Preparation and Properties of Crosslinked Chitosan Thermosensitive Hydrogel for Injectable Drug Delivery Systems

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ABSTRACT: The aim of this study was to prepare and investigate the physical properties of a thermosensitive crosslinked chitosan pregel solution, and evaluate the *in vitro* release profiles of macromolecules from this sol–gel transition system. Chitosan and poly (vinyl alcohol) were used to form an interpenetrating polymeric network with glutaraldehyde as the crosslinker, and glycerophosphate (GP) was added to transform the pH-dependent solutions into thermosensitive pH-dependent solutions. Rheological study showed that the gelation was dependent on the crosslink degree and GP concentration of the solution. The crosslinked gel had excellent mechanic properties and no apparent "pores" and formed an integrated hydrogel texture according to scanning electronic micrograph. Gas chromatography test guaranteed the medication safety with no detection of glutaraldehyde remnants in the hydrogels. *In vitro* release study showed that the gelation does not significantly affect the macromolecules diffusion but the crosslinking degree does. These results indicated that the hydrogel formed an intensified three-dimensional hybrid network with interpenetrating molecules, which effectively buffered or delayed the macromolecules diffusion. The hydrogels sustained the drug release over 30 days and could be potentially used as *in situ* gelling implants. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 101: 1892–1898, 2006

Key words: hydrogels; drug delivery systems; chitosan; crosslinking; interpenetrating networks

INTRODUCTION

Biodegradable thermosensitive hydrogels have been extensively explored in drug delivery systems, artificial tissues, and biomedical devices.[1,2] These hydrogels may have the properties of soft rubbery consistency and low interfacial tension.[3] They may exhibit a sol–gel transition at body temperature, which enables them to be subcutaneously injected to function as drug reservoir or to resemble the soft tissues, instead of surgical implanting. They also can be biodegraded automatically avoiding surgery taking out afterwards.[4] Recently, chitosan (CTS)-based pH-dependent, thermosensitive, biodegradable, and injectable neutral solution has shown potential to be used as *in situ* gel-forming biopolymer. This thermosensitive CTS hydrogel has been reported mostly as carrier for long-term drug delivery or cell transport.[5,6]

Nevertheless, one of the disadvantages of this sensitive hydrogel is their low mechanical strength because of the high water content. Another disadvantage is that the gel formed mainly from synergistic forces including hydrogen bonding, electrostatic interactions, and hydrophobic interactions between cationic polysaccharide, and polyol–phosphate salts.[5] The linear-cationic polysaccharide molecules will form a relatively loose three-dimensional (3D) network. Drug molecules will easily diffuse from this configuration, which results in a burst drug release from this biopolymer network.

Crosslinking of these cationic polysaccharides may form a 3D network, whereas this network still has a low mechanical strength. The use of an interpenetrating (IPN) agent has been reported to alleviate this problem. The IPN agent can polymerize to form a double crosslinked structure, or entangles to form a hybrid polymeric network. The network was maintained by covalent bonds, interspace resistance, electrostatic, or hydrophobic interactions, etc. The IPN polymers always have better mechanical properties than the principal polymer and can improve the mechanical properties of the final hydrogel.[3,7]

In this article, we describe the synthesis and characterization of a biodegradable, pH-dependant thermosensitive hydrogel with high mechanical properties and intensified hybrid network by using CTS as the principal polymer, poly(vinyl alcohol) (PVA) as the IPN agent, glutaraldehyde as the crosslinker, and β-glycerophosphate (GP) as the hydrophobicity modifier.

