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Monolithic gelation of chitosan solutions via enzymatic hydrolysis of urea

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Abstract

A novel process for the formation of physical hydrogels of chitosan from injectable aqueous solutions is investigated. Uniform neutralization of chitosan solutions with ammonia generated in situ from enzymatic hydrolysis of urea is shown to produce pH-induced hydrogels with monolithic and homogeneous coherent 3D structure. Hydrogel formation was characterized by rheology. We found that the gelation time decreases as temperature increases from 15 to 45 °C as a consequence of synergistic effect of increased reactant diffusion and increased urease activity (maximum activity at 37 °C). The effects of urea and urease concentration are also presented.
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Keywords: Chitosan; Monolithic hydrogel; Rheology; pH-induced gelation; Sol–gel transition

1. Introduction

The gelled state of biopolymers enhances their biotechnological usefulness as biocompatible and biodegradable biomaterials. Polysaccharides represent a significant portion of natural biopolymers and are of particular interest for forming stable hydrated gels or hydrogels as evidenced by their use in a myriad of industrial applications in the food, cosmetics, pharmaceutical and biomedical industries (Einerson et al., 2003; Peppas, 1985; Rathna & Chatterji, 1996). Many polysaccharide aqueous solutions are environmentally sensitive as they can be physically cross-linked into hydrated gels in response to environmental changes such as temperature, pH or solvent. The scope of interest is usually restricted to aqueous solutions, since the aqueous environment is often essential to meet cytocompatibility and biological activity requirements in vivo.

It is well known that the solutions of many biopolymers which dissolve in hot water, such as agarose (Nishinari et al., 1992), gelatin (Higgs & Ross-Murphy, 1990) and carrageenan (Nishinari & Watase, 1992; Richardson & Goycoolea, 1994), can form reversible gels if cooled down below a critical gelation temperature. Conversely, other biopolymers, which form aqueous solutions at cold temperature, such as methylcellulose (Haque & Morris, 1992; Nishinari, 1997) and hydroxypropyl cellulose (Guenet, 1992) can form reversible gels upon heating. Similarly, biopolymers that dissolve in water in a particular pH range may gel upon subsequently changing the pH. Since pH-induced gelation takes place instantly and locally upon the addition of the acid or the base, the resulting gel is non-uniformly hydrated and is essentially formed of aggregated or precipitated gelled particulates. In contrast, since the heating and cooling processes can be more spatially uniform than diffusion of acid or base, thermosensitive solutions can often provide greater structural homogeneity of resulting gels, and permits them to be shaped into desired forms.

Chitosan is a polysaccharide that requires specific pH conditions to be water-soluble. Its unique cationic characteristics and the gel- and film-forming properties (Chandy & Sharma, 1990; Felt, Buri, & Gurny, 1998; Illum, 1998) have attracted considerable attention. Chitosan is obtained by alkaline deacetylation of chitin, a cellulose-like polysaccharide that is extracted from the shells of crustaceans such as crabs, shrimps and lobsters (Muzzarelli, 1973). While chitin is completely insoluble in aqueous media, chitosan can be dissolved under acidic conditions that provide sufficient protonation of its amino groups. The resulting aqueous solutions are usually stable as long as the pH is below 6.2. Neutralisation of chitosan solutions by common alkali solutions then leads systematically to the formation of a hydrated gel-like precipitate when the pH exceeds approximately 6.2.

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Physical hydrogels of chitosan have been induced through complex formation with multivalent anions (Draguet, Vårum, Moen, Gynnild, & Smidsrød, 1992; Hirano, Yamaguchi, Fukui, & Iwata, 1990) and anionic polyelectrolytes (Fukuda & Kikuchi, 1979; Sakiyama, Takata, Kikuchi, & Nakanishi, 1999; Takahashi, Takayama, Machida, & Nagai, 1990) as well as by simply increasing pH above the pK_a (Cairns et al., 1992; Montembeault, Viton, & Domard, 2005; Rodriguez-Sanchez & Rha, 1981) or by increasing the temperature in the presence of a polyol buffer (Chenite, Buschmann, Wang, Chaput & Kandani, 2001; Chenite et al., 2000).

Current processes for forming chitosan gels via pH sensitivity (Cairns et al., 1992; Montembeault, Viton, & Domard, 2005; Rodriguez-Sanchez & Rha, 1981) include (i) neutralizing an acidic solution of chitosan with alkaline solutions (NaOH, KOH or NH_4OH), (ii) treating chitosan solutions with gaseous NH_3 or (iii) dialyzing chitosan against alkaline media. However, due to the sluggishness of diffusion-mediated processes, these three approaches fail to produce a structurally homogeneous chitosan hydrogels due to pH gradients present at the time of gel formation. In contrast, a procedure that could homogeneously modulate pH and minimize gradients of pH at the time of gel formation could provide a monolithic and homogeneous hydrogel structure with superior biotechnological performance.

We report here the formation of monolithic chitosan hydrogels via the neutralization of chitosan solutions through gradual in situ generation of base from an alkaline source that is uniformly incorporated and dissolved within the polymeric solutions. This source of base is the hydrolysis of urea, a reaction catalyzed by the introduction of a specific enzyme, urease. Resulting gels have been investigated by performing rheological measurements.

