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Preparation and properties of a novel thermosensitive N-trimethyl chitosan hydrogel

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Abstract A novel injectable thermosensitive hydrogel system composed of *N*-trimethyl chitosan chloride (TMC) and β-glycerophosphate (β-GP), coded as TMC/β-GP, was designed. The morphology and rheological behavior of hydrogels were characterized by scanning electron microscopy and rheometer, respectively. Their swelling properties were carefully studied. The results revealed that the TMC/β-GP system was liquid with low viscosity at low temperature, which allowed it to be an ideal injectable material for biomedical applications. It was interesting that the system kept in liquid status for a long time near 4 °C and transformed rapidly to gel status within 1 min upon heating to 37 °C. The hydrogel could be dissolved at acid pH, while it absorbed water at neutral and basic conditions. The release of BSA from TMC/β-GP gels was slow at neutral pH. The TMC/β-GP hydrogel is a promising vehicle for the drug release, tissue repairing and regeneration.

Keywords *N*-trimethyl chitosan · Thermosensitivity · Hydrogel · Controlled release

Introduction

The thermosensitive polymer hydrogels which show sol–gel transition at human body temperature have gained increasing attention for drug delivery, cell encapsulation, and tissue engineering. Many polymer materials have been used to form thermosensitive gels [1]. Among them, chitosan, a nontoxic amino-polysaccharide derived from chitin [2], is especially interesting. As a polycationic biopolymer, chitosan has many unique advantages such as high availability, high biocompatibility, and biodegradability,

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which attract scienti c and industrial interests in pharmaceutical and biomedical area [3–7]. Chitosan is insoluble at neutral and alkaline pH values. However, solubility of chitosan in aqueous solutions is attained in acidic environments via protonation of its amino groups. Raising pH of chitosan solutions to above 6.2 leads to the formation of a hydrated gel-like precipitate [8]. The thermosensitive chitosan hydrogels were rst described by Chenite et al [9]. These formulations, composed of chitosan and β-glycerophosphate (C/β-GP), possess a physiological pH and can keep in liquid status below room temperature. At human body temperature, they form monolithic gels. The percentages of amino and acetyl groups on chitosan chain are important parameters in uencing the gelation process. The solution of chitosan with degree of deacetylation (DD) of 84% can remain in sol status for at least three months at 4 C, but it takes 140 min to complete sol–gel transition at 37 C. The mixture [1:1 (w/w)] of chitosan with DD of 95% and DD of 84% can transfer from solution to gel in 5 min at 37 C, but it is stable only within 3 days when the solution is kept at 4 C [10]. In general, the solution stability and sol–gel transition time in response to temperature for those systems mentioned above are not satisfactory. In recent years, a number of new chitosan-based thermosensitive hydrogels have been developed [11–15]. However, these formulations complete sol–gel transition slowly at human body temperature and can not keep liquid status at low temperature for a long time.

In order to obtain a novel hydrogel system that is injectable, keeps in stable solution status at 4 C and has fast sol–gel transition at human body temperature, we modi ed chitosan through methylation reaction. *N*-trimethyl chitosan chloride (TMC), a partially quaternized derivative of chitosan, possesses superior water solubility compared to chitosan [16]. It has excellent capacity to transfer hydrophilic macromolecules across mucosal epithelia even at neutral and basic pH values [17, 18]. Some studies have demonstrated the safety of using TMC as peptide drug permeation enhancer [19]. Therefore, TMC based materials show potential to be used as selective and effective delivery systems for peptide and protein drugs. Although TMC has many advantages as mentioned above, limited work has been conducted to develop their applications for drug delivery [20–22]. Reports on TMC thermosensitive hydrogels for the drug delivery are scarce.

Recently studies in our group have found that injectable thermosensitive system composed of β-glycerophosphate and TMC of low quarternizaton degree could keep in stable liquid status at 4 C and transfer to gel immediately when temperature is raised to 37 C. In this work, the thermosensitivity and other properties of the TMC/β-GP hydrogel were investigated in comparison to C/β-GP formulation. Bovine serum albumin (BSA) was entrapped into the hydrogel as a model drug to investigate the release property of the complex gel system.

Materials and methods

Materials

Chitosan having weight-average molecular weight (M_w) of 5.3×10^5 and DD of 93% was supplied by Yuhuan Ocean Biochemistry Co. Ltd. (Zhejiang, China).

Hydrated β-glycerophosphate disodium salt (β-GP) was purchased from Fluka Chemie (Switzerland). All other reagents were of analytical grade.

