A pH- and ionic strength-responsive polypeptide hydrogel: Synthesis, characterization, and preliminary protein release studies

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Abstract: A novel polypeptide hydrogel has been synthesized by crosslinking poly(L-glutamic acid) (PLG) with poly(ethylene glycol) (PEG). The PLG-PEG hydrogel was shown to be highly hydrophilic, and the extent of swelling varied with pH, increasing at higher ionization of the PLG. Aside from electrostatic effects, such as ion–ion repulsion and internal ion osmotic pressure, circular dichroism studies showed that swelling response to pH also is affected by secondary structural attributes associated with the polypeptide backbone. Modification of the polypeptide by changing its hydrophobicity and degree of ionization was an effective method for altering the overall extent of pH-responsive swelling. Rapid de-swelling (contraction) was observed when the PLG-PEG hydrogel was transferred from high to low pH buffer solution, and this swelling/de-swelling behavior was reversible over repeated cycles. Drug release from swollen hydrogels was examined using the model protein lysozyme. Rapid de-swelling of the hydrogel was found to be an effective means of facilitating lysozyme release. The crosslinking of synthetic polypeptides with PEG appears to be a highly versatile approach to the preparation of pH-responsive biodegradable hydrogels. © 1999 John Wiley & Sons, Inc. J Biomed Mater Res, 47, 595–602, 1999.

Key words: hydrogels; polypeptide hydrogels; pH-responsive swelling; polyglutamic acid; polyethylene glycol; crosslinking; protein release

INTRODUCTION

Crosslinked hydrogel networks are being investigated as drug delivery systems due to their potential to control the transport and release of macromolecular drugs such as peptides, proteins, and oligonucleotides.[1] Acrylate polymers often are used to prepare ionic crosslinked hydrogels for these applications.[2] Copolymer hydrogels containing hydroxyethyl methacrylate (HEMA) and methacrylate acid (MAA) have been characterized with respect to their swelling kinetics[3] and drug release properties.[4,5] Such crosslinked ionic hydrogels, which contain weakly ionizable acidic or basic groups, typically exhibit swelling behavior that can vary with pH, ionic strength, and buffer conditions.[6] Factors contributing to the overall swelling forces normally include the polymer–solvent interaction parameter, electrostatic interactions, internal ion osmotic pressure, and the transport and distribution of protons and ions through the hydrogel.[7,8]

While considerable effort has been made to synthesize and characterize acrylate-based ionic hydrogels, these materials are not biologically degradable by either hydrolytic or enzymatic mechanisms. As a result, acrylate systems are limited in their potential as biodegradable drug-delivery platforms. To overcome this liability, a variety of novel chemistries has been explored that incorporate biodegradability into the crosslinked hydrogel network.[9] For example, unsaturated polyesters have been used to prepare crosslinked beads, and the release of bovine serum albumin from these beads was shown to be facilitated by hydrolysis and erosion of the polymer network.[10] The synthesis of hydrolytically degradable hydrogels also has been attempted by crosslinking block copolymers of poly(ethylene glycol) with either poly(lactic acid) or poly(glycolic acid).[11]

Polymers containing naturally occurring monomers, such as amino acids and saccharides, represent

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a broad category of potentially biodegradable materials with chemistries amenable for crosslinking.[9] Crosslinked dextran hydrogels susceptible to biodegradation by the enzyme dextranase have been prepared.[12] Release of high molecular weight proteins from these hydrogel networks was shown to be controlled by enzymatic degradation of the polymer network rather than by diffusion through the hydrogel. Alternatively, crosslinked hyaluronic acid has been prepared and evaluated as a possible biodegradable drug delivery platform.[13]

Recently, crosslinked polypeptide hydrogels containing collagen[14] and gelatin[15,16] have been synthesized. Synthetic polypeptides also have received interest because they possess a more regular arrangement and a smaller diversity of amino acid residues than those derived from natural proteins. Examples of such synthetic polypeptide hydrogels include poly(hydroxyethyl-L-glutamate),[17] poly(L-ornithine),[18] poly(aspartic acid),[19] poly(L-lysine),[20] and poly(L-glutamic acid).[21]

