



Reaction kinetics and gel properties of blocked diisocyanate crosslinked chitosan hydrogels

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Abstract

Cure kinetics and network properties of hydrogels prepared by the reaction of chitosan and a water-soluble blocked diisocyanate cross-linker have been measured using swelling experiments and rheometry. The gel time measured by two different rheological methods, the rate equivalence of the change in modulus as a function of reaction time ($G'G''$ crossover) and the critical gel via the Winter–Chambon approach, are comparable. The reaction rate increased with increased temperature following the Arrhenius behavior, from which apparent activation energies can be determined. The network moduli can be tailored by adjusting the ratio of chitosan to cross-linker or the solution concentration. This allows for the formation of processable chemically cross-linked chitosan hydrogels with controlled kinetics and mechanical properties.

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Keywords: Chitosan; Hydrogels; Crosslink kinetics; Gel modulus

1. Introduction

Chitin is the second most abundant natural biopolymer on earth and is composed of β (1 $\rightarrow$ 4)-linked 2-acetamido-2-deoxy- β -D-glucose (*N*-acetylglucosamine). The principle derivative of chitin, chitosan, is obtained by *N*-deacetylation, to a varying extent. The result is a co-polymer of *N*-acetylglucosamine and glucosamine, characterized by the degree of deacetylation (Fig. 1(a)). Due to their biocompatibility, biodegradability and other unique properties, chitin and its derivatives have found wide application in a variety of areas such as: medicine, pharmaceuticals, paper production, textiles, metal chelation, food additives, antimicrobial agents, adhesives, and other industrial applications (Struszczyk, 2002a,b,c). We are especially intrigued by the structural similarities between chitosan and the glycosaminoglycans (GAGs) found in the extracellular matrix (ECM) of mammalian tissue. These similarities give rise to many possible uses of chitosan-based hydrogels as scaffolds for tissue engineered medical products (TEMPs) (Nettles, Elder, & Gilbert, 2002;

Suh & Matthew, 2000; Tan, Krishnaraj, & Desai, 2001; Zhang & Zhang, 2001).

A number of methods have been developed for the cross-linking of chitosan, including chemical cross-linking with glutaraldehyde (Hirano, Yamaguchi, Matsuda, Miura, & Kondo, 1977) and with Mo(VI) polyoxyanions (Draget, Varum, Moen, Gynnild, & Smidsrod, 1992). We recently prepared a water-soluble, blocked diisocyanate as a cross-linking agent for the network formation (Welsh, Schauer, Qadri, & Price, 2002). This cross-linker remains soluble in water and therefore allows for easy processability of chitosan gel formations. With greater control over cross-link efficiency, specifically the chemical cross-linking of amine groups, the physical, mechanical, and biological properties of chitosan gels can be tailored for a given application. Furthermore, the cross-linking chemistry allows for a straightforward, covalent attachment of amine-containing biochemical moieties such as collagen and fibronectin. Before this class of chitosan-based hydrogels can realize their full potential, a detailed understanding of the network structures and properties is required.

Rheological measurements have been used by various groups to characterize chitosan solutions, gels and cross-linked networks (Arguelles-Monal, Goycoolea,

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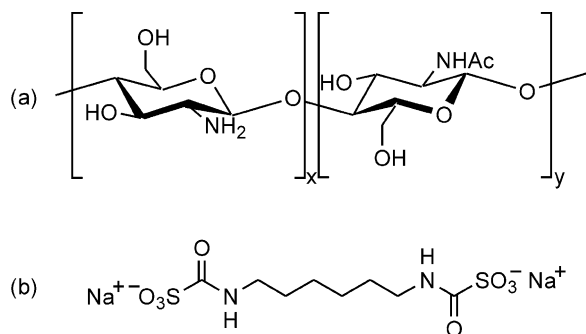


Fig. 1. Structure of chitin ($x = 0$) and chitosan (a), and hexamethylene-1,6-di-(aminocarboxysulfonate) (b).