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PVA has been widely used owing to its excellent mechanical properties. It is also biodegradable under certain conditions, which makes it suitable to be used as an IPN agent in this study. Solubility of PVA in water depends on the degree of hydrolysis and polymerization. PVA with hydrolysis of 98.5% or higher can be dissolved in water at 70°C, which is a common practice for preparing its solution.[8]

Glutaraldehyde is a common crosslinker used in polypeptide and protein crosslinking because of the high activity of the aldehyde groups, which readily form Schiff's base with amino groups of CTS. The nucleophilic nitrogen of the amino group (—NH_2) attacks the carbon of the aldehyde and displaces the oxygen of the aldehyde. And then the C═N bond was formed, which is defined as the Schiff's base.[9]

Polyol salts could help transform purely pH-dependent CTS solutions into thermosensitive pH-dependent CTS solutions. GP was used here to modify the synergistic forces between crosslinked chitosan (CCS) and PVA.

EXPERIMENTAL

Preparation of CCS–PVA–GP solutions

CTS solutions were prepared by dissolving CTS of 87.1% deacetylation and molecular weight about 300,000 (Fluka Chemie GmbH, Germany) in 0.1 mol L^{-1} acetic acid solution at room temperature with stirring for overnight. The solution was filtered before use. PVA powder (average molecular weight 66,000, Sigma) was dissolved in the CTS solutions, and heated in 80°C distilled water with stirring until completely dissolved and formed a clear blend. The CTS to PVA weight ratio was 2 : 1 (optimized ratio). The final polymer concentration was 2.0% (w/w) for CTS, and 1.0% (w/w) for PVA. The blend was cooled to 25°C and diluted glutaraldehyde (50% v/v, Aldrich Chemical) was slowly added under constant stirring. The final concentration of glutaraldehyde in the pregel solution varied from 10 μM to 100 μM, which roughly equals one linear CTS chain to aldehyde mole ratio of 0.5–5. This solution was then shifted into ice bath and 50% GP solution was added in dropwise with its final concentration of 3–5%. The system was incubated to undergo mixing for 30–60 min. Saturated disodium hydrogen phosphate was then added into the mixture. The final pH of this system was adjusted to 7.15.

Zeta potential measurements

Zeta potential measurements were carried out using a Zeta potential analyzer (Brookhaven). Various CTS colloid solutions were tested. The move rates of colloids are different in an electric field according to their different charge densities on the surface. Zeta potential reveals the charge properties of CTS colloid samples.

Rheological properties of the pregel solution

The viscosity of the polymer solution was measured by DV-E viscometer (Brookfield). To investigate the rheological properties of the pregel solutions, a ThermoHaake RV20 rheometer was used with concentric cylinder C-21 geometry. Oscillatory tests were performed with a frequency of 1 Hz. To limit evaporation during the measurements, we covered the samples with mineral oil. The changes in elastic modulus were recorded as a function of time at 37°C. The acquisition rate was set up at one point per 13 s.

Mechanical properties of the hydrogel

Samples for this test were prepared by gelling the pregel solution in a cylindrical mold (diameter = 1 cm; height = 1 cm) for 12 h at 37°C with various final glutaraldehyde and GP concentrations in the hydrogel. The compressive strength of the hydrogel samples was determined by using a uniaxial compression testing device equipped with a constant force (F) load cell. The strain, $\lambda = \Delta L/L$, of hydrogels after compressing 30 s was traced, and the compression stress, G, was calculated according to the following equation:[10]

$$G = \frac{F}{A \tan^{-1}(1 - \lambda)}$$

where A refers to the sectional area of the mold.

FTIR spectra

The FTIR spectrum of sample was obtained by the flowing method: the sample solutions (immediately after preparation or 48 h later) were poured onto a plastic surface, dried in an oven at 80°C, peeled off and stored in a desiccator. The resulting thickness of the film was between 0.5 and 1.0 mm with microscope observation. The different regions of the film were directly mounted in the light path of the FTIR spectrometer (NEXUS, Thermo Nicolet) for scanning. The scanning was performed between 400 and 4000 cm^{-1} with resolution of 4 cm^{-1} and 32 scans/sample.

