass ratio of drug released, M_t/M_o (where M_t is the accumulated mass of drug released at time t and M_o is the original mass of drug loaded), was quantified as a function of time.

IN VITRO STABILITY OF HYDROGELS

The dissolution of the matrices and the degradation of MPEG–PDLLA copolymers were investigated *in vitro* during the drug release study. *In vitro* dissolution was quantified by measuring the weight of the remaining matrices as a function of time. At each predetermined time point, the weights of the matrices remaining in the vials were measured gravimetrically after the buffer was removed. For the degradation studies, the removed PBS buffer samples were centrifuged and the dry insoluble component was obtained by vacuum drying. The dry samples were dissolved in chloroform and centrifuged to remove gelatin. The supernatants were dialyzed for 2 days against chloroform using Spectra/Por® 7 regenerated cellulose membrane with MWCO 2 kDa (Spectrum Laboratories, Rancho Dominguez, CA, USA), and then lyophilized. The lyophilized samples were dissolved in $CDCl_3$ and characterized by ^1H NMR spectroscopy. The data points in mass change of the matrices, cumulative release of gentamicin, and gentamicin concentration in the remained matrices are expressed as means ± SD.

RESULTS AND DISCUSSION

Effects of Molecular Weight of MPEG, Weight Ratio of MPEG to DLLA, and Copolymer Concentration on Solvation Capability

Solvation of MPEG–PDLLA copolymers, a critical parameter in gel formulation, was tested. Such a parameter for MPEG–PDLLA has not been established in the literature. As molecular weight and hydrophilicity affect the rheological properties of hydrogels (29), MPEG–PDLLA copolymers of various hydrophilicity and molecular weight were synthesized by varying the molecular weight of MPEG and the weight ratio of MPEG to DLLA. The MPEG–PDLLA diblock copolymers we synthesized were as follows: MPEG (5K)–PDLLA (4:1), MPEG (5K)–PDLLA (1:1), MPEG (5K)–PDLLA (1:4), MPEG (2K)–PDLLA (4:1), MPEG (2K)–PDLLA (1:1), and MPEG (2K)–PDLLA (1:4). As the hydrophobic PDLLA content decreased, the copolymer became more hydrophilic. For copolymers with the same weight ratio as MPEG/DLLA, for example, MPEG (5K)–PDLLA (4:1) and MPEG (2K)–PDLLA (4:1), the decrease in molecular weight of MPEG from 5 to 2 kDa led to the decrease in the molecular weight of the copolymer. However, the overall hydrophilicity was the same for both copolymers. Based on our results (Table I), the hydrophilicity and the molecular weight both influenced the solvation of MPEG–PDLLA copolymers. MPEG (5K)–PDLLA (4:1) and MPEG (2K)–PDLLA (4:1) were highly hydrophilic because of a large portion of MPEG segments. Thus, MPEG (5K)–PDLLA (4:1) and MPEG (2K)–PDLLA (4:1) could completely dissolve in water even at the selected highest concentration, i.e., 30 wt.%. When hydrophilic MPEG segments and hydrophobic PDLLA segments were equal in weight (e.g., MPEG–PDLLA, 1:1) or when PDLLA segments dominated in weight (e.g., MPEG–PDLLA, 1:4), the molecular weight and the concentration of the copolymer affected solvation more than hydrophilicity. Specifically, MPEG (2K)–PDLLA (1:4) and MPEG (2K)–PDLLA (1:1) of low molecular weight were soluble at 15, 20, 25 and 30 wt.%. MPEG (5K)–PDLLA (1:4) and MPEG (5K)–PDLLA (1:1) were completely solvated in water at a low concentration of 15 wt.%. MPEG (5K)–PDLLA (1:1) could only be partially solvated at concentrations higher than 15 wt.%. Although MPEG (5K)–PDLLA (1:4) at 20 wt.% could be fully solvated at 25 and 30 wt.%, the mixture could not be fully solvated. Interestingly, at 20 wt.%, MPEG (5K)–PDLLA (1:4) of less hydrophilicity was completely solvated and MPEG (5K)–PDLLA (1:1) of more hydrophilicity was partially solvated. In this case, we hypothesized that swelling ratio may increase with increasing hydrophilicity of MPEG–PDLLA and that more water was needed to completely solvate the copolymer. After prescreening the synthesized copolymers through solvation tests, MPEG (2K)–PDLLA (1:4) was chosen for formulating hydrogels with gelatin because of the good solvation in water as well as the reasonable concentration required to undergo gelation.

Composition Effect on Phase Transition Temperature of the Diblock Copolymers

Thermal properties of the copolymers were investigated using TGA. Samples were heated from 20 to 200°C at a rate of 10°C/min. A typical curve of MPEG (2K)–PDLLA (1:4) is presented in Fig. 1. MPEG (2K)–PDLLA (1:4) lost 21–22 wt.% when the sample was heated up to 200°C. The curve was reformatted based on temperature differences, and a peak identified at 84.8°C indicated a transition from solid to melt phase. The composition affected the thermal behavior of the copolymers, especially the starting phase transition