Peniche, & Higuera-Ciapara, 2003; Draget, 1996; Jiang, Su, Mather, & Bunning, 1999; Johnson, Dunstan, & Franks, 2002; Khalid, Ho, Agnely, Grossiord, & Couarraze, 1999). Physical gelation has been observed for chitosan solutions at high solution concentrations (Iversen et al., 1997) and high in N -acetyl glucosamine residues (Draget, 1996). The degree of cross-linking for chemically cross-linked chitosan has been correlated with the rheology of the gel (Brack, Tirmizi, & Risen, 1997). Several studies have provided details on the cure kinetics (Chenite, Buschmann, Wang, Chaput, & Kandani, 2001; Li & Xu, 2002). For the gelation behavior of chitosan–glutaraldehyde systems, several variables including the concentration of reagent, temperature and the acetic acid concentration have been examined (Roberts & Taylor 1989). To our knowledge, no systematic kinetic study using the widely accepted Winter–Chambon criteria for gel point assessment has been conducted on chemically cross-linked chitosan hydrogels. In the present study, we investigate the cure kinetics and macroscopic properties of chitosan network formation processes by investigating the gel time (onset of gelation) and the gel modulus (elastic modulus of the cured networks), and compare the results with those obtained using the Winter–Chambon approach. This investigation provides a basis from which cell activity on scaffolds with multivariate material properties can be explored.

2. Experimental

Unless indicated otherwise, deionized water was used to prepare chitosan hydrogels.

2.1. Deacetylation of chitosan

Seacure chitosan (Vanson-Halosource¹) was further deacetylated (Mima, Miya, Iwamoto, & Yoshikawa,

1983) by suspending 200 g of the commercial preparation in 20% by volume fraction NaOH (2.5 l) at 60 °C for 2 h under a nitrogen atmosphere, followed by washing with 20 l deionized water. The alkali treatment was repeated once, and the remaining solids were washed with deionized water until the pH matched that of the wash water. The product was then dissolved in 2% acetic acid (4 l) from which the acid-insoluble material was removed by filtration and the chitosan was recovered by precipitation of the soluble fraction into 1 N NaOH (6 l). After extensive dialysis against water (5 d), the chitosan was neutralized by addition of dry ice and decolorized by refluxing in acetone for 2 h under a nitrogen atmosphere. Dried solids were pulverized in a mill to 200 μm sieve pass. The final product (122 g) was obtained after drying in vacuo at 50 °C for 48 h.

2.2. Synthesis of hexamethylene-1,6-di-(aminocarboxysulfonate)

In a 100 ml round-bottom flask containing a magnetic stir bar, 6.73 g (40 mmol) hexamethylene diisocyanate was added to 8.36 g $\text{Na}_2\text{S}_2\text{O}_5$ (44 mmol) dissolved in 15.53 ml H_2O and was stirred for 20 h at room temperature. The product was precipitated in acetone and dried in vacuo. Insoluble polymeric byproducts were removed by dissolving the product in water (30 ml) followed by filtration. The product was isolated from the filtrate by precipitation in acetone and dried in vacuo, resulting in a white powder with a 75% yield.

2.3. Swelling measurements

Chitosan solution (10% chitosan by mass fraction, 5% acetic acid by mass fraction) and the calculated amount of cross-linker were combined into centrifuge tubes. Additional water was added to adjust the solution concentration to 5% chitosan by mass fraction. The reagents were mixed thoroughly, and then centrifuged to remove air bubbles. Curing was achieved by heating the mixtures in the sealed tubes at 60 °C for 48 h. Upon cooling, gels were carefully removed from the tubes, cut into three sections, and submerged in RO water (50 ml), which was changed three times over a 24 h period. The hydrated mass (m_h) was determined by weighing the gels that had been blotted with tissue paper to remove excess surface moisture. These gels were then dried for 72 h in vacuum at room temperature with the last 24 h of drying over P_2O_5 . The dry mass (m_d) was subsequently determined. Equilibrium water content (EWC) was calculated as $(m_h - m_d)/m_h \times 100\%$. Reported values are averages of three measurements, and the root mean square standard deviation between measurements is reported as the relative standard uncertainty.

¹ Certain commercial materials and equipment are identified in this paper in order to specify adequately the experimental procedure. In no case does such identification imply recommendation by the National Institute of Standards and Technology nor does it imply that the material or equipment identified is necessarily the best available for this purpose.

2.4. Rheology sample preparation and measurements

Chitosan solution (10% chitosan by mass fraction, 5% acetic acid by mass fraction) and the calculated amount of cross-linker were weighed into clean vials. Additional water was added to adjust the chitosan concentration to obtain the desired concentration. The reagents were mixed thoroughly and then centrifuged to remove air bubbles. Samples were stored in a refrigerator and were generally used within three days of preparation. Rheological measurements were performed on a Rheometric Scientific ARES instrument with a parallel plate geometry (25 mm diameter) or a Rheometric Scientific SR-5000 instrument with a parallel plate geometry (40 mm diameter). Solvent (water) evaporation was minimized by covering the edges of the sample in a pool of low viscosity silicone oil. In general, no water evaporation was detected for at least 15 h with adequate oil coverage. Duplicate experiments showed excellent reproducibility with relative standard uncertainty of 3%.