Previously we reported on the synthesis of a variety of polypeptides and demonstrated that release of a small hydrophobic drug from these polymers could be regulated by varying the amino acid composition and hydrophobicity of the polypeptide backbone.[22] Recently, we also demonstrated that polymers synthesized by block copolymerization of polypeptides with poly(ethylene glycol) (PEG) could yield a complete release of entrapped macromolecular protein drugs.[23] In this paper we report the synthesis of a novel pH-responsive polypeptide hydrogel produced by covalently crosslinking poly(L-glutamic acid) (PLG) with poly(ethylene glycol) (PEG). The swelling/deswelling behavior of the PLG-PEG hydrogel, as well as factors affecting these properties, has been characterized. In addition, the possibility of utilizing the deswelling property of the hydrogel to facilitate the release of the entrapped model protein lysozyme has been explored.

MATERIALS AND METHODS

Materials

γ-Benzyl-L-glutamic acid (BLG) was purchased from Sigma Chemical Co. (St. Louis, Missouri). Anhydrous hydrogen bromide (HBr) was supplied by Matheson Gas. Diamino-terminated poly(ethylene glycol)s (diamino-PEG) of different molecular weights (300, 1500, and 3400 daltons) were purchased from Shearwater Polymers (Huntsville, Alabama). They were converted to their hydrochloride salts by adjusting their pH in solution to 4.0–4.5 using dilute hydrochloric acid and isolating the product by lyophilization. All other chemicals and solvents were obtained from Aldrich Chemical Company (Milwaukee, Wisconsin). Water was distilled and deionized, and dimethylformamide (DMF) was stored over sodium sulfate.

Polypeptide synthesis

Poly(γ-benzyl-L-glutamic acid) (PBLG) was prepared from the BLG N-carboxyanhydride according to the method described previously.[23] Debenzylation of PBLG was carried out by dissolving 1.0 g of PBLG in 100 mL of anhydrous benzene and then slowly bubbling HBr through the vigorously stirred solution for 3–4 h. Precipitates of the partially debenzylated polymer began to form after 12–18 h. Stirring was continued until the desired extent of debenzylation was achieved, as determined by NMR analysis. Complete debenzylation of PBLG to poly(L-glutamic acid) (PLG) required approximately 48 h. The partially or fully debenzylated polymer was washed extensively with fresh portions of acetone, followed by thorough drying under vacuum over phosphorus pentoxide.

Polypeptide characterization

NMR analysis was performed in deuterated dimethyl sulfoxide. The extent of debenzylation was estimated by comparing integrals for benzyl phenyl protons at 7.3 ppm (5 protons) to the acidic proton at 12.1 ppm (1 proton). Intrinsic viscosity of PBLG was calculated from viscosity measurements performed at 25°C in dichloroacetic acid using a Cannon–Ubbelohde capillary viscometer. PBLG molecular weights were estimated according to the Mark–Houwink relationship described by Doty et al.[24] In a similar fashion, the molecular weight of PLG was estimated by viscosity measurements in 0.2*M* of aqueous NaCl at pH 7.3, following the method of Idelson and Blout.[25]

Hydrogel synthesis

Synthesis of the polypeptide hydrogel is illustrated in Figure 1. As shown, poly(L-glutamic acid) (PLG) and diamino-PEG (PEG) were mixed in DMF and allowed to equilibrate overnight to insure intimate blending and interpenetration of the two polymers. Unless otherwise specified, hydrogels were prepared using an acid:amine molar ratio of 5:1 and a diamino-PEG having a molecular weight of 1500 daltons. When partially debenzylated polypeptides were utilized, the same acid:amine ratio was used except that the moles of acid were replaced by the total moles of amino acid monomers within the polypeptide. It has been reported in the literature[26] that at a total polymer concentration below about 10% by weight, the PLG-PEG-PLG ternary system existed as an isotropic, homogeneous solution. When polymer concentrations exceeded this limit, however, a mixed isotropic–cholesteric suspension was observed. It should be