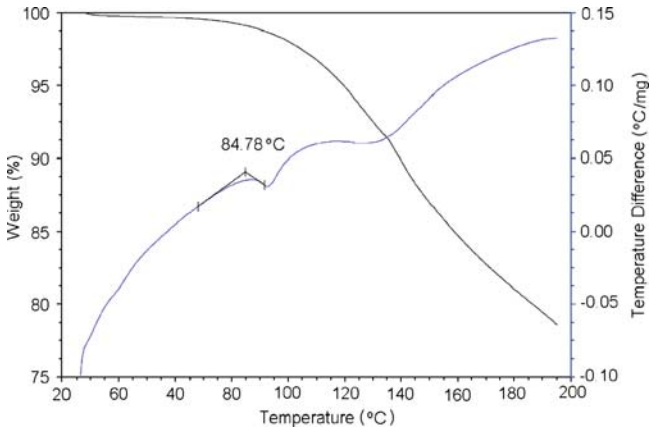

Fig. 1. Thermogravimetric analysis curve of MPEG (2K)–PDLLA (1:4) copolymer.

temperature. MPEG (2K)–PDLLA (1:1) and MPEG (2K)–PDLLA (4:1) showed a lower starting phase transition temperature at 39.5 and 45.9°C, respectively. A similar trend was found in MPEG (5K)-based copolymers, but with an average of 4–6°C higher than MPEG (2K)-based copolymers of the same weight ratio. The starting phase transition temperatures were 88.9, 45.4, and 49.5°C for MPEG (5K)–PDLLA (1:4), MPEG (5K)–PDLLA (1:1), and MPEG (5K)–PDLLA (4:1), respectively.

Effects of Temperature and Formulation on Sol–Gel Phase Transition Kinetics of Gelatin/MPEG–PDLLA Binary Hydrogels

Sol–gel phase transition is an important parameter that determines the injectability and the formulation condition (30). Sol–gel phase transition temperatures can be determined by test tube inverting method (26,30), falling ball method (30,31), or dynamic mechanical analysis (30,32). We employed the test tube inverting method to determine sol–gel phase transition kinetics or time scales for the gelation of hydrogels. The results for the hydrogel based on MPEG (2K)–PDLLA (1:4) and gelatin are tabulated in Table II. Several formulations of the hydrogel showed different sol–gel transition kinetics compared to pure gelatin and pure MPEG (2K)–PDLLA (1:4). At 37°C, when 100 mg/mL gelatin was mixed with 15, 20, or 25 wt.% MPEG–PDLLA, the hydrogels flowed up to 20 h and beyond. However, hydrogels underwent gelation in less than 15 min when 100 mg/mL gelatin was mixed with 30 wt.% MPEG (2K)–PDLLA (1:4). Gelatin-based hydrogels flowed at 50 mg/mL when mixed with 15 or 20 wt.% MPEG–PDLLA at 37°C. An increase in polymer concentration to 25 wt.% resulted in the hydrogel undergoing gelation within 20 h. However, hydrogel gelation occurred within 15 min when gelatin was mixed with 30 wt.% MPEG (2K)–PDLLA (1:4). Except for when 10 mg/mL gelatin was mixed with 30 wt.% MPEG (2K)–PDLLA (1:4) and underwent quick gelation within 15 min, the other 10 mg/mL gelatin-based hydrogels still flowed at 37°C. We also observed hydrogels undergoing a faster phase transition, when 100 mg/mL gelatin was mixed with 15, 20, or 25 wt.% MPEG (2K)–PDLLA (1:4), as temperature changed from 37°C to RT. Gelatin-based hydrogel at 100 mg/mL containing 15, 20 or 25 wt.% MPEG (2K)–PDLLA (1:4) could form gelation completely within 15 min as soon as they are taken out from the 37°C water bath and cooled to RT.

MPEG–PDLLA copolymers consist of a mixture of hydrophilic and hydrophobic segments. In such polymers, hydrogen bonding dominates at low temperatures, thus enhancing polymer dissolution in water (7). At high temperatures, hydrophobic interactions become more significant than hydrogen bonding. Micelles can form at certain concentrations when hydrophobic interactions begin to dominate and the subsequent packing of the hydrophobic segments occurs (7). The critical micelle concentration decreases as temperature increases, whereas micelle size and association number increase (29). Further association of micelles leads to sol–gel phase transitions for MPEG–PDLLA. In our MPEG–PDLLA systems, 25 and 30 wt.% MPEG–PDLLA could form "no-flow" gels within 10–20 and 1–5 h, respectively. Gelatin forms transparent elastic thermoreversible gels at temperatures below 35°C (9). We showed that the combination of these two classes of polymers, gelatin and MPEG–PDLLA, produced hydrogels with a variety of sol–gel transition kinetics different from the individual polymer components.

Effects of Temperature and Composite Concentration on Viscosity of Gelatin/MPEG–PDLLA Binary Hydrogels

The rheological behavior of the hydrogels was quantified in terms of viscosity. The viscosities of gelatin gel, MPEG–PDLLA gel, and gelatin/copolymer hydrogels at 21 and 37°C were measured as a function of frequency as shown in Figs. 2–4. The hydrogels displayed decreasing viscosity with increasing frequency. As seen in Fig. 2, gelatin gel and MPEG–PDLLA gel have opposite viscosity trends with temperature changes. MPEG–PDLLA's viscosity of 428 mg/mL increased as temperature increased, for example, from 4.0×10^2 Pas at 21°C to 1.1×10^3 Pas at 37°C when the frequency was 6.28×10^{-2} rad/s. In contrast, the viscosity of gelatin gel (100 mg/mL) decreased as temperature increased, for