3. Results and discussion

The chitosan prepared for the current study is readily soluble in dilute acetic acid solutions, has a degree of deacetylation of 88.6% (Domszy & Roberts 1985), and has a viscosity-average molecular weight of 2.2×10^5 g/mol. The intrinsic viscosity was determined from a dual Huggins–Kraemer plot to be (243 ± 0.76) cm³/g. These numbers correspond to a molecular formula given by Fig. 1(a), where $x = 1203$ and $y = 154$, or an amine concentration of 5.3 mmol/g of chitosan. A bisulfite-protected diisocyanate that is also readily soluble in aqueous solutions serves as the cross-linking agent (Fig. 1(b)). Protecting the diisocyanate reduces its reactivity and imparts water solubility, thus enabling better control over aqueous based cure reactions. While stable under acidic aqueous conditions at room temperature, the diisocyanate adduct readily reacts with amines at increased pH or elevated temperature to form a urea linkage. Furthermore, the diisocyanate–amine reaction rate is much greater than that characteristic of competing alcohol- or water–amine reactions (Wicks & Wicks 2001). The reactivity of blocked diisocyanates with amines can also be tuned by selecting, as appropriate, the less reactive aliphatic or the more reactive aromatic diisocyanates. Therefore, the amenability to stable storage and well-controlled cure reactions with chitosan makes the cross-linker used in the current study an attractive choice.

A direct and simple method for assessing the cross-link density is by measuring the degree of swelling in water, where the amount of water uptake is related to the extent of cross-link of a network (Flory, 1953). Fig. 2 shows the equilibrium water content measured for chitosan gels cured with various amounts of cross-linker. The equilibrium water

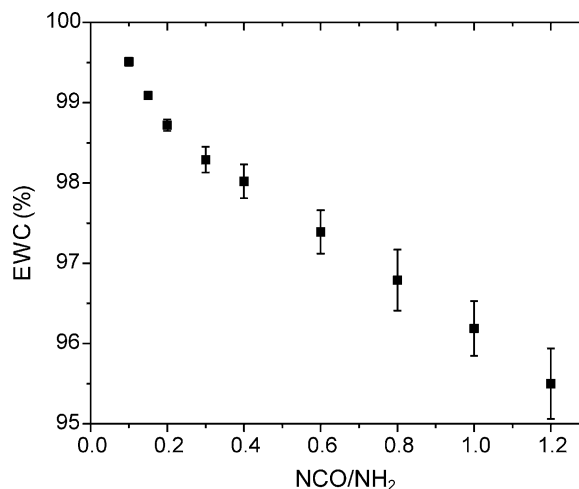


Fig. 2. Equilibrium water content (EWC) measured for chitosan gels cured with various amounts of cross-linker, solution concentration is 5% chitosan by mass fraction.

content decreased with increased cross-linker suggesting the formation of denser networks. It is interesting to note that the equilibrium water content does not show a minimum at the stoichiometric ratio as would be expected, assuming full conversion. The data suggest that the network density continues to increase with an increasing NCO/NH₂ ratio up to at least 1.2. This indicates that the cross-linker does not react stoichiometrically with all NH₂ groups. This could be due to a combination of factors that include: insufficient reaction time; decreased mobility, accessibility and proximity of reacting species; or side reactions, such as hydrolysis of the blocked isocyanate and further deacetylation of the chitosan, which would generate more reactive sites than was originally considered.

In order to gain a more quantitative understanding of the gelation kinetics and gel properties, we used rheological measurements in which the storage modulus (G') and loss modulus (G'') were monitored. To determine an appropriate method for assessing the gelation time, we used two different techniques, dynamic time sweeps and dynamic frequency sweeps at various cure times using an SR-5000 instrument. A small stress (1 Pa) was applied at low frequencies ($\omega = 1$ –10 rad/s) for these measurements to minimize disturbance to the gelation process. Fig. 3(a) shows the dynamic time sweep results from which the gelation time was obtained and was defined as the time at which G' and G'' intersected. Prior to gelation, G'' was greater than G' indicating liquid-like behavior; solid-like behavior dominates after gelation as G' was significantly greater than G'' . Fig. 3(b) shows the results obtained by dynamic frequency sweep measurements at various reaction times by plotting the loss tangent ($\tan \delta$) measured at various frequencies as a function of time. According to the Winter–Chambon criterion (Winter, Morganelli, & Chambon, 1988), the time at which $\tan \delta$ curves converge is defined as the gel point. The corresponding

