Soft Matter

PAPER

View Article Online
View Journal | View Issue

Cite this: *Soft Matter*, 2014, **10**, 8245

Thermal gelation of chitosan in an aqueous alkali–urea solution†‡

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Received 20th June 2014
Accepted 10th August 2014

DOI: 10.1039/c4sm01336k

www.rsc.org/softmatter

Chitosan can readily dissolve in a precooled aqueous alkali–urea solution, a solvent that has previously been developed to dissolve cellulose. Upon heating, the resulting solutions quickly become a gel. The thermal gelling of the chitosan solutions was studied by rheology. Initially, a temperature ramp test was used to determine the gelation temperatures (T_{gel}). It was found that T_{gel} does not significantly change with chitosan concentration. The *in situ* formed gels liquefy on cooling, but the liquefication temperature (T_{liq}) is considerably lower than T_{gel}, indicating a large hysteresis in the cooling process. In addition, T_{liq} decreases with increasing polymer concentration. The kinetics of thermal gelation was then studied by isothermal curing. The solution gels were cured not only at temperatures above the T_{gel}, which was determined in the temperature ramp test, but also at temperatures far below the T_{gel}, provided that the solution is cured at the temperature for a long enough time. The solutions become gel faster when cured at higher temperatures. When cured at the same temperature, higher concentration solutions become gel faster. The apparent activation energy for the thermal gelation of the chitosan solutions was determined to be ~200 kJ mol^{-1}. Physical gels of pure chitosan were obtained by repeated soaking the *in situ* formed gels in water. Preliminary test shows that new gels are highly biocompatible.

Introduction

Chitosan, the only known cationic pseudonatural polymer, is a linear copolymer of β-(1–4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β-D-glucopyranose. It is obtained by the deacetylation of chitin, the second most abundant polysaccharide in nature.[1] Similar to many biopolymers, chitosan is innately biocompatible, biodegradable, and non-toxic to living tissues. It also shows antibacterial, antifungal and antitumor activity. Therefore, chitosan has been widely exploited for biomedical/pharmaceutical applications,[1] including tissue engineering,[2] drug delivery, wound dressing, gene therapy, and bioimaging.

Many of these applications require chitosan to be engineered into hydrogels, and numerous methods have been proposed for this purpose.[3–9] Chemical gels were synthesized by crosslinking chitosan with glutaraldehyde,[4,5] genipin,[6] and other crosslinkers. Considering that crosslinkers are usually toxic, physical hydrogels are highly desirable for biomedical applications. One of the ways to physically gel a chitosan solution is *via* the reacylation of chitosan in a dilute acidic solution because the solubility of polymer chains progressively decreases with increasing *N*-acylation degree.[8] By grafting with PEG,[9] poly(*N*-isopropylacrylamide) (PNIPAM),[10] or homogeneously reacetylated to a deacetylation degree of about 50%,[11] the chemically modified chitosan becomes soluble at neutral pH and becomes capable to gel on heating. In particular, A. Chenite *et al.*[12–15] found that chitosan/β-glycerophosphate (GP) solutions undergo sol–gel transition around body temperature. These approaches either chemically modify the structure of chitosan or achieve gelation in the presence of an additive. In this context, Christophe Viton *et al.*[16] achieved the gelation of chitosan solutions in an acetic acid–water–propanediol mixture by the evaporation of the solution. However, this approach is time-consuming, taking usually several days for the solution to evaporate to reach the gel point.

In the present work, we describe another approach to prepare physical chitosan hydrogels, in which chitosan is first dissolved in an aqueous alkali–urea solution which was previously developed by L. Zhang *et al.*[17,18] to dissolve cellulose. It is well-known that the stable crystalline structure of solid chitosan prevents it from getting dissolved in aqueous solutions above pH 7.[19,20] However, chitosan readily dissolves in this new cellulose solvent, possibly due to the similarity in their structures. This solution undergoes sol–gel transition on heating. In this work, we thoroughly studied the thermal gelation of the chitosan solution. We have shown that the gelation temperature

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† The authors wish to dedicate this work to Professor Weixiao Cao, Peking University, China, for his 80th birthday in November 2014.

‡ Electronic supplementary information (ESI) available: XRD pattern of chitosan hydrogel, gelation temperature as a function of heating rate and photograph of a solution after 16 day-storage. See DOI: 10.1039/c4sm01336k

of the chitosan solution, determined by dynamic temperature ramp test, does not show significant concentration-dependency. The gelation is reversible, despite the large hysteresis in cooling process. The kinetics of thermal gelation was also studied by isothermal curing. Thus, new chitosan gels consisting of only chitosan, without any crosslinker or additive, were prepared. Preliminary tests show that new chitosan gel is biocompatible and supports the growth of cells. We expect that this new hydrogel will find biomedical applications such as wound dressing.

Experimental section

Materials

Chitosan (85% deacetylated, medium molecular weight) was purchased from Sigma-Aldrich. NaOH (96%) and urea (99%) were obtained from Tianjin Chemical Reagent Company, China. The reagents were used without further purification.