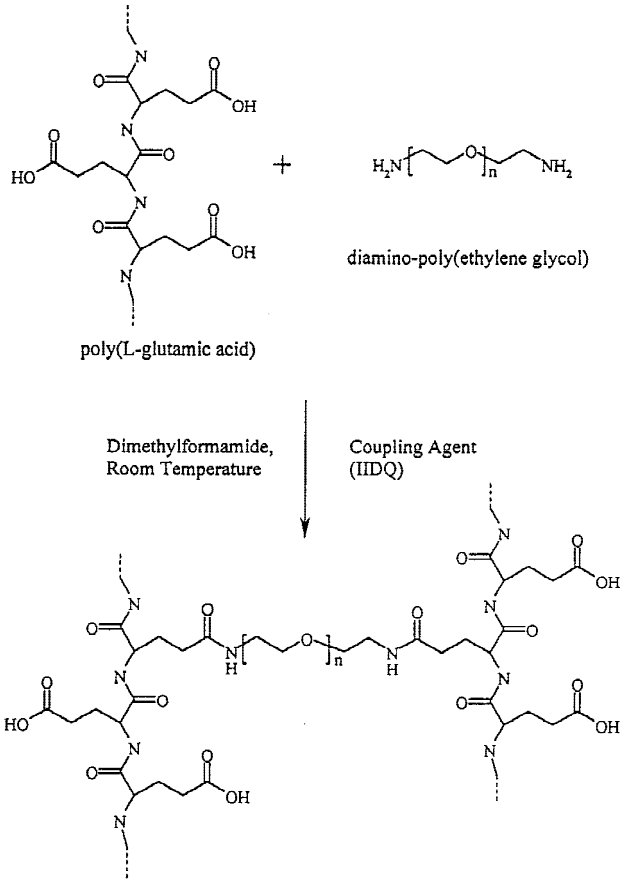

Figure 1. Scheme of the synthesis of the crosslinked polypeptide hydrogel.

pointed out that in our studies all hydrogels were prepared from the isotropic solution.

The peptide coupling agent 2-isobutoxy-1-isobutoxycarbonyl-1,2-dihydroquinoline, isobutyl 1,2-dihydro-2-isobutoxy-1-quinolinecarboxylate (IIDQ) was dissolved in a small volume of DMF, which then was added to the blended polymer solution. IIDQ was used at a 20% excess relative to the moles of the amine groups of the diamino-PEG. The selection of IIDQ over traditional coupling agents, such as dicyclohexylcarbodiimide (DCCI) and carbonyldiimidazole (CDI) was due to its comparatively slower activation and coupling kinetics. The slower kinetics permitted sufficient time for mixing the coupling agent into the polymer solution and subsequent handling of the mixture.[27]

After a brief but thorough mixing and removal of entrapped air bubbles, the solution was placed in a suitable mold. To form a cylindrical hydrogel platform, the solution was drawn within a glass pipette with an inside diameter of approximately 5.5 mm. To produce a thin-film hydrogel system, however, the solution was placed on a glass plate surrounded by a 30 mm diameter glass ring. After curing for at least 48 h at room temperature, the crosslinked hydrogel platforms were removed from their molds. The cylindrical hydrogel was cut into individual systems of approximately 10 mm in length. All hydrogel platforms were washed extensively by soaking first in water for 24 h (to insure hydrolysis of excess coupling agent) and then in DMF for several days. The washing cycle was repeated until a constant weight was obtained in distilled water. After washing, the hydrogel systems were dried in air (no vacuum) for a minimum of 3 days, followed by a more rigorous drying under vacuum until a constant weight was achieved.

Hydrogel swelling experiments

Swelling experiments were conducted by equilibrating the selected dry hydrogel platform in buffer until a constant weight was obtained. Phosphate buffer was used for pH values of 3.0 and 7.4 whereas citrate buffer was used for pH from 2.5 to 7.0. The total buffer concentration was maintained at 0.01*M*, and the total ionic strength (μ) was adjusted by addition of sodium chloride. The buffer solution was replaced frequently throughout the swelling process to insure complete equilibration at the desired pH. Typically a complete equilibration was obtained within 1 week. The equilibrium swelling ratio (SR) was calculated as the ratio of the mass of wet hydrogel (m_{wet}) to the dry mass (m_{dry}):

$$SR = \frac{m_{wet}}{m_{dry}} \quad (1)$$

whereas the solvent weight fraction within a hydrogel (w_s) was calculated using Equation (2):

$$w_s = \frac{(m_{wet} - m_{dry})}{m_{wet}} = \frac{m_s}{m_{wet}} \quad (2)$$

where m_s is the mass solvent absorbed in the hydrogel at any time (t).