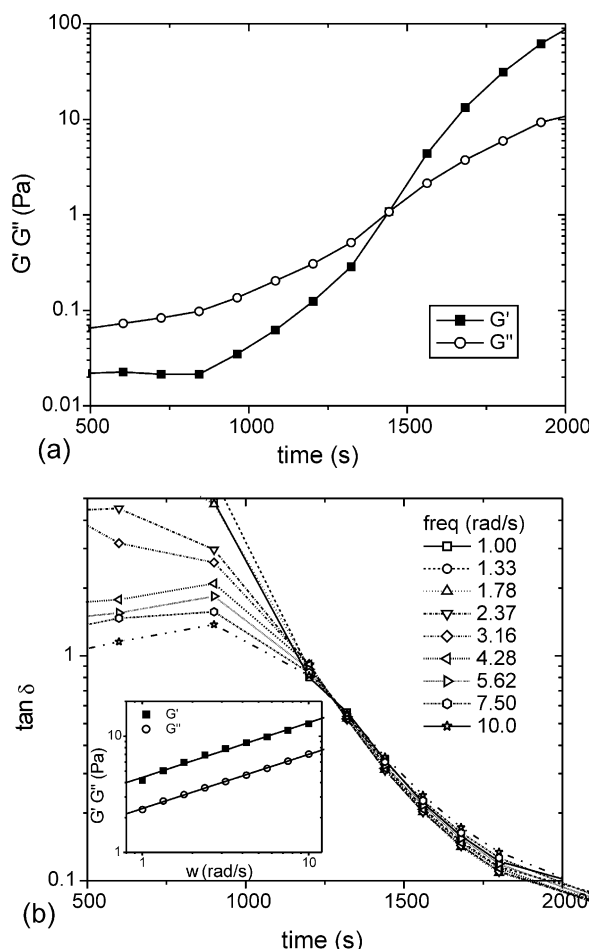


Fig. 3. (a) Isothermal reaction at 80 °C, $\omega = 1$ rad/s, (b) $\tan \delta$ versus reaction time for different frequencies at 80 °C reaction. Inset shows the frequency sweep at 1320 s.

frequency-dependent measurement at the gel point should exhibit the same slope for G' and G'' . The insert of Fig. 3(b) shows the frequency sweep measured at 22 min, the closest measurement to the $\tan \delta$ convergence time (21 min). The slope of G' and G'' are essentially the same, again confirming the gelation process.

The gel time measured by the rate equivalence of the change in modulus as a function of reaction time (G'/G'' crossover) and the critical gel (convergence of $\tan \delta$) are 1430 s (24 min) and 1250 s (21 min), respectively. The small differences observed by the two methods are expected and are attributed to the differences in the imposed strain and frequencies applied during the measurements. In addition, both experimentally determined gel times are expected to differ slightly from the static gel time where the system is unperturbed. However, it is difficult to assess the exact difference since experimental conditions could both promote gelation by mixing and disrupt gelation by perturbation (Smith & Ishida 1999). Nonetheless, the current experiments do provide reasonable gel times for the experimental conditions studied.

In order to investigate gelation within the larger parameter space of cure temperatures, reactive group ratios (amine:isocyanate), and solution concentrations, we limited further measurements to dynamic time sweep experiments. From these experiments, both the gelation time and gel modulus (if accessible within a reasonable experimental time frame) were determined. A small strain-amplitude (2%) was applied at a frequency of 1 rad/s to ensure that the experimental conditions did not interfere with the gelation process.

The effect of temperature on the cure kinetics and the resultant gel modulus was measured for chitosan cured between 60 and 90 °C, with 20% cross-linker (i.e. NCO/NH₂ = 0.2), and with a solution concentration of 5% chitosan by mass fraction. Fig. 4(a) shows an example of the G' and G'' measured as a function of cure time at 70 °C. The data collected before gelation was near the sensitivity limit of the transducer and were therefore noisy. Both G' and G'' increased beyond the gel point and reached a plateau at high cross-link conversions.