Dissolution of chitosan

The solvent used to dissolve chitosan is an aqueous solution containing NaOH/urea/H_2O in the ratio 7 : 12 : 81 by weight. Pre-weighed chitosan was added into the solution and dispersed by stirring. The mixture was then transferred to a refrigerator and stored overnight at −20 °C. The frozen solution was removed and gently stirred. It gradually thawed and turned into a clear solution.

Rheological characterization

Rheological characterization of the chitosan solutions was performed on an AR2000ex rheometer (TA Instruments). Parallel plate geometry with a diameter of 40 mm was used. The sample gap was set to be 1.0 mm. The temperature was controlled by a Peltier system in the bottom plate connected with a water bath.

The freshly prepared chitosan solutions were degassed by centrifugation prior to the measurements. After a sample was set between the plates, silicon oil was placed around the rim to prevent water evaporation. Temperature-dependent changes in elastic (storage) modulus, G', and viscous (loss) modulus, G'', were recorded in a dynamic temperature ramp test (DTRT). For kinetics study, the temperature of the bottom plate was first set to −10 °C. The sample was loaded and then heated to a predetermined temperature. It was then maintained at that temperature, and change in storage modulus (G') and loss modulus (G'') during the gelation process was monitored as a function of time. The frequency and the strain were set at 1 rad s^{-1} and 1%, respectively. All The rheological experiments were performed within the linear viscoelastic region.

NMR and FTIR measurements

^{1}H NMR measurements of the chitosan solution were performed on a Varian UNITY-plus 400 NMR spectrometer. The solvent is 7% NaOH/12% urea/D_2O or D_2O/DCl. FTIR spectra were recorded on a Bio-Rad 6000 FTIR instrument.

Cell culture

To each well of a 24-well cell culture plate, 2.0 mL of freshly prepared 4.0 wt% chitosan solutions were added. After *in situ* gelation, the hydrogel films were repeatedly washed with water. NIH 3T3 cells were seeded at about 5×10^5 cells per mL and cultured in a media containing 90% Dulbecco's modified Eagle's medium (DMEM), 10% heat-inactivated fetal calf serum and 100 units per mL of penicillin/streptomycin. The cultures were maintained at 37 °C in an incubator in a humidified atmosphere of 5% CO_2. The appearance of the cells was observed by an Olympus LX70-140 inverted fluorescence microscope. The cells were stained with acridine orange (AO) and ethidium bromide (EB) before imaging. The excitation wavelength used was 450 ± 20 nm.

Results and discussion

Dissolution of chitosan in aqueous alkali–urea solution

In general, chitosan can only dissolve in an acidic solution by the protonation of –NH_2 group on the C-2 position of D-glucosamine repeat unit.[19,20] It is notable that chitosan quickly dissolves in precooled NaOH/urea aqueous solution that was previously developed to dissolve cellulose.[17] The ^{1}H NMR spectra of chitosan dissolved in the new solvent are shown in Fig. 1. For comparison, ^{1}H NMR spectra of chitosan dissolved in an acidic solution are also included. According to previous studies, the peaks at 1.75 and 2.89 ppm in the measured spectra of acidic solution are assigned to the protons in the acetyl group of the acetylated unit and H-2 in the deacetylated unit, respectively.[21–24] However, when measured in the alkaline solution, the H-2 peak shifts to 2.36 ppm, while the acetyl peak remains at 1.75 ppm. The shift of the H-2 peak indicates its different chemical environment in the two different solvents: the –NH_2 group attached on the same carbon atom is protonated in the acidic solution, while it does not get protonated in the alkaline solution. The result indicates that chitosan dissolves in acidic solutions *via* the protonation of the –NH_2 group, while it maintains its pristine structure when dissolved in alkali solution. From the ratio of the integral areas of the two peaks, the deacetylation degree of the chitosan sample can be estimated.

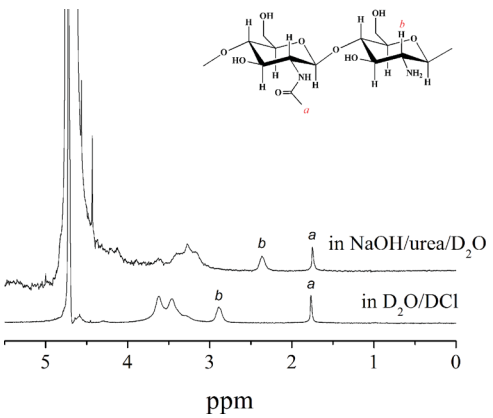

Fig. 1 ^{1}H NMR spectra of chitosan dissolved in different solvents.

In both cases, the same deacetylation degree of ~84% was determined (the deacetylation degree of the sample is 85% according to the provider). The result indicates that no deacetylation occurs, when the chitosan sample dissolves in the new solvent. In addition, the FTIR spectra of the regenerated chitosan are almost identical to that of the original chitosan (Fig. 2). The mechanism for the dissolution of chitosan in the aqueous alkali–urea solution may be similar to that for the dissolution of cellulose in the same solvent. L. Zhang *et al.*[25] previously proposed that cellulose, urea and NaOH could form a relatively stable inclusion complex at low temperatures, leading to the dissolution of cellulose. The structure of chitosan is similar to that of cellulose. It is possible that chitosan can also form an inclusion complex with urea and NaOH, therefore it dissolves in the solvent.