Circular dichroism

The dried hydrogel was swollen in buffer and ground to a fine powder in liquid nitrogen using a ceramic mortar and pestle. The ground hydrogel particles then were resuspended in an appropriate buffer (either 0.01*M* of phosphate or citrate, depending on the pH) and was subjected to circular dichroism (CD) measurements. CD spectra were obtained as the accumulated average of four scans collected at 20 nm/min using a 1-mm cell path length, 50-mdeg sensitivity, 1-nm bandwidth, and a response time of 0.5 s. Spectra were collected between 190 and 260 nm and were treated with solvent subtraction and noise suppression.

Drug loading and release experiments

Lysozyme was selected as a model protein drug. The drug was loaded into the hydrogel platform by a swelling-diffusion method. Drug solutions of known concentrations were prepared in 0.01*M* of phosphate buffer (pH 7.4) at low ionic strength (μ = 0.04*M*). A dried and weighed cylindrical hydrogel platform then was placed in 20 mL of the drug solution and allowed to swell for 4 days at 4°C under gentle

agitation. The swollen hydrogel system was removed, wiped dry using a laboratory tissue, and weighed.

Release rate experiments were performed by placing the swollen, drug-loaded hydrogel systems into vials containing 20.0 mL of 0.01*M* of phosphate buffer (pH 7.4) (μ = 0.04*M*). The vials were placed onto an oscillating shaker maintained at 25°C and gently agitated. At selected time intervals, individual hydrogel systems were removed from solution, gently wiped dry to remove excess buffer on the surface, weighed, and then placed into new vials containing fresh buffer solution. To examine the effect of volume change of the hydrogel on drug release, selected swollen hydrogel systems were transferred into high ionic strength 0.01*M* phosphate buffer (pH 7.4) containing 0.9 wt. % sodium chloride (μ = 0.15*M*). The drug release studies then were performed using the same procedures described above. Drug concentrations in the solutions were determined by UV absorbance at 280 nm using a spectrophotometer.

The fractional change in swelling was calculated as the ratio of the hydrogel mass at time t relative to the initial, fully swollen mass determined immediately prior to the start of release experiments. The results of drug release were presented as the fraction of the drug dose remaining within the hydrogel at time t (f_t) according to Equation (3):

$$f_t = \frac{m_o - m_t}{m_o} \tag{3}$$

where m_o is the total mass of drug loaded within the swollen hydrogel at t = 0 and m_t is the cumulative mass drug released at time t . It should be pointed out that the value of m_o was determined based on the final cumulative mass of drug extracted from the hydrogel.

RESULTS AND DISCUSSION

Synthesis of PBLG was carried out using the BLG N-carboxyanhydride in order to obtain polymers of high molecular weights. Reprecipitation also was performed in an attempt to remove low molecular weight fractions. Viscosity measurements indicated that PBLG debenzylation by HBr resulted in a loss of polymer molecular weight; in one instance, a loss of approximately 25% was observed. This decrease in molecular weight was a result of polymer main-chain degradation, as observed by other investigators.[25] Typically, PLG polymers with molecular weights above 100,000 daltons were obtained. Hydrogels were prepared using these PLG polymers and the coupling agent IIDQ according to the procedures described in Figure 1.