Preparation of samples

TMC was synthesized by a modified method proposed by Sieval et al. [23]. A mixture of chitosan (2.0 g), sodium iodide (4.8 g), 15% aqueous sodium hydroxide (NaOH) solution (11 mL), methyl iodide (11.5 mL) and 1-methyl-2-pyrrolidinone (80 mL) were stirred on a water bath at 60 °C for 90 min, the mixture turned into transparent solution. The product was precipitated by adding ethanol (200 mL), and then isolated by centrifugation. After washing with ethanol (100 mL) and centrifugation, the material was dissolved in 10% NaCl aqueous solution (40 mL) to exchange the iodide-ion with chloride-ion. The obtained polymer was purified by dialysis against distilled water for 5 days and then freeze-dried, and the yield of the reaction was 87%.

TMC/β-GP hydrogel was prepared by the following steps. TMC solution was obtained by dissolving TMC (300 mg) in 0.1 M aqueous lactic acid solution (5 mL) at room temperature. TMC solution and β-GP solution (1,100 mg in 5 mL water) were co-cooled in a refrigerator at 4 °C for 15 min respectively. Then β-GP solution was slowly dropped into the stirring TMC solution in an ice bath. The resulted solution was stirred for 10 min to gain homogeneous mixture, coded as TMC/β-GP solution. The pH value of the final solution was 7.0. The hydrogel was formed by heating TMC/β-GP solution (10 mL) in a water bath at 37 °C for a few minutes. To compare, the CS/β-GP hydrogel was prepared according to a previously described method [9]. Chitosan (200 mg) was dissolved in 0.1 M HCl solution (10 mL). β-GP solution (800 mg in 2 mL water) was dropped into the stirring chitosan solution in an ice bath to obtain a clear and homogeneous liquid solution, coded as CS/β-GP solution. The pH value of the final solution was 7.0. The hydrogel was formed by heating CS/β-GP solution (12 mL) in a water bath at 37 °C for several minutes.

The gelation point was determined by test tube inverting method [18]. The obtained formulation in solution state (2 mL) was added into a tube (10 mL) with a cap and kept in a water bath at 37 °C. At predetermined interval, the tube was taken out and inverted to observe the state of the sample. The gelation point was determined by flow or no-flow criterion over 30 s with the test tube inverted. The sol–gel transition behaviors of the TMC/β-GP and CS/β-GP solutions were further illustrated by viscosity measurement at 37 °C by NDJ-1 viscometer. Shear viscosity measurements were made at a fixed shear rate of 1 min^{-1}.

Characterization

FT-IR spectra of chitosan and TMC were recorded with KBr pellets on Nicolet 5700 spectrophotometer (Minnesota, USA). ^{1}H-nuclear magnetic resonance (NMR) spectroscopy was used to confirm substitutions of quaternary amino groups on chitosan. The NMR spectrum of the TMC in D_2O at 80 °C was recorded with Mercury 300 NMR spectrometer (Varian Inc., USA). The degree of quaternization

(DQ) and dimethylation (DM) was calculated according to the following equation: [20, 24].

$$DQ = [[(CH_3)_3]/[H] \times 1/9] \times 100 \quad (1)$$

$$DM = [[(CH_3)_2]/[H] \times 1/6] \times 100 \quad (2)$$

where DQ and DM are the degree of quaternization and dimethylation, respectively, in mole percentage of the chemical shift of free amine; $[(CH_3)_3]$ are the integral of trimethyl amino group at 3.7 ppm and the $[(CH_3)_2]$ are the integral of dimethylated amino group at 3.3 ppm; [H] is the integral of the ^{1}H peaks between 4.7 and 5.7 ppm.

The morphological measurements of TMC-GP and CS-GP gels were observed after lyophilization to maintain the porous structure without any collapse. The samples were plunged in liquid nitrogen and the vitri ed samples were cut with a cold knife. Then they were mounted on the base plate and coated with gold. The morphology was investigated by using scanning electron microscope S-570 (Hitachi, Japan).

Rheological measurements

The rheological measurement was performed on an ARES-RFS rheometer (TA. Inc., New Castle, USA) according to the procedure described by Lue et al. [25]. The dynamic viscoelastic parameters such as the dynamic shear storage modulus (G') and the loss modulus (G'') were measured as functions of temperature, time and frequency. The rheometer was equipped with two force transducers, allowing the torque measurement in the range from 0.004 to 1,000 g cm. The values of the strain amplitude were checked to ensure that all measurements were set at 15%, which is within a linear viscoelastic regime. For each measurement, solution was poured into the Couette geometry instrument, which had been kept at desired temperature without pre-shearing or oscillating. Temperature control was established by connection with a Julabo FS18 cooling/heating bath kept within ±0.2 °C of the desired temperature. To prevent dehydration during rheological measurements, a thin layer of low-viscosity paraf n oil was spread on the exposed surface of the measured solution. For the temperature sweep measurements to determine the gelation temperature, oscillating measurements were performed at a frequency of 1 rad/s, while the temperature was increased at the rate of 1 °C/min between 0 and 50 °C. For the time sweep measurements, time = 0 min was de ned when the temperature reached the desired value. The sweep of frequency was from 0.1 to 100 rad/s.