Glutaraldehyde residue examination

The use of glutaraldehyde in CCS may raise problems because of its toxicity. There might be residual of glutaraldehyde in the crosslinked networks, which may cause necrosis and bring risks in medication. It can be theoretically estimated that the residual of glutaraldehyde would be none because the —NH_2 group

is quantitatively excessive in the crosslink reaction. The residual glutaraldehyde was extracted by accelerated solvent extraction method with acetone as solvent.[11] The assays were performed by a capillary gas chromatograph (Shimazhu). The column was C-18, 30 mm × 0.25 mm. The carrier gas was nitrogen at the flow rate of 0.5 mL min^{-1} and 1.0 μL on column injections were made. The operating temperatures were gasification, 250°C; detector, 250°C; oven, initial 25°C (no hold time) with a ramp of 6°C min^{-1} up to the final temperature of 170°C.

Morphological characteristics

It is difficult to observe the "pores" in the wet hydrogels. But for CTS hydrogels, they change their states between sol state and gel state, and there will be no visible water in or out. The water in sol and gel state are mostly free-water existing in the "pores" of the hydrogels. So the structural difference between wet hydrogel and freeze-dry hydrogel is not so typical. Scanning electron microscopy (SEM) was used to obtain sectional texture information of the hydrogels. After 24 h of exposure to the release medium (phosphate buffered saline, PBS, pH = 7.4) at (37 ± 0.1)°C, the already formed gels were lyophilized (−55°C, 20 Pa, Sanyo freeze-dry system, with mannitolum as cryoprotectant) to maintain the structure without any collapse.[12] Then they were immerged in liquid nitrogen and the vitrified samples were cut with a cold knife. The samples were mounted onto aluminum stubs with double-face glue tape and sputter-coated with gold (50 Å). The microstructure of the samples were imaged using a scanning electron microscope, KYKY 2800, at 25 kV accelerate voltage.

In vitro release

Lysozyme was used as model compound. CTSs of high deacetylation degree were resistant to the action of the lysozymes. CTS can be relatively stable in our experimental condition (CTS deacetylation degree 87%, pH = 7.1–7.4).[13,14] Lysozymes were dissolved in the CCS–PVA–GP solution (pH = 7.0 and degassed by sonication) to form a homogeneous formulation. Each formulation has a total lysozyme load of 3 mg. Four parallel samples were performed for each formulation. Samples of 3 mL of homogeneous formulation were injected into a thermostated permeable cell with surface covered with membrane (mean pore size 22 μm). The cell was immersed in the release medium (40 mL PBS, pH = 7.4) at (37 ± 0.1)°C. At the presetting time, the release buffer was sampled and changed with fresh PBS buffer. Lysozyme activities were determined using freeze-dried cells of *Micrococcus lysodeikticus* as the substrate.[15]

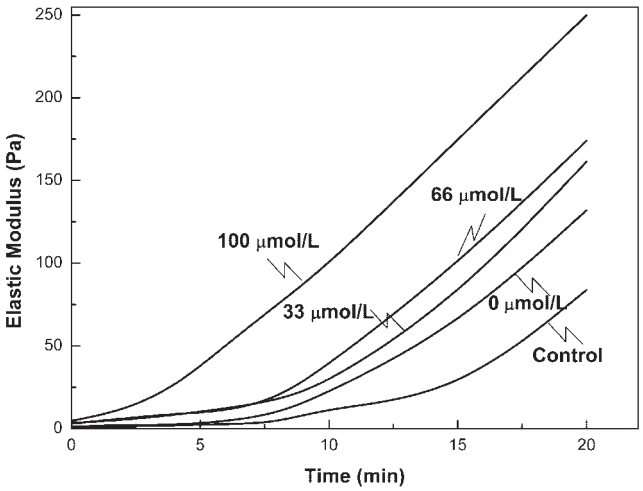

Figure 1 Elastic modulus as a function of time at (37 ± 0.1)°C for CCS–PVA–GP solutions of different glutaraldehyde concentration. GP concentration 3% (w/w), pH = 7.15. The frequency of oscillation is 1 Hz and the acquisition rate is 1 point every 13 s.