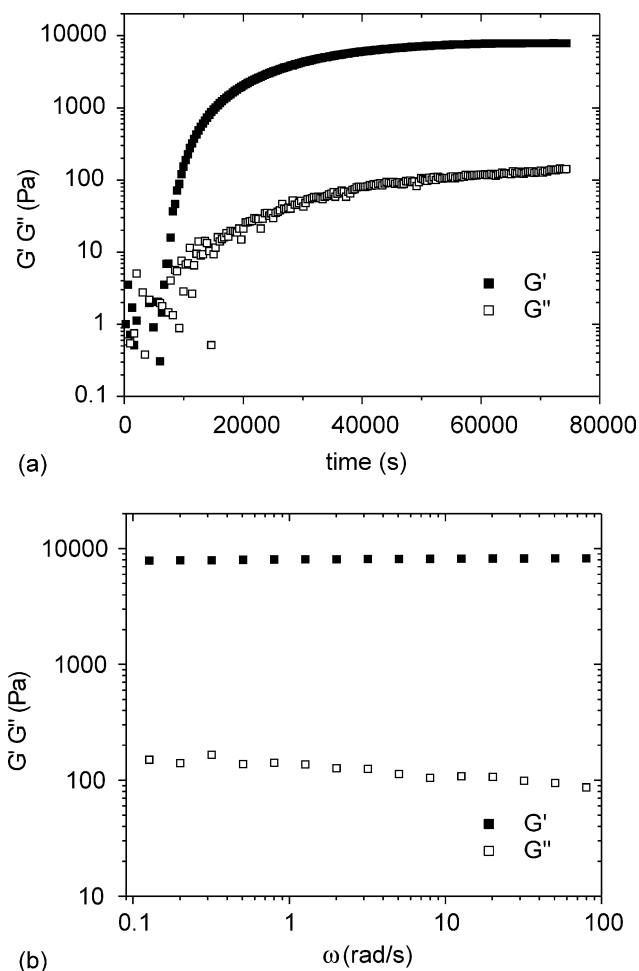


Fig. 4. (a) Dynamic time sweep of chitosan cured with 20% cross-linker measured at 70 °C. (b) Dynamic frequency sweep of the fully reacted chitosan cured with 20% cross-linker at 70 °C.

Dynamic frequency sweeps were measured following each dynamic time sweep to assess the degree of cross-linking. Fig. 4(b) shows the corresponding G' and G'' for the material cured in Fig. 4(a) where $G' \gg G''$ and both moduli were nearly independent of frequency in the frequency region probed. This verifies solid-like behavior in the cured systems and suggests that a high degree of reaction conversion was obtained (Macosko, 1994). All networks cured at various temperatures showed similar frequency sweep profiles.

From the dynamic time sweep experiments and the subsequent dynamic frequency sweeps, we determined the cure temperature dependence of both the gel time and the gel modulus. The gel time is defined as the time at which G' intersects G'' from the dynamic time sweep measurements, and the gel modulus is the modulus observed at 1 rad/s from the corresponding dynamic frequency sweeps. As expected, the gel time decreased as the cure temperature increased. The gel moduli of networks cross-linked at different temperatures (between 80 and 90 °C) were comparable and were (9700 ± 300) Pa. This indicates that the reaction temperature only affects the rate of the cure but not the structure of the cured networks. The gel moduli cured at lower temperatures could not be determined accurately due to the extremely long time required to reach high cross-link conversions.

Fig. 5 shows a semi-logarithmic plot of the gel time versus inverse reaction temperature. This Arrhenius analysis was used to calculate the apparent activation energy for the gel forming processes. The linear relationship suggests that the underlying cure mechanism does not change as a function of reaction temperature. An apparent activation energy of 103 kJ/mol was calculated from the slope of the line. Using the apparent activation energy and the corresponding pre-exponential factor, the gelation time at any given temperature can be calculated. The apparent activation energy for chitosan/blocked-diisocyanate reaction is significantly higher than those observed for

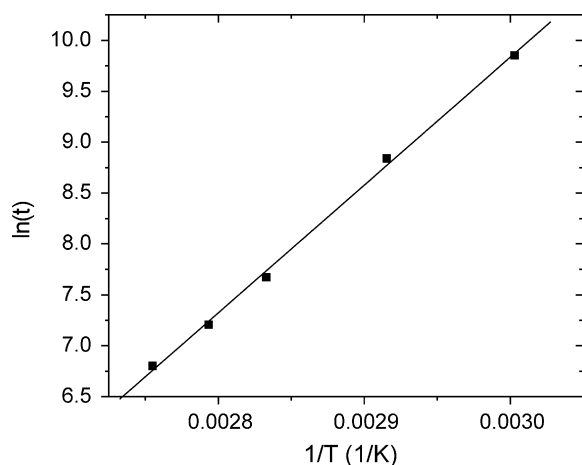


Fig. 5. Arrhenius plot for chitosan cured with 20% cross-linker, the line is a linear fit of the data points.