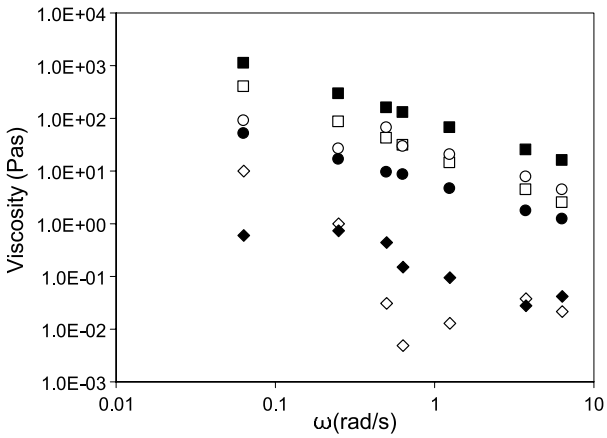

Fig. 2. Changes in the viscosities of single component gel solution as a function of frequency at 21 and 37°C, respectively. 428 mg/mL of MPEG (2K)–PDLLA (1:4), □ (21°C), ■ (37°C); 100 mg/mL gelatin, ○ (21°C), ● (37°C); 50 mg/mL gelatin, ◊ (21°C), ◆ (37°C).

example, from 9.2×10^1 Pas at 21°C to 5.1×10^1 Pas at 37°C when the frequency was 6.28×10^{-2} rad/s. Gelatin gel displayed lower viscosity at 50 mg/mL than at 100 mg/mL, and the viscosity of gelatin gel at 50 mg/mL was not sensitive to temperature changes.

The effect of gelatin concentration on the viscosity of hydrogels was studied at 21°C (Fig. 3A) and at 37°C (Fig. 3B) by keeping the concentration of MPEG–PDLLA constant at 428 mg/mL. At 21°C, the viscosity of hydrogels increased when gelatin concentration changed from 0 to 10 mg/mL, then to 50 mg/mL. However, hydrogel viscosity began to decrease when the concentration of gelatin was further increased to 100 mg/mL. At 37°C, the same hydrogels showed opposite viscosity trends: viscosity decreased when gelatin concentration was increased from 0 to 10 mg/mL, then to 50 mg/mL; conversely, viscosity began to increase as the concentration of gelatin was further increased to 100 mg/mL. The results implied that when the sample was at 21°C and when the gelatin concentration was below 50 mg/mL, the increase in viscosity of hydrogels may be attributable to the addition of gelatin. However, when the gelatin concentration exceeded 50 mg/mL, gelatin dominated in determining the viscosity of the hydrogel. At 37°C, the incorporation of gelatin with the concentration below 50 mg/mL decreased the viscosity of hydrogels, whereas gelatin with the concentration over 50 mg/mL increased the viscosity of hydrogels.

The effect of MPEG–PDLLA concentration on the viscosity of hydrogels was also studied at 21°C (Fig. 4A) and at 37°C (Fig. 4B) by keeping the concentration of gelatin constant at 100 mg/mL. At 21°C, the viscosity of hydrogels increased as more gelatin was added. At 37°C, the addition of MPEG–PDLLA with a concentration below 200 mg/mL decreased the viscosity of hydrogels. However, when the concentration of MPEG–PDLLA was over 200 mg/mL, the viscosity started increasing with the increase in the MPEG–PDLLA concentration. The viscosities of M5, M6, M7, M8, and M9 at 37°C were lower than the viscosities of other matrices. This could be used to explain why M5, M6, M7, M8, and M9 did not undergo gelation. Therefore, M5, M6, M7, M8, and M9 were not considered for the drug release study at 37°C. The viscosity of hydrogels depended on the frequency applied in the rheological tests, the environment temperature, and the concentration of both polymer components. Although higher frequency corresponded with lower viscosity, the effects of temperature and concentration on hydrogel viscosity were complex because of the opposing thermal characteristics of gelatin and MPEG–PDLLA.

The gelatin/PEG–PDLLA hydrogels are formed only through physical cross-links in this study. Most of the existing

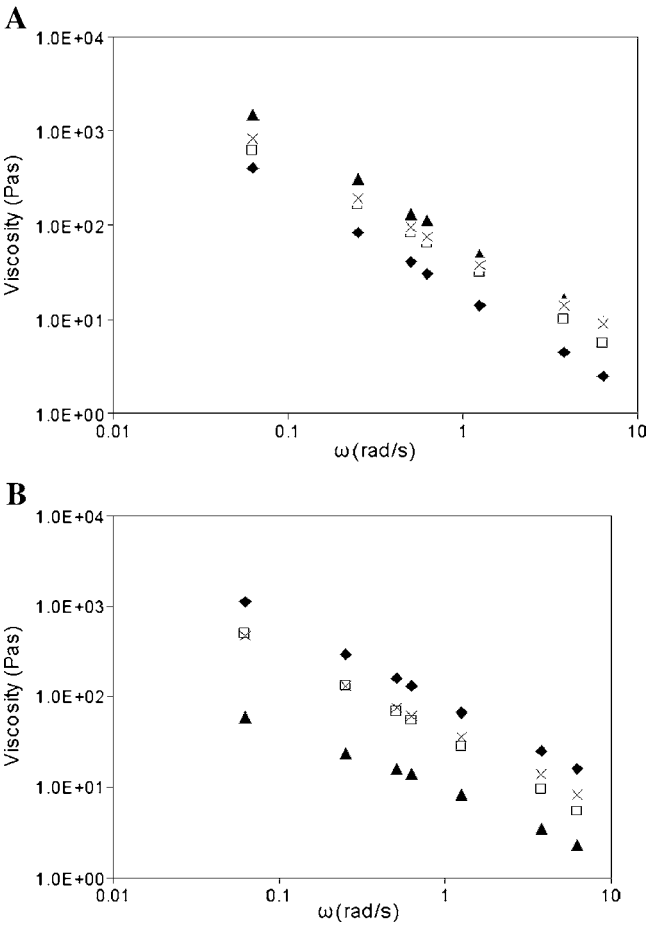

Fig. 3. Effect of gelatin concentration on viscosity of gelatin/MPEG–PDLLA hydrogels at 21°C (A) and 37°C (B), respectively. M1 (♦), M2 (□), M3 (▲), M4 (×).

