Preparation and Characterization of a Novel Thermosensitive Hydrogel Based on Chitosan and Gelatin Blends

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Received 18 July 2008; accepted 22 December 2008
DOI 10.1002/app.29954
Published online 19 March 2009 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: In this study, a novel injectable *in situ* gelling thermosensitive hydrogel system based on chitosan and gelatin blends was designed and investigated. The addition of gelatin provides the correct buffering and other physicochemical conditions including control of hydrophobic interactions and hydrogen bonding, which are necessary to retain chitosan in solution at neutral pH near 4°C and furthermore to allow gel formation upon heating to body temperature. The chitosan/gelatin hydrogels were studied by FTIR, swelling, and rheological analysis. The rheological analysis evidenced the endothermic gelation of chitosan/gelatin solutions, which indicated their gelation temperatures and reflected the effect of gelatin concentration on the thermosensitive properties of gels. The morphology of this system was examined with laser scanning confocal microscopy and scanning electron microscopy. The images indicated that the gels were quite heterogeneous and porous. The investigation of these gels as vehicles for delivering bovine serum albumin as a model drug of protein showed that the system could sustain the release of the protein drug. These results show that chitosan/gelatin solutions can form gels rapidly at body temperature and have promising perspective for their use in local and sustained delivery of protein drug.
 J Appl Polym Sci 113: 400–407, 2009

Key words: chitosan/gelatin; blends; thermosensitivity; hydrogels; drug delivery systems

INTRODUCTION

Hydrogels are hydrophilic three-dimensional polymer networks capable of absorbing large amounts of water or biological fluids.[1,2] Because of their relatively high water contents and their soft, rubbery consistency, hydrogel materials more closely resemble in their physical properties to living tissue.[3] In recent years, an increasing number of *in situ* forming hydrogel systems have been reported in the literature for various biomedical applications such as drug delivery, cell carriers, and tissue engineering.[4–6] There are several possible mechanisms leading to *in situ* gel formation: solvent exchange, UV-irradiation, ionic crosslinkage, pH change, and temperature modulation.[7] Among all of the gel systems, thermosensitive hydrogels are very favorable members and have been widely investigated. Thermosensitive hydrogels are formed from aqueous polymer solutions with temperature changes, so that they can avoid toxic organic crosslinkers or solvents usually employed to form hydrogels.[7,8]

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Journal of Applied Polymer Science, Vol. 113, 400–407 (2009)

Chitosan, originated from chitin, the second most abundant natural biopolymer only to cellulose, is a copolymer consisting of β-(1,4)-2-acetamido-2-deoxy-D-glucopyranosyl and β-(1,4)-2-amino-2-deoxy-D-glucopyranosyl units.[9] Chitosan has many desirable properties, including nontoxicity, biocompatibility, biodegradability, and so on, that attract scientific and industrial interests for numerous applications such as drug delivery, tissue engineering, and gene therapy.[10–12] Chenite et al.[13] reported in an approach the preparation of thermosensitive chitosan hydrogels using polyol salt to adjust pH of the solutions. These formulations possessed a physiological pH and could be held liquid below room temperature for encapsulating living cells and therapeutic proteins; they formed gels at body temperature.[13,14] Chitosan as one of the most favorable thermosensitive hydrogel materials has recently attracted attention in pharmaceutical and biomedical fields.[15–18] Gelatin is a heterogeneous mixture of hot water-soluble proteins of high-average molecular weight, obtained by the thermal denaturation of collagen.[19] Gelatin contains carboxyl groups on its chain backbones and has the potential to mix with chitosan because of its ability to form hydrogen bonding with chitosan.[20] Gelatin and chitosan blends have been studied in some specific areas, such as sponges,[21,22]

scaffolds,[23–25] and films.[20,26] Gelatin is blended with chitosan to improve the biological activity since gelatin contains Arg-Gly-Asp (RGD)-like sequence that promotes cell adhesion and migration.[24] The safe reliability as surgical biomaterials in clinical applications of chitosan and gelatin blends has been proved.[22] Although some blends of gelatin and chitosan have been prepared, there are scarcely any reports about the thermosensitive hydrogel nature of chitosan and gelatin blends.