Figure 2(a) shows that the equilibrium swelling ratio (SR) of the PLG-PEG hydrogel increased as the pH increased, mainly in the range of pH 4–6 where the acidic moieties of PLG became increasingly ionized.[28] In addition, the SR was influenced by the solvent ionic strength; increasing ionic strength resulted in a reduction of the swelling ratio of the ionized hydrogel by nearly 50%. Furthermore, polypeptide composition was found to exert a significant impact on the swelling characteristics of this hydrogel formulation. As seen in Figure 2(a), the hydrogel prepared with a partially debenzylated polypeptide containing only 40% free L-glutamic acid residues yielded no appreciable

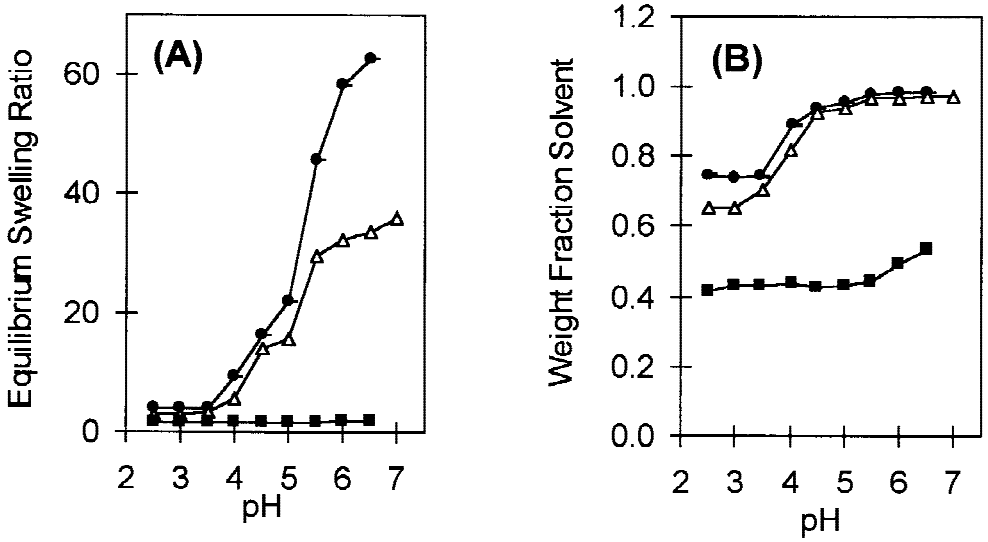

Figure 2. Equilibrium swelling of the polypeptide hydrogel as a function of pH. A hydrogel was synthesized using a 1500-Da diamino-PEG and an acid/amine loading ratio of 5. Swelling data were presented as (A) the equilibrium swelling ratio and (B) the weight fraction solvent within the swollen hydrogel. Hydrogels containing the fully debenzylated PLG were swollen at either low (●) (0.04*M*) or high (△) (0.15*M*) ionic strength whereas those containing a partially debenzylated PLG (40% free acid moieties) were swollen at only low ionic strength (■) (0.04*M*). Error bars represent the standard deviation (n = 3).

change in swelling across the pH range and over the time frame studied. Measurement of the solvent fraction [Fig. 2(b)], however, indicated that this partially debenzylated polypeptide hydrogel still contained over 40% by weight of buffer. Hence, modification of the hydrophobicity and ionic composition of the hydrogel appears to be a simple and yet useful strategy for manipulating the pH-dependent swelling properties of the hydrogel system.

While swelling increased consistently with pH, an obvious inflection in equilibrium SR was observed at pH 5.0 under both low and high ionic strength conditions [Fig. 2(a)]. If the pH-induced changes in swelling were only a function of ionization, polymer relaxation, and swelling mechanisms, a smooth change in swelling in response to pH would have been anticipated.[8] The inflection seen in Figure 2(a) therefore suggested that additional mechanisms might have contributed to the pH-dependent swelling of these hydrogels.