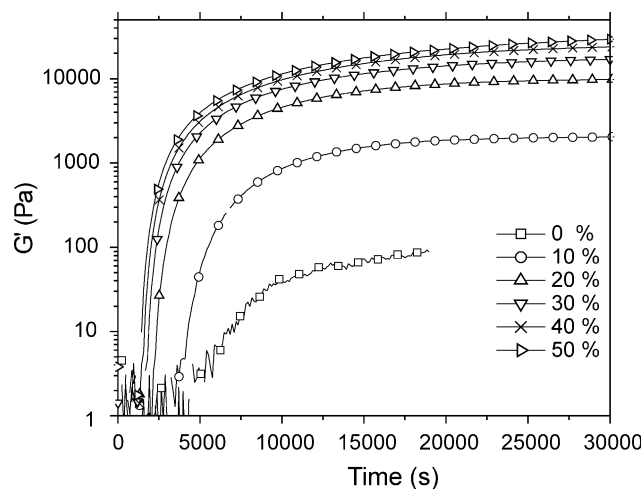


Fig. 6. G' measured as a function of reaction time for chitosan reacted with various amount of crosslinker at 80 °C, solution concentration is 5% by mass fraction.

chitosan–glutaraldehyde reaction (63.1 kJ/mol) (Roberts & Taylor 1989) and for *N*-acetylchitosan (68 kJ/mol) (Moore & Roberts 1980).

The effect of cross-linker composition was also investigated. To vary the degree of cross-linking, the blocked-diisocyanate was added to acidic chitosan solutions (5% by mass fraction) with isocyanate to amine percentages of 0, 10, 20, 30, 40 and 50. Fig. 6 shows the G' observed from dynamic time sweeps for chitosan cured with various amount of cross-linker. Interestingly, the modulus for the chitosan solution without cross-linker increased after exposure to elevated temperatures. However, the modulus of the pure chitosan was significantly lower than the chemically cross-linked networks. The increased modulus was presumably due to the formation of an associated or physically cross-linked system as previously observed (Draget, 1996). Chitosan reacted with 5% blocked diisocyanate (not shown) did not form a chemically cross-linked network. At this large stoichiometric offset, there was not enough diisocyanate to form a three-dimensional network through a percolation type transition. For chitosan cured with (10–50%) cross-linker, gelation occurred more rapidly with increased cross-linker. The gel modulus also increased with increased cross-linker due to the formation of more highly cross-linked networks. These results are consistent with those obtained using the swelling measurements and with statistical treatment of idealized networks from rubber elasticity theory.

Fig. 7 plots the gelation time and gel modulus as a function of cross-linker composition. The decreased gelation time with increased cross-linker content is a result of higher reactant concentration, which leads to an increased reaction rate. The gel modulus increased with increased cross-linker content because more highly cross-linked networks were formed. The increase in gel modulus

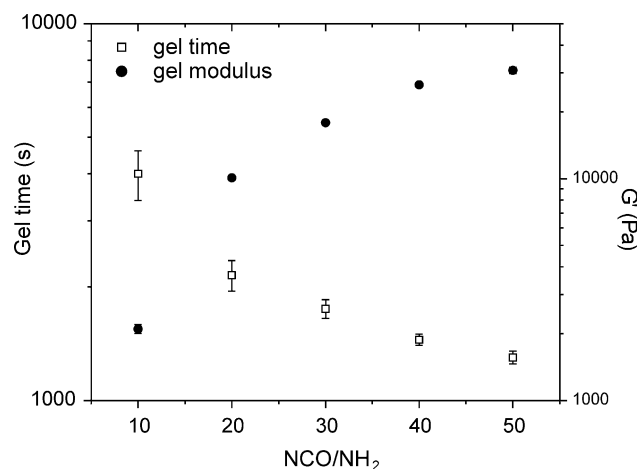


Fig. 7. Gelation time and gel modulus determined for chitosan cross-linked with various amount of cross-linker (%) at 80 °C.

appeared to reach a plateau as the NCO to NH₂ ratio approaches a stoichiometric equivalence.

