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Synthesis of biodegradable thermo- and pH-responsive hydrogels for controlled drug release

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ABSTRACT

Novel intelligent hydrogels composed of biodegradable and pH-sensitive poly(L-glutamic acid) (PGA) and temperature sensitive poly(*N*-isopropylacrylamide-*co*-2-hydroxyethyl methacrylate) (PNH) were synthesized and characterized for controlled release of hydrophilic drug. The influence of pH on the equilibrium swelling ratios of the hydrogels was investigated. A higher PNH content resulted in lower equilibrium swelling ratios. Although temperature had little influence on the swelling behaviors of the hydrogels, the changes of optical transmittance of hydrogels as a function of temperature were marked, which showed that the PNH part of hydrogel exhibited hydrophobic property at temperature above the lower critical solution temperature (LCST). The biodegradation rate of the stimuli-sensitive hydrogels in the presence of enzyme was directly proportional to the PGA content. Lysozyme was chosen as a model drug and loaded into the hydrogels. The *in vitro* drug release experiment was carried out at different pH values and the release data suggested that both the pH and PNH content played important roles in the drug release behaviors of the hydrogels.

1. Introduction

Recent interest in stimuli-sensitive materials has promoted numerous efforts in preparing intelligent hydrogels [1–5]. The "smart" hydrogels have various potential applications in biomedical materials field, especially in the controlled drug delivery system [6,7]. There are many kinds of stimuli-responsive hydrogels that can response to the external changes in environmental conditions such as temperature, pH, photo and electric field [8–12]. Among all of the above-mentioned stimuli, temperature and pH are the most common physical and chemical ones used in biotechnological and biomedical applications, respectively. Accordingly, double-responsive hydrogels that are sensitive to both pH and temperature have attracted more and more attentions [13–21].

Poly(*N*-isopropylacrylamide) (PNIPAM) is one of the most popular thermosensitive polymers for biorelated applications. The PNIPAM chain undergoes a reversible coil-to-globule transition at about 32 °C (lower critical solution temperature, LCST). It is well soluble in water below the LCST. However, when the temperature increases above the LCST, the polymer becomes insoluble and precipitates out from its aqueous solution. The LCST can be appropriately elevated or reduced by copolymerizing NIPAM with a more hydrophilic monomer or a more hydrophobic monomer [22,23]. According to its unique hydrophilic/hydrophobic change, many thermo-responsive PNIPAM-based hydrogels have been prepared for controlled delivery of hydrophilic drugs [24,25]. The loading of hydrophilic drugs can be carried out in aqueous condition at low temperatures, which reduces the risk of denaturation of protein drugs in a hostile environment [26]. Further, the release rate can be modulated by the hydrophilic to hydrophobic change of the PNIPAM induced by temperature increase.

Polymers containing pendent carboxylic groups, such as poly(acrylic acid) (PAA), are typical acidic pH-responsive polymers [6,27]. Hydrogels containing carboxylic groups exhibit pH-sensitive swelling-deswelling behaviors and are widely used in controlled drug delivery systems [28,29]. However, most of synthetic pH-sensitive polymers are not biodegradable, which becomes a serious limitation in some applications [22]. Therefore, more attention has been focused on the development of

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biodegradable and pH-sensitive hydrogels based on polypeptides and natural macromolecules [30–32]. As compared to natural macromolecules, polypeptides have more regular structures and their molecular weights can be controlled more precisely [33]. Poly(L-glutamic acid) (PGA) and its derivatives are the most widely studied pH-responsive polypeptides due to their biocompatibility and biodegradability. For example, V. C. Yang et al. prepared pH-responsive PGA hydrogels using PEG as the crosslinker and investigated their release behaviors of protein drugs at different pH [34]. M. Akashi et al. reported the synthesis of pH-sensitive poly(γ-glutamic acid) (γ-PGA) hydrogels and semi-interpenetrating polymer network like hetero-gels composed of γ-PGA and partly sulfonated γ-PGA [35]. The pH-sensitive controlled release of fibroblast growth factor-2 (FGF-2) from the biodegradable hydrogel without any denaturation of the FGF-2 was successfully performed.

The combination of pH and temperature response is particularly useful to optimize the control of drug release and a variety of hydrogels being sensitive to both pH and temperature have been synthesized. Most of these double-responsive hydrogels are IPN or semi-IPN being composed of pH-responsive part and temperature-responsive part. However, the main problem of these networks is that they are not biodegradable [36,37]. An improved method is using biodegradable crosslinker [15]. For example, one temperature-responsive monomer (called "A") was crosslinked by a biodegradable pH-responsive crosslinker yielding a double sensitive hydrogel. But the problem is that it may cause long-term accumulation of high molecular weight "polymer A" *in vivo* after the degradation of crosslinker because its molecular weight is not controllable in the crosslinking reaction.

Recently, two kinds of biodegradable thermo- and pH-responsive hydrogels that can be fully biodegradable or composed of eliminable nondegradable part were successfully synthesized, but their biodegradability and drug release behaviors were not investigated [38,39]. Moreover, studies of hydrogels comprising thermosensitive components and polypeptides are relatively limited. In this paper, copolymer of NIPAM and HEMA was prepared to endow the copolymer with not only temperature-responsive property but also pendent −OH functional groups for a further modification. Then a series of thermo- and pH-responsive hydrogels composed of PGA and poly(NIPAM-*co*-HEMA) were synthesized through chemical coupling between the carboxylic groups in the PGA and the hydroxyl groups in the PNH. The as-prepared materials exhibited different temperature and pH response capabilities based on the variation of hydrogels compositions. The degradation properties of the hydrogels were assessed to be dependent on the different weight percentages of poly(NIPAM-*co*-HEMA). The molecular weight of poly(NIPAM-*co*-HEMA) is controlled ($Mn < 10{,}000$), so that it can be eliminated *in vivo* after the degradation of PGA [40,41]. In addition, preliminary drug release studies were studied using lysozyme as the model protein drug. The pH and temperature depending release behaviors indicated the promising application of these materials as controlled drug delivery vehicles.