The polymer PLG is known to undergo a helix-to-coil transition within the pH range where the above inflection was observed.[28] In addition, the PLG-PEG hydrogels also have been reported to possess organized structural attributes in the solid state.[29] Our results from CD spectra (Fig. 3) show that hydrogels maintained at pH $\leq$ 4.5 exhibited a single minimum at about 229 nm, implicating the presence of organized beta structure within the polymer network.[30] When the pH was raised above pH 4.5, however, a single maximum was seen near 218 nm, suggesting a pH-induced relaxation towards the formation of a random coil structure. Apparently protonation of the carboxyl groups on the polypeptide at pH $\leq$ 4.5 was able to produce hydrogen bonding which stabilized the organized structural features observed in the CD spectra. Similar to the helix-coil transition of free PLG in solution,[28] ionization of the carboxyl groups at higher pH (pH > 4.5) appears to destabilize these organized structural features within the hydrogel, permitting relaxation of the polymer chains towards the random-coil configuration.

The reversibility of the swelling/de-swelling behavior was demonstrated by repeatedly cycling the PLG-PEG hydrogel between buffers at pH 6.5 and pH 5.5 over a 24-h period. As shown in Figure 4, the hydrogel rapidly contracted when placed in a pH 5.5 buffer but slowly returned to nearly the original swollen volume at pH 6.5 over the remainder of the 24-h period. This process appeared to be completely reversible, as the same swelling/de-swelling behavior continued across several cycles. The hydrogel returned to its original volume when allowed to swell to equilibrium at the end of the experiment.

The swelling properties of the hydrogel appeared to be influenced by the PLG concentration, PEG loading, and PEG molecular weight. As shown in Figure 5(a), crosslinking of the polypeptide using progressively increased PLG concentrations produced ionized networks that exhibited an appreciably reduced degree of swelling. This phenomenon is believed to be due to the formation of a higher intermolecular crosslinking density as the PLG concentration is increased. In contrast, swelling at pH 3.0 of the unionized hydrogel did not show any appreciable dependence on the PLG concentration. This finding suggests that swelling at low pH is not significantly influenced by the crosslink-

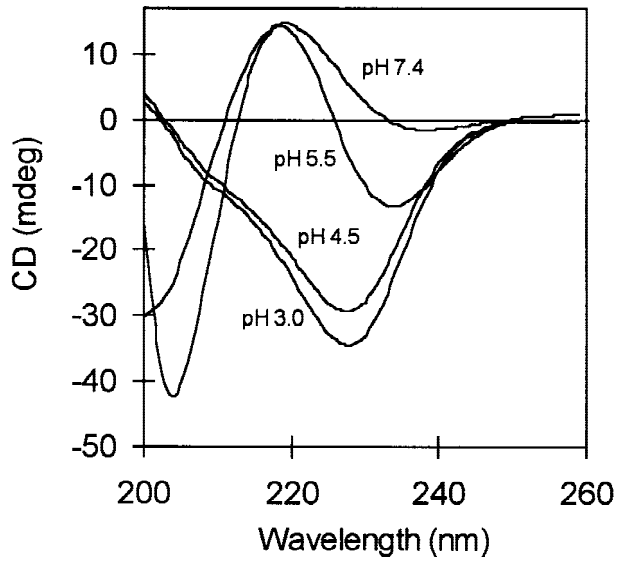

Figure 3. Circular dichroism spectra of the PLG-PEG hydrogel suspension in buffer at various pH levels (ionic strength of 0.1*M*). A hydrogel was synthesized using 1500 Da diamino-PEG at an acid/amine loading ratio of 5.

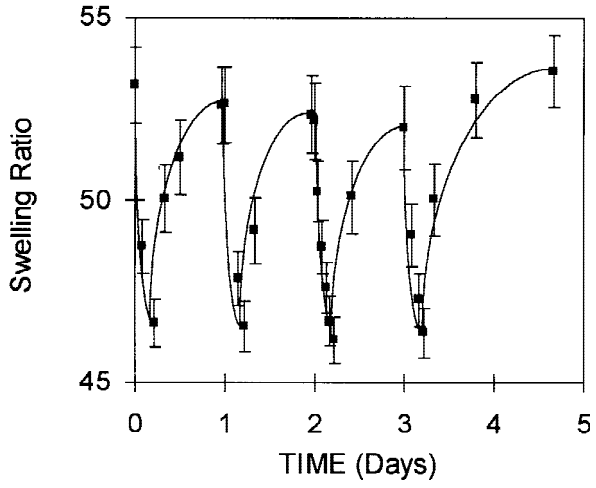

Figure 4. Reversible effect of pH on the swelling/de-swelling behavior of the PLG-PEG hydrogel. Swollen hydrogels were placed in pH 5.5 buffer for 4 h and then placed back into buffer at pH 6.5 for 19 h. Error bars represent the standard deviation (n = 3).