In this study, a novel thermosensitive hydrogel was obtained by simply mixing chitosan and gelatin with a small amount of $NaHCO_3$. The addition of small amount of gelatin into chitosan solutions can prevent immediate precipitation of chitosan solutions at neutral pH near 4°C and control the hydrogel formation when the surrounding temperature is up to body temperature. We investigated the physical and mechanical properties of thermosensitive chitosan/gelatin hydrogel and established the *in vitro* release profiles of bovine serum albumin (BSA) as a model drug of protein.

EXPERIMENTAL

Materials

Chitosan (molecular weight 160,000, 87% deacetylated) was supplied by Yuhuan Ocean Biochemistry Co., Ltd. (Taizhou, China). Gelatin was obtained from Shanghai Chemical Reagent Co. (Shanghai, China). Fluorescein isothiocyanate (FITC) was purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were of analytical grade.

Preparation of the hydrogels

Chitosan solution was obtained by dissolving 200 mg of chitosan in 0.1*M* HCl (10 mL) and chilled in an ice bath for 15 min. Gelatin was added to deionized water and heated at 50°C for 2 h to make solutions containing 1, 2, 3, 4, and 5% w/v gelatin. The gelatin solution (1 mL) was dropped into the chitosan solution in an ice bath under magnetic stirring and was stirred for 15 min to gain homogeneous mixture. Then, the cooled 1 mL $NaHCO_3$ (1.0*M*) was dropped into the stirring chitosan/gelatin solution in an ice bath to neutral pH. The obtained solution was stirred for another 30 min to gain homogeneous mixture. The hydrogel was formed by heating chitosan/gelatin solution in a water bath at 37°C for a few minutes.

Characterization of thermosensitivity

The gelation time was determined by test tube-inverting method.[27] The obtained formulation in solution state (12 mL) was added into a tube (25 mL) with a glass 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 for more than 30 s with the test tube inverted.

Rheological measurements

The rheological properties were performed on an ARES rheometer (TA, New Castle, DE). The measure system was Couette (two concentric cylinders) cell geometry, requiring about 10 mL of the solution as volume for the sample. Samples were covered with mineral oil to prevent water evaporation during the measurements. The dynamic viscoelastic parameters such as the dynamic shear storage modulus (G') and loss modulus (G'') were measured as functions of the temperature and frequency. 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 4 and 50°C. To determine the mechanical strength of the gel, the sweep of frequency was performed from 0.1 to 100 rad/s, at controlled constant temperature of 37°C (gel). The values of the strain amplitude were checked at 20% to ensure that all measurements were carried out within the linear viscoelastic regime, where the G' and G'' were independent of the strain amplitude according to the result of dynamic strain sweep.

FTIR spectra

FTIR spectra of chitosan, gelatin, and dried chitosan/gelatin gel were recorded in KBr pellets (the samples were triturated with KBr in the ratio of 1 : 100 and pressed to form pellets) in the range of 4000–400 cm^{-1} on a FTIR spectrophotometer (Nicolet, Model Impact 410; Madison, WI) at room temperature.

Scanning electron microscopy

Scanning electron microscopy (SEM) was performed on hydrogels after freeze-dried to maintain the porous structure without any collapse. The samples were plunged in liquid nitrogen, and the vitrified samples were cut with a cold knife. They were mounted on the base plate and coated with gold. The morphology was imaged on a Hitachi S-570 SEM (Tokyo, Japan) using an accelerating voltage of 20 kV.

Laser scanning confocal microscopy

Laser scanning confocal microscopy (LSCM) was performed on hydrogels directly. 0.1 mL of sample

solution with FITC was pipetted onto a microscope slide with a 0.5-mm spacer, before a glass cover slip was placed on top, sealing the space and preventing syneresis. The sample was then heated at 37°C for 2 min. Then, the samples were observed using LSCM (Leica, Wetzlar, Germany).

Swelling characterization

The degree of swelling (D_s) was determined by immersing the freeze-dried gels in phosphate-buffered saline (PBS) solutions at room temperature. At predetermined time intervals, they were removed from the solution, gently wiped to remove excess surface solution with filter paper, and then weighed and returned to the same container until equilibrium was achieved. The D_s was determined according to the following equation:

$$D_S = (W_S - W_0)/W_0,$$

where W_0 is the weight of dry gel and W_s is the weight of gel at different swelling time.