2. Experimental section

2.1. Materials

N-isopropylacrylamide (NIPAM, 99%, Sigma) was recrystallized from hexane and dried under vacuum for 24 h prior to use. 2,2′-Azoisobutyronitrile (AIBN, Beijing Chemical Co., China) was recrystallized twice from methanol. γ-Benzyl-L-glutamate *N*-carboxyanhydride (BLG-NCA) was prepared according to our previous method [42]. 2-Aminoethanthiol hydrochloride (AET·HCl, 98%, Acros), 33 wt% HBr solution in acetic acid (Acros), 4-dimethylaminopyridine (DMAP, 98%, Fluka), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl, Shanghai GL Biochem, China), proteinase K (38.6 U/mg at 37 °C, Amresco) and dichloroacetic acid (Shanghai Laize Chemical Co., China) were used as received. 2-Hydroxyethyl methacrylate (HEMA, 96%, Acros) was distilled under reduced pressure before use. Dimethyl sulfoxide (DMSO) was purified by vacuum distillation over CaH_2. *N*,*N*′-dimethylformamide (DMF) was purified by vacuum distillation. Triethylamine was dried over CaH_2 and distilled prior to use.

2.2. Synthesis of poly(NIPAM-co-HEMA) (PNH)

NIPAM-based copolymer with NIPAM/HEMA = 80:20 was synthesized by radical polymerization in DMF using AIBN as the initiator. To control the molecular weight of poly(NIPAM-*co*-HEMA), AET·HCl was used as a chain transfer reagent. In brief, NIPAM (9.04 g, 80 mmol), HEMA (2.6 g, 20 mmol), AIBN (0.164 g, 1 mmol), AET·HCl (0.23 g, 2 mmol), and DMF (40 mL) were added into a flask with a magnetic bar. After being degassed by three freeze-vacuum-thaw cycles, the flask was sealed under Ar atmosphere and then immersed in an oil bath at 60 °C under stirring. After 24 h, the product was precipitated by pouring the reaction solution into excessive diethyl ether, purified by precipitating twice into diethyl ether from THF solution, and eventually dried under vacuum at room temperature for 24 h. The yield was 90.4%.

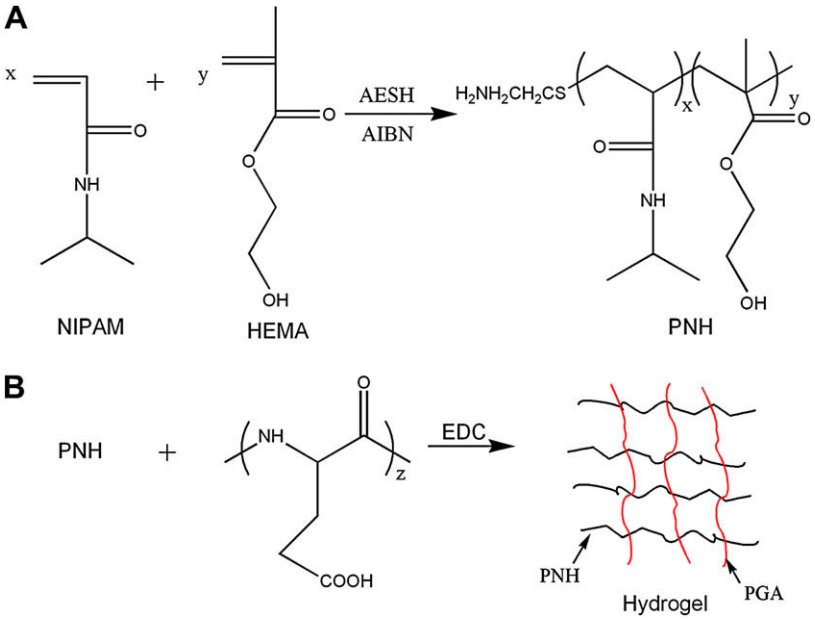
2.3. Synthesis of poly(L-glutamic acid) (PGA)

Firstly, poly(γ-benzyl-L-glutamate) (PBLG) was prepared by the ring-opening polymerization (ROP) of BLG-NCA. In brief, BLG-NCA (6 g) was dissolved in 180 mL anhydrous dioxane. Then 92.4 mg triethylamine was added with vigorous stirring at room temperature. After 3 days, the reaction mixture was added to a 10-fold excess of ethanol. The precipitated polypeptide was isolated by filtration and dried under vacuum at 40 °C for 24 h.

Poly(L-glutamic acid) was prepared by debenzylation of PBLG using HBr. Briefly, PBLG (5 g) was dissolved in dichloroacetic acid (50 mL) at ambient temperature. After excessive 33 wt% HBr solution in acetic acid [HBr/benzyl (mol/mol) = 8] was added, the solution was stirred at 30 °C for 1 h. The product was precipitated by pouring the solution into excessive acetone, isolated by filtration, and repeatedly washed using acetone. Then the product was dried under vacuum at room temperature for 24 h.