Thermal gelation of the chitosan solution

It is interesting that the chitosan solution undergoes sol–gel transition on heating. As shown in Fig. 3, the freshly prepared solution flows when tilted. However, after a brief heat treatment at 45 °C the solution immediately becomes a gel. The resulting gel is stable to inversion.

The rheology characterization also indicates a sol–gel transition. Fig. 4 shows the dynamic moduli of a chitosan solution before and after the heat treatment. For the freshly prepared solution, the loss modulus G'' is larger than the storage modulus G' for entire measured frequency range. In addition, both G' and G'' increase with increasing frequency, a typical rheological behaviour of a semi-dilute polymer solution.[26] However, after being treated at 45 °C for 5 min, G' dominates G'' and both moduli become relatively frequency-independent. These results clearly indicate the formation of gel networks possessing long-lived cross-links.[27]

Temperature ramp test: determination of gelation temperature

To further study the thermal gelling behaviour of the chitosan solutions, a series of rheological measurements were carried out. Initially dynamic temperature ramp tests (DTRT), which measure the changes of dynamic moduli in a heating process, were carried out with an aim to determine the gelation temperature of the system. As an example, Fig. 5A shows the thermal gelling behaviour of a 2.5 wt% chitosan solution. Initially, G'' dominates G', indicating that the solution is in liquid state. On heating, both G' and G'' initially slightly decreases with increasing temperature. As temperature continuously increases, a sharp increase in both G' and G'' is observed. Because G' increases faster than G'', a crossover of G' and G'' was observed at ~41.5 °C. Beyond this point, G' is always larger than G'', indicating the formation of a physical network. Similar thermal gelling behaviours were observed for solutions with concentrations varying from 2.0 to 3.5 wt%.

The temperature where G' and G'' crossover during the heating process is defined as the gelation temperature (T_{gel}). As shown in Fig. 5B, T_{gel} was measured to be 40.3(±1.2), 42.0(±1.0), 37.3(±0.6), and 37.0(±1.0) °C for chitosan solutions with concentration of 2.0, 2.5, 3.0 and 3.5 wt%, respectively. We can observe that the T_{gel} of the chitosan solution is relatively concentration-independent in this concentration range.

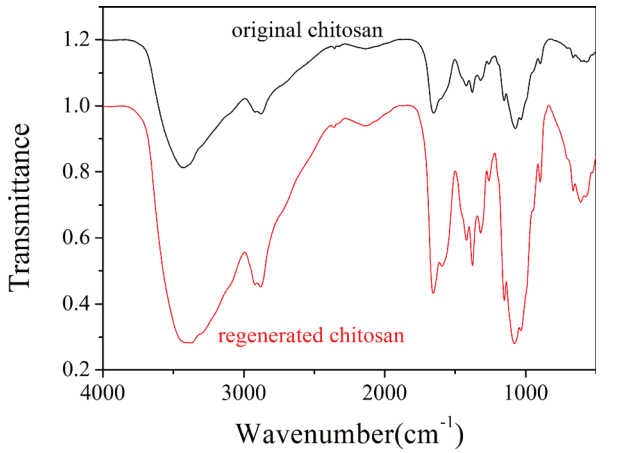

Fig. 2 FTIR spectra of the original chitosan and the regenerated chitosan. The regenerated chitosan was obtained by neutralization of the chitosan solution with HCl.

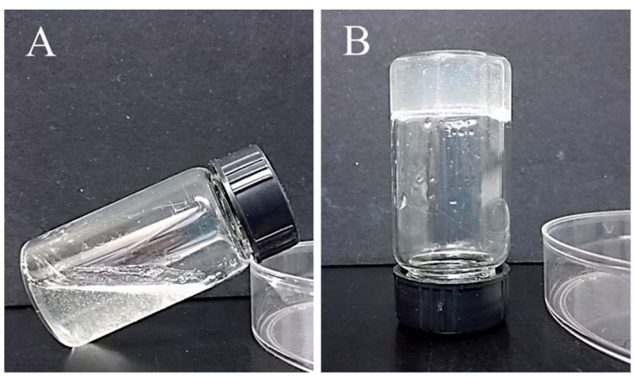

Fig. 3 Images of a 3.0 wt% chitosan solution before (A) and after a 2 min immersion in a water bath at 45 °C (B).

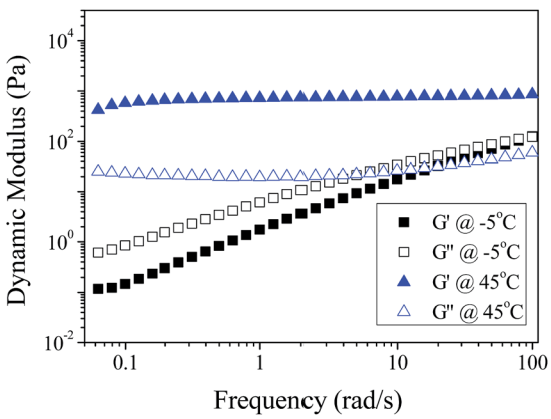

Fig. 4 Dynamic moduli of a 2.0 wt% chitosan solution measured at −5 °C (before gelation) and 45 °C (after gelation).

