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Controlled Release of Protein from Biodegradable Multi-sensitive Injectable Poly(ether-urethane) Hydrogel

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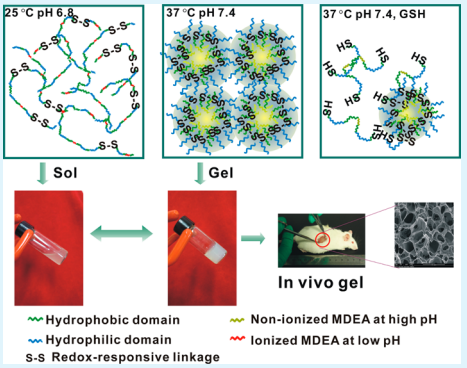
ABSTRACT: The synthesis and characterization of multi-sensitive polymers for use as injectable hydrogels for controlled protein/drug delivery is reported. A series of biodegradable multi-sensitive poly(ether-urethane)s were prepared through a simple one-pot condensation of poly(ethylene glycol), 2,2′-dithiodiethanol, *N*-methyldiethanolamine, and hexamethylene diisocyanate. The sol-gel phase transition behaviors of the obtained copolymers were investigated. Experimental results showed that the aqueous medium comprising the multi-segment copolymers underwent a sol-to-gel phase transition with increasing temperature and pH. At a certain concentration, the copolymer solution could immediately change to a gel under physiological conditions (37 °C and pH 7.4), indicating their suitability as in situ injectable hydrogels in vivo. Insulin was used as a model protein drug for evaluation of the injectable hydrogels as a site-specific drug delivery system. The controlled release of insulin from the hydrogel devices was demonstrated by degradation of the copolymer, which is modulated via the 2,2′-dithiodiethanol content in the poly(ether-urethane)s. These hydrogels having multi-responsive properties may prove to be promising candidates for injectable and controllable protein drug delivery devices.



KEYWORDS: *poly(ether-urethane), multi-sensitive hydrogels, biodegradable, injectable, drug delivery*

INTRODUCTION

For the past few decades hydrogels have attracted considerable research attention in various fields as drug carriers, biomaterials for tissue repair, and numerous other medical devices because of their favorable biocompatible properties.[1−4] Hydrogels, consisting a three-dimensional network, can absorb a large amount of water while maintaining their shape, making it easier for drugs, proteins, and cells to be encapsulated within their framework.[5−8] One of the more recent trends in hydrogels research has been directed at in situ-formed injectable hydrogels, which have potential applications in site-specific drug delivery systems.[9−12] An advantage offered by in situ-formed injectable hydrogels is that they can deliver the drugs into the specific sites without surgical or implantation procedures.

Temperature-sensitive hydrogels, which undergo reversible sol-gel transitions in response to temperature changes, have been extensively studied as injectable drug/protein delivery systems.[13−18] However, when they are injected into deep anatomical sites, temperature-sensitive hydrogels tend to undergo a premature phase transition due to body temperature, thereby blocking the needle.[19] To overcome this problem, a number of pH/temperature-responsive injectable hydrogels have been reported.[20−31] These hydrogels have pH-sensitive moieties, which can exist in their ionized states; a change in the hydrophilic/hydrophobic equilibrium can be achieved simply by decreasing the pH value, thereby allowing for modulation of phase transition temperature to avoid the formation of gels and clogging during injection.[28,31] In addition, the positive charges of protonated amino groups in these polymers could form reversible electrostatic linkages with the drugs or proteins to allow a predictable and customizable mechanism for loading and release.

Polyurethanes are an important class of polymers that have found many applications as biomaterials, owing to their excellent physical properties and good biocompatibility.[32,33] A variety of in situ forming temperature- and pH-responsive poly(ether-urethane)-based hydrogels were developed by Lee's group.[34−36] However, most of the copolymers mentioned above are not suitable for short-term drug delivery applications due to their slow biodegradation by enzymes in vivo. Recently, we have reported a series of multi-responsive degradable poly(ether-urethane) nanoparticles with a tunable structure, which are easily prepared by a facile one-pot approach by incorporating functional segments into the poly(ether-urethane) backbone.[37−39] As a further demonstration of this approach toward functional and tunable hydrogels, a series of pH, temperature, and redox potential multi-sensitive poly(ether-urethane)s were prepared for in situ forming hydrogels with fast degradation. In this study, (1) different ratios of poly(ethylene glycol) (PEG) and *N*-methyldiethanolamine (MDEA) possessing a tertiary amine group were used to

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Table 1. Summary of the Random Poly(ether-urethane) Copolymers

sample no.	polymer	PEG:(MDEA+DiT)	MDEA:DiT	DiT content (%)	Mn	PDI
P1	(PEG2000-MDEA-DiT)	1:6	5:1	13.3	16700	1.56
P2	(PEG2000-MDEA-DiT)	1:4	7:1	10	19700	1.97
P3	(PEG2000-MDEA)	1:6	1:0	0	27200	1.36

tune the sol-to-gel window under physiological conditions (37 °C, pH 7.4) in order to prevent clogging during injection; (2) different ratios of 2,2′-dithiodiethanol (DiT) containing a disulfide bond were used to modulate the degradation time of the hydrogels in vivo; (3) insulin was used as a model protein to test its controlled release using the prepared multi-sensitive poly(ether-urethane) hydrogels.