2.4. Hydrogel synthesis

The method of preparing the pH- and temperature-responsive hydrogel is shown in Scheme 1. Typically, PGA and PNH (total amount of 100 mg) at different weight ratios, e.g. PGA: PNH = 6:4, 5:5, 4:6, and 2:8 (w/w), were dissolved in 1.5 mL of DMSO. After dissolution, a solution of EDC·HCl (50 mg) and DMAP (6 mg) in DMSO was added as the coupling agent and catalyst, respectively. The mixture was mixed thoroughly and then quickly poured into a glass mold. The gelation was allowed to proceed at room temperature for 24 h. Then, an excess amount of acetone was added to the mold and the reaction product was isolated. To obtain a swelled hydrogel, the product was kept in a pH 7.4 phosphate buffer solution which was changed once a day for 4 days. Then the gel was placed into deionized water and washed by changing the water once a day for 4 days. After removal of the excess water, the swelled gel was lyophilized to obtain a dry gel. Hydrogels were denoted as Gel 6/4, 5/5, 4/6, and 2/8 corresponding to their initial PGA: PNH feeding ratios of 6:4, 5:5, 4:6, and 2:8 (w/w).



Scheme 1. Synthetic route of PNH (A) and hydrogel (B).

2.5. Characterization

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded by a Bruker 400 MHz spectrometer. FTIR spectra were measured on a Bruker Vertex 70 Fourier Transform Infrared spectrometer using the KBr disk method.

The morphology of the hydrogel was investigated by environmental scanning electron microscopy (ESEM) on an XL 30 ESEM FEG Scanning Electron Microscope (Micrion FEI PHILIPS). The hydrogel samples were swollen in water for 48 h to reach equilibrium, and then quickly put into liquid nitrogen for 10 min and transferred to a freeze-dryer for 72 h. The samples were then loaded on the surface of a copper SEM specimen holder and sputter coated with gold before observation.

SEC-MALLS were carried out by combining a Waters 515 GPC instrument with a light scattering apparatus at 25 °C. The system included a Styragel HMW6E column, a 515 HPLC pump, an IR OPTILAB DSP detector, and a DAWN EOS multiangle laser-light scattering (MALLS) detector (Wyatt Technology). The mobile phase was DMF containing 0.01 M LiBr at a flow rate of 1.0 mL·min^{-1}.

Phase transition measurements were carried out on a UV/Vis spectrometer (Shimadzu UV-2401PC) equipped with a Temperature Controller (Shimadzu S-1700) with a hydrogel slab mounted in a glass cuvette. The heating rate was 0.5 °C/min over the temperature range of 20–45 °C.

The molecular weight of the PGA was estimated by viscosity measurement in 0.4 M NaCl and 0.01 M NaH_2PO_4 solution at pH 6.8 and 25.5 °C using the intrinsic viscosity-molecular weight relationship derived from Haekins et al. [43]:

$$[\eta] = 2.93 \times 10^{-5}\, M^{0.923} \quad (1)$$

2.6. Swelling of hydrogel

To measure the swelling ratio of the hydrogels, the dried sample was immersed in various solutions with certain pH and temperature for 2 days. The buffer solution was replaced frequently throughout the swelling process to insure complete equilibration at the desired pH. The swelling ratio (gram per gram) SR of the hydrogels was calculated from the following equation:

$$SR = (W_t - W_0)/W_0 \quad (2)$$

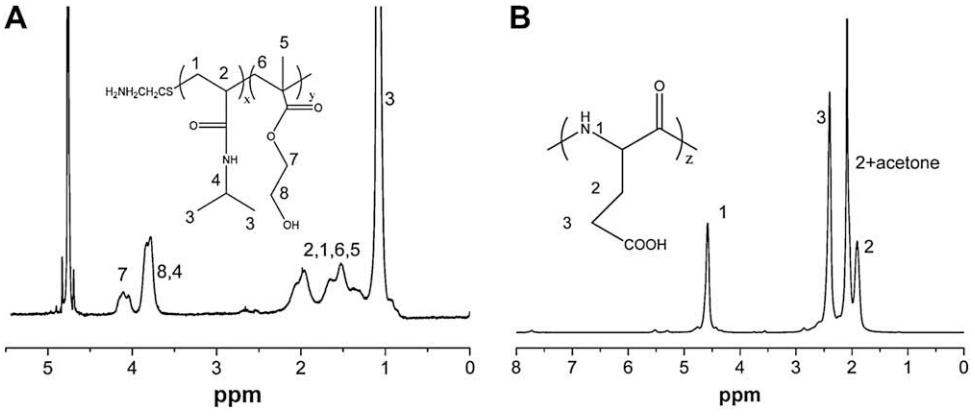


Fig. 1. ^{1}H NMR spectra of PNH (A) and PGA (B).

Table 1
Feed and result compositions of hydrogels.

Samples	Feed compositions				PGA%(w/w)[a]	Yield(%)[b]
	PGA/mg	PNH/mg	EDC/mg	DMAP/mg		
Gel 6/4	60	40	50	6	66.6	96.2
Gel 5/5	50	50	50	6	54.4	91.1
Gel 4/6	40	60	50	6	44.2	86.0
Gel 2/8	20	80	50	6	27.7	61.5

[a] Weight content of PGA in dry gels, calculated from elemental analysis results.
[b] Calculated from the following equation: Yield (%) = $W_h/(W_g + W_n)$, where W_h is the weight of dried hydrogel, W_n is the feed weight of PNH and W_g is the weight of poly(sodium glutamate) derived from feed weight of PGA.

where W_t and W_0 are the weights of the swollen gels and dried samples, respectively. All the experiments were carried out in triplicate, and the average values were reported.

2.7. In vitro enzymatic degradation of hydrogels

Biodegradation of hydrogels was carried out in a small vial containing a small piece of dry hydrogel sample and PBS buffer (pH = 7.4, 0.01 M) with proteinase K at a concentration of 0.2 mg/mL. The mixture was then incubated at 37 °C with constant shaking (100 rpm). At different time interval, the samples were taken out and rinsed thoroughly with deionized water; then they were lyophilized to determine the dry weights of the hydrogels. The solution was replaced once a day in order to maintain enzymatic activity. The percentage of weight loss [W_l(%)] was calculated based on the following equation:

$$W_l(\%) = (W_0 - W_d)/W_0 \times 100 \quad (3)$$

where W_0 is the original weight of the dried gel sample before immersion, and W_d is the weight of the dried sample after degradation at predetermined days.