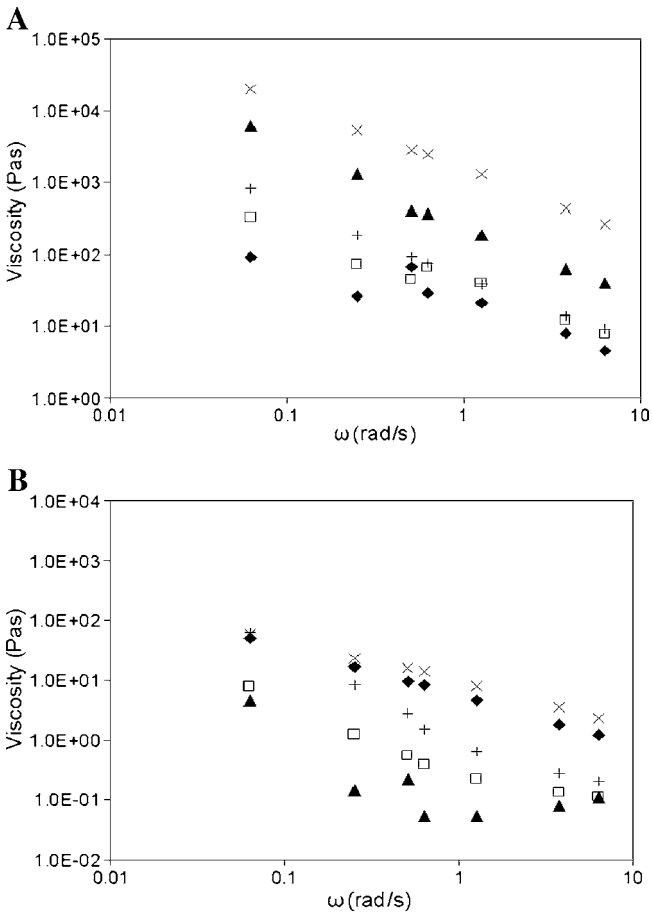

Fig. 4. Effect of MPEG–PDLLA concentration on viscosity of gelatin/MPEG–PDLLA hydrogels at 21°C (A) and 37°C (B). M4 (×), M5 (♦), M7 (□), M8 (▲), M9 (+).

gelatin-based hydrogels are made through either modification with photosensitive cross-linking groups or being mixed with photosensitive-group-containing polymers, then photocured to trigger cross-linking to form hydrogels (11–17). The gelatin hydrogels were also prepared through chemical cross-link (33), gamma ray, and electron beam (34). The incorporation of a second polymer of opposite thermal response to modify the thermal characteristic of gelatin and to produce hydrogels with thermal characteristics different from the individual polymer components is unique as compared to other gelatin-based hydrogels. Through characterization of gelatin/MPEG–PDLLA hydrogels, we have found that several formulations based on this new type of hydrogels could rapidly undergo gelation at room temperature and 37°C or when the temperature drops from 37°C to room temperature.

Effects of Temperature and Composite Concentration on Stability of Hydrogels and Gentamicin Release

The dissolution of hydrogel matrices in PBS buffer (pH 7.4) was investigated at RT and 37°C for the quantification of the stability of physically cross-linked hydrogels. A high temperature (37°C) disrupted the weakly bonded matrices (Fig. 5B). By 168 h, a mass loss of matrices up to 80 wt.% was observed. In contrast, the integrity of the matrices was not disturbed at RT, and the matrices continuously increased in weight and swelled as a result of water absorption up to 40 days (Fig. 5A).

Gentamicin sulfate—a mixture of the sulfates of gentamicin C1, gentamicin C1A, and gentamicin C2—is a water-soluble antibiotic of the aminoglycoside group, which has activity against a wide spectrum of pathogenic gram-negative and gram-positive bacteria, and mycoplasma. The gelatin/MPEG (2K)–PDLLA (1:4) hydrogel was studied as matrix for the delivery of gentamicin in PBS buffer (pH 7.4) at RT and 37°C. Formulations of drug-loaded matrices are listed in Table III. At RT, all matrices showed a delayed gentamicin release. The time needed for 50% drug release was 5 days or longer (Fig. 6A). Gentamicin was continuously released up to 40 days at RT. Interactions between hydrophilic gentamicin and the hydrophilic compositions of the matrix were believed to be a main factor causing a significantly sustained release of gentamicin. At 37°C, 50% gentamicin was released from M1