The effect of solution concentration on the cure kinetics and gel properties was examined using solutions of chitosan mixed with 20% cross-linker diluted with various amounts of water. Fig. 8 shows G' measured as a function of reaction time for solution concentrations of (2.5–10%) chitosan by mass fractions. For the more concentrated solutions, (5, 6.6 and 10%) chitosan by mass fraction, reactions between chitosan and cross-linker led to chemically cross-linked hydrogels. The gelation time decreased and the gel modulus increased with increased solution concentration (Table 1), which is consistent with both network theory and chemical kinetics. The observed differences in the gel modulus could be the result of different degrees of swelling, if the reaction conversion is the same, or of different degrees of conversion arise from different solution concentration. Interestingly, at the same chitosan to cross-linker ratio, the most dilute

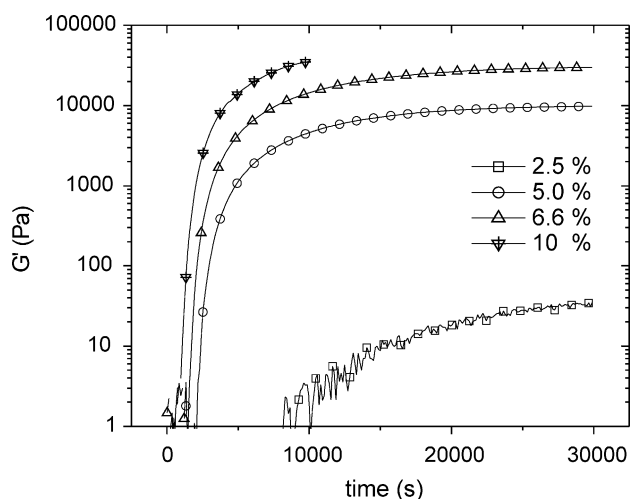


Fig. 8. G' measured as a function of reaction time for chitosan cured with 20% cross-linker at 80 °C with various solution concentrations.

Table 1

Gel time and gel modulus measured as a function of solution concentration for chitosan cured with 20% cross-linker cured at 80 °C

Solution concentration (%)	Gel time (s)	Gel modulus (Pa)
2.5	11000 ± 600	55 ± 5
5	2150 ± 200	10100 ± 300
6.6	1570 ± 100	30000 ± 1000
10	1100 ± 50	>41000

solution (2.5% by mass fraction) did not form chemically cross-linked networks. This is presumably due to the dilute nature of the solution, which drastically reduced the probability of chitosan to cross-linker reaction.

4. Conclusions

Swelling measurements and rheometry were used to assess the cure kinetics and network properties of hydrogels prepared by the reaction of chitosan and a water-soluble blocked diisocyanate cross-linker. Both techniques showed increased cross-link density with increased amount of diisocyanate cross-linker up to 1.2 equivalence cross-linker per reactive site on chitosan. Comparable gel times were observed by two different rheological methods, the rate equivalence of the change in modulus as a function of reaction time ($G'G''$ crossover) and the critical gel via the Winter–Chambon approach. For a given composition, the gel time decreased, and therefore, the reaction rate increased with increased temperature. The reaction kinetics followed the Arrhenius behavior from which apparent activation energies could be determined. The network moduli can be tailored by adjusting the ratio of chitosan to cross-linker or the solution concentration where the former affects the degree of cross-link and the later is a concentration effect. The present study provides parameters, which will allow for the formation of processable chemically cross-linked chitosan hydrogels with well-defined reaction kinetics and mechanical properties. The suitability of the present cross-linker in biomedical applications will be a subject of future research.

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References

- Arguelles-Monal, W., Goycoolea, F. M., Peniche, C., & Higuera-Ciupara, I. (2003). Rheological Study of the Chitosan/Glutaraldehyde Chemical Gel System. *Polymer Gels and Networks*, 6, 429–440.