2.8. Drug release studies

A hydrophilic model drug, lysozyme, was loaded into the hydrogel by a swelling-diffusion method. The drug solution (5 mg/mL) was prepared in a 0.01 M phosphated buffer at pH 7.4. Then a dried and weighted cylindrical hydrogel was placed in 20 mL of the drug solution and allowed to swell for 3 days at 4 °C. After swelling equilibrium, the hydrogel was taken out and rinsed thoroughly with 0.01 M PBS (pH = 7.4). The left drug solution and the PBS used to rinse the drug-loaded hydrogel were collected together and diluted to 50 ml in a volumetric flask. Then the amount of lysozyme left in the loading medium was determined by a UV/Vis spectrophotometer (Shimadzu UV-2401PC) at a wavelength of 280 nm.

For the drug release studies, the drug-loaded hydrogels were immersed in 10 ml of 0.01 M PBS (pH = 7.4) or 0.01 M citrate-buffered saline (pH = 4.0). The samples were incubated at 37 °C with constant shaking (100 rpm). At selected time intervals, 1 mL of the release medium was withdrawn and replaced with 1 mL of the fresh solvent. The amount of released lysozyme was quantified by the UV/Vis spectrophotometer.

3. Results and discussion

3.1. Synthesis and characterization

PNH was prepared by free radical polymerization using AET·HCl as the chain transfer reagent, as shown in Scheme 1(A). The composition of the copolymer was calculated from the ^{1}H NMR spectra. The molar ratio of NIPAM and HEMA was calculated from the integration ratio between the methenyl proton (1H) ($(CH_3)_2CH$NHCO−) of NIPAM and the methylene protons (2H) ($HOCH_2CH_2OCO$−) of HEMA appearing at 3.8 and 4.1 ppm (shown in Fig. 1A), respectively, by the following equation:

$$\text{HEMA mol.} - \% = (I_{4.1}/2)/[(I_{3.8} - I_{4.1}) + (I_{4.1}/2)] \times 100 \quad (4)$$

where $I_{4.1}$ and $I_{3.8}$ are the integral values of the peaks at $\delta = 4.1$ and 3.8 ppm, respectively. The HEMA mol.% in the PNH was 18.5%. In addition, we also prepared PNH with different HEMA mol.% (11.4% and 27.8%, Table S1).

It has been reported that PNIPAM with lower molecular weight (such as $M_n < 10{,}000$) can be excreted by glomerular filtration without long-term accumulation *in vivo* [40,41]. Accordingly, the molecular weight of PNH was controlled by the amount of introduced chain transfer agent. From the GPC result in THF [Waters 410GPC, flow rate: 1 mL/min, at 35 °C. The molecular weights were calibrated against polystyrene (PS) standards], the weight and number average molecular weight for PNH were determined to be 1.2×10^4 and 9.2×10^3, respectively (PDI = 1.28).

PBLG was synthesized by the ring-opening polymerization of BLG-NCA. In order to prepare high molecular weight PBLG, which cannot be obtained by primary amine initiators, triethylamine was chosen as the initiator. In addition, a higher molecular weight of the polypeptides could be obtained by a lower monomer concentration, longer reaction time and lower reaction temperature. The weight and number average molecular weight of PBLG were 4.4×10^5 and 4.2×10^5, respectively (PDI = 1.04). The debenzylation of PBLG was carried out in dichloroacetic acid using HBr. It was

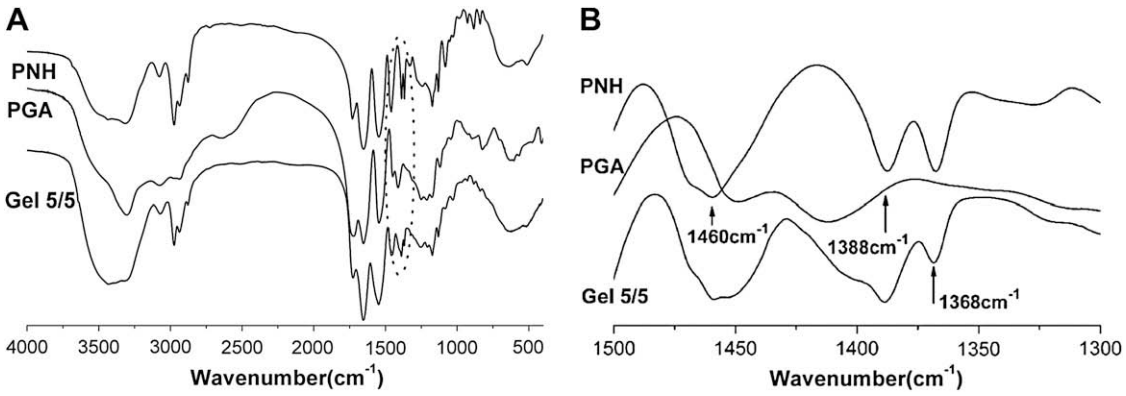


Fig. 2. Representative FTIR spectra of PNH, PGA and Gel 5/5 (A) and its selected details (B).