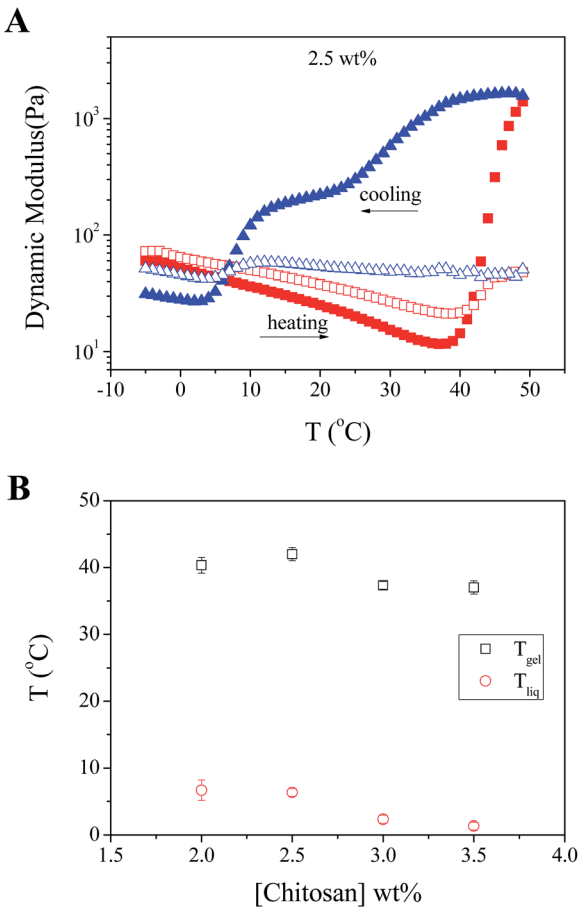

Fig. 5 (A) Evolution of dynamic moduli of a 2.5 wt% chitosan solution in heating and cooling cycle under a stress of 0.7958 Pa and a frequency of 1.0 Hz. Solid and open squares are for storage and loss moduli (G' and G'') during the heating process, while solid and open triangles are for G' and G'' during the cooling process. (B) T_{gel} and T_{liq} of chitosan solution as a function of chitosan concentration.

The thermal gelation of the chitosan solutions may be attributed to the balance between solvophobic and solvophilic interactions.[28] As mentioned above, chitosan dissolves in the aqueous alkali–urea solution due to the formation of an inclusion complex of chitosan, urea and NaOH. This complex may collapse at an elevated temperature. Without the protection of urea and NaOH, the 'bare' chitosan chains lose their solubility. Therefore, they associate with each other to form a physical network. The association of chitosan chains also results in an increased turbidity. The resulting hydrogels are usually opaque, as shown in Fig. 3. It is noteworthy that cellulose solutions in similar solvents also undergo a sol–gel transition on heating.[18]

Many thermosensitive polymers undergo thermal gelation on heating. For "strong" thermosensitive polymers, such as PNIPAM, their T_{gel} is relatively concentration-independent. We[29–31] previously found that the T_{gel} of PNIPAM microgel dispersions is close to the volume phase transition temperature (VPTT) of microgels because the driving force for the conversion of dispersion to gel is the hydrophobic interactions among the microgel particles, which emerge only at temperatures close to VPTT.[32] For many thermosensitive polymers that can be termed as "weak" thermosensitive polymers, T_{gel} is concentration-dependent.[33,34] For example, S. A. Arvidson *et al.*[34] found that the gelation temperature of aqueous methylcellulose solutions decreases with increasing concentration. In particular, for the thermal gelling of cellulose in similar solvents, T_{gel} is highly concentration-dependent.[18,35,36] It was reported that T_{gel} of cellulose solutions in 6 wt% NaOH/5 wt% thiourea decreases from 38.6 to 20.1 °C, when the cellulose concentration increases from 4 to 6 wt%.[36] In another study, it was found that the sol–gel transition temperature of cellulose in 7 wt% NaOH/12 wt% urea decreased from 60.3 to 30.5 °C with an increase of its concentration from 3 to 5 wt%.[18] In this context, the chitosan solution behaves more like a "strong" rather than a "weak" thermosensitive polymer.

To study the reversibility of gelling process, the rheological properties of the *in situ* formed gels in a cooling process were also studied. As shown in Fig. 5A, when cooled to a certain temperature, both G' and G'' decrease with decreasing temperature. G' decreases faster than G'', such that they crossover again. Beyond this point, G' is always smaller than G'', indicating that the hydrogel transforms back to a liquid state. The result suggests that the gelation of chitosan solution is reversible. This is again considerably different from cellulose solutions in similar solvents.[18,35] Cai and Zhang[18] reported that the gelation of dissolved cellulose in the same solvent is irreversible. The formed gel does not liquefy even when it is cooled back to −10 °C, at which it was previously dissolved.

The temperature where G' and G'' crossover during the cooling process is defined as the liquefaction temperature (T_{liq}). As shown in Fig. 5B, for all the solutions studied here, T_{liq} is much lower than T_{gel}. For example, the 2.0 wt% chitosan solution gels at ~40.3 °C during the heating process, but does not liquefy until cooled to ~6.7 °C. These observations indicate that the thermal gelation of the chitosan solutions is not fully reversible.