EXPERIMENTAL SECTION

Chemicals and Reagents. Poly(ethylene glycol) (PEG, M_w = 2000 Da), hexamethylene diisocyanate (HDI, 98%), 2,2′-dithiodiethanol (DiT), N-methyldiethanolamine (MDEA) insulin and glutathione (GSH) were purchased from Sigma-Aldrich (Shanghai, China). Tetrahydrofuran (THF) and 1, 2-dichloroethane were distilled over sodium and calcium hydride, respectively. All the other reagents were obtained from Tianjin Chemical Reagent Co. (Tianjin, China) and used without further purification.

Synthesis of the Random Poly(ether-urethane) Compolymers. A series of random poly(ether-urethane)s (**P1**−**P3**) were synthesized by a one-pot condensation of PEG, DiT, MDEA, and HDI. The condensation reaction was conducted at a certain molar ratios (as shown in Table 1). The typical reaction procedure is as follows: certain amounts of PEG, DiT, and MDEA were dissolved in a mixture of 1, 2-dichloroethane and THF with a molar ratio of 5:1. Then, the solution was added dropwise to 1, 2-dichloroethane with a certain amount of HDI (molar ratio of HDI to (PEG+DiT+MDEA) is 1:1) containing a catalytic amount of dibutyltin dilaurate (0.5 wt %, with respect to the reactant). The flask was kept under a dry nitrogen atmosphere, and then, the reaction mixture was heated to 80 °C. After stirring for 24 h, an excess amount of methanol was added, and the mixture was reacted for another hour to eliminate the dibutyltin dilaurate residue and oligomers. The resulting products were collected by precipitating in diethyl ether. The resulting product was collected through filtration, followed by drying under vacuum to constant weight, affording a yield of over 92%.

Measurements. Fourier transform infrared spectra (FT-IR) were measured using Bio-Rad FTS6000 spectrophotometer at room temperature. ^{1}H NMR spectra were recorded on a Varian UNITY-plus 400 NMR spectrometer using DMSO-d_6 as the solvent. The molecular weight and polydispersity index (PDI) were determined by gel permeation chromatography (GPC, Waters 2414 system Milford, MA). THF was used as eluent at a flow rate of 1.0 mL/min at 35 °C. The average molecular weights were calibrated with standard polystyrene samples.

In Vitro Sol−Gel Phase Transition Measurement. The sol−gel phase transition was recorded by the inverting test method using a 4 mL (10 mm diameter) vial test tube at a temperature interval of 2 °C. The random copolymers were dissolved in 0.01 M phosphate buffered saline (PBS) buffer solution at a given concentration for 24 h at 2 °C. The pH of these samples was then adjusted with 5 M NaOH and the solutions were maintained at 2 °C. The sol-gel phase transition behavior of the sample at each pH value was determined by inverting the vial after keeping it at a constant temperature for 15 min. It is defined as a gel state if no fluidity is visually observed by inverting the vials for 1 min, or a sol state if it flows.

Rheology Test. The rheological properties of hydrogels were performed on an AR2000ex rheometer (TA Instruments). Temperature-dependent changes in elastic modulus (G′), viscous modulus (G″), and viscosity changes were recorded using the aluminum parallel plate with a diameter of 40 mm. The sample gap was set to be 1.0 mm. The temperature was controlled by a Peltier system in the bottom plate connected with a water bath. The heating rate was 1 °C min^{-1}.

In Vivo Gel Formation. The in vivo gel formation and injectability of the poly(ether-urethane) hydrogels was investigated. In this experiment, 200 μL 10 wt % **P1** solution was subcutaneously injected into a male Sprague-Dawley (SD) rat at pH 6.8, 25 °C. After 20 min, the rat was sacrificed, the injection site was cut open and the imorphology of the in situ formed hydrogel was observed.

In Vitro Biodegradability of the Multi-sensitive Hydrogels. The multi-sensitive hydrogels at a concentration of 8 wt % were prepared by dissolving them in 0.01 M PBS solution at pH 1.0 and kept for 24 h at 2 °C. The pH value of the sample was adjusted to 7.4 using 5 M NaOH solution. The sample (1 mL) at a sol state was injected into a vial (4 mL, with 1 cm diameter) and then heated to 37 °C to form a gel. The formed gel was incubated at 3 mL 0.01 M PBS solution at pH 7.4, 37 °C under continuous shaking (90 strokes/min). The PBS solution with different glutathione concentration ranging from 0 to 50 mM was removed and changed at a fixed time intervals, and the height of the gel was measured each time to calculate the remaining volume of the hydrogel. The experiments were conducted in triplicate, and the results presented are the average data.

Scanning Electron Microscopy (SEM). Scanning electron microscopy (SEM) was used to observe the morphologies of pristine hydrogels and degraded hydrogels. The SEM samples were prepared as below: The hydrogels and hydrogels treated with 500 mM GSH were incubated at 37 °C for 12 h and then were freeze-dried and then gold-coated. The digital images were recorded on a SS-550 SEM (SHIMADZU, Japan).