Swelling test

The degree of swelling (D_s) of dry gels was measured according to Chen et al. [26]. The dry gels were immersed in phosphate buffered saline (PBS) solutions with different pH values at room temperature. At predetermined time intervals, they were removed from the solutions, gently wiped with lter paper to remove the surface

solution, weighed and returned to the same container until equilibrium was achieved. The D_s was calculated according to the following equation:

$$D_s = (W_s - W_0)/W_0 \quad (3)$$

where W_0 is the weight of dry gel, W_s is the weight of gel at different swelling time. All of the experiments were performed in triplicate.

BSA incorporation and release

Bovine serum albumin (BSA) (10 mg) as a model drug of protein was dissolved in the stirring 10 mL TMC/β-GP solution (300 mg TMC in 5 mL 0.1 M aqueous lactic acid solution and 1,100 mg β-GP in 5 mL water) or 10 mL CS/β-GP solutions (167 mg chitosan in 8.3 mL 0.1 M HCl solution and 667 mg β-GP in 1.7 mL water) in an ice bath. Each sample (about 1,000 mg) was placed into 10 mL plastic tube with a cap and incubated at 37 °C for 15 min to form hydrogel. 5 mL PBS buffer (pH 7.4, 0.1 M sodium phosphate containing 0.145 M NaCl) was added to each tube. Samples were incubated at 37 °C in a thermostated shaker rotating at 100 rpm. At predetermined intervals (1 day), 1 mL of the solution was taken out and the release of BSA from hydrogel was estimated by UV-9100 spectrophotometer at 595 nm with Coomassie Brilliant Blue G-250 according to the procedure described by Zhao et al. [27]. With each sample, the solution was changed with fresh medium, maintaining the total volume constant. The buffer from hydrogel without BSA was used as the blank sample to erase the disturbance of the hydrogel itself. The percentage of cumulative amount of released BSA was determined from standard curves. All of the experiments were performed in triplicate.

Results and discussion

Structure and morphology

TMC was synthesized through methylation of chitosan using methyl iodide as a reaction reagent in the presence of sodium hydroxide. The synthetic route is presented in Fig. 1. Figure 2 shows FTIR spectra of chitosan and TMC. Compared to the spectrum of chitosan, there is a new band at 1,474 cm^{-1} in TMC spectrum, which is attributed to the methyl groups ($-CH_3$) of the ammonium, indicating the existence of $-N(CH_3)_2$ and $-N^+(CH_3)_3$ groups in TMC [22, 28]. Characteristic peaks of alcohol and second alcohol between 1,160 and 1,030 cm^{-1} are not changed, confirming the lack of the introduction of an alkyl group at C-3 and C-6 of the chitosan [28, 29]. The degree of quaternization and dimethylation of TMC was calculated from ^1H NMR analysis (Fig. 3). Since excess substituted quaternary amino groups resulted in non-gelling solution at 37 °C, TMC with a quaternization degree of about 11% and the di-methylation degree of about 35% was used to prepare hydrogel in the present study.

Compared to chitosan, a pronounced decrease in the viscosity of TMC is observed, which is resulted from the degradation of the polymer chain during the

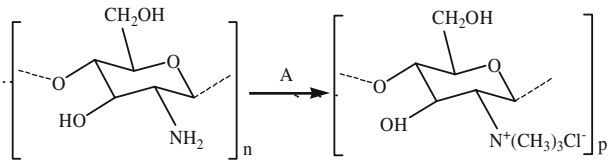

Fig. 1 Synthetic route for preparation of *N*-trimethyl chitosan chloride (TMC) (*A*) Methyl iodide, 1-methyl-2-pyrrolidinone, NaOH, 60°C, NaCl

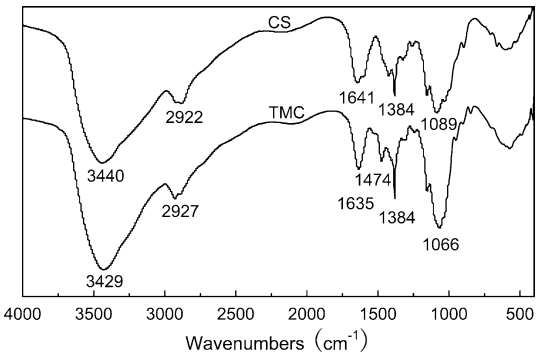

Fig. 2 FTIR spectra of chitosan (CS) and *N*-trimethyl chitosan chloride (TMC)