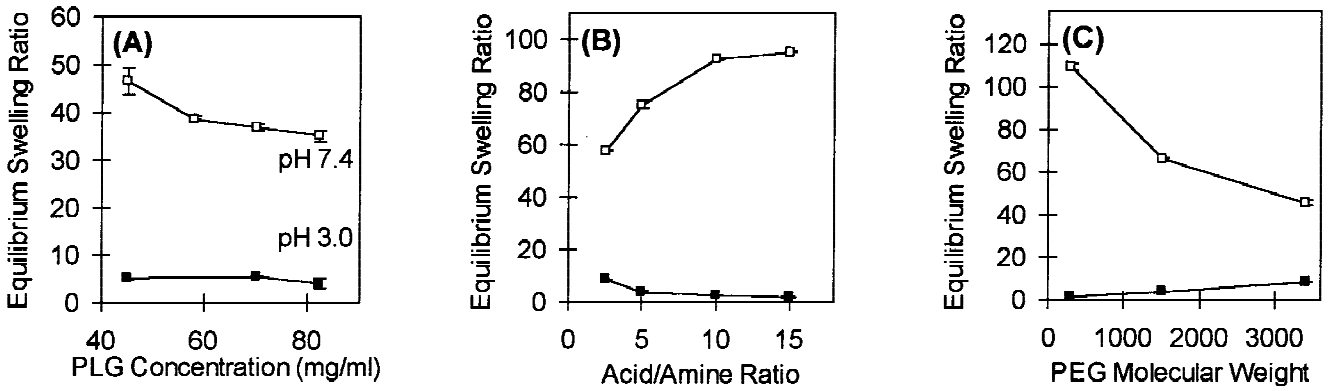

Figure 5. Effects of variables on the swelling characteristics of the PLG-PEG hydrogel at pH 3.0 (■) and pH 7.4 (□). Studies were performed by varying (A) PLG concentration, (B) the acid:amine crosslinking ratio (refer to description in the Materials and Methods section), and (C) PEG molecular weight during hydrogel synthesis. Buffer solutions were prepared at low ionic strength (0.04*M*).

ing density but rather by other factors, such as hydrophobicity of the polymer or hydrogen bond interactions.

Figure 5(b) shows that decreasing the molar ratio of PEG within the formulation produced hydrogels with a higher overall extent of swelling at high pH. Similar trends in swelling were obtained as the molecular weight of PEG was lowered [Fig. 5(c)]. These results suggest that both the PEG loading and its molecular weight are important factors in regulating the effective crosslink density of the polypeptide hydrogel. It is not surprising that higher PEG molecular weight produced greater crosslinking density since crosslinking is performed in polymer solutions where PEG chains having a larger hydrodynamic radius are more likely to span between, and thereby crosslink, adjacent PLG chains.

While both the loading and molecular weight of PEG could influence swelling via changes to the crosslinking density, they also could impart such effects via changes to the ratio of PEG to PLG in the hydrogel. As the content of PEG is increased, a dilution of the electrostatic swelling forces within the hydrogel is produced as the relative concentration of the acidic moieties within the hydrogel formulation is reduced. Consequently, the overall magnitude of the ion–ion repulsion and internal ionic osmotic pressure swelling forces is diminished and the overall degree of swelling is reduced. Interestingly, swelling of the unionized hydrogel at a low pH followed exactly the opposite trend as that obtained at a high pH; that is, formulations containing a higher PEG ratio produced a higher degree of swelling [Fig. 5(b,c)]. Although the mechanism contributing to such differences is not yet clear, it is hypothesized that increasing the ratio of PEG to PLG within the unionized hydrogel may increase bulk hydrophilicity or provide steric shielding to intermolecular hydrogen bonding, thereby resulting in the observed swelling trends.