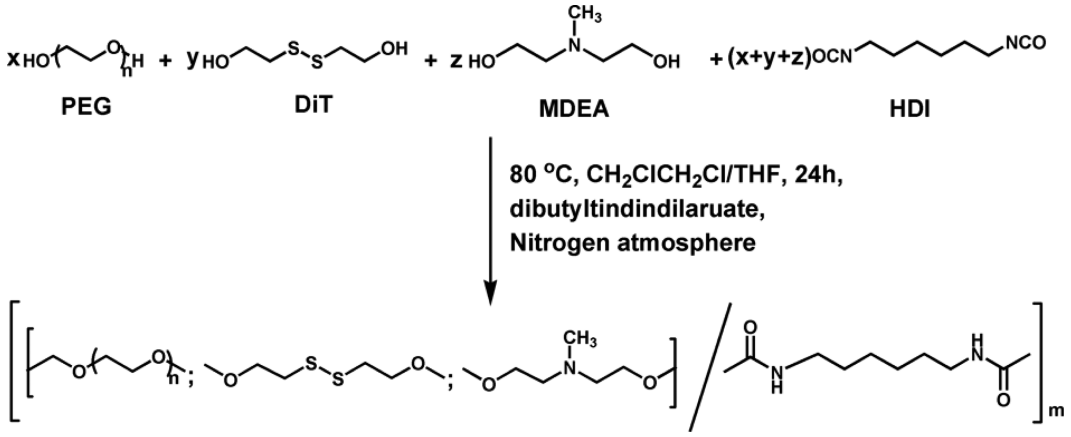
Insulin Loading and Release from the Hydrogels In Vitro. The insulin-loaded hydrogels were prepared as follows: The **P1**, **P2**, and **P3** solutions were prepared by dissolving them in 0.01 M PBS solution at pH 1.0 with a concentration of 8 wt % and 20 wt %, respectively. The solutions were and kept at 2 °C for 24 h. Insulin was loaded by mixing into copolymer solutions at 2 °C and pH 3.0. The pH of these solutions could be adjusted by 5 M NaOH and 5 M HCl at 2 °C. The mixture (5 mg/mL insulin in poly(ether-urethane) solution) was then adjusted to pH 7.4 and changed to a gel state after incubating at 37 °C for 30 min. The prepared insulin-loaded hydrogels (0.5 g) were incubated in 6 mL 0.01 M PBS solution at pH 7.4 with different GSH concentration. The solutions were placed in a shaking water bath at 37 °C. At desired time intervals, 3 mL of release media was taken out and 3 mL of fresh media was added to refill the incubation solution to 6 mL. The amount of insulin released from the insulin-loaded hydrogels was determined by using a UV-2450 spectrophotometer (Shimadzu Co. Japan). The cumulative insulin release was calculated as

$$\text{cumulative release (\%)} = (V_e \sum_{1}^{n-1} C_i + V_0 C_n)/m_{drug} \times 100$$

where V_e is the amount of release media took out every time (3 mL), V_0 is the amount of release medium (6 mL), C_i is the concentration of insulin released from hydrogel at displacement time of i, m_{drug} is the mass of drug used for release, and n is the displacement time. Three replicates were measured for each sample, and the results presented are the average data.

To confirm the conformational stability of the native insulin and insulin released from the insulin-loaded hydrogels, circular dichroism (CD) spectra of the insulin were recorded on JASCO-J-715 (Jasco, Tokyo, Japan) equipped with a temperature control system operated at 25 °C at an insulin concentration of 100 μg/mL. The spectra were scanned from 190 to 250 nm.

Scheme 1. Synthesis Route of the Random Poly(ether-urethane)s



Cytotoxicity Evaluation. The biocompatibility of the poly(ether-urethane) hydrogels was evaluated using NIH-3T3 cells. The hydrogels at a concentration of 8 wt % at pH 7.4 was added into the 24-well cell culture plate at room temperature, and then thermostated at 37 °C to form a gel. NIH-3T3 cells were seeded into the 24-well plates on top of the hydrogel at an initial density of 1 × 10^5 cells per well in DMEM complete medium. After incubated under 5% CO_2 atmosphere at 37 °C for 0, 12, 24, and 72 h, AO/EB was added into the 24-well cell culture plate to stain the cells. After incubated for another 1 h, the cell was observed by an inverted microscope.

The cytotoxicity of the poly(ether-urethane)s at different concentration were also investigated by the MTT assay. The **P1** solutions at concentrations ranging from 0 to 8 × 10^4 μg/mL (0, 5, 10, 50, 100, 200, and 80 000 μg/mL) were added into the 24-well cell culture plate at room temperature, and then thermostated at 37 °C, when the **P1** solution at 80 000 μg/mL concentration would form a gel. NIH-3T3 cells were seeded into the 24-well plates on top of the hydrogel at an initial density of 1 × 10^5 cells per well in DMEM complete medium and then incubated under 5% CO_2 atmosphere at 37 °C for 24 h. The cell proliferation and viability were determined using the MTT assay. Briefly, the media were replaced with 100 μL of MTT solution (0.5 mg/mL final concentration) diluted with growth media and incubated for a further 4 h. The medium was removed and 150 μL of DMSO were added to each well to dissolve the formazan by mildly shaking for 15 min. The absorbance of each well was measured using a microplate reader (Labsystem, Multiskan, Ascent, Model 354 Finland) at 490 nm. The relative cell viability was determined by comparing the absorbance at 490 nm with control wells containing only cell culture medium. Three replicates were measured for each sample, and the dates are shown as the mean value plus a standard deviation (±SD).