RESULTS AND DISCUSSIONS

The thermal gelation was mainly caused by GP,[5,6] the chemical crosslinking and PVA do not directly lead to the thermal gelation; but they also contributed to the gel formation process, either by changing the physical or chemical interactions between components.

Zeta potential of CTS colloid particles in a nondilute solutions describe the charge properties on their particulate surface,[16] even because of the weak crosslink in the CCS–PVA hydrogels, the particulate properties were still supposed. First of all, liquidity of the CCS–PVA sol do not change that much when compared with CTS sol according to the initial rheological properties (Fig. 1). And also, based on the percolation theory,[17] the bulk crosslinking will happen only when the crosslinker's concentration is over the critical value (the experimental critical value of this system may be over 500 μM of glutaraldhehyde), over which the sol–gel transition occurs. So it means the bulk crosslinking did not happen yet. Secondly, the sine wave of the dynamic moving of the colloid particles was found for each sample in Table I, which demonstrates that the particulate properties of each sample were not changed. Table I gives the pH and zeta potential of different CTS colloid solutions. The weakly acidic NH_3^+ groups contribute to the high positive zeta potentials in solution state. As expected, the crosslinking, the presence of PVA and the use of GP have resulted in increased pH and decreased zeta potentials. All these three approaches have the effect of decreasing or shielding the apparent charge density of the colloids.

Figure 1 shows the rheological properties of four CCS–PVA–GP solutions at (37 ± 0.1)°C. The increase of the elastic modulus clearly indicates that the liquid

TABLE I
pH and Zeta Potential of Different Chitosan Colloid Solutions

	pH	Zeta potential (mV)
CTS	4.67	44.61 ± 1.57
CCS	5.0	35.96 ± 1.45
CTS–PVA	5.03	28.19 ± 1.83
CCS–PVA	5.34	17.54 ± 0.88
CTS–GP	7.02	10.71 ± 0.77
CCS–GP	7.0	12.17 ± 0.52
CTS–PVA–GP	7.0	9.77 ± 0.44
CCS–PVA–GP	7.09	10.57 ± 0.61

solution is turning into a semisolid gel. This transformation from solution to gel took approximately 3–10 min (the lag time of gelation) for the CCS: PVA solution made of a 2 : 1 (w/w) mixture using different glutaraldehyde concentration. The CTS–GP solution with the same CTS concentration was compared as control. PVA in the CTS solution did not significantly change the viscosity. For example, the viscosities of CTS–PVA solution (1.5%, w/w, solvent 1% acetic acid) and the CTS solution (1.0%, w/w, solvent 1% acetic acid) were 192 and 190 mPas at 20°C, respectively. However, the dynamic rheological property, elastic modulus, was definitely enhanced because of the excellent mechanical property of PVA in the hybrid. With the increase of the crosslinkage, the lag time of gelation decreased and the elastic modulus reached higher values within the first 20 min. This is because of the formation of Schiff base participated in the formation of hydrophobic junctions between polymer chain segments.[18] Higher crosslinking degree hydrogels have lower apparent charge density on the CTS molecules, which resulted in quick gelation of the CTS pregel solution (Table I).

GP used here was to decrease the polymer ionization and the apparent charge density. The increased GP concentration in the pregel system decreased the apparent charge density and enhanced hydrophobic interaction between CTS molecules (Table I). Therefore, the time taken in phase transformation from solution to gel was decreased when the GP concentration increased from 3 to 4% (Fig. 2). It can also be found that the CCS–PVA–GP pregel solution (GP concentration 4%) approximately showed the same rheological properties as the control (GP concentration 5%). This is caused by the formation of Schiff base, which decreased the number of the —NH_2 groups. Thus, the amount of GP in the crosslinked network is reduced to reach the same balance between hydrophilic and hydrophobic interactions as the control.