Retardation in the cooling process *versus* the heating process has previously been observed in other thermosensitive systems, such as PNIPAM.[37–43] We recently found that the T_{liq} of PNIPAM microgel dispersions, which also undergo thermal gelation upon heating, is usually 4–5 °C lower than the corresponding T_{gel}.[44] For PNIPAM-based systems, it is believed that during the heating process the polymers become hydrophobic and form aggregates *via* hydrophobic interactions, which are further stabilized by additional intra- and inter-chain interactions, including hydrogen bonding and hydrophobic interactions.[42,43] Therefore, the dissolution of these aggregates in the cooling process is kinetically slow, resulting in the observed hysteresis. The lower T_{liq} of the chitosan solution may be explained in the same way. It is possible that additional hydrogen bonds will be formed among the chitosan chains, which would further stabilize the junctions, and result in a kinetically slow dissolution during the cooling process.

Close examination of Fig. 5A reveals a two-stage decrease of G' in the cooling process. When cooled from 49 °C to 38 °C, G' remains almost unchanged, indicating that the physical network is almost intact. A sharp decrease in G' begins at ~38 °C, which is also the onset temperature for G' to start

increasing during the heating process. This fact may suggest that the decrease in G' is a result of the breakage of junctions which are formed *via* hydrophobic interactions among the chitosan chains. When cooled to ~23 °C, the decreasing of G' becomes very slow. When temperature further drops to ~12 °C, the decrease of G' again becomes fast; it crossovers with G'' at ~8 °C and stops decreasing at ~3 °C. In addition, Fig. 5B shows that T_{liq} slightly decreases from 8 °C for the 2.0 wt% solution to 2.7 °C for 3.5 wt% solution.

To explain the two stage decrease of G' and the concentration-dependency of T_{liq}, we suspect that some junctions formed during the heating process may not only be stabilized by additional inter-chain hydrogen bonds, but also by crystallization. It is well-known that chitosan is a semi-crystalline polymer in the solid state.[45] The polymer chains in some junctions will possibly rearrange and form crystallites. These junctions will be destructed with more difficulty during the cooling process. Therefore, G' does not significantly change, when temperature drops to the range of ~23 °C to ~12 °C. These junctions begin to dissolve when temperature drops further to below 12 °C. As a result, a second stage decrease of G' was observed. The concentration-dependence of T_{liq} can also be explained by the formation of crystallites. It is expected that larger crystallites will form in solutions with higher concentrations, which are more difficult to be destructed. Therefore, the T_{liq} of the solutions decreases with increasing chitosan concentrations. As shown in Fig. S1 in the ESI,‡ a small peak appears at $2\theta \approx 20°$ in the XRD pattern of the *in situ* formed chitosan gel, demonstrating the formation of low amounts of small crystalline.[46,47] The formation and destruction of crystalline structures has been previously used to explain the observed hysteresis in the sol–gel transition of methyl cellulose.[48]

It is noteworthy that the measured T_{gel}s and T_{liq}s are a function of the thermal history. As an example, Fig. 6 shows the rheological properties of a 2.0 wt% chitosan solution in continuous three heating and cooling cycles. In the first cycle, the solution gels at ~40.7 °C during the heating process. This value decreases to ~38 °C in the second cycle and further decreases to ~37.5 °C in the third cycle. T_{liq} also decreases in the cooling process from ~8 °C in the first cycle to ~5 °C in the second cycle, and to ~3.5 °C in the third cycle. It appears that some structures formed during the heating process are not fully destructed in the cooling process. When heated again, these pre-organized structures facilitate the formation of a physical network. As a result, T_{gel} shifts to a lower temperature. These pre-organized structures also facilitate the formation of crystallites in the junctions, leading to larger crystallites and therefore, a lower T_{liq}. With regards to these observations, freshly-prepared samples were used in all the rheological measurements in this study. We used a same heating and cooling rate (1 °C min^{-1}) in the dynamic temperature ramp tests to determine T_{gel}s and T_{liq}s. To examine the effect of heating and cooling rate on the determined T_{gel} and T_{liq}, a 2.5 wt% chitosan solution was tested at various heating and cooling rates. As shown in Fig. S2 in the ESI,‡ the effect of heating and cooling rate is negligible.

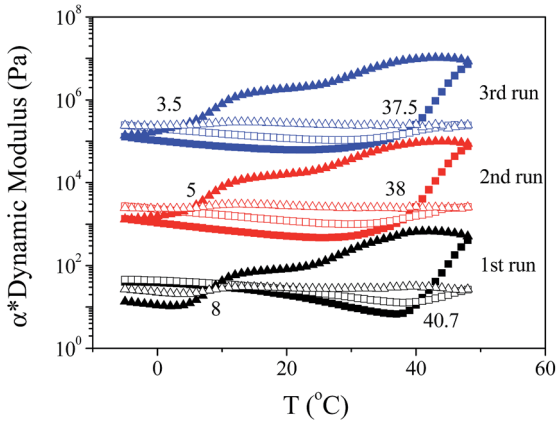

Fig. 6 Evolution of dynamic moduli of a 2.0 wt% chitosan solution in three heating and cooling cycles under a stress of 0.7958 Pa and a frequency of 1.0 Hz. Solid and open squares are for storage and loss moduli (G' and G'') during the heating process, while solid and open triangles are for G' and G'' during the cooling process. For the clarity of presentation, the curves are shifted vertically along their ordinate axis. Ordinate shift factor $\alpha = 10^0$, 10^2, and 10^4 from bottom curves to top curves.