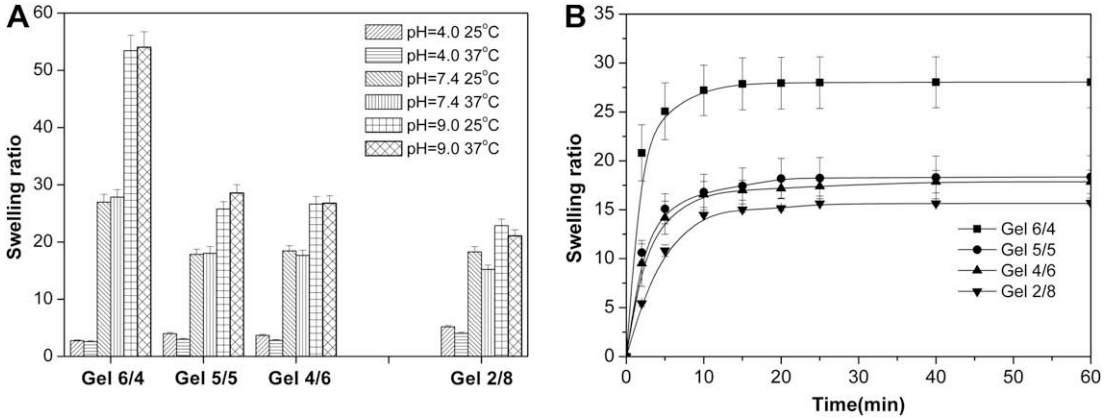


Fig. 3. Equilibrium swelling ratios of hydrogels at different pH and temperature (A), and swelling kinetics of hydrogels at 37 °C and pH 7.4 (B).

established by us that a higher debenzylation temperature such as 50 °C depicted by others resulted in severe cleavage of the PBLG backbone [44]. Therefore, the debenzylation temperature was set at 30 °C. The ^{1}H NMR spectrum of PGA is shown in Fig. 1B. The molecular weight of PGA determined from the intrinsic viscosity was 1.2×10^5.

Hydrogels were prepared by the esterification between PGA and PNH using a water-soluble carbodiimide as the coupling reagent according to the procedures described in Scheme 1(B). Interestingly, the crosslinking reaction did not occur in water, but it successfully proceeded in DMSO in the presence of DMAP. Similar phenomenon was also observed by S. Murakami and his coworkers [45].

The PGA contents (weight percent) in the gels were estimated from the elemental analysis results according to the following equation:

$$\text{PGA wt.\%} = (R_1 - R_3)/(R_1 - R_2) \quad (5)$$

where R_1 is the C/N (weight ratio) of the PNH, R_2 is the C/N (weight ratio) of the PGA, and R_3 is the C/N (weight ratio) of the hydrogel calculated from the elemental analysis results. As shown in Table 1, the PGA contents in the gels decreased in the order according to the feeding ratio. The yields of Gel 6/4, 5/5 and 4/6 were relatively high (>86%). However, the yield of Gel 2/8 was only 61.5%. Since the PGA content of Gel 2/8 (27.7%) was higher than the feeding ratio (20%), it could be safely concluded that a certain amount of PNH did not react with PGA and was washed away.

The FTIR spectra of PGA, PNH and Gel 5/5 are shown in Fig. 2. In the FTIR spectrum of hydrogel, the absorption peak of amide group at 1650 cm^{-1} (νCO) and 1545 cm^{-1} (νCO–NH) were strengthened. The characteristic divided bands of symmetric C–H bending from the $-CH(CH_3)_2$ group at 1368 and 1388 cm^{-1} from PNH of hydrogels were also observed (Fig. 2B). All the results clearly confirmed that the biodegradable pH and temperature sensitive hydrogels were prepared.

3.2. Swelling behaviors

The swelling behaviors of Gel 6/4, 5/5, 4/6 and 2/8 were measured at different pH and temperatures, as shown in Fig. 3A. As expected, the hydrogels had a lower swelling ratio in an acidic medium, and the corresponding swelling ratios increased with increase in pH value at both 25 °C and 37 °C because of the deprotonation of PGA. For example, at 25 °C or 37 °C, the swelling ratios of all hydrogels were less than 5 at pH 4.0. While in the alkaline medium (pH 9.0), the highest swelling ratio reached to 10 times higher than that in the acidic medium.

At pH 4.0, the equilibrium swelling ratios were higher at 25 °C than the values observed at 37 °C for all hydrogels due to the phase transition of PNH. Similar results were also got from the hydrogels prepared by PNH containing different HEMA mol.%, which were shown in Table S2, Figs. S1 and S2. However, the equilibrium swelling ratios had no marked difference between the two temperatures at pH = 7.4 or 9.0. This was probably due to the hydrophilic property of PGA is much stronger than PNH, which resulted in the swelling ratio mainly being controlled by pH. As shown in Fig. 3A, the equilibrium swelling ratio of Gel 6/4 at pH 9.0 was much higher than others. This fact was mainly attributed to the reason that more −COOH groups exist in the gel compared with others, which led to a stronger intermolecular repulsion between $-COO^-$ in the basic buffer than that in the acid buffer and create more space for solvent to come in. Another reason may be that the pore size of Gel 6/4 was bigger than those of others (Fig. 5A). It is known that the average pore size of the hydrogel is related to the capacity of hydrogel to keep water. Thus, the decrease of average pore size would result in the decrease of swelling ratio. Fig. 3B shows the swelling rate of hydrogels at pH 7.4. It was found all hydrogels exhibited a fast swelling and reached equilibrium within 20 minutes.

The pH-responsive swelling-deswelling property of hydrogels was demonstrated by Gel 5/5 between pH 4.0 and 7.4 at 37 °C. As shown in Fig. 4, the swelling-deswelling process was repeatable as pH changes across the cycles. It was found that the deswelling rate

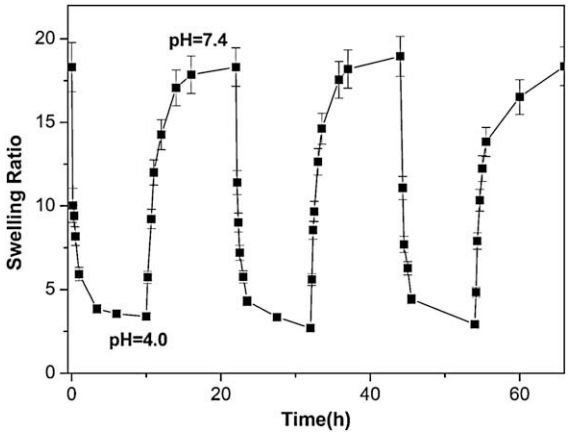


Fig. 4. Reversible swelling/deswelling behaviors of Gel 5/5 between pH 4.0 and pH 7.4.

was faster than the swelling rate in each cycle. This behavior made it possible to control drug release by a feed-back mechanism [46].