As reported, PVA was shown to modify the mechanical properties of the CTS hydrogel. However, the compressive stress for CCS–PVA–GP hydrogels of different crosslinking degree exhibited significant differences (Fig. 3). There was a relatively linear increase in the gel compressive stress with the increase in glutaraldehyde concentration. The increase in GP concentration in the gel also contributed to the increase of its compressive stress. The increase of the compressive stress of a gel means the decrease of the compressibility of this gel, which was mainly caused by the decrease of water volume in the hydrogel. The interpenetrating behavior of PVA molecules in the 3D hybrid network helped the crosslinked CCS form a tight 3D hybrid network, which induced decreased water volume in the hydrogel body.

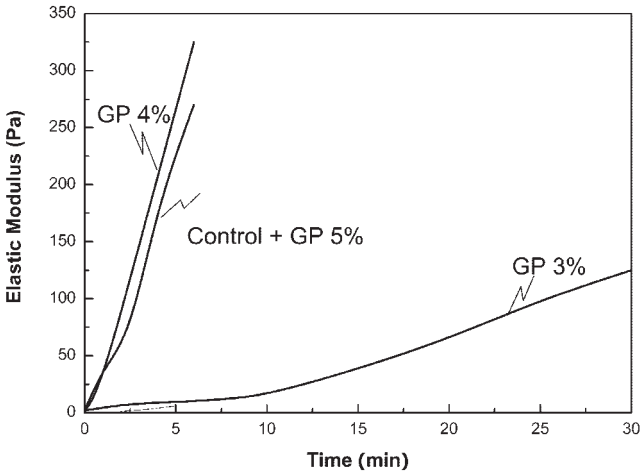

Figure 2 Elastic modulus as a function of time at (37 ± 0.1)°C for CCS–PVA–GP solutions of different GP concentration. glutaraldehyde concentration 66 μM, pH = 7.15. The frequency of oscillation is 1 Hz and the acquisition rate is 1 point every 13 s.

The FTIR spectrum of CTS [Fig. 4(a)] showed the absorption peak at about 1547 cm^{-1} for the —NH_2 groups. Figure 4(b, c) shows the absorption peaks at

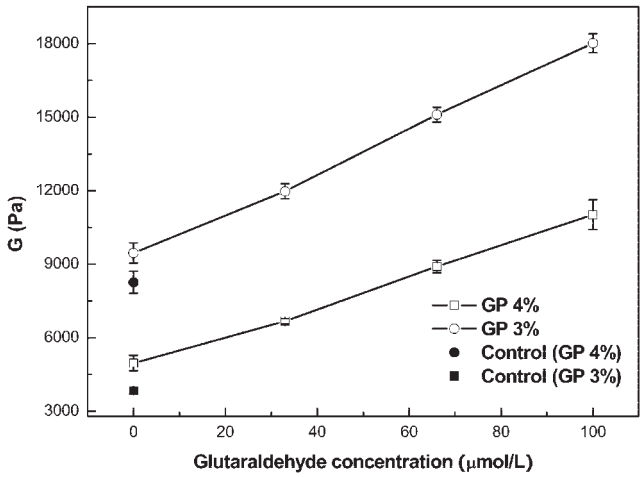

Figure 3 Compressive stress as a function of glutaraldehyde concentration in CCS–PVA–GP solutions. pH = 7.15. Each point represents the mean of Triplicate samples.