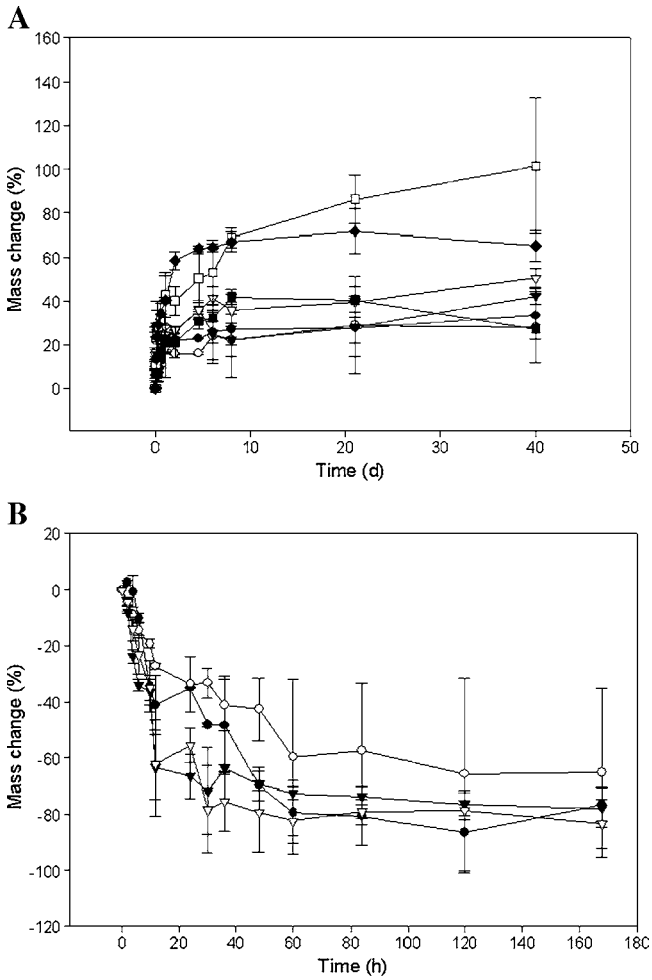

Fig. 5. Mass change of gentamicin-loaded matrices in pH 7.4 PBS buffer at RT (A) and 37°C (B). A: M3 (♦), M4 (□), M5 (●), M6 (○), M7 (▼), M8 (▽), M9 (■); B: M1 (●), M2 (○), M3 (▼), M4 (▽).

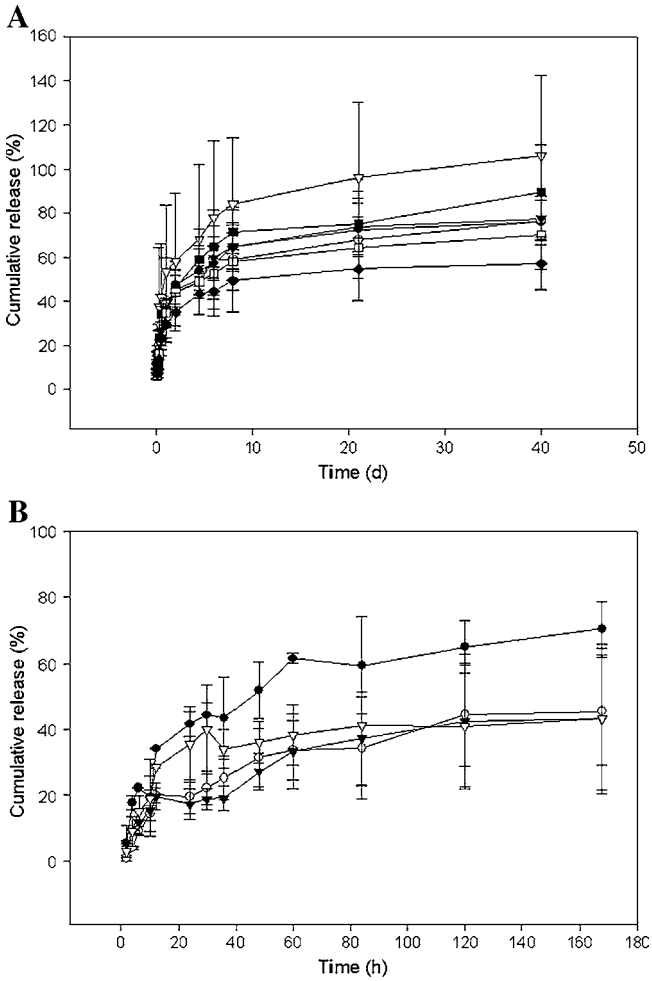

Fig. 6. Cumulative release of gentamicin from gentamicin-loaded matrices in pH 7.4 PBS buffer at (A) RT and (B) 37°C. A: M3 (♦), M4 (□), M5 (●), M6 (○), M7 (▼), M8 (▽), M9 (■); B: M1 (●), M2 (○), M3 (▼), M4 (▽).