RESULTS AND DISCUSSION

The purpose of this work was to develop an injectable and biodegradable multi-sensitive hydrogel for potential use in drug delivery applications. The hydrogel design was based on the following guidelines: first, equal amounts of HDI and the diols PEG, DiT, and MDEA were employed to obtain the random poly(ether-urethane)s by a one-pot condensation reaction; the PEG moieties of the copolymers serve as the hydrophilic portions, while the remainding components with both pH and reduction sensitivities comprise the hydrophobic parts. Second, the excellent injectability of the hydrogels was introduced to the copolymer by incorporation of the pH-sensitive MDEA moiety; the tertiary amine of the MDEA moiety can be protonated or deprotonated at different pH values, which could modulate the hydrophilic/hydrophobic properties. Third, the disulfide bond containing DiT can serve as the redox-sensitive fragment; cleavage of the disulfide linkage in the DiT moiety would enable the hydrogels to degrade more easily in the presence of a mild reducing agent such as glutathione (GSH).

The synthetic route toward random poly(ether-urethane)s **P1**−**P3** is illustrated in Scheme 1; it should be noted taht poly(ether-urethane) **P3** was synthesized in the same manner as illustrated in Scheme 1, but in the absence of the reactant DiT. The synthesized copolymers (**P1**−**P3**) were characterized using FT-IR, ^{1}H NMR, and gel permeation chromatography (GPC) techniques. Figure 1 shows the representative FT-IR

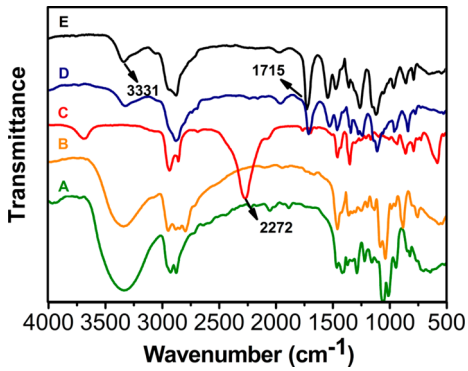


Figure 1. FTIR spectra of DiT (A), MDEA (B), HDI (C), PEG (D), and **P1** (E).

spectra of **P1** as well as the reactants MDEA, DiT, and HDI. By comparison with the spectra of reactants, the absorbances at 1715 and 3331 cm^{-1} in the spectrum of **P1** are assigned to the C═O and N−H stretching of urethane groups, respectively. The absence of any absorbance at around 2272 cm^{-1} indicates that no unreacted isocyanate groups remain in the resulting polymers. The ^{1}H NMR spectrum of **P1** is shown in Figure 2, which indicates the coexistence of PEG, MDEA, and DiT segments. GPC measurements were performed to determine the molecular weights and polydispersity indices of the synthesized copolymers. The typical GPC traces of PEG and poly(ether-urethane) **P1** are shown in Figure 3. The observation of a unimodal GPC peak for **P1** indicates formation of the copolymers via polymerization. The results of characterization for all synthesized multiblock copolymers (**P1**−**P3**) are summarized in Table 1.

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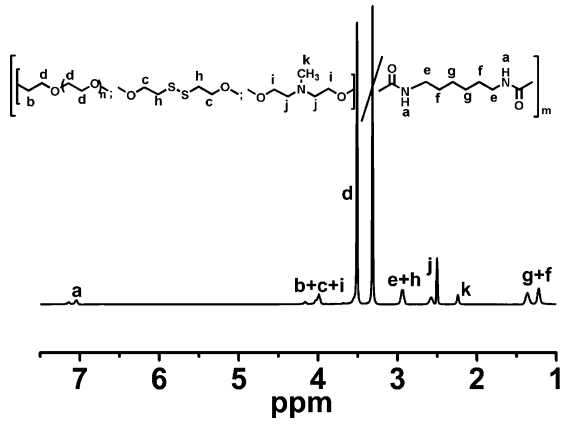

Figure 2. ^{1}H NMR spectrum of **P1**.

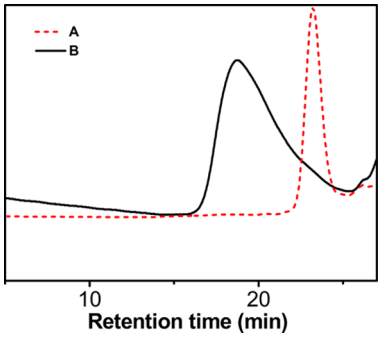

Figure 3. GPC traces of the parent PEG 2000 (A) and **P1** (B).