Kinetics of the thermal gelation: isothermal curing

To study the kinetics of the thermal gelation of the chitosan solutions, the solutions were cured at a predetermined temperature, and the changes in dynamic moduli were followed with time. Fig. 7 shows the results obtained from chitosan solutions with a concentration of 3.0 wt%. The curing temperature ranges from 28 °C to 48 °C. At all these temperatures, the initial G'' value is larger than G', suggesting a liquid state. They remain almost unchanged for certain period, and then they begin to increase with time. As G' increases faster than G'', it surpasses G'' and remains larger than G'' thereafter. The curing time at which G' and G'' crossover is defined as the gelation time

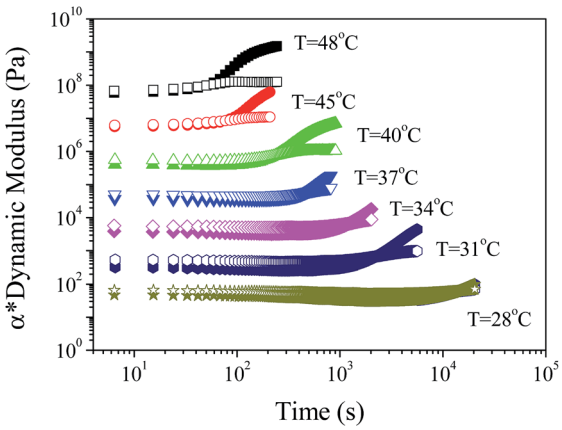

Fig. 7 Evolution of the dynamic modulus of 3.0 wt% chitosan solution with time. The solutions were cured at marked temperatures. The solid and open symbols are G' and G'', respectively. For the clarity of presentation, curves are vertically shifted along their ordinate axis. Ordinate shift factor $\alpha = 10^0$, 10^1, 10^2, 10^3, 10^4, 10^5 and 10^6 from bottom curves to top curves.

(t_{gel}). Note that in the above temperature ramp test, the gelation temperature T_{gel} for 3.0 wt% solution was determined to be 37.3 °C (Fig. 5B). Interestingly, here we found that the solution gels when cured either at temperatures above the determined T_{gel}, or at a temperature below it, provided it is cured at the temperature a long enough time. Similar results were obtained from 2.0 wt% chitosan solution (data not shown).

Although the chitosan solution gels at all the temperatures we studied, it takes a significantly different time for the solution to gel. As shown in Fig. 8A, gelation time increases with decreasing curing temperature. For example, it takes only ~59 s for 3.0 wt% chitosan solution to gel at 48 °C. Whereas, when cured at 28 °C, it takes ~5160 s.

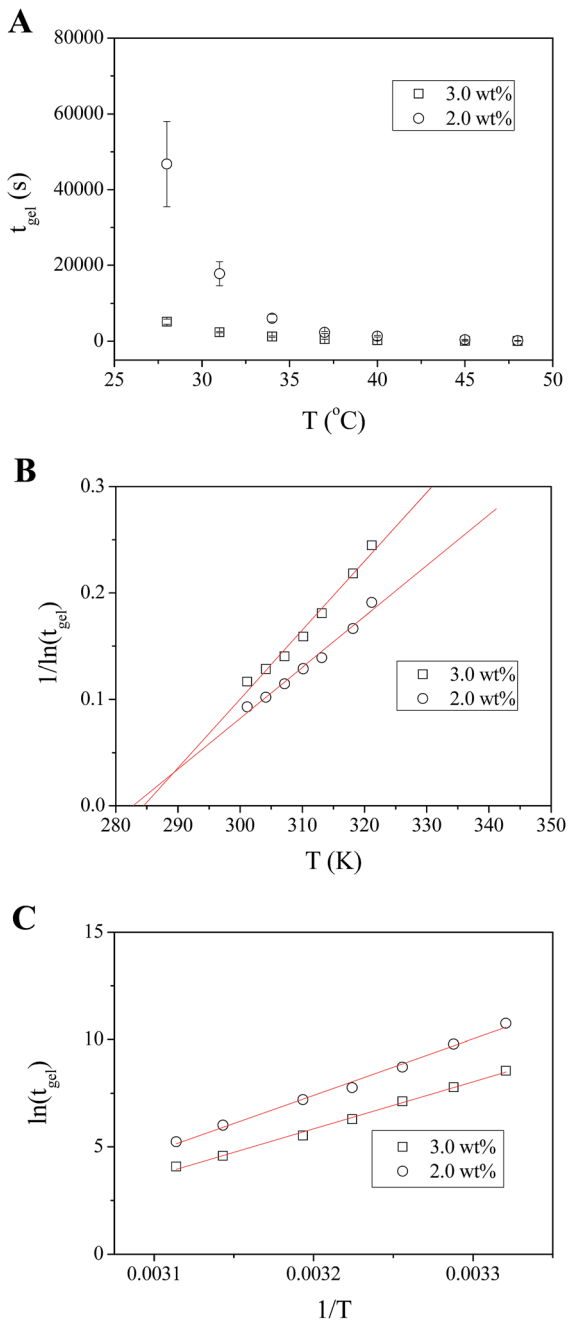

Fig. 8 (A) Plot of gelation time t_{gel} *versus* curing temperature *T*. (B) Plot of 1/ln (t_{gel}) *versus T*. (C) Plot of ln (t_{gel}) *versus* 1/T. [Chitosan] = 2.0 wt% (○) and 3.0 wt% (□), respectively.