3.3. Hydrogel morphology

To examine the surface and interior structure of the hydrogel in swollen state, ESEM measurement was performed. As shown in Fig. 5A, all of the hydrogels exhibit porous structures, while the pore sizes and structures are different as the content of PNH changes. It was found that the pore size of Gel 6/4 was bigger and more irregular than others. As the content of PNH increased, the pore sizes decreased in the order of Gel 5/5 > Gel 4/6 > Gel 2/8 (50 μm, 12 μm and 10 μm, respectively). However, the difference in pore structure of Gel 5/5, Gel 4/6 and Gel 2/8 was not great compared with Gel 6/4. The different composition ratios of PNH to PGA resulted in dramatic pore structure change from an irregular and loose appearance of Gel 6/4 to a very well-defined and tight structure of Gel 2/8. For example, the Gel 6/4 had an irregular porous structure and the pores were very large, but the Gel 2/8 had a regular structure and the average pore size was around 10 μm.

The changes in pore size and structure were mainly attributed to the increased crosslinking level from Gel 6/4 to Gel 2/8. It was known that the hydrogels were synthesized by the crosslinking reaction between the hydroxyl groups of PNH and carboxyl groups of PGA, but in all of our experiments the carboxyl groups were in excess of the hydroxyl groups. So increasing the weight percentage

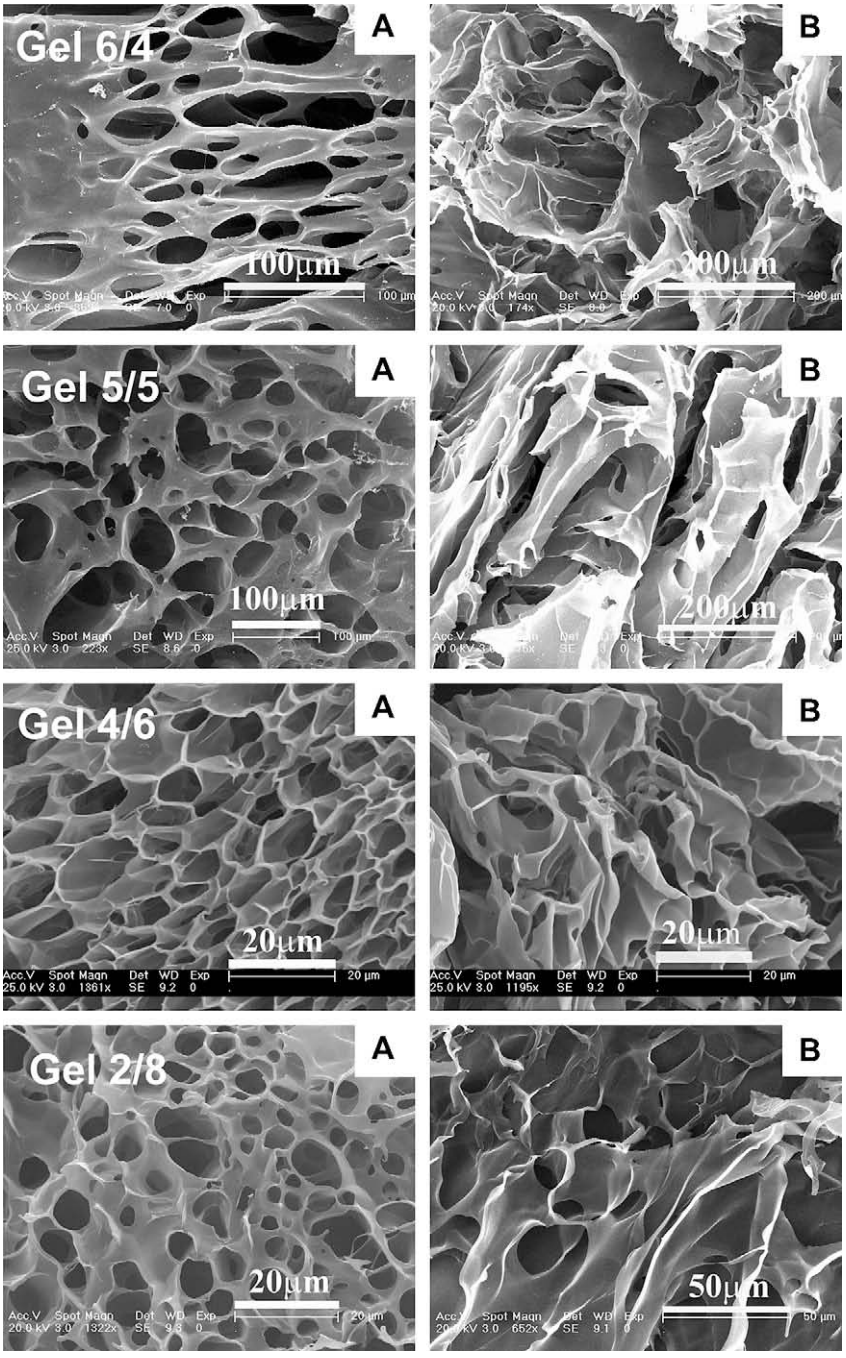


Fig. 5. SEM micrographs of hydrogels before degradation (A), after enzymatic degradation for 2 days (with the enzyme concentration of 0.2 mg/mL, at pH 7.4 and 37 °C) (B).

of PNH in the hydrogel meant increasing the crosslinking point of hydrogel. Thus more PNH in the hydrogels would result in higher crosslinking density.