Sol–Gel Transition Phase Diagrams. The sol to gel transition behavior was in an abrupt way, it could happen within 5 s. The sol–gel phase transition behaviors of poly(ether-urethane)s **P1** and **P2** in response to pH and temperature were assessed. Figure 4A shows the sol–gel phase diagram of poly(ether-urethane) **P1** at various pH and temperatures at different concentrations. The poly(ether-urethane) solutions exhibited a sol-to-gel and a sol-to-gel-to-sol phase transition within a certain pH range with increasing pH and temperature. However, the poly(ether-urethane) solutions could not form a gel in the tested temperature range at low pH values (pH < 6.8). Variations in viscosity, elastic modulus (G′), and viscous modulus (G″) of poly(ether-urethane) solution **P1** at pH 7.4 (Figure 4C) and pH 5.0 (Figure 4D) were examined as a function of temperature using dynamic rheological analysis. As shown in Figure 4C, at pH 7.4, an apparent increase in viscosity during heating from 25 °C was observed, and the elastic modulus (G′) was higher than the viscous modulus (G″) over the experimental temperature range 31–59 °C, indicating the poly(ether-urethane) solution existed in a gel state under these conditions. The fast decrease in viscosity at temperatures higher than 50 °C was attributed to the dehydration of the PEG segment at high temperatures. According to Figure 4D, no apparent increase in viscosity of the poly(ether-urethane) solution was observed during heating, and the viscosity at 37 °C with an acidic pH (pH 5.0) was much lower than that at 37 °C with a neutral pH (pH 7.4). In addition, the elastic modulus (G′) was lower than the viscous modulus (G″) at pH 5.0 over the entire tested temperature range, indicating that it was in the sol state and lowering the pH value of the poly(ether-urethane) solution could prevent the clogging during injection. This is because the tertiary amines in the MDEA moiety are protonated under the acidic conditions, which increases the hydrophilicity of the copolymer and leads to the poly(ether-urethane) solutions existing in a sol state despite an increase in temperature, which could increase the hydrophobicity of the block copolymer. The tertiary amine groups could be deprotonated with an increase in pH value, gradually transforming the MDEA moiety from hydrophilic to hydrophobic. The hydrophobicity of the block copolymer increased with the increasing temperature and showed a typical

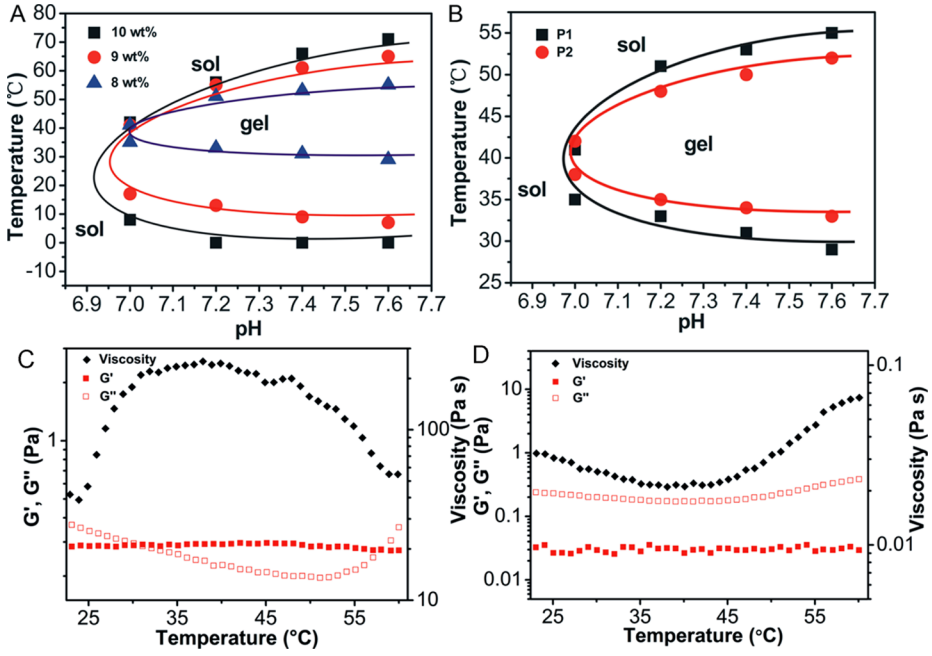

Figure 4. Sol-gel phase diagrams of (A) **P1** at different concentration, (B) **P1** and **P2** at a concentration of 8 wt %, and the rheological properties of poly(ether-urethane)s solution **P1** at (C) pH 7.4 and (D) pH 5.0.

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a sol-to-gel-to-sol phase transition behavior. The increase in pH could also decrease the sol-to-gel transition temperature and broaden the gel temperature region. Figure 4A also shows the concentration dependence of poly(ether-urethane) solutions (**P1**); as the concentration of poly(ether-urethane) solutions increased from 8 to 10 wt %, the sol-to-gel transition temperature decreased and the gel region became broader and shifted to a lower pH value. This could be attributed to the increase in intermolecular hydrophobic interactions between hydrophobic segments.

The sol−gel phase diagrams of **P1** and **P2** block copolymer solutions at a concentration of 8 wt % were also recorded to investigate the effects of the hydrophilic segment (PEG) ratio on the sol-gel phase transition behavior. As shown in Figure 4B, poly(ether-urethane) solutions **P1** showed a similar sol-gel phase transition behavior as a function of pH and temperature value. Comparing poly(ether-urethane) solutions **P1** and **P2**, a lower hydrophilic (PEG) molecular ratio (**P1**) was found to induce a broader gel range due to the stronger hydrophobic interaction.