It is expected that there exists a critical gelation temperature, below which the solution will not gel. This temperature can be determined by extrapolating the plot to infinite gelation time.[31,49] For this purpose, a plot of 1/ln(t_{gel}) against curing temperature *T* was drawn, which shows a linear relationship between 1/ln(t_{gel}) and *T* (Fig. 8B). By linear regression and extrapolating to infinite gelation time, the critical gelation temperatures were determined to be 11.2 °C for the 3.0 wt% solution and 9.9 °C for the 2.0 wt% solution. It is noticeable that the critical gelation temperature determined for the two solutions are almost the same. To avoid the gelation of the chitosan solution, it could be stored at temperatures lower than this critical temperature. For example, a 3.0 wt% solution was stored at 4 °C and still can flow after 16 day-storage (Fig. S3 in ESI‡).

The gelation process can be described as a chemical reaction which forms junctions among the polymer chains. Therefore, the gelation time t_{gel} is inversely proportional to the reaction rate constant *k*, which relates to the temperature *T* according to the Arrhenius equation as follows:[50]

$$k = A \exp\left(\frac{-E_a}{RT}\right)$$

where E_a is an apparent activation energy and *R* is the gas constant. Therefore, the relationship between t_{gel} and temperature *T* is deduced as follows:

$$\ln(t_{gel}) \sim \frac{E_a}{RT}$$

As shown in Fig. 8C, when ln(t_{gel}) is plotted against 1/*T*, a linear relationship is observed. From the slope of the fitted line, the apparent activation energies of the gelling reaction were determined to be ~182 kJ mol^{-1} for the 3.0 wt% solution, and ~218 kJ mol^{-1} for the 2.0 wt% solution. The activation energy for the thermal gelling of cellulose in 7 wt% NaOH/12 wt% urea was previously reported to be 77.1 kJ mol^{-1} (in the temperature range from 0 to 30 °C). E_a for chitosan is therefore considerably larger than that of cellulose. In the Arrhenius equation, E_a is used to define the sensitivity of a particular reaction to temperature. The larger E_a for chitosan indicates the chitosan solution is more temperature-sensitive than cellulose.

To study the effect of chitosan concentrations on the kinetics of the thermal gelation, chitosan solutions with various concentrations were investigated. Their gelation kinetics was studied at two curing temperatures: 31 and 40 °C, and the results measured at 40 °C are shown in Fig. 9. Crossover of G′ and *G″* was observed in all the cases. The gelation time, t_{gel}, was plotted against chitosan concentration (Fig. 10A). At each curing temperature, t_{gel} dramatically increases with decreasing chitosan concentration. For example, when cured at 40 °C, it takes only ~160 s for 3.5 wt% solution to gel, whereas it takes ~1340 s for a 2.0 wt% solution to gel. As mentioned above, the gelation process can be described as a chemical reaction which forms junctions among the polymer chains.[50] A higher polymer

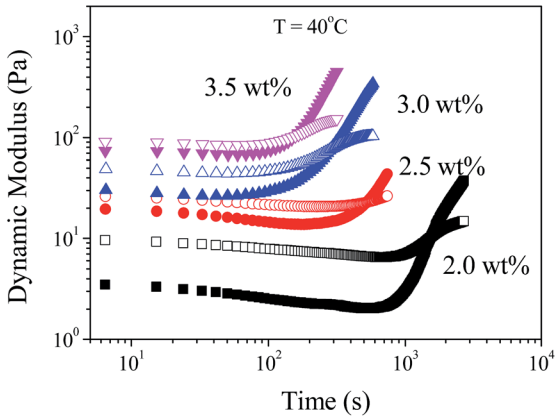

Fig. 9 Evolution of dynamic modulus with the time of the chitosan solutions with concentration as marked. The solutions were cured at 40 °C. The solid and open symbols are G' and G'', respectively.

concentration increases the chance for the polymer chains to collide and form junctions, therefore, the gelation rate increases with increasing chitosan concentration. Similar results were reported for the thermal gelation of PNIPAM microgel dispersions,[31] globular proteins[50] and also cellulose in similar solvents.[36] It is reasonable to expect that there will be a critical gelation concentration below which the solution will not gel, which can be determined by extrapolating to infinite gelation time.[49,51] In Fig. 10B $1/t_{gel}$ is plotted against chitosan concentration and it can be observed that there is a linear relationship between them. By linear regression, the critical gelation concentration was determined to be 1.90 wt% at 40 °C and 1.97 wt% at 31 °C.