The pH-dependent swelling/de-swelling properties of an ionic hydrogel have been utilized in the delivery of a variety of drugs, including insulin[31–33] and other macromolecules.[34] To assess whether the de-swelling property of the hydrogel could be employed as a means of facilitating release of the entrapped drug, the model protein lysozyme (molecular weight 16,000) was loaded within the PLG-PEG hydrogel. Drug loading was performed by swelling the hydrogel at a low ionic strength ($\mu = 0.04M$), and drug release was monitored while de-swelling the hydrogel at a high ionic strength ($\mu = 0.15M$). As shown in Figure 6, the control hydrogel systems that were not subject to changes in swelling exhibited no appreciable release of lysozyme. In sharp contrast, however, a rapid and extensive lysozyme release was obtained once de-swelling of the swollen hydrogels was initiated. Over a 2-h period, a 40% decrease in swelling accompanied by approximately a 50% release of the total entrapped dose of lysozyme was observed. Further studies of regulating the release rate of protein drugs from this PLG-PEG hydrogel by manipulating the pH change in the release environment currently are underway in our laboratory.

CONCLUSIONS

In summary, a novel amide-linked pH-responsive polypeptide hydrogel containing PLG and diamino-PEG was synthesized. Swelling of this PLG-PEG hydrogel was observed to vary significantly as the degree of ionization along the polypeptide chain was modified. This was accomplished via changes of the external pH or by modifying the hydrophobicity of the polypeptide. Secondary structural attributes were shown to exist within the unionized PLG-PEG hydrogel, presumably the result of hydrogen bond forma-

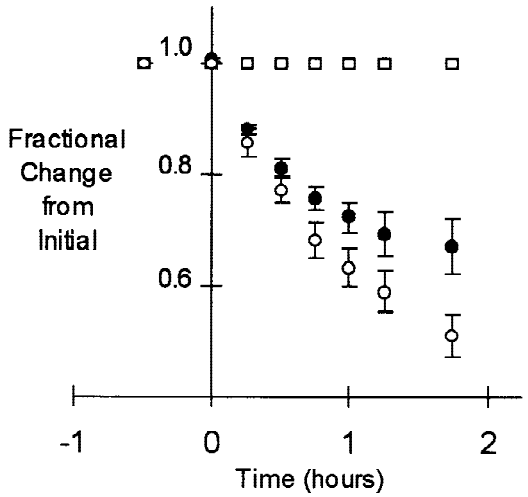

Figure 6. Effects of hydrogel de-swelling on the facilitation of lysozyme release. Cylindrical hydrogel systems were swollen in 0.01*M* phosphate buffer (pH 7.4) (μ = 0.04*M*) containing 1 mg/mL lysozyme. At time $t = 0$, the system was transferred into a buffer solution at high ionic strength (μ = 0.15*M*) to produce a rapid contraction of the swollen hydrogel. The weight percent of lysozyme based on the swollen weight of the hydrogel at the start of the release experiments was 3.26 ± 0.83% ($n = 3$). The fractional change in swelling (●) and the fraction of the lysozyme remaining within the hydrogel (○) are presented as a function of time. For comparison, lysozyme release from the control hydrogel system that did not undergo any change in swelling also is presented (□). Error bars represent the standard deviation of $n = 3$.

tion between the PLG residues or between the acid moieties of the PLG and the oxygen atoms on the PEG crosslinker. Variables involved during the synthesis of the PLG-PEG hydrogel, such as the PLA concentration, PEG loading, and molecular weight of the PEG, were found to influence significantly the swelling properties of the polypeptide hydrogel. For the highly swollen and ionized gel, the extent of swelling was dependent on factors affecting the density of crosslinking obtained during synthesis. For the unionized hydrogel, however, swelling appeared to be influenced by factors affecting the bulk hydrophilicity of the polymer network. The rapid contraction of the PLG-PEG hydrogel in response to external conditions was found to be an effective means for facilitating release of the entrapped protein lysozyme. Overall, the use of polypeptides in the synthesis of hydrophilic hydrogels appears to be a highly versatile and effective approach for producing biodegradable drug delivery systems.

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