In Vivo Gel Formation. A male Sprague−Dawley (SD) rat was used to examine the injectability and in vivo gel formation ability of the poly(ether-urethane) hydrogels. As shown in Figure 5, a hydrogel was observed to form in situ 20 min after injection, which can be attributed to the change in pH and temperature under the physiological conditions (pH 7.4, 37 °C) in accordance with the sol-to-gel transition experiment in vitro. The in vivo injection experiment suggests that the multiblock poly(ether-urethane) solution can be easily injected into the body and form a gel in situ with a short gel formation time.

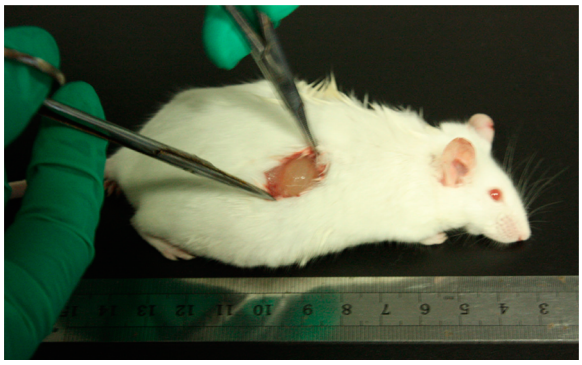

Figure 5. In vivo gel formation. Photograph taken 20 min after subcutaneous injection of the 10 wt % **P1** block copolymer solution into mouse.

In Vitro Biodegradability of the Multi-sensitive Hydrogels. The reducible DiT segment contains a disulfide bond that can be rapidly degraded via bond cleavage in the presence of a reducing reagent (such as GSH).[40] These processes always occur in a rapid fashion, enabling a fast rate of hydrogel degradation. The degradation behaviors of poly(ether-urethane) hydrogels **P1**, **P2**, and **P3** under different GSH concentrations over time are presented in Figure 6. As evidenced from the degradation of **P1**, as GSH concentration was increased from 0 to 500 μL, the remaining mass decreased from 72% to 16% over the tested 28 d (Figure 6A). Hydrogel **P2** showed a similar trend under the influence of GSH (Figure 6B). In contrast, the remaining mass of poly(ether-urethane) hydrogel **P3** did not show significant differences under the two different GSH conditions examined, only losing 12% of its mass over 28 d, as no DiT segment is present in **P3** (Figure 6C). GSH concentrations of 10 mM and 50 mM were also investigated in the degradation of **P1** (Figure 6A), which showed a rapid degradation within 1 and 2 d, respectively. These experiments indicate that hydrogel degradation can be tuned by incorporating different DiT segment ratios in the copolymers.

The surface and interior morphologies of hydrogels treated with or without GSH were observed by scanning electron microscopy (SEM). As seen in Figure 7, the pristine hydrogel immersed in 0.01 M phosphate-buffered saline (PBS) solution at pH 7.4 showed a porous three-dimensional network structure, which is essential for capturing drug molecules. The intermolecular hydrogen bonding between the tertiary amine groups in the MDEA moieties gives rise to the formation of a stable gel. In contrast, the hydrogel treated with 500 mM GSH existed as an irregular network structure with varying pore sizes bigger than the original hydrogel; this is because cleavage of the disulfide bonds of the poly(ether-urethane) copolymers caused by the presence of GSH results in erosion of the hydrogel structure, speeding up degradation and enhancing the drug release from the hydrogels.

Insulin Loading and Release from the Multi-sensitive Hydrogels. To evaluate the ability of the multi-sensitive poly(ether-urethane) hydrogels to effectively deliver protein drugs, in vitro insulin release from **P1**, **P2**, and **P3** gels (pH 7.4) at 37 °C with or without GSH was studied. Figure 8 shows the release profiles of insulin from the multi-sensitive hydrogels. As shown in Figure 8A, almost all of the insulin was completely released from hydrogel **P1** in the presence of 500 μM GSH over the tested 28 d period, as a result of disulfide bond cleavage in the DiT segments, while only 55% of the insulin was released from the insulin-loaded **P1** hydrogel in the absence of GSH; these results indicate that the release of insulin from insulin-loaded hydrogel **P1** is closely dependent on the GSH concentration. As the ratio of disulfide bonds in hydrogel **P2** is slightly lower than that in **P1**, a slightly slower insulin release from hydrogel **P2** was observed over the same time period (Figure 8B). However, the GSH concentration-dependence in hydrogel **P3** was not significant (Figure 8C), with greater than 50% of the initial insulin loading remaining in the hydrogel after 28 d under 500 μM GSH. Comparing the release profiles of **P1**, **P2**, and **P3** hydrogels, it can be concluded that introducing a greater DiT content ratio into the hydrogel material is a viable strategy for tunable drug release from hydrogels via disulfide bond cleavage in the presence of GSH.