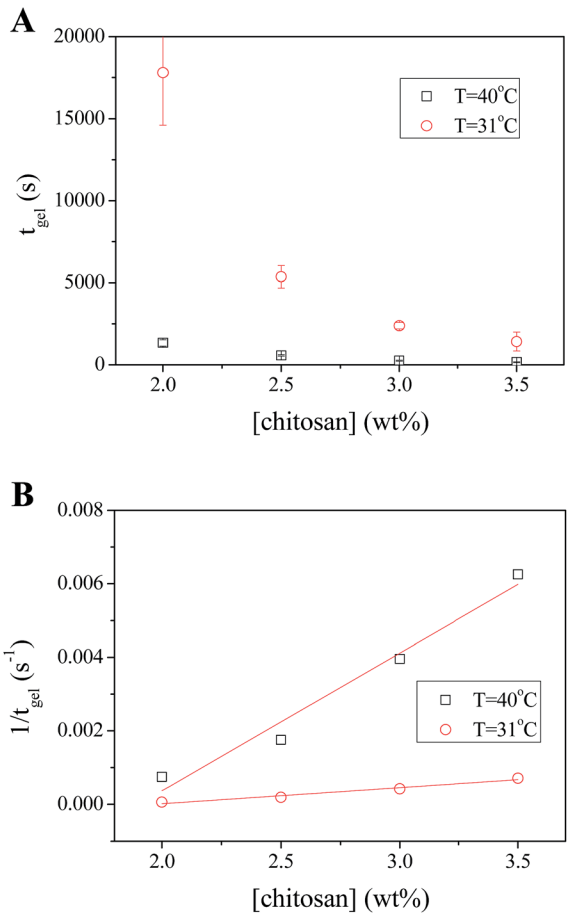

Fig. 10 (A) Plot of gelation time t_{gel} *versus* chitosan concentration [chitosan]. The curing temperature T is 31 °C (○) and 40 °C (□), respectively. (B) The plot was redrawn as a semi-reciprocal plot to determine the critical gelation concentration.

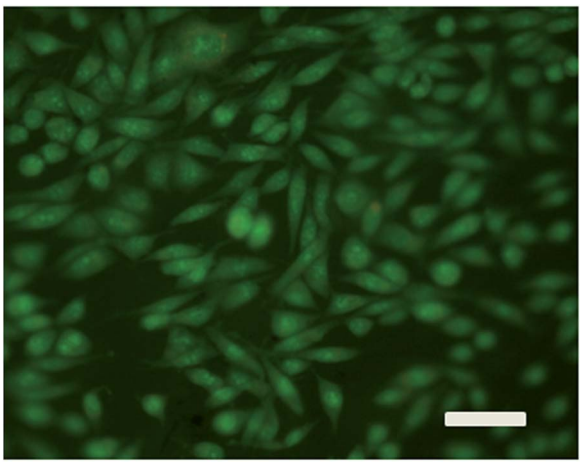

Fig. 11 Fluorescence image of NIH 3T3 cells cultured on chitosan hydrogel. The cells were double stained with AO/EB. Scale bar: 100 μm.

Cell culture

It is well-known that chitosan is biocompatible. In a preliminary test, the chitosan hydrogels obtained by *in situ* thermal gelation were repeatedly washed with water to remove NaOH and urea; NIH 3T3 cells were then seeded on the gel. Fig. 11 shows the image of the cells after being cultured for 12 h. Before imaging the cells were double stained with AO/EB. Thus, the live cells were stained green while the dead ones were red. As shown in Fig. 11, the cells can attach on the gel surface. Most of the cells are alive, suggesting that the gel can support the growth of cells.

Conclusions

In conclusion, chitosan can be dissolved in a precooled aqueous alkali–urea solution, a solvent which was previously developed to dissolve cellulose. The resulting solutions are thermosensitive and can undergo a sol–gel transition on heating. The solvent of the *in situ* formed gel can be changed to water, by repeated soaking in water. Preliminary test shows the new chitosan gel is biocompatible and supports the growth of cells. The new gel consists of only chitosan, without any crosslinker or additive. It is expected that this hydrogel will find biomedical applications such as wound dressing.

The thermal gelling behaviours of chitosan in the aqueous alkali–urea solution were thoroughly studied by rheology. In temperature ramp test, the determined gelation temperature

does not show significant concentration-dependency, behaviour close to that of strong thermosensitive polymers, such as PNIPAM. The gelation is reversible, despite a large hysteresis existing in the cooling process. Although gelation temperature in the heating process is relatively concentration-independent, liquefication temperature in the cooling process decreases when the polymer concentration increases. The kinetics of the thermal gelation was also studied by isothermal curing. It was found that the thermal gelation can be achieved by isothermal curing at a temperature even below the gelation temperature, determined in the temperature ramp test, provided that the solution is cured at that temperature for long enough. The curing time required for the thermal gelation decreases with increasing curing temperature and the concentration of chitosan.

Acknowledgements

We thank financial support for this work from the National Natural Science Foundation of China (Grants nos 21174070, 21274068, 21228401 and 21374048), Tianjin Public Health Bureau(13KG110), Program for New Century Excellent Talents in University (NCET-11-0264), and PCSIRT program (IRT1257).

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