BSA incorporation and release

BSA (10 mg) was dissolved in the stirring chitosan/gelatin/$NaHCO_3$ solutions (10 mL) in an ice bath. Each sample (about 1000 mg) was placed into 10-mL plastic tube with a cap and incubated at 37°C for 10 min to form hydrogel. PBS buffer (5 mL) with pH 7.4 was added to each tube. Then, the sample was incubated at 37°C in a thermostated shaker rotating at 100 rpm. At predetermined intervals, 1 mL of the PBS buffer was taken out, and then the release of BSA from them was estimated. With each sample, the solution was changed with fresh medium, maintaining the total volume constant. The hydrogel without BSA was the blank and it was measured at the same way as the comparable sample.

The release of gelatin from the hydrogel blank was not found using Coomassie Brilliant Blue G-250, suggesting that the strong interaction existed between chitosan and gelatin chains; however, the gelatin in hydrogel did not influence the measurement of BSA release. BSA released from the hydrogel could be measured by UV-9100 spectrophotometer (Beijing, China) at 595 nm with Coomassie Brilliant Blue G-250. The percentage of cumulative amount of released BSA was determined from standard curves.

RESULTS AND DISCUSSION

Thermosensitivity analysis

Chitosan is not soluble in water, but chitosan solutions can be obtained in acidic aqueous media, which protonate chitosan amino groups, rendering the polymer positively charged and thereby overcoming associative forces between chains. When adding a base to such solutions, chitosan remains in solution up to a pH in the vicinity of 6.2. Further basification, to pH $>$ 6.2, systematically leads to the formation of a hydrated gel-like precipitate. This precipitation is due to the neutralization of chitosan amine ions and the consequent removal of repulsive interchain electrostatic forces, which subsequently allows for extensive hydrogen bonding and hydrophobic interactions between chains.[14] In this study, the precipitate could be prevented by the addition of gelatin, and the hydrogel would be formed by adjusting the environment temperature from 4 to 37°C. The pH of chitosan/gelatin solution was raised to around 7.0 with $NaHCO_3$ in ice bath. The mixed solution was placed at 37°C, and then the gel was formed after a few minutes.

The influence of chitosan concentration on the gel formation was determined by adding the gelatin solutions to chitosan solutions with different concentrations. The results in Figure 1(a) clearly showed that chitosan concentration had a crucial influence on the gelling time. The gelation time decreased quickly with the increase in the concentration of chitosan when its concentration was more than 1% w/v. When chitosan concentration was less than 1% w/v, the system failed to form gels at 37°C (data not shown) and hence the blends of 2% w/v chitosan solutions were investigated in the following research.

To investigate the influence of the concentration of gelatin on the gel formation, the gels were prepared by adding the gelatin solutions with different concentrations to chitosan solutions. Figure 1(b) displayed the results of the variation in the gelation time as a function of the gelatin concentration. The results indicated that the concentration of gelatin had an apparent influence on the gelation time. The gelation time of chitosan/gelatin solutions varied with the concentration of gelatin. The gelation time decreased first and then increased with the increase of gelatin concentration. There was minimized gelation time at 3% w/v gelatin solution. The gelation time changes were probably due to the result of competition between intermolecular forces such as hydrophobic interactions and hydrogen bonding.

To investigate the effect of pH value of solution on the gel formation, the gels were prepared by adding the different concentration of $NaHCO_3$ solutions to chitosan/gelatin solutions. Chitosan/gelatin solutions can be neutralized to pH values between 6.6 and 7.3 via the addition of $NaHCO_3$, without inducing immediate precipitation or gelation, provided the temperature is maintained about 4°C. The ability to maintain system in solution at nearly neutral pH is due to the mild alkalinity of $NaHCO_3$ and the