The release mechanism of insulin from the degradable matrix is influenced both by the degradation of the matrix and diffusion of the drug.[41] The Ritger−Peppas equation was used to study the mechanism of insulin release from hydrogel **P1** at different GSH concentrations.[42] According to the Ritger−Peppas equation:

$$M_t/M_\infty = kt^n$$

where t refers to the drug release time, M_t/M_∞ is the drug fraction released at time t, and k and n are the constant and kinetic exponent of drug release, respectively. The n value calculated according to the equation for the initial several hours of hydrogel **P1** degradation without GSH was 0.404, indicating that the kinetics of insulin release corresponds to that of typical Fickian diffusion. The n value for hydrogel **P1** at 500 μM GSH was 0.508, indicating that the release of insulin follows random

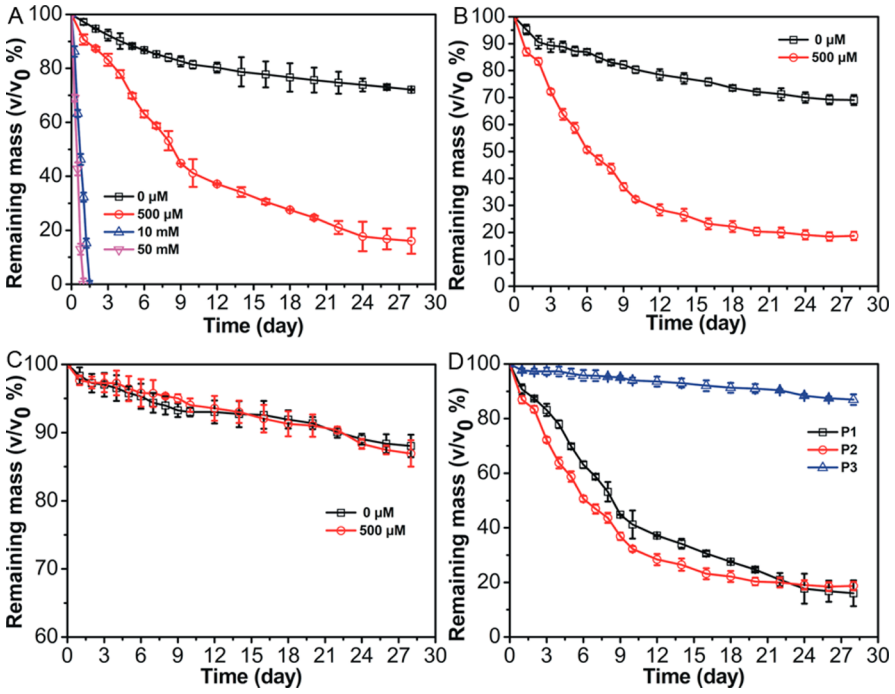

Figure 6. Degradation of in-situ-formed 8 wt % **P1** (A), 8 wt % **P2** (B), and 20 wt % **P3** (C) gel as a function of glutathione concentration in the medium, and **P1**, **P2**, and **P3** gel with 500 μM glutathione (D).

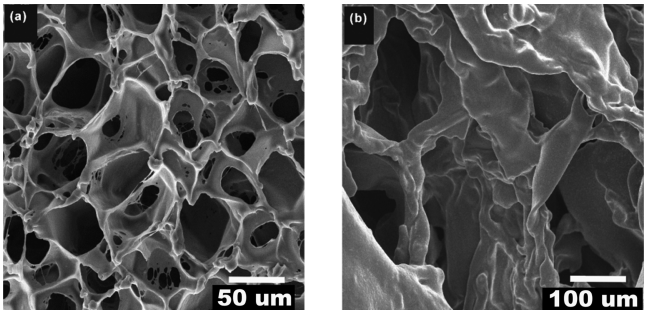

Figure 7. SEM micrographs of originally formed hydrogel at pH 7.4 (a) and the formed hydrogel after immersion in buffer pH 7.4 with 10 mM GSH (b) of the 10 wt % **P1** block copolymer.

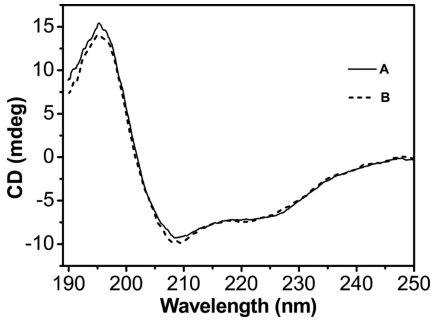

Figure 9. CD spectra of native insulin (A) and insulin released from insulin-loaded block copolymer hydrogel **P1** at a concentration of 8 wt % (B).

diffusion controlled kinetics, which is combined with Fickian diffusion and polymer chain loosecning caused by the cleavage of the disulfide bond.

The conformation of the insulin released from the hydrogels in PBS was confirmed by circular dichroism (CD) measurements (Figure 9). The bands at 208 and 223 nm in the CD spectra are assigned to the α-helical structure and β-structure of insulin, respectively. The CD spectrum of insulin released from the hydrogels revealed no significant difference in the secondary structure compared with that of native insulin, indicating the conformational stability of insulin during the loading and releasing processes.