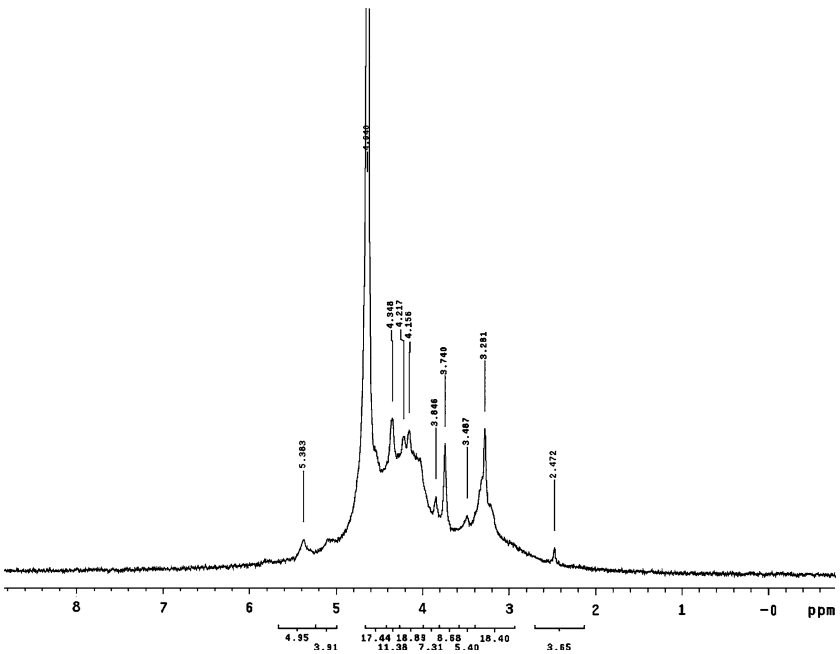

Fig. 3 ^{1}H NMR spectrum of *N*-trimethyl chitosan chloride (TMC)

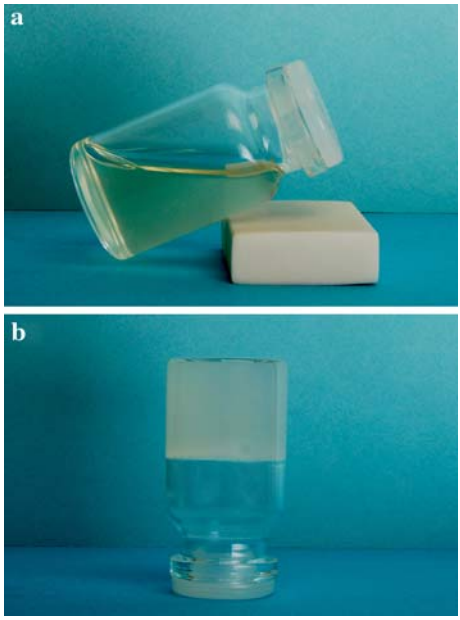

Fig. 4 Photographs of TMC/β-GP solution at 4 C (a) and gel at 37 C (b)

Table 1 Characterization of CS/β-GP and TMC/β-GP gels

Hydrogel	Concentration (% w/v)	pH	Viscosity (4 C) (mpa s)	Gelation time	
				4 C	37 C (min)
CS/β-GP	1.7	7.0	220	12 h	12
TMC/β-GP	3.0	7.0	25	>6 months	1

synthesis according to the reports from van der Merwe et al. [18] and Snyman et al. [24]. Figure 4 shows the photographs of solution and gel of TMC/β-GP system. It is liquid at 4 C, when the temperature is elevated to 37 C, the solution transfers to a gel. Compared to CS/β-GP solution, TMC/β-GP solution is more stable with a better fluidity at 4 C, and forms gel more rapidly at 37 C, as shown in Table 1 and Fig. 5. The low viscosity of the TMC/β-GP solution allows it to be used as an ideal injectable material. At 4 C, TMC/β-GP solution retains liquid status as long as 6 months, while CS/β-GP solution transfers to gels after 12 h, suggesting that chitosan quaternization significantly slows down the gelation speed at 4 C. When temperature is increased to 37 C, the TMC/β-GP solutions transfers to solid status within 1 min, while CS/β-GP solutions need 12 min to complete the phase transition (tube inverting method).