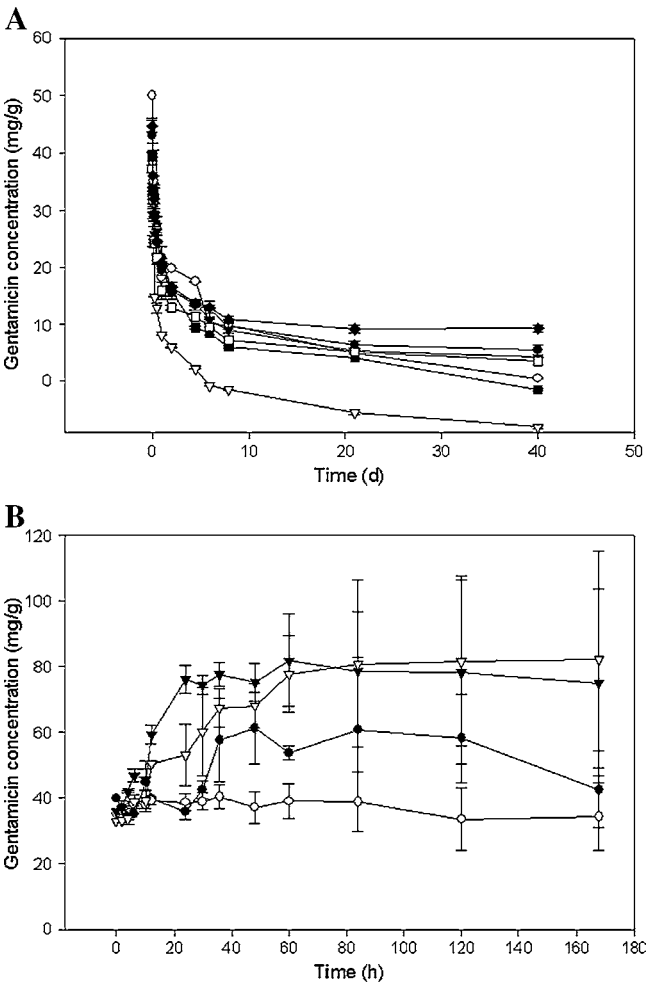

Fig. 7. Gentamicin concentration in remaining gentamicin-loaded matrices at RT (A) and 37°C (B). A: M3 (♦), M4 (□), M5 (●), M6 (○), M7 (▼), M8 (▽), M9 (■); B: M1 (●), M2 (○), M3 (▼), M4 (▽).

within 30 h, and M1 had a distinctively faster drug release rate compared to gelatin-containing M2, M3, and M4 (Fig. 6B). As M1 is composed of MPEG (2K)–PDLLA (1:4) only, the results suggest that gentamicin may have a weaker affinity to single-component copolymer matrices (i.e., MPEG–PDLLA only) over gelatin-containing matrices. Gentamicin release was not detectable after 168 h at 37°C because of a significant mass loss of the matrices. Gentamicin, actively transported across the bacterial cell membrane, binds to the ribosomal A site and causes a misreading of the genetic code and inhibits translocation (35). Consequently, nonfunctional proteins are produced; polyribosomes are split apart and are unable to synthesize protein. During this process, the positive charges of the amine groups of gentamicin at biological pH contribute to RNA binding (36). In the drug release study, the amino groups of the released gentamicin were derivatized with OPA to yield chromophoric products. This indicates the amine groups of gentamicin were functionally available and suggests that gentamicin may maintain the antibiotic activity after being released from the hydrogels.

To obtain a complete understanding of drug delivery and release using such hydrogels, the amount of gentamicin remaining in the matrices was also quantified based on mass balance. Antibiotic concentration is plotted against time as shown in Fig. 7. A significant decrease in antibiotic concentration was observed at RT (Fig. 7A) because of the swelling of the matrix and gentamicin release. The antibiotic concentration dropped from 37.7–50.0 mg/g to below 10.0 mg/g within 10 days as a consequence of gel swelling and drug release. At 37°C, the mass loss of the matrices was faster than drug release, and thus the remaining gentamicin maintained a higher antibiotic concentration compared to initial gentamicin concentrations (Fig. 7B).

The degradation of MPEG–PDLLA copolymers within the hydrogels was investigated. The ester bonds in the repeat units of PDLLA can break randomly along the polymer chain in PBS buffer (pH 7.4), and the degraded PDLLA segments were observed as an indication of the copolymer degradation. The hydrolyzed products were separated from the copolymers using dialysis bags with membrane of molecular weight cut off 2 kDa and characterized via ^{1}H NMR. Because MPEG has a molecular weight of 2 kDa, those PDLLA fragments with molecular weight near or below 2 kDa as well as most MPEG fragments would come out of the dialysis bag if PDLLA broke at the sites near