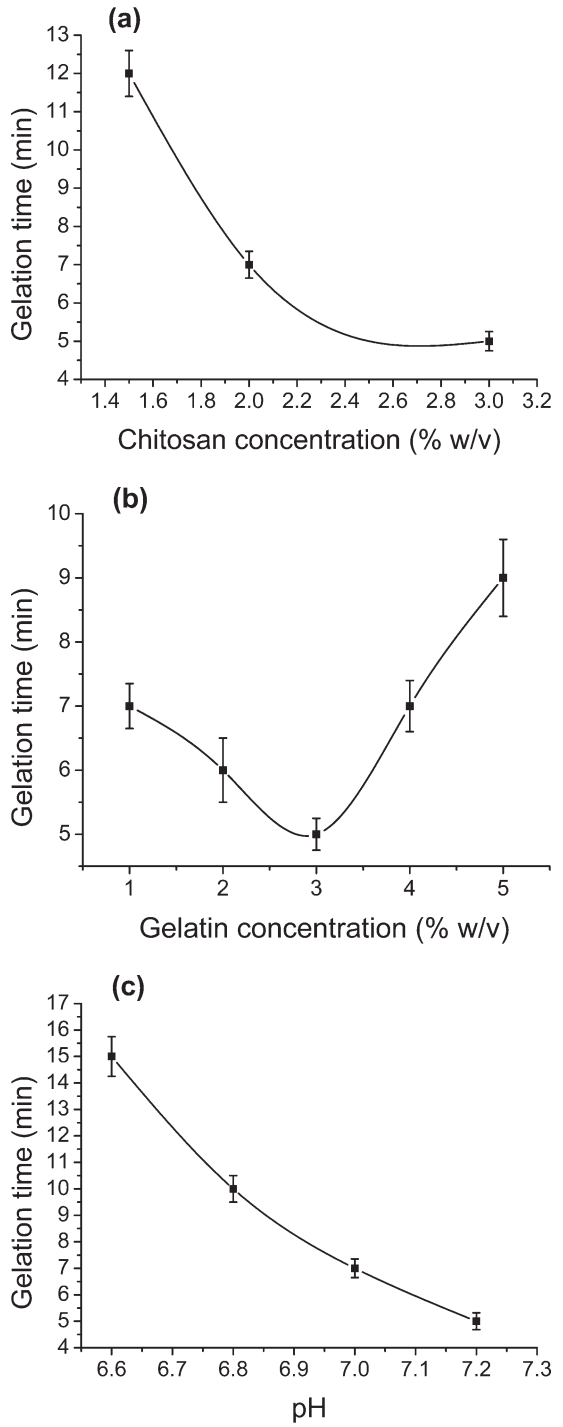
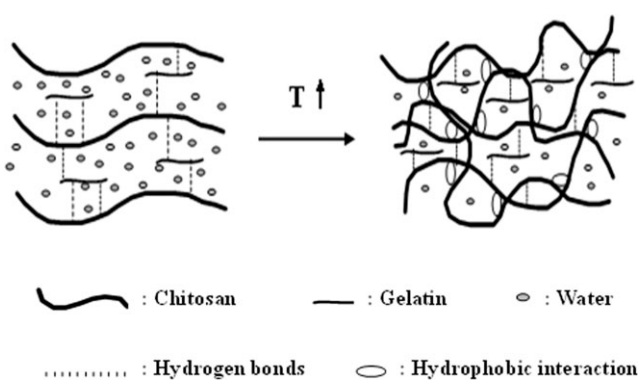


Figure 1 Gelation time as a function of (a) chitosan concentration of the chitosan/gelatin solutions (gelatin, 1% w/v; pH ~ 7.0) ($n = 3$), (b) gelatin concentration of the chitosan/gelatin solutions (chitosan, 2% w/v; pH ~ 7.0) ($n = 3$), and (c) pH of the chitosan/gelatin solutions (chitosan, 2% w/v; gelatin, 1% w/v) ($n = 3$).

presence of gelatin. Figure 1(c) showed the results of the influence of pH value on the gelation time of chitosan/gelatin solutions. The results indicated that pH value of solutions had an apparent influence on the gelation time. The gelation time reduced with the increase of pH value.

The mechanism of the thermosensitive sol–gel transition for chitosan/gelatin system is illustrated in Scheme 1. In acidic environment, free amino groups of chitosan are positively charged. Negatively charged molecules can be ionically bound to the chitosan. Chitosan can form a polyelectrolyte complex with negatively charged moieties of gelatin in an aqueous solution.[26] The amine ions of chitosan decrease with increasing pH value of the solution, which results in the reduction of electrostatic attractions and the increase of hydrogen bonding between chitosan and gelatin. Besides, the hydroxy polymer can stabilize certain compounds in aqueous solutions and promote the formation of a shield of water around some macromolecules or polymer chains.[13] Gelatin is a hydrophilic material and can promote the protective hydration of chitosan chains, keeping the chitosan chains stretched freely in solution at low temperature. Raising the temperature will increase the internal energy and break the hydrogen bonds between chitosan and water, so the water molecules bound to chitosan chains are released. The movement of free water molecules increases the entropy in system, and to decrease the entropy change, hydrophobic chitosan chains tend to aggregate and gelation occurs. The addition of gelatin is to prevent immediate precipitation of chitosan solution and to control the hydrogel formation when the temperature is increased. Our results suggested that the addition of gelatin provided the correct buffering and other physicochemical conditions including control of hydrophobic interactions and hydrogen bonding, which were necessary to retain chitosan in solution at neutral pH near 4°C and furthermore to allow gel formation upon heating to body temperature. This type of thermosensitive gelation has also been observed in other cases.[13,27] Based on these previous studies and considering the observation that chitosan/gelatin solutions gel upon heating, we



Scheme 1 Schematic of the formation mechanism of chitosan/gelatin hydrogel.