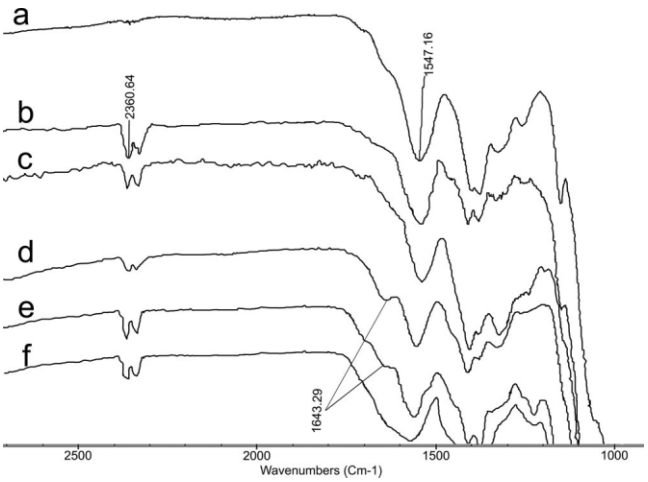

Figure 4 FTIR spectrum of various CTS/PVA blend hydrogel membranes: (a) pure CTS, (b) CTS with GP, (c) CTS with PVA, (d) CCS–PVA, (e) CCS–PVA–GP, and (f) CCS–PVA–GP after 72 h.

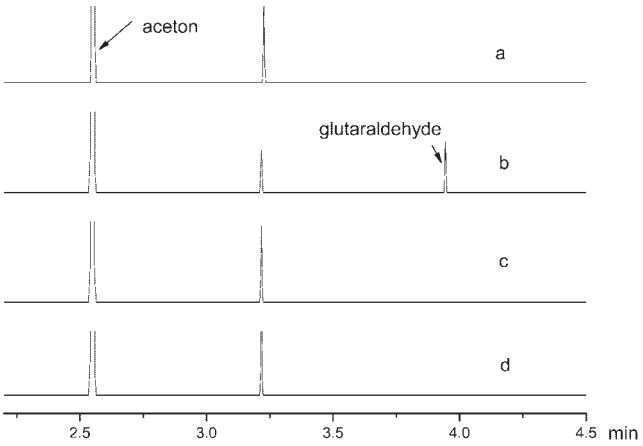

Figure 5 Chromatogram of (a) blank acetone solution, (b) standard glutaraldehyde acetone solution, 20 μM, (c) extractant from CCS–PVA hydrogel, and (d) extractant CCS–PVA hydrogel.

2340 cm^{-1} for —OH group because of the adding of GP or PVA, and decreases of the absorption peaks at —NH_2 groups in CTS–GP and CTS–PVA films were found. The descreases indicate that part of the apparent —NH_2 groups in the CTS surface were coved by PVA and GP molecules. The covering effect could also be explained according to Figure 1. Compared with Figure 4(c–e) illustrate the effect of glutaraldehyde on the CTS due to the decrease of absorption peaks of —NH_2 groups and the formation of N═C at about 1643 cm^{-1}. Although the probability of the reverse reaction is low due to the quantitatively excessive —NH_2 groups, N═C is not going to be stable under physiological environment. Figure 4(f) revealed that for the sample of CCS–PVA solution of 72 h after preparation, the peak at about 1643 cm^{-1} diminished. This suggests that the Schiff's is easy to be reduced, which often changes into a stable C—N bond resulting in the safety of the polymers for clinic application.

Compared with the blank and standard solution, the chromatogram of the extractant shows that the amount of residual free glutaraldehyde in the CCS–PVA or CCS–PVA–GP hydrogel was under trace detection limit or none. The residue determination approved the crosslinked hydrogels satisfy the safety need and have lower risks in medication (Fig. 5).

Figure 6 presents the textures of different CTS hydrogels. The SEM graphs of dried hydrogels roughly reflect the pores and water information in the hydrogels, because of the small difference of free water between the sol and gel state of weak-swollen or non-swollen CTS hydrogels. The observed pores means there had been once existing of water. The structure of numerous pores was observed in CTS–GP sample [Fig. 6(a)]. The porous structure created a substantive water environment and resulted in burst release of drugs and low compression stress, because this was caused by the local aggregation of the colloid particles. Figure 6(b) showed continuous texture for the CCS–GP hydrogel section, but there are particle-like blocks distributed on it. The localized conglobation may cause the risks of gel fracture or collapse. PVA changed the porous CTS–GP gel into a continuous gel with few pores, and eliminated the formation of localized blocks [Fig. 6(c)]. As shown in Figure 6(d), the thermosensitive CCS–PVA–GP hydrogel exhibited integrated gel texture. The hybrid CCS–PVA network intensified the gel structure. The chemical and physical crosslink both helped to modulate the charge properties, avoid local aggregation of the hydrophobic or deionized parts of the CTS colloid particles, and form