3.4. Phase transition studies

The phase transition behaviors of hydrogels were characterized by monitoring the turbidity of the hydrogels as the temperature changed. The LCST of the hydrogel was determined as the temperature at 50% transmittance. Although the temperature had a little influence on the swelling behaviors of hydrogels, the changes of optical transmittance of hydrogels as a function of temperature was obvious. As shown in Fig. 6, the LCSTs of hydrogels and the remained transmittance at 37 °C for hydrogels decreased as the weight percentage of PNH increased. And the phase transition became sharper with the increase of PNH component. Furthermore, the LCSTs of Gel 6/4 and Gel 5/5 were slightly higher than that of the PNH (about 31.5 °C). But the LCSTs of Gel 4/6 and Gel 2/8 were slightly lower than that of the PNH. The results showed that the PNH part of hydrogels exhibited hydrophobic property when the temperature was above their LCSTs. Based on this temperature sensitive property, intelligent drug release system can be achieved. For example, hydrophilic drug can be loaded into hydrogel at a temperature below LCST. When the temperature is higher than the LCST, the PNH part becomes hydrophobic and will restrict the diffusion of drug. Thus, the drug loading and release can be controlled by temperature.

3.5. In vitro enzymatic biodegradability of hydrogels

PGA is one of the most studied synthetic polypeptide and has been proved to be biodegradable *in vitro* and *in vivo*. However, the prepared hydrogels had no significant weight loss when they were placed into pH = 7.4 PBS (0.01 M) at 37 °C for a month. This suggested that the hydrolytic degradation was very slow under this situation. Therefore, in this study proteinase K was used to accelerate the degradation of PGA chain in hydrogels. Proteinase K is a broad-spectrum proteolytic protease. It normally hydrolyzes amide bonds and also attacks ester bonds [47]. So the presence of proteinase K can accelerate the brokenness of both the crosslinking sites (ester bonds) and PGA main chains (amide bonds) of network. The enzymatic degradation behavior of hydrogels in the presence of proteinase K is shown in Fig. 7. In our experiments, all the hydrogels showed various extents of weight loss. It was found that there was obvious disintegration after 1 day of enzymatic degradation for Gel 6/4. However, there was little weight loss for Gel 2/8 after 3 days.

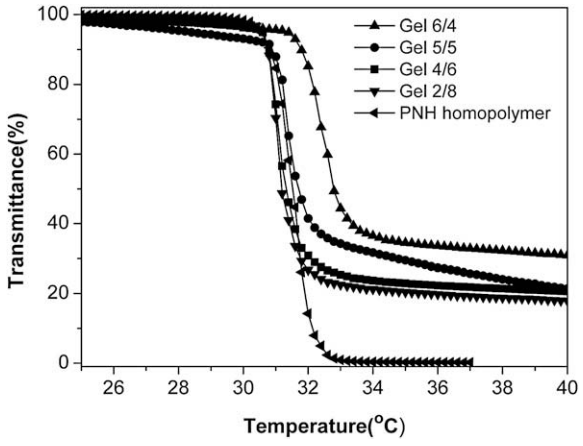


Fig. 6. Effect of temperature on the phase transition behavior of hydrogels.

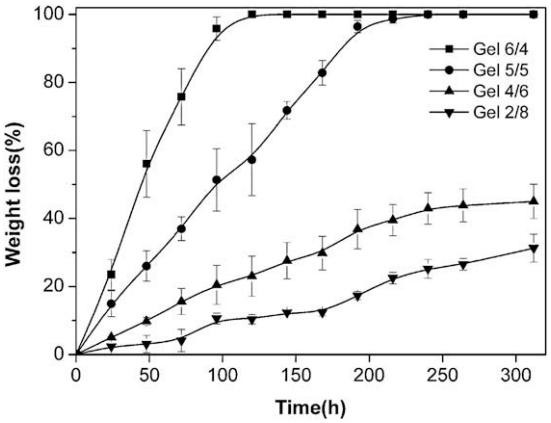


Fig. 7. Degradation of hydrogels in buffer solution of pH 7.4 (PBS) in the presence of proteinase K (0.2 mg/mL) at 37 °C.

The degradation rate decreased with the order of Gel 6/4 > Gel 5/5 > Gel 4/6 > Gel 2/8. As the degradation proceeding, all hydrogels showed a crack surface and the pore became larger (Fig. 5B).

The degradation of hydrogel is linked to network parameters such as crosslinking density and the proportion of hydrophobic part in hydrogel. The degradation behavior of hydrogel at same condition revealed that hydrogels with higher crosslinking densities and PNH contents exhibited slower degradation properties. As shown in Fig. 7, Gel 6/4 degraded completely in 4 days, but Gel 2/8 only lost 10% of its weight, and Gel 5/5 and Gel 4/6 were in the middle, with 51% and 21% weight loss after 4 days, respectively. As the hydrogel degrading, the soluble part including PGA and its degraded residue