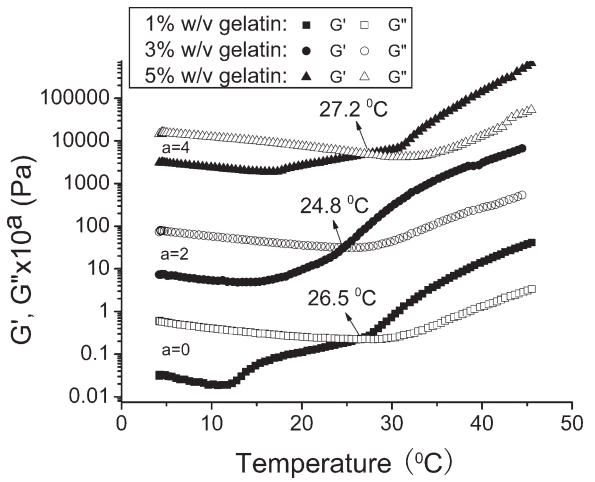


Figure 2 Temperature dependence of storage modulus G' and loss modulus G'' of chitosan/gelatin solutions with different gelatin concentrations at a heating rate of 1°C/min and at a frequency of 1 rad/s (chitosan, 2% w/v). The data is shifted along the axes by 10^a with given a value to avoid overlapping.

hypothesized that hydrophobic forces play an important role in the gelation process.

Rheological analysis

The rheological properties of chitosan/gelatin solutions were measured to understand the formation mechanism of the hydrogels. Samples were first subjected to a strain sweep test to define the linear viscoelastic region in which the storage modulus (G') and loss modulus (G'') were independent of the applied strain. In our experiment, the strain amplitude was set as 20%. Figure 2 showed the change in the G' and G'' as a function of temperature for chitosan/gelatin solutions containing 1, 3, and 5% w/v gelatin. The storage modulus G' reflects the elastic modulus, and loss modulus G'' reflects the viscous of the system. The gelation temperature is determined as the temperature at which G' is equal to G''. The crossover point of the G' and G'' gives an indication of the transition from the initial dominant viscous liquid-like behavior to elastic solid or gel-like behavior. The storage modulus G' is lower than the loss modulus G'' below the gelation temperature, which shows viscoelastic behavior of a liquid. G' sharply rises and exceeds G'' upon heated to the vicinity of gelation temperature. When the system temperature is higher than the gelation temperature, G' exceeds G'', which shows viscoelastic behavior of a solid. All composition exhibited a crossover point in the temperature range of 24–28°C. For the solution composed of 1% w/v gelatin, the gelation temperature was about 26.5°C, and the cures were divided into two parts. The first region was below 26.5°C, where G' was lower than G'', which improved the polymer chain mobility, promoted less resistance to macromolecular rearrangements, which showed linear correlation, and displayed typical solution rheological behavior. Subsequently, the sharp increase to about 26.5°C in G' was considered as a result of the partial formation of chitosan clusters through hydrophobic interaction. In the second region, G' and G'' increased dramatically with an increase of temperature, G' was higher than G'', indicating that an elastic gel network had been formed. Similar behavior was observed in chitosan/gelatin solutions based on 3 and 5% w/v gelatin solutions, but the gelation temperatures were 24.8°C and 27.2°C, respectively. Gelation temperature of chitosan/gelatin solution reduced first and then increased with the increase of gelatin concentration. Besides, reheological characterizations of chitosan/gelatin solutions showed that their viscous modulus (G'') increased as gelatin concentration increased at low temperature, and it was difficult to inject when gelatin concentration was more than 5% w/v. These results may be due to the differences in the hydrophobic interactions and hydrogen bonding of polymer chains between chitosan and gelatin.