The mechanism of the thermosensitive sol–gel transition of the CS/β-GP system has been already reported [8–10]. The gelation mechanism of TMC/β-GP system should be similar to that of CS/β-GP system, but it is more complex, as illustrated in Fig. 6. In TMC/β-GP systems, three types of interactions may be involved in the gelation process including the electrostatic attraction between positively charged

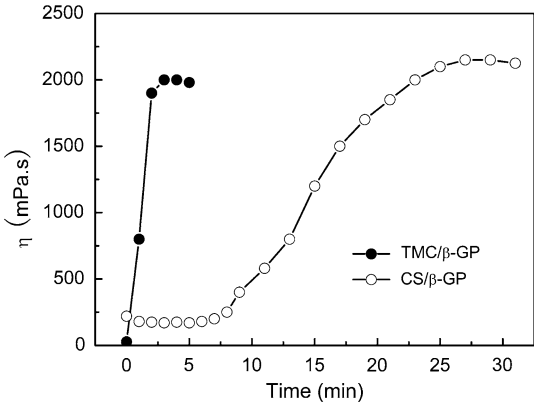

Fig. 5 Viscosities of TMC/β-GP and CS/β-GP systems as a function of time at 37 C

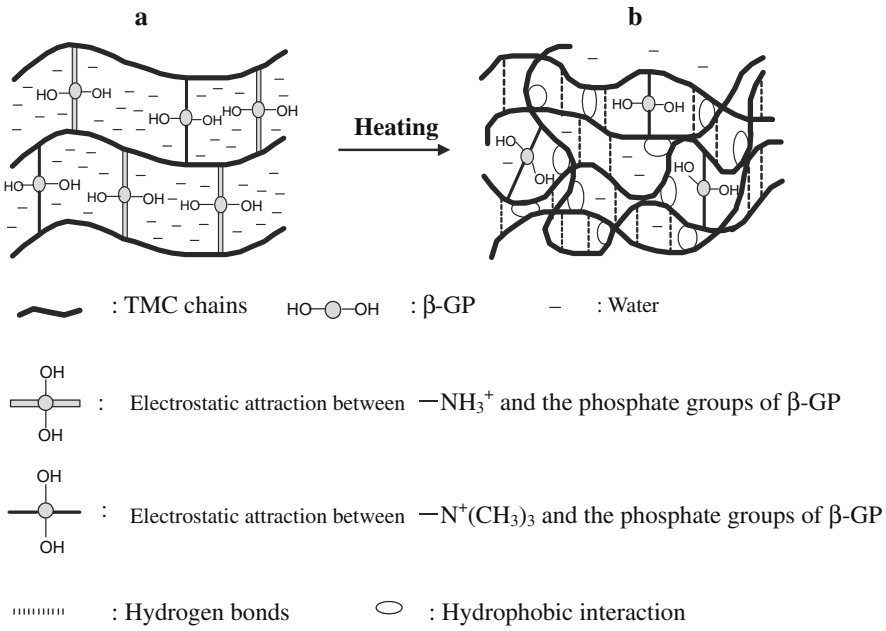

Fig. 6 The schematic illustration of the formation mechanism of TMC/β-GP gel: a solution at low temperature, b gel at high temperature

quaternary amino groups and the negatively charged phosphate groups of the glycerophosphate, hydrophobic and hydrogen bonding between TMC chains. The gel formation is the competition result of these three types of interactions between TMC chains. The precipitation of chitosan upon increasing the pH above a critical value (such as 6.2 when using a strong base) can be explained by a reduction of charge density along the polymer backbone reducing inter-chain electrostatic repulsion and allowing the attractive hydrophobic and hydrogen-bonding forces to predominate and precipitate chitosan [8]. When TMC acid solution is neutralized to pH 7.0 at 4 C by adding of β-GP, attraction between negatively charged phosphate

moieties of β-GP and positively charged $-N^{+}(CH_3)_3$ and $-NH_3^{+}$ groups of TMC inserts β-GP between TMC chains and separates them. The glycerol moiety of GP can promote the protective hydration of TMC chains, keeping the polymer chains stretched in solution at low temperature and maintaining TMC soluble. When the system temperature increases, movement of molecular chain becomes quicker. Protons from $-NH_3^{+}$ of TMC are transferred to the phosphate moiety of β-GP, and the portions of β-GP are disengaged from the network of TMC chains. Dehydrating leads to the increase of hydrophobic and hydrogen bonding forces between TMC chains. At the same time, the phosphate moiety of β-GP has strong electrostatic attraction with the $-N^{+}(CH_3)_3$ of TMC, and the forces can not be destroyed at higher temperature. Ionic cross-linking between TMC chains by β-GP increases junction zones and shortens the distance between the TMC chains. The hydrophobic methyl groups on TMC also play a signi cant role on aggregation. Hydrophobic and hydrogen bonding forces between chains in short distance induces aggregation fast. Therefore, compared to the CS/β-GP system, the TMC/β-GP system can keep liquid status at 4 C for a longer time, and it transfers to hydrogel more rapidly at 37 C.