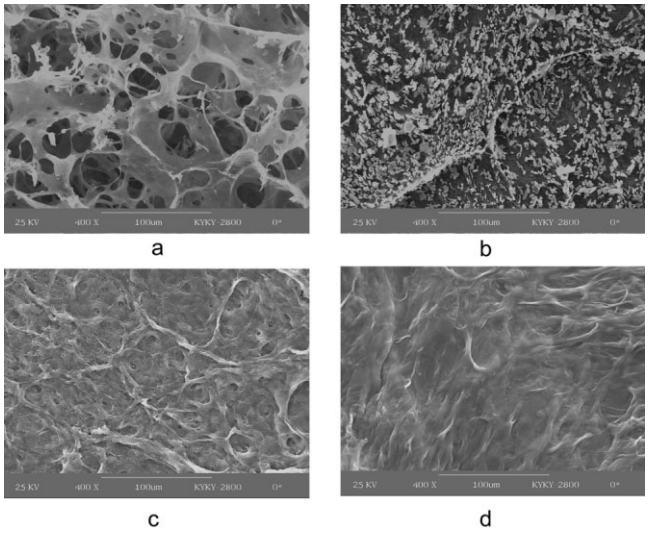

Figure 6 Scanning electronic micrograph of various CTS/PVA blend lip lyophilized hydrogel: (a) CTS with GP, (b) CCS, (c) CTS–PVA–GP, and (d) CCS–PVA–GP.

a bulk integrated hydrogel networks. The free water volume may be decreased, as may directly result in lower compressibility and drug dissolving rate than the noncrosslinked and noninterpenetrating networks. In terms of their applications in drug delivery systems, the drug release may be better controlled from the integrated structure than from the porous structure.

Figure 7 shows the *in vitro* release of lysozyme from CCS–PVA–GP hydrogels prepared at various glutaraldehyde concentrations. For the noncrosslinked CTS–PVA–GP hydrogel, a quick initial lysozyme release (about 75%) was found in the first 5 days and, afterwards, a plateau (about 82%) was reached in the following two weeks. The remaining protein was trapped in the gel, which may be released along with the degradation of the hydrogel network.[6] This will be discussed in our further work. The initial lysozyme release from hydrogels could be definitely reduced with the increase in glutaraldehyde amount in the CCS–PVA–GP hydrogel. Figure 8 reveals that the lysozyme release profiles from CCS–PVA–GP hydrogels were not significantly different for each GP concentration ($P > 0.05$, student's *t*-test). However, CCS–PVA–GP hydrogels have significantly lower initial release than CTS–GP hydrogels ($P < 0.025$, student's *t*-test), though their gelation kinetics are comparable.

The release profiles from these hydrogels (Figs. 7 and 8) were typical for monolithic hydrogel release devices,[19] governed by the diffusion of molecules through the hydrogel. Figures 1 and 2 indicated that the presence of glutaraldehyde and GP is essential for a quick organization of the polymeric network to retain the drug molecules. In the absence of PVA, the CTS–GP solution may form porous gel structures as revealed by SEM [Fig. 6(b)], which led to burst release.