The frequency dependence of the viscoelastic properties of chitosan/gelatin hydrogels was revealed at 37°C in Figure 3. For each sample, the G' and G'' were measured as a function of frequency from 0.1 to 100 rad/s. When the ambient temperature was higher than the gelation temperature, the storage modulus G' was higher than the loss modulus G'' over the whole frequency range. It was evident that the storage modulus G' showed almost no dependence with frequency. These features are characteristic of a "strong gel."

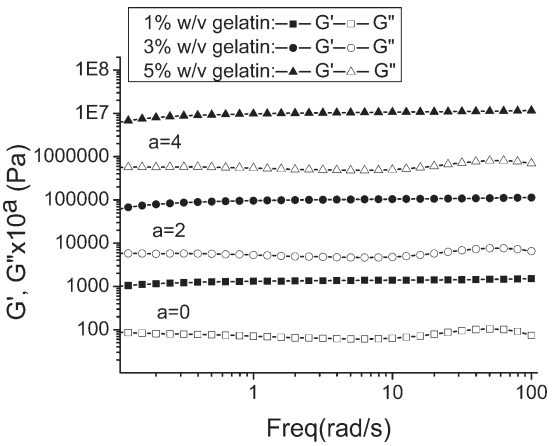


Figure 3 Frequency dependence of storage G' and loss modulus G'' of chitosan/gelatin solutions with different gelatin concentrations (chitosan, 2% w/v). The data is shifted along the axes by 10^a with given a value to avoid overlapping.

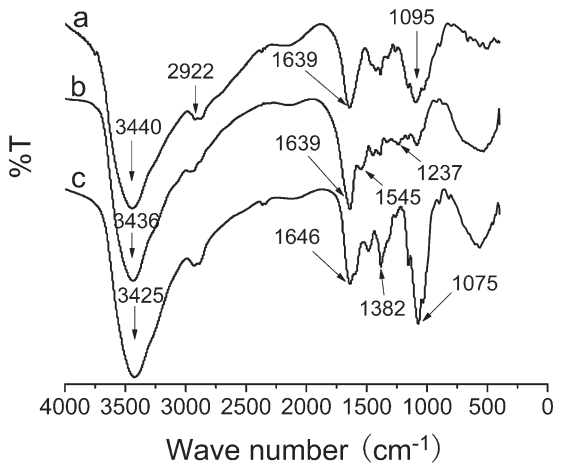


Figure 4 FTIR spectra of (a) chitosan, (b) gelatin, and (c) chitosan/gelatin gel.

FTIR analysis

FTIR studies were performed to confirm the nature of gel. Figure 4 shows the FTIR spectra of chitosan, gelatin, and chitosan/gelatin gel. Chitosan exhibited a broad peak at 3440 cm^{-1}, which was assigned to the stretching vibration of N—H and O—H bond. Peaks at 2922 cm^{-1} were due to the C—H stretch vibrations. A peak at 1639 cm^{-1} was due to the C=O stretch of amide bond. FTIR spectrum of pure gelatin showed N—H stretching at 3436 cm^{-1} and C=O stretching at 1639 cm^{-1}. The peak at 1545 cm^{-1} was due to N—H bending vibrations of the amide II band and the peak at 1237 cm^{-1} was due to amide III band. The spectrum of chitosan/gelatin showed that C=O groups of the gelatin interacted with N—H groups and O—H groups of chitosan and resulted in the increased frequency of peak about 3425 cm^{-1}. For the chitosan/gelatin sample, the peak at 1639 cm^{-1} belonging to chitosan amide bond was shifted slightly to lower frequency with the addition of gelatin. These results suggested that the functional groups of chitosan in the chitosan/gelatin gel interacted with carboxyl groups of gelatin.

Scanning electron microscopy analysis

The morphology of the chitosan/gelatin gels can be examined using SEM after freeze drying when the material has an adequate modulus to avoid the structural collapse during dehydration. Figure 5 shows the SEM of chitosan/gelatin gel with 1 and 5% w/v gelatin. These images indicated that chitosan/gelatin gels had similar interconnection pore structure forming a three-dimensional network structure. Besides, the morphology of the gels was insensitive to the gelatin concentration. The pore size of gel of 5% w/v gelatin was smaller than the gel composed of 1% w/v gelatin. This mainly resulted from the increasing entanglings between gelatin and chitosan with the increase of gelatin content.