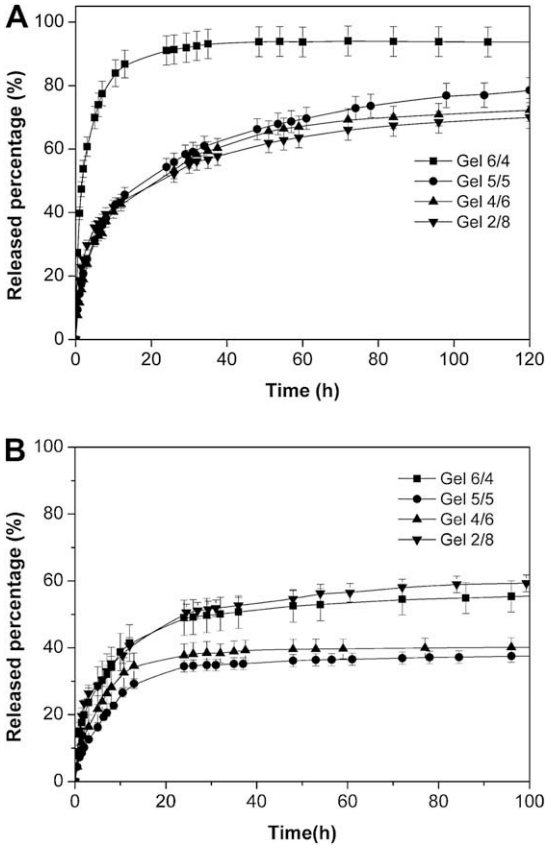


Fig. 8. Cumulative release of lysozyme from the hydrogels at pH 7.4 (A) and pH 4.0 (B), 37 °C as a function of time.

were released and diffused into the buffer solution. So the weight of the remaining crosslinked network decreased. It was established that the PNH exhibited hydrophobic property at 37 °C. So with more soluble PGA diffusing from the hydrogel, the remained hydrogel networks became more hydrophobic. Thus, it was more difficult for the enzyme to access the polymer chain and the degradation rate of hydrogels became slower.

3.6. *In vitro* release of lysozyme

In vitro lysozyme release profiles of hydrogels at 37 °C were investigated in pH 7.4 and 4.0 buffers, as shown in Fig. 8A and B, respectively. It was found that there were burst releases during the initial stage at pH 7.4. This relative constant rapid release at the initial stage was attributed to the release of drug that located at the hydrogel surface. When the hydrogel was placed into the buffer solution, the drug at the hydrogel surface could be dissolved immediately, leading to the burst release. This result was also reported by others [48]. After the initial burst, the hydrogels served as diffusion barriers and the drugs were mainly released by the diffusion mechanism. As shown in Fig. 8A, the release rate decreases in the order of Gel 6/4 > Gel 5/5 > Gel 4/6 > Gel 2/8, which is consistent with the crosslinking density change of the hydrogels. The hydrophobic property of PNH at 37 °C also affected the diffusion of lysozyme and a higher PNH content resulted in a slower release rate. Compared with that of other hydrogels, the release rate of Gel 6/4 was the fastest due to its lowest crosslinking density and PNH content. For example, about 85% lysozyme was released for Gel 6/4 after 14 h later, whereas the corresponding cumulative releases for Gel 5/5, Gel 4/6 and Gel 2/8 were all around 45%. After about 4 days, all hydrogels exhibited no or very slow release of lysozyme. That was probably attributed to the interactions between the remained lysozyme and the hydrogel network, which prevented the release of the drugs. The low drug concentration preserved in the hydrogels, which leads to a low drug concentration gradient and weak release driving force, may be another reason.

PGA and its derivatives are most widely studied polypeptides for their pH-sensitive property, so it is necessary to examine the lysozyme release profile under an acidic condition. Fig. 8B shows the *in vitro* cumulative release of lysozyme from the hydrogels at pH 4.0. It was found that the initial burst was suppressed compared with that at pH 7.4. The release profiles at pH 4.0 were quite different from those at pH 7.4. All hydrogels exhibited lower amounts of the cumulative releases at pH 4.0 than those at pH 7.4 at corresponding release time. For instance, at the 4th day, Gel 6/4 had 55% drug released at pH 4.0 but 94% at pH 7.4; Gel 5/5 had 38% at pH 4.0 but 77% at pH 7.4; Gel 4/6 had 41% at pH 4.0 but 71% at pH 7.4 and Gel 2/8 had 59% at pH 4.0 but 68% at pH 7.4. As a polypeptide bearing carboxylic side groups, PGA was protonated under an acidic condition. Therefore, the diffusion of drugs was restricted by the constrained network at pH 4.0. In addition, the PNH was hydrophobic at 37 °C and the hydrophobic interaction in the network would also hinder the release of lysozyme. It was noticed that the release rate of Gel 2/8 was fast despite its highest PNH content. This could be ascribed to the reason that the PGA content in Gel 2/8 was too low to provide enough hindrance at pH 4.0, leading to its apparent release rate faster than other hydrogels. Therefore, it could be concluded that the PGA and PNH part had cooperative effect on the drug release rate at pH 4.0.

4. Conclusion

A series of pH and temperature sensitive hydrogels were synthesized by the esterification between PGA and PNH using a water-soluble carbodiimide as the coupling reagent. The swelling ratios of hydrogels increased with the pH increase and these processes were reversible. Although there was no marked difference between the equilibrium swelling ratios at 25 °C and 37 °C, the temperature had great influence on the optical transmittance of hydrogels, which showed that the PNH part of hydrogel exhibited a hydrophobic property at temperature above its LCST. ESEM observations revealed that all the hydrogels had similar porous structures but hydrogel with a lower crosslinking density had larger pore size. In the presence of enzyme, all hydrogels exhibited biodegradability and the degradation rate could be controlled by changing the composition ratio. The *in vitro* release of lysozyme from the hydrogels at pH 7.4 demonstrated that the hydrogel with a lower crosslinking density and PNH content had a faster release rate. The drug release rate at pH 4.0 was slower than that at pH 7.4 due to the protonation of the PGA part at an acidic condition. The results display the possibility of using this pH and temperature sensitive hydrogel as an intelligent drug delivery carrier.

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Appendix. Supplementary information

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.polymer.2009.07.010.

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