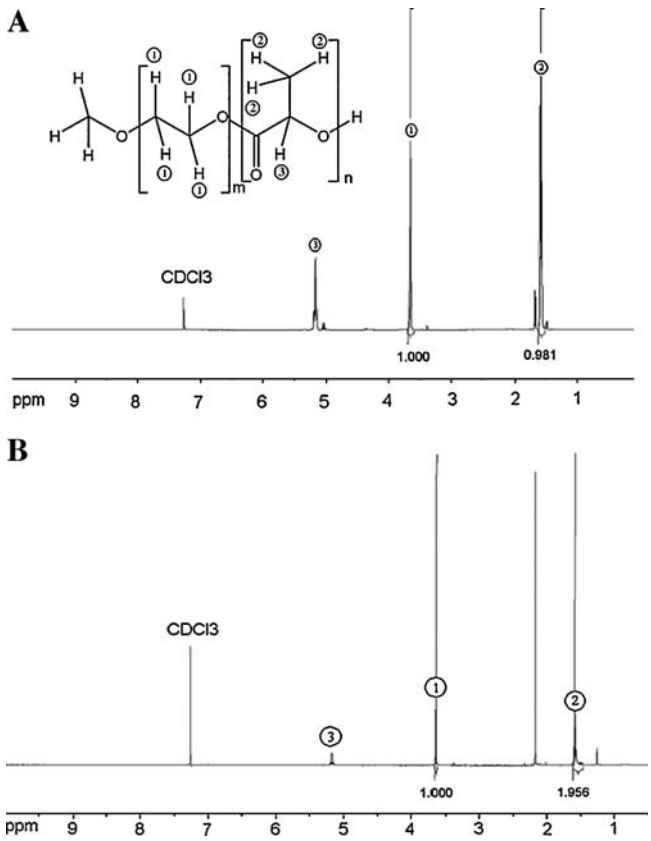

Fig. 8. ^{1}H NMR spectra of MPEG (2K)–PDLLA (1:4) copolymer before incubation (A) and after 120 h incubation in pH 7.4 PBS buffer at 37°C (B). (For the chemical structure of MPEG–PDLLA, *m* and *n* are the numbers of the repeat units for MPEG and PDLLA, respectively. *m* is 45 for MPEG 2 kDa and 114 for MPEG 5 kDa; *n* varies from 7 to 111 for MPEG 2 kDa-based copolymers and from 14 to 278 for MPEG 5 kDa-based copolymers according to the starting feed ratio of MPEG to DLLA. Protons in methylene, methyl, and methine are labeled as ①, ②, and ③, respectively, for ^{1}H NMR analysis.)

MPEG. Based on Fig. 8A, the proton intensity ratio of the methylene of MPEG to the methyl of PDLLA is 1:0.981. The methylene of MPEG and the methyl of PDLLA have four protons and three protons, respectively. Because the number of the repeat unit of MPEG 2K was 45, the number of repeat unit of PDLLA was calculated as 59. Therefore, the average weight ratio of MPEG to PDLLA was 1:2.12. Based on the proton intensity integration from the ^{1}H NMR spectra (Fig. 8A and B), the average weight ratio of MPEG to PDLLA changed from the initial 1:2.12 to 1:4.22 after 120 h. The change of weight ratio of MPEG to PDLLA indicated that MPEG–PDLLA copolymers hydrolyzed during drug release. Increase in the weight percentage of PDLLA was due to the PEG-dominating fragments after hydrolysis became less soluble in chloroform, resulting in an appreciable loss of PEG fragments when the hydrolyzed product was redissolved in chloroform for ^{1}H NMR analysis.

CONCLUSIONS

Hydrogels were prepared using gelatin and MPEG–PDLLA. MPEG–PDLLA copolymers were synthesized using a ring-opening polymerization method to obtain a wide range of molecular weight and hydrophilicity. Thermal gelation strategy was applied to formulate gelatin-based binary component scaffolds (gelatin/MPEG–PDLLA) *in situ*. A second polymer, i.e., MPEG–PDLLA, with opposite thermal response would modify the thermal characteristic of gelatin and the combination of these two polymers provided a unique effect to produce hydrogels with thermal characteristics different from the individual polymer components.