Laser scanning confocal microscopy analysis

Chitosan/gelatin hydrogels composed of 1 and 5% w/v gelatin were examined by LSCM to study their fine aggregate structure. Chitosan/gelatin hydrogel was found to have a beaded, open structure (Fig. 6), forming a network by linking polymeric aggregates in agglomerates and chains. The confocal images showed that the polymer-rich phase increased with increasing of gelatin content. The overall appearance of the gel structure observed with LSCM was quite

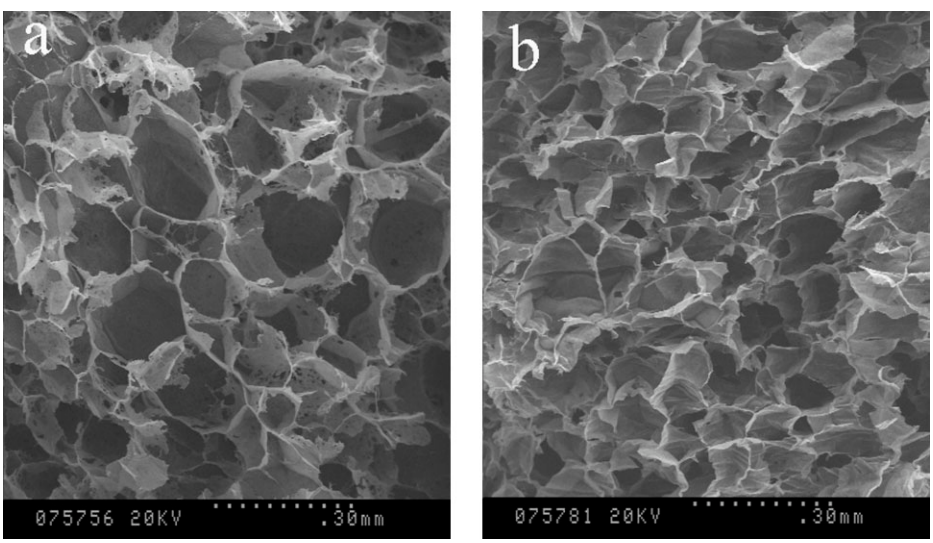


Figure 5 The SEM of chitosan/gelatin gels with (a) 1% w/v and (b) 5% w/v gelatin (chitosan, 2% w/v).

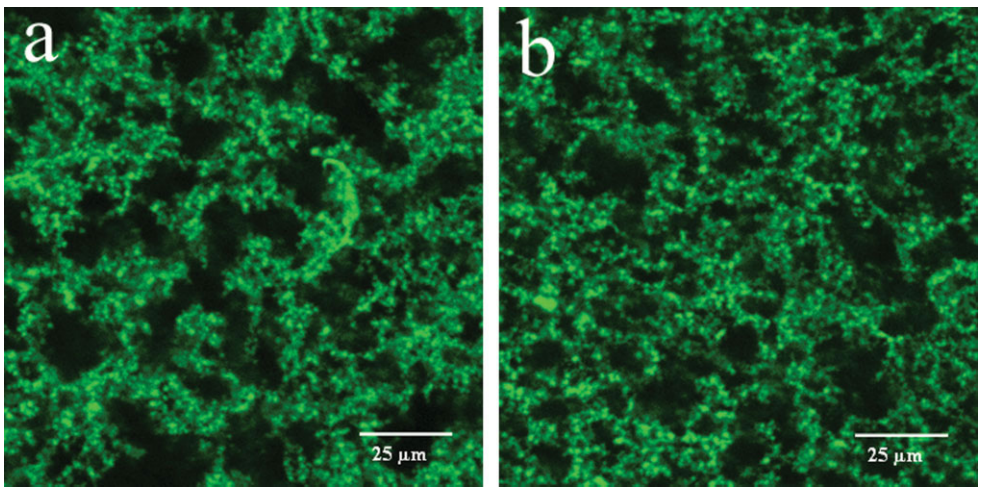


Figure 6 Microstructural images from LSCM of chitosan/gelatin gels with (a) 1% w/v and (b) 5% w/v gelatin (chitosan, 2% w/v). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

different to the images provided by SEM. SEM failed to detect the fine aggregate structure. The confocal images demonstrated that the gelation mechanism of chitosan/gelatin solution was nucleation and growth. Nucleation and growth occurs when the temperature change causes the single-phase solution to move into a metastable, rather than unstable, region. At this point, a second phase develops, which over time grows in size but does not change in composition. During nucleation, the polymeric phase is composed of many small regions, which during the growth phase will expand and possibly connect. This type of aggregate structure has also been observed in thermosensitive chitosan/glycerophosphate salt hydrogel system.[28]

Swelling experiments analysis

The swelling behavior of chitosan/gelatin dry gel was studied as a function of the hydrogel properties. The results of swelling experiments of chitosan/gelatin dry gel under pH 7.4 PBS buffer are shown in Figure 7. The equilibrium swelling values were found to be higher at lower gelatin concentration when the chitosan content is the same. This can be explained by the fact that low gelatin concentration leads to weak hydrogen bonding and consequent facilitation of the entrance of solvent into the material.