Figure 7 shows the morphology of TMC/β-GP and CS/β-GP gels. The SEM photographs clearly illustrate the pore structure of gels. Compared to CS/β-GP gels, TMC/β-GP gels are more porous with smaller pore diameters. This phenomenon is corresponding to the gel formation mechanism. TMC chains with positive charges can form steady junctions with β-GP rapidly, resulting in a more compact porous structure. The SEM results support the conclusion above.

Rheological behavior

The rheological behaviors of TMC/β-GP and CS/β-GP solutions were investigated for an understanding of the hydrogel formation mechanism and the identi cation of their applications. To determine the gel point, the crossover of the storage modulus (G') curve and the loss modulus (G'') curve has been used as an indicator. Gelation temperature is usually de ned as the sol/gel transition temperature at which G' is equal to G'' [14]. Below the gelation temperature, G' is lower than G'', showing viscoelastic behavior of a liquid. G' sharply rise and over G'' upon heated to the vicinity of gelation temperature. When the system temperature is higher than the gelation temperature, G' is over G'', indicating that an elastic gel network has formed. It should be pointed out that the gel point determined by this method is frequency dependent [30]. Oscillating measurements were performed at a frequency of 1 rad/s, while the temperature was increased at the rate of 1 C/min between 0 and 50 C. Figure 8 shows the temperature dependence of G' and G'' of CS/β-GP and TMC/β-GP solutions. For TMC/β-GP solution, the gelation temperature is 14.0 C, while the gelation temperature of CS/β-GP solution is 38.7 C. The gelation temperature of TMC/β-GP solution is lower than that of CS/β-GP solution. This indicates that TMC/β-GP solution is more easily to form the gel below 37 C compared to CS/β-GP solution.

To determine gelation time at 37 C, the time sweep measurements were performed (Fig. 9). The solutions were directly heated at 37 C in the rheometer, and each time sweep took 30 min. The changes of G' and G'' during the gelation

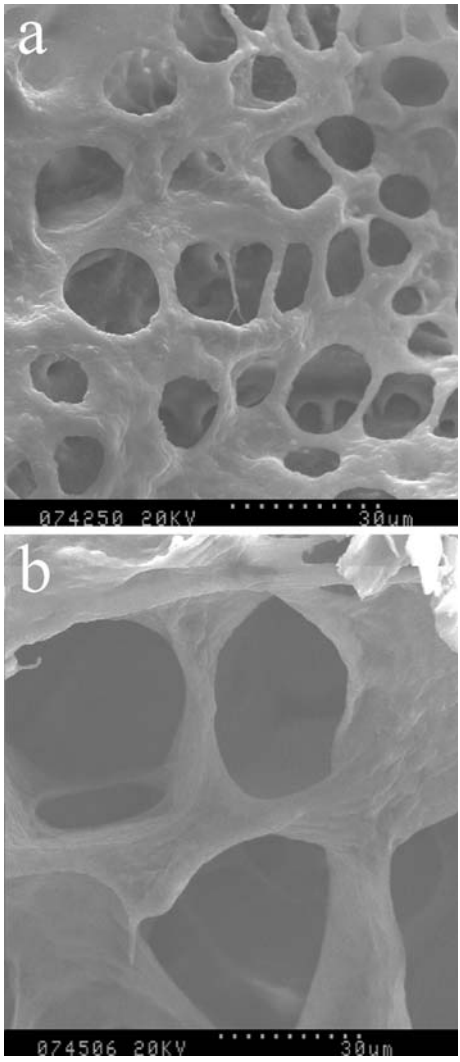

Fig. 7 SEM of TMC/β-GP (a) and CS/β-GP (b) gels

process at a constant temperature re ect the gelation speed and the gel intensity. Since the solution transfers to gel within 1 min at 37 in TMC/β-GP system, the steady gel formed prior to the measurement (before the temperature reached the desired value). For CS/β-GP system, both G' and G'' increase gradually. After 7 min, G' values outgrow those of G'', indicating that an elastic gel network has formed. Compared to CS/β-GP system, the gelation in TMC/β-GP system is more rapid. The G' of CS/β-GP gel is slightly higher than that of TMC/β-GP gel, indicating that the strength of CS/β-GP gel is slightly higher than that of TMC/β-GP gel.