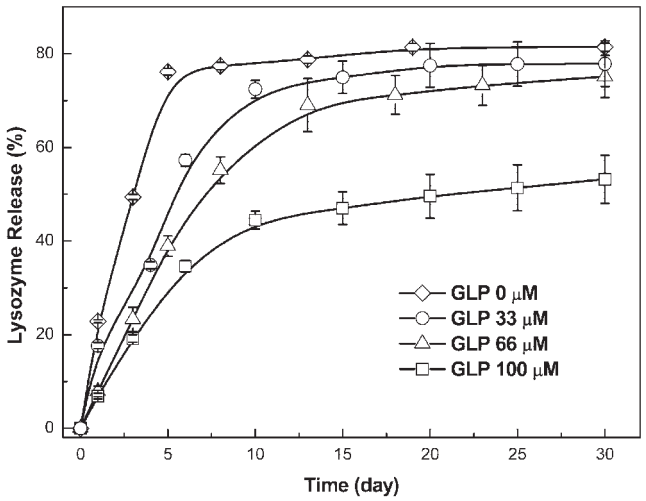

Figure 7 Lysozyem release from CCS–PVA–GP solutions of different glutaraldehyde concentration. GP concentration 4% (w/w), PVA concentration 1.0% (w/w). Each point represents the mean value ± SD (standard deviation) ($n = 4$).

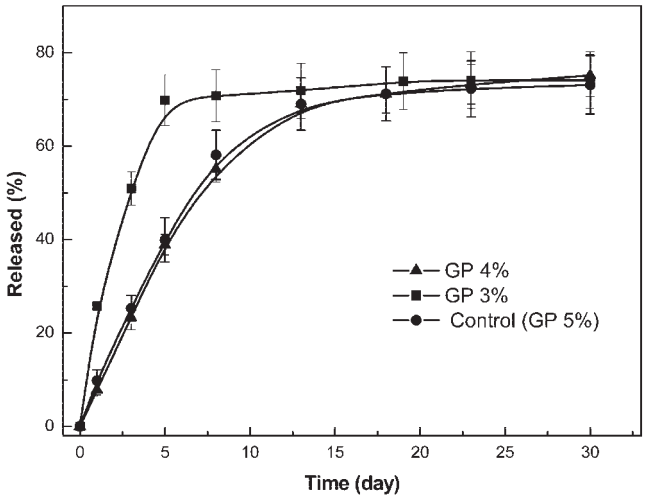

Figure 8 Lysozyem release from CCS–PVA–GP solutions of different GP concentration. Glutaraldehyde concentration 66 μM, pH = 7.15. Each point represents the mean value ± SD ($n = 4$).

In the absence of glutaraldehyde, the CTS–PVA–GP solution formed nonporous gel [Fig. 6(c)], but the structure formed was not organized enough to avoid the quick initial release. The CCS–PVA–GP hydrogel formed an intensified 3D network with interpenetrating molecules, which effectively retained or buffered the macromolecules diffusion. Despite a difference in the gelation rate, the release kinetics between the GP 3% (w/w) and GP 4% (w/w) CCS–PVA–GP systems were comparable, probably because the two gels have similar structures. This phenomenon occurs because GP does not contribute to the physical crosslinking of the gel.[6] It seems that the release from the hydrogels cannot reach 100% because of the enzyme entrapment in the system, and the remnants release may be induced along with degradation of the networks.

CONCLUSIONS

The results presented here showed that CCS–PVA–GP solutions possess advantages over CTS–GP hydrogel and have potentials to be used as injectable *in situ* gelling thermosensitive formulations. They can gel at body temperature within 10 min, and the gelation was dependent on the crosslink degree and GP concentration of the solution. The chemical crosslink with glutaraldehyde and the physical crosslink with PVA do not directly lead to sol–gel transition. However, they both helped to modulate the charge properties, diminish local aggregation of the hydrophobic or deionized part of the CTS colloid particles to the apparent "pores" and form a bulk integrated hydrogel networks, as leads to the good mechanic properties of the crosslinked gel. The CCS–PVA–GP can release macromolecules in a low and sustained rate over a period of

30 days. It may be potentially used as *in situ* gelling implants for sustained and controlled drug delivery.

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