Drug delivery analysis

Currently, protein drugs are increasingly becoming a very important class of therapeutic agents with the

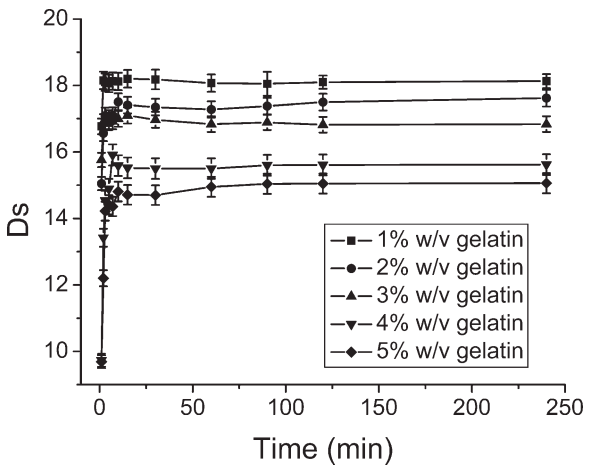


Figure 7 Swelling ratio of chitosan/gelatin gels with different gelatin concentrations in pH 7.4 PBS buffer (chitosan, 2% w/v) ($n = 3$).

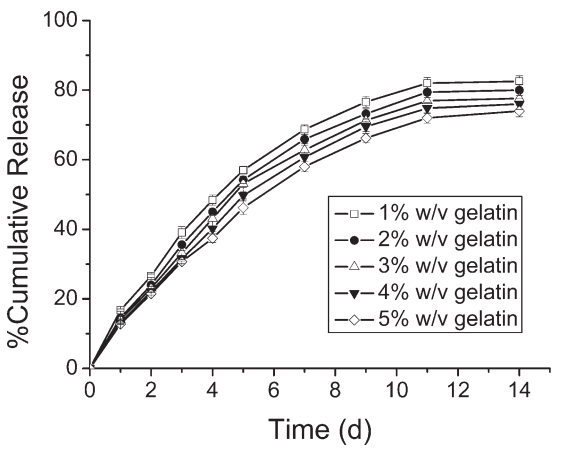


Figure 8 Accumulative release of BSA from chitosan/gelatin gels with different gelatin concentrations in pH 7.4 PBS buffer (chitosan, 2% w/v) ($n = 3$).

rapid advances in the field of biotechnology. These drugs are mostly delivered by parenteral administration. However, repeated injections are required due to extremely short acting of this kind of drugs. To minimize the health hazard by constant injection, sustained delivery is the ideal alternate route of administration.[29] Figure 8 shows the BSA cumulative release profiles from chitosan/gelatin gels in pH 7.4 buffer at 37°C. The release rate of BSA was affected by the gelatin content of gels. The higher the gelatin content, the slower is the release rate of BSA. The mechanism of drug release may be due to the diffusion of BSA through the three-dimensional network, and the smaller pore size of the network results in the slower release rate of the drug loaded. *In vitro* release behaviors of BSA from these polymer hydrogels were sustained for more than 10 days without a distinct initial burst. These results demonstrated that thermosensitive chitosan/gelatin gels were potential to be used as a vehicle for the delivery of proteins.

CONCLUSIONS

In this study, thermosensitive chitosan/gelatin formulation was developed by neutralizing chitosan/gelatin solutions with a small amount of $NaHCO_3$ to retain the formulation in aqueous solution at physiological pH. These formulations could transform from solutions to hydrogels when they were heated to body temperature. The addition of gelatin provides the correct buffering and other physicochemical conditions including control of hydrophobic interactions and hydrogen bonding, which are necessary to maintain chitosan in solution at neutral pH near 4°C and furthermore to allow gel formation upon heating to body temperature. The polymer solutions are biocompatible, biodegradable, and adhesive to human tissue, providing new opportunities in local and sustained delivery of proteins, cell encapsulation, and tissue engineering.

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