- Brack, H. P., Tirmizi, S. A., & Risen, W. M. (1997). A spectroscopic and viscometric study of the metal ion-induced gelation of the biopolymer chitosan. *Polymer*, 38, 2351–2362.
- Chenite, A., Buschmann, M., Wang, D., Chaput, C., & Kandani, N. (2001). Rheological characterisation of thermogelling chitosan/glycerol-phosphate solutions. *Carbohydrate Polymers*, 46, 39–47.
- Domszy, J. G., & Roberts, G. A. F. (1985). Evaluation of infrared spectroscopic techniques for analyzing chitosan. *Makromolekulare Chemie—Macromolecular Chemistry and Physics*, 186, 1671–1677.
- Draget, K. I. (1996). Associating phenomena in highly acetylated chitosan gels. *Polymer Gels and Networks*, 4, 143–151.
- Draget, K. I., Varum, K. M., Moen, E., Gynnild, H., & Smidsrod, O. (1992). Chitosan cross-linked with Mo(VI) polyoxyanions—A new gelling system. *Biomaterials*, 13, 635–638.
- Flory, P. J. (1953). *Principles of polymer chemistry*, 576–589.
- Hirano, S., Yamaguchi, R., Matsuda, N., Miura, O., & Kondo, Y. (1977). Chitosan–aldehyde gel a novel polysaccharide gel produced from chitosan and aldehydes. *Agricultural and Biological Chemistry*, 41, 1547–1548.
- Iversen, C., Kjoniksen, A. L., Nystrom, B., Nakken, T., Palmgren, O., & Tande, T. (1997). Linear and non-linear rheological responses in aqueous systems of hydrophobically modified chitosan and its unmodified analogue. *Polymer Bulletin*, 39, 747–754.
- Jiang, H., Su, W., Mather, P. T., & Bunning, T. J. (1999). Rheology of highly swollen chitosan/polyacrylate hydrogels. *Polymer*, 40, 4593–4602.
- Johnson, S. B., Dunstan, D. E., & Franks, G. V. (2002). Rheology of cross-linked chitosan–alumina suspensions used for a new gelcasting process. *Journal of the American Ceramic Society*, 85, 1699–1705.
- Khalid, M. N., Ho, L., Agnely, F., Grossiord, J. L., & Couarraze, G. (1999). Swelling properties and mechanical characterization of a semi-interpenetrating chitosan/polyethylene oxide network—Comparison with a chitosan reference gel. *Stp Pharma Sciences*, 9, 359–364.
- Li, J. J., & Xu, Z. H. (2002). Physical characterization of a chitosan-based hydrogel delivery system. *Journal of Pharmaceutical Sciences*, 91, 1669–1677.
- Macosko, C. W. (1994). *Rheology: Principles, measurements, and applications*.
- Mima, S., Miya, M., Iwamoto, R., & Yoshikawa, S. (1983). Highly deacetylated chitosan and its properties. *Journal of Applied Polymer Science*, 28, 1909–1917.
- Moore, G. K., & Roberts, G. A. F. (1980). Chitosan gels. 2. Mechanism of gelation. *International Journal of Biological Macromolecules*, 2, 78–80.
- Nettles, D. L., Elder, S. H., & Gilbert, J. A. (2002). Potential use of chitosan as a cell scaffold material for cartilage tissue engineering. *Tissue Engineering*, 8, 1009–1016.
- Roberts, G. A. F., & Taylor, K. E. (1989). Chitosan Gels. 3. The formation of gels by reaction of chitosan with glutaraldehyde. *Makromolekulare Chemie—Macromolecular Chemistry and Physics*, 190, 951–960.
- Smith, M. E., & Ishida, H. (1999). Critical gel phenomena in the rheology of epoxide network formation. *Journal of Applied Polymer Science*, 73, 593–600.
- Struszczyk, M. H. (2002a). Chitin and chitosan—Part I. Properties and production. *Polimery*, 47, 316–325.
- Struszczyk, M. H. (2002b). Chitin and chitosan—Part II. Applications of chitosan. *Polimery*, 47, 396–403.
- Struszczyk, M. H. (2002c). Chitin and chitosan—Part III. Some aspects of biodegradation and bioactivity. *Polimery*, 47, 619–629.
- Suh, J. K. F., & Matthew, H. W. T. (2000). Application of chitosan-based polysaccharide biomaterials in cartilage tissue engineering: A review. *Biomaterials*, 21, 2589–2598.
- Tan, W., Krishnaraj, R., & Desai, T. A. (2001). Evaluation of nanostructured composite collagen-chitosan matrices for tissue engineering. *Tissue Engineering*, 7, 203–210.
- Welsh, E. R., Schauer, C. L., Qadri, S. B., & Price, R. R. (2002). Chitosan cross-linking with a water-soluble, blocked diisocyanate. 1. Solid state. *Biomacromolecules*, 3, 1370–1374.
- Wicks, D. A., & Wicks, Z. W. (2001). Multistep chemistry in thin films; the challenges of blocked isocyanates. *Progress in Organic Coatings*, 43, 131–140.
- Winter, H. H., Morganelli, P., & Chambon, F. (1988). Stoichiometry effects on rheology of model polyurethanes at the gel point. *Macromolecules*, 21, 532–535.
- Zhang, Y., & Zhang, M. Q. (2001). Synthesis and characterization of macroporous chitosan/calcium phosphate composite scaffolds for tissue engineering. *Journal of Biomedical Materials Research*, 55, 304–312.