The frequency dependence of the viscoelastic properties of solution and gel was measured at temperature 4 and 37, respectively G' and G'' were measured as a

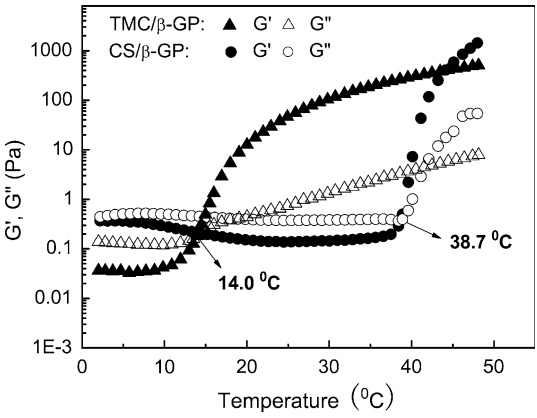

Fig. 8 Temperature dependence of storage modulus G' and loss modulus G'' of TMC/β-GP and CS/β-GP solutions at a heating rate of 1 °C/min and at a frequency of 1 rad/s

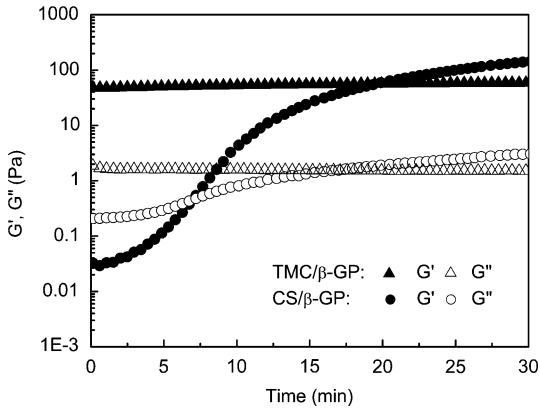

Fig. 9 Time dependence of storage modulus G' and loss modulus G'' of TMC/β-GP and CS/β-GP solutions at a frequency of 1 rad/s at 37 °C

function of frequency in the range between 0.1 and 100 rad/s. At 4 °C, G' is lower than G'' within the frequency range for both solutions. Those features are characteristic of the stable viscous liquid. Compared to CS/β-GP solutions, TMC/β-GP solutions have better liquidity at 4 °C (Fig. 10a). When the temperature is higher than the gelling temperature, G' exceeds G'' over the whole frequency range, which indicates that the gel had formed (Fig. 10b). The G' of CS/β-GP and TMC/β-GP shows almost no dependence of frequency, suggesting a "strong gel" had formed [14].

pH-sensitive swelling

The swelling behaviors of TMC/β-GP and CS/β-GP dry networks were studied at different pH. The results are shown in Fig. 11. CS/β-GP dry network is dissolved rapidly at low pH (1.27) (data not shown). At pH 3.0–8.0, it swells first and then shrinks. The gel dissolution is caused by protonation of amino groups on chitosan molecular chains in strong acidic solutions. With the pH increasing, the amount of protonated amino groups decreases. Excess free GP in

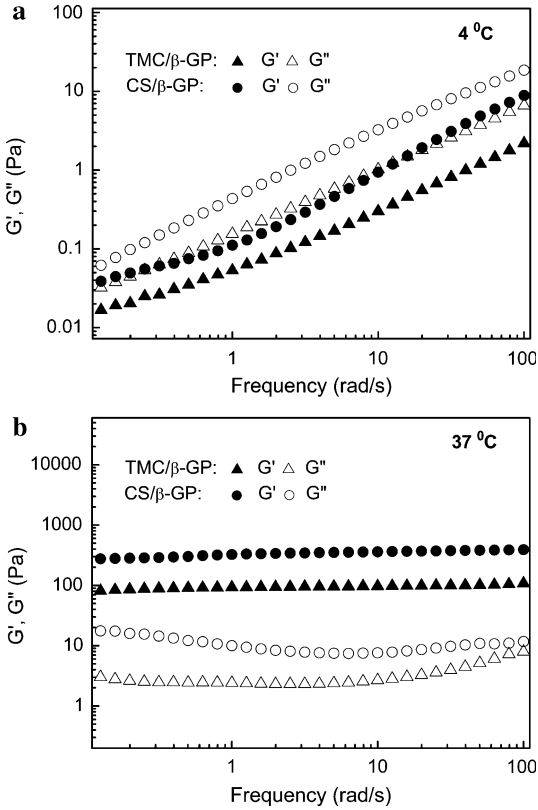

Fig. 10 Frequency dependence of storage modulus G' and loss modulus G'' of TMC/β-GP and CS/β-GP solutions at 4 C (a) and gels 37 C (b)