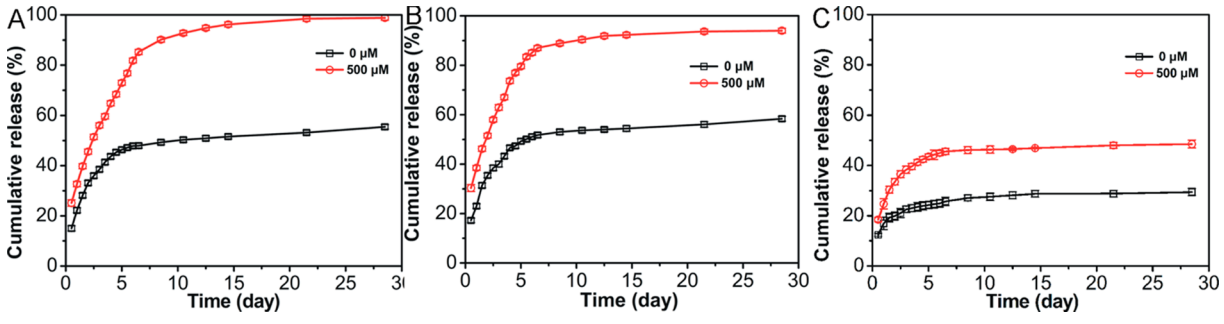

Figure 8. In vitro release of insulin from 8 wt % **P1** gel (A), **P2** gel (B), and 20 wt % **P3** gel (B) as a function of glutathione concentration in the medium.

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Cytotoxicity of the Poly(ether-urethane) Hydrogels. To investigate the biocompatibility of the prepared hydrogels, cell culture studies were used to evaluate their in vitro cytotoxicities by seeding NIH-3T3 cells on the hydrogel surface. After incubated with the hydrogel **P1** for 0, 12, 24, and 72 h, NIH-3T3 cells were stained using acridine orange/ethidium bromide (AO/EB). Fluorescence micrographs were obtained under an inverted microscope to distinguish the living cells (green fluorescence) from the dead (red fluorescence), and are presented in Figure 10. Cells were observed to flourish

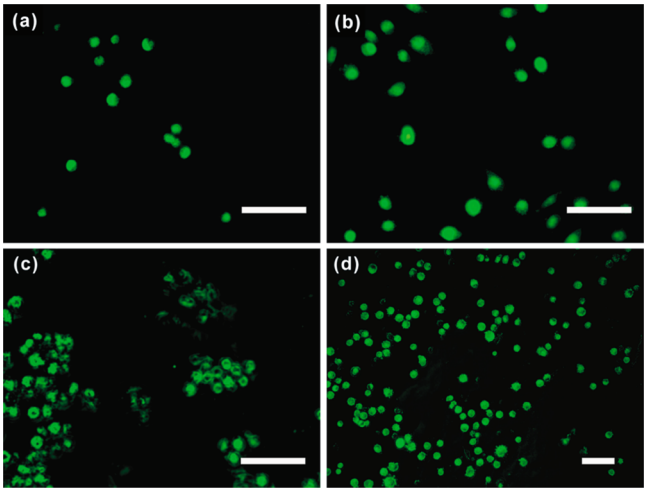

Figure 10. Fluorescence images of NIH-3T3 cells cultured in the in situ formed hydrogel **P1** for 0 (a), 12 (b), 24 (c), and 72 (d) hours. The cells were double stained with AO/EB. Bars = 200 μm.

on the hydrogel surface (as indicated by a green fluorescence) at all the incubation times examined; hardly any red fluorescence could be seen in the micrographs even after a prolonged incubation time of 72 h. These results indicate that the poly(ether-urethane) hydrogel can be used as a low-cytotoxic biomaterial.

The cytotoxicities of the blank poly(ether-urethane)s **P1** at different concentrations were also evaluated in NIH-3T3 cells using the MTT colorimetric assay. First, the cells were incubated with the polymers at concentrations ranging from 0 to 200 $\mu g \cdot mL^{-1}$ (0, 5, 10, 50, 100, and 200 $\mu g \cdot mL^{-1}$) for 24 h. The cytotoxicity of **P1** at a concentration of 8 wt % at pH 7.4, which could form a gel at 37 °C, was also examined. As shown in Figure 11, after incubation for 24 h, all experiment groups showed practically no cytotoxicity toward NIH-3T3 cells (cell viability >85%) even at a high polymer concentration, up to 8 wt %, indicating the good biocompatibility of these poly(ether-urethane)s.

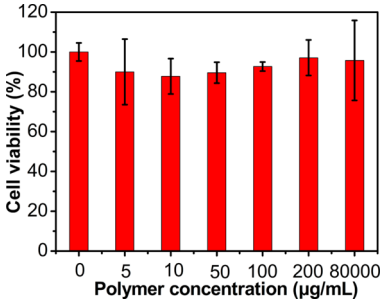

Figure 11. In vitro cytotoxicity of the copolymers **P1** to NIH-3T3 cells following 24 h incubation.

CONCLUSIONS

A variety of injectable and biodegradable hydrogels were successfully prepared based on a series of multi-sensitive poly(ether-urethane)s, which show promise as biomaterials for controlled release of proteins by subcutaneous injection. There are various advantages to this system, including facile one-pot polymerization, direct injection without any surgical procedures, no clogging during injection, straightforward drug loading to the polymer solution, simple dose adjustment, system biocompatibility, and controllable drug release and system degradation rates.

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Notes

The authors declare no competing financial interest.

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