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REFERENCES

1. K. Y. Lee and D. J. Mooney. Hydrogels for tissue engineering. *Chem. Rev.* **101**:1869–1879 (2001).
2. A. S. Hoffman. Hydrogels for biomedical applications. *Adv. Drug Deliv. Rev.* **54**:3–12 (2002).
3. Y. Qiu and K. Park. Environment-sensitive hydrogels for drug delivery. *Adv. Drug Deliv. Rev.* **53**:321–339 (2001).
4. N. A. Peppas, P. Bures, W. Leobandung, and H. Ichikawa. Hydrogels in pharmaceutical formulations. *Eur. J. Pharm. Biopharm.* **50**:27–46 (2000).
5. M. W. Davis and J. P. Vacanti. Toward development of an implantable tissue engineered liver. *Biomaterials* **17**:365–372 (1996).
6. Q. Hou, P. A. De Bank, and K. M. Shakesheff. Injectable scaffolds for tissue regeneration. *J. Mater. Chem.* **14**:1915–1923 (2004).
7. B. Jeong, Y. H. Bae, D. S. Lee, and S. W. Kim. Biodegradable block copolymers as injectable drug-delivery systems. *Nature* **388**:860–862 (1997).
8. B. Jeong, Y. K. Choi, Y. H. Bae, G. Zentner, and S. W. Kim. New biodegradable polymers for injectable drug delivery systems. *J. Control. Release* **62**:109–114 (1999).
9. Y. Tabata and Y. Ikada. Protein release from gelatin matrixes. *Adv. Drug Deliv. Rev.* **31**:287–301 (1998).
10. E. S. Gil and S. M. Hudson. Stimuli-responsive polymers and their bioconjugates. *Prog. Polym. Sci.* **29**:1173–1222 (2004).
11. G. J. Martinez-diaz, D. Nelson, W. C. Crone, and W. J. Kao. Mechanical and chemical analysis of gelatin-based hydrogel degradation. *Macromol. Chem. Phys.* **204**:1898–1908 (2003).
12. Y. Otani, Y. Tabata, and Y. Ikada. Rapidly curable biological glue composed of gelatin and poly(L-glutamic acid). *Biomaterials* **17**:1387–1391 (1996).
13. J. L. Zilinski and W. J. Kao. Tissue adhesiveness and host response of *in situ* photopolymerizable interpenetrating networks containing methylprednisolone acetate. *J. Biomed. Mater. Res., A* **68**:392–400 (2004).
14. J. A. Burmania, G. J. Martinez-Diaz, and W. J. Kao. Synthesis and physicochemical analysis of interpenetrating networks containing modified gelatin and poly(ethylene glycol) diacrylate. *J. Biomed. Mater. Res., A* **67**:224–234 (2003).
15. J. A. Burmania, K. R. Stevens, and W. J. Kao. Cell interaction with protein-loaded interpenetrating networks containing modified gelatin and poly(ethylene glycol) diacrylate. *Biomaterials* **24**:3921–3930 (2003).
16. K. R. Stevens, N. J. Einerson, J. A. Burmania, and W. J. Kao. *In vivo* biocompatibility of gelatin-based hydrogels and interpenetrating networks. *J. Biomater. Sci., Polym. Ed.* **13**:1353–1366 (2002).
17. M. Changez, K. Burugapalli, V. Koul, and V. Choudhary. The effect of composition of poly(acrylic acid)-gelatin hydrogel on gentamicin sulphate release: *in vitro*. *Biomaterials* **24**:527–536 (2003).
18. M. C. Tate, D. A. Shear, S. W. Hoffman, D. G. Stein, and M. C. LaPlaca. Biocompatibility of methylcellulose-based constructs designed for intracerebral gelation following experimental traumatic brain injury. *Biomaterials* **22**:1113–1123 (2001).
19. A. Chenite, C. Chaput, D. Wang, C. Combes, M. D. Buschmann, C. D. Hoemann, J. C. Leroux, B. L. Atkinson, F. Binette, and A. Selmani. Novel injectable neutral solutions of chitosan form biodegradable gels *in situ*. *Biomaterials* **21**:2155–2161 (2000).
20. C. E. Kast, W. Frick, U. Losert, and A. Bernkop-Schnurch. Chitosan–thioglycolic acid conjugate: a new scaffold material for tissue engineering? *Int. J. Pharm.* **256**:183–189 (2003).
21. B. Jeong, K. M. Lee, A. Gutowska, and Y. H. An. Thermogelling biodegradable copolymer aqueous solutions for injectable protein delivery and tissue engineering. *Biomacromolecules* **3**:865–868 (2002).
22. R. A. Stile and K. E. Healy. Thermo-responsive peptide-modified hydrogels for tissue regeneration. *Biomacromolecules* **2**:185–194 (2001).
23. H.-H. Lin and Y.-L. Cheng. *In-situ* thermoreversible gelation of block and star copolymers of poly(ethylene glycol) and poly(*N*-isopropylacrylamide) of varying architectures. *Macromolecules* **34**:3710–3715 (2001).
24. R. A. Stile, W. R. Burghardt, and K. E. Healy. Synthesis and characterization of injectable poly(*N*-isopropylacrylamide)-based hydrogels that support tissue formation *in vitro*. *Macromolecules* **32**:7370–7379 (1999).
25. A. Lucke, J. Tessmar, E. Schnell, G. Schmeer, and A. Gopferich. Biodegradable poly(D,L-lactic acid)–poly(ethylene glycol)–monomethyl ether diblock copolymers: structures and surface properties relevant to their use as biomaterials. *Biomaterials* **21** 2361–2370 (2000).
26. J. C. Gilbert, J. L. Richardson, M. C. Davies, K. J. Palin, and J. Hadgraft. The effect of solutes and polymers on the gelation properties of Pluronic F-127 solutions for controlled drug delivery. *J. Control. Release* **5**:113–118 (1987).
27. X. Zhang, U. P. Wyss, D. Pichora, and M. F. Goosen. Biodegradable controlled antibiotic release devices for osteomyelitis: optimization of release properties. *J. Pharm. Pharmacol.* **46**:718–724 (1994).
28. S. S. Sampath and D. H. Robinson. Comparison of new and existing spectrophotometric methods for the analysis of tobramycin and other aminoglycosides. *J. Pharm. Sci.* **79**:428–431 (1990).
29. M. J. Song, D. S. Lee, J. H. Ahn, D. J. Kim, and S. C. Kim.

Thermosensitive sol–gel transition behaviors of poly(ethylene oxide)/aliphatic polyester/poly(ethylene oxide) aqueous solutions. *J. Polym. Sci., Part A: Polym. Chem.* **42**:772–784 (2004).
30. Y. M. Chung, K. L. Simmons, A. Gutowska, and B. Jeong. Sol–gel transition temperature of PLGA-g-PEG aqueous solutions. *Biomacromolecules* **3**:511–516 (2002).
31. T. Yoshida, M. Takahashi, T. Hatakeyama, and H. Hatakeyama. Annealing induced gelation of xanthan/water systems. *Polymer* **39**:1119–1122 (1998).
32. G. Wanka, H. Hoffmann, and W. Ulbricht. The aggregation behavior of poly(oxyethylene)–poly(oxypropylene)–poly(oxyethylene) block copolymers in aqueous solution. *Colloid Polym. Sci.* **268**:101–117 (1990).
33. J. W. Mwangi and C. M. Ofner. Crosslinked gelatin matrices: release of a random coil macromolecular solute. *Int. J. Pharm.* **278**:319–327 (2004).
34. K. Terao, N. Nagasawa, H. Nishida, K. Furusawa, Y. Mori, F. Yoshii, and T. Dobashi. Reagent-free crosslinking of aqueous gelatin: manufacture and characteristics of gelatin gels irradiated with gamma-ray and electron beam. *J. Biomater. Sci., Polym. Ed.* **14**:1197–1208 (2003).
35. S. Yoshizawa, D. Fourmy, and J. D. Puglisi. Structural origins of gentamicin antibiotic action. *EMBO J.* **17**:6437–6448 (1998).
36. R. E. Botto and B. Coxon. Nitrogen-15 nuclear magnetic resonance spectroscopy of neomycin B and related aminoglycosides. *J. Am. Chem. Soc.* **105**:1021–1028 (1983).