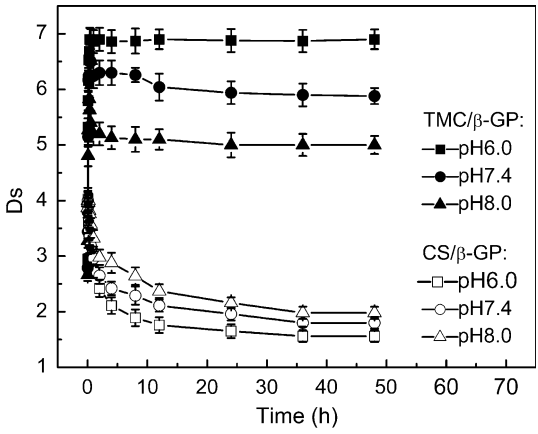

Fig. 11 Swelling ratio of TMC/β-GP and CS/β-GP gels at different pH. Each point represents mean ± SD ($n = 3$)

hydrogel interacts with protonated amino groups to form an ionic cross-linking. Therefore CS/β-GP dry network swells rstly due to osmosis pressure, and then shrinks in the range of pH 3.0–8.0 due to the interaction between protonated

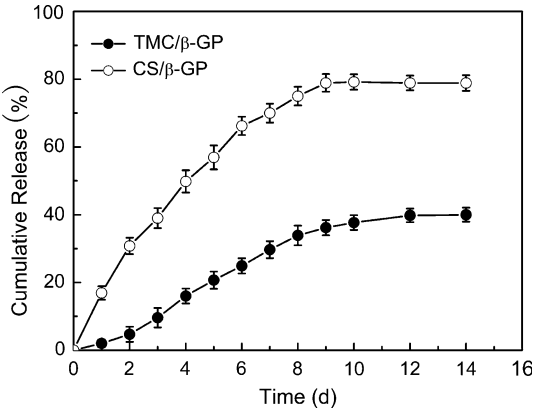

Fig. 12 Accumulative release of BSA from TMC/β-GP and CS/β-GP gels at pH 7.4. Each point represents mean ± SD ($n = 3$)

amino groups and β-GP. The TMC/β-GP dry network behaves quite differently, it is dissolved quickly in strong acidic solution (pH 1.27), but swells first and then dissolves gradually at pH lower than 5.0. The speed of dissolution decreases with the increase of pH (data not shown). TMC/β-GP dry network is not dissolved in the neutral and basic conditions. At high pH (>5.0), TMC/β-GP dry network absorbs water and swells rapidly. The swelling ratio decreases with the increase of pH. The swelling properties of TMC/β-GP dry network under different pH conditions can be explained as follows. The hydrogel swells quickly because the hydrophilic property of quaternized groups. The swelled network allows more acid solutions to penetrate into the gel. With the increase of pH, the amount of protonated amino groups on quaternized chitosan chains decreases gradually, resulting in decrease of the net charge on chitosan molecular chains and lower swelling degree. The TMC/β-GP hydrogels are suitable for use at physiological pH (6–8).

Release behavior of BSA

Protein drugs have been developed for therapeutic purposes. However, repeated injections are required due to extremely short acting of this kind of drugs. A number of approaches have been reported on the controlled release of protein drugs using polymeric carriers [31]. Thermosensitive hydrogels show potential to be used for sustainable protein release systems that can increase therapeutic effects and lower the cost. Using BSA as a model protein, BSA releases from TMC/β-GP and CS/β-GP gels were studied respectively, by immersing the BSA-incorporated gels in pH 7.4 buffer at 37°C. BSA release profiles from TMC/β-GP and CS/β-GP gels are shown in Fig. 12. BSA release from TMC/β-GP gel is apparently slower than from CS/β-GP gels. Approximately 40% of BSA was released from the hydrogel in 14 days. The interaction between BSA and positively charged TMC may play an important role to decrease the release rate of BSA.

Conclusions

The TMC/β-GP system composed of TMC and β-GP has a noticeable thermo-responsive behavior. The formulations possess a physiological pH, and can keep liquid status as long as 6 months at 4 °C without apparent change in viscosity. Interestingly, upon heating to 37 °C, the solutions transfer rapidly to gels within 1 min. The release rates of BSA from TMC/β-GP hydrogel are apparently slower than from CS/β-GP hydrogel. These interesting features make the novel TMC/β-GP system a promising vehicle for local sustainable release of drugs, as well as tissue repairing and regeneration materials.

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