2. Experimental section

2.1. Materials

Chitosan with medium viscosity and a degree of deacetylation, DDA, of 88% was produced in house. The raw material, chitosan with high molecular weight and DDA of 80% from Marinard Ltd was further deacetylated and purified at Biosyntech. This finished chitosan was fully characterized and certified as an ultra pure chitosan for medical and pharmaceutical use. The final average molecular weight of 3.5×10^5 and the DDA of 88% were determined by size exclusion chromatography (SEC) (Brugnerotto, Desbrières, Roberts, & Rinaudo, 2001) and ^{1}H NMR (Lavertu, Xia, & Serreqi, 2003) spectroscopy, respectively.

Urea, Urease and HCl (0.1 M) were purchased from Sigma. Urease activity established by the supplier was 66.3 u/mg.

2.2. Preparation of solutions

Chitosan solutions (2.25% w/v) were prepared by dissolving 200.0 mg of chitosan in 8.9 mL of 0.1 M HCl at room temperature with a resulting pH of 5.3. This clear solution was supplemented with 0.1 mL of urease solution (1 mg/mL) and then cooled down to ~4 °C, in order to inhibit urease enzymatic activity. This cooled solution was then supplemented with 1.0 mL of urea solution (0.5 M) under vigorous stirring. The stirring was maintained for an additional 30 min, after which the mixture was poured into a rheometer cell for the rheological measurements. The composition of the final solution was then 2% w/v of chitosan with 43 mM urea and 17.2 μg/mL urease.

The effects of urea and urease were further investigated by varying concentrations between 23.4 and 71.4 mM for urea, and from 9 to 67.8 μg/mL for urease, while maintaining a constant chitosan concentration.

2.3. Rheological measurements

Viscoelastic properties were measured with a CVO rheometer (Bohlin Instrument Inc. Crandbury, NJ) equipped with SSC25 concentric cylinders. The solution sample volume was 3 mL of solution, which were covered with mineral oil to prevent evaporation during measurements.

The time evolution of elastic and viscous moduli, G' and G'', were measured at constant temperature within the linear viscoelastic region at a fixed frequency of 1 Hz. The gelation time (t_g) was taken as the time at which $G'=G''$, following the approach proposed by Winter and Chambon (Chambon & Winter, 1987; Winter & Chambon, 1986). The frequency dependence of G' and G'' of solutions and gels was also characterized in the frequency range 0.01–10 Hz, after equilibrating samples at 37 °C for about 30 min.

3. Results and Discussion

It is well known that chitosan hydrogels can be obtained by simply adding alkali to raise the pH of chitosan solutions. However, the resulting gels lack cohesion since they consist of aggregated particulate gels, for example when treated with a basic solution (Onsoyen & Skaugurd, 1991). These diffusion-controlled processes have been shown to result in local precipitation when dialyzed against sodium hydroxide solution for 24 h (Cairns et al., 1992; Rodriguez-Sanchez & Rha, 1981) or to form a layer of gel that gradually expands from the surface of the sample towards the bottom when exposed to gaseous ammonia (Montembeault, Viton, & Domard, 2005).

The purpose of the present study was to investigate whether a more uniform neutralization of a chitosan solution leads to the preparation of a monolithic and homogeneous gel of chitosan with greater cohesive structure. Such neutralization may be possible if an alkaline source is dissolved and uniformly distributed at molecular level within the chitosan solution. Urea, a neutral substance whose hydrolysis generates ammonia is the example of alkaline source used in the current study. The hydrolysis reaction is depicted in Fig. 1. However, as this hydrolysis is extremely slow under normal conditions, it is necessary to use urease, a well-known enzymatic catalyst of urea hydrolysis. The accompanying acidification of

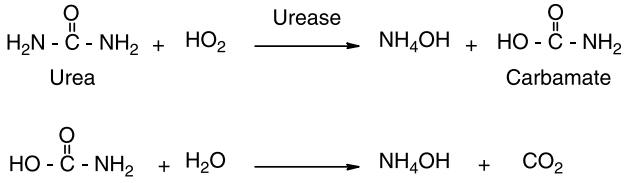

Fig. 1. Illustration of urea hydrolysis in presence of urease.

the solution due to CO_2 formed during the hydrolysis reaction is expected to be negligible.

We found that an initially transparent chitosan solution containing urease that is admixed with urea became progressively more turbid and more viscous (data not shown), leading to the formation of a very dense and opaque gel. The increase of both turbidity and viscosity was a consequence of increasing pH from a progressive release of NH_4OH molecules upon the enzymatic hydrolysis of urea. This clearly suggests the formation of pH-induced monolithic hydrogel of chitosan with a truly homogeneous 3D structure.

3.1. Rheological characterization

Progressive gelation of chitosan solutions (2% w/v), admixed with urea (43 mM) and urease (17.2 μg/mL) was evidenced by rheologically assessing the time evolution of G' (elastic modulus) and G'' (viscous modulus) at 37 °C (Fig. 2). Initially, during the first 15 min, G' is inferior to G'' indicating that the released NH_4OH has increased the pH from 5.3 to near 6.2 where chitosan is still soluble. Subsequently, continued NH_4OH release increases pH above 6.2, thus entering a domain where chitosan tends to precipitate or gel. A sudden increase of the elastic modulus is then observed, that slows down to eventually reach a plateau. This dramatic increase of G' is attributed to the initial fast rate of network formation, facilitated by the high concentration of reactants including chitosan. The following decrease in the rate of change of G' finally approaches completion due to depletion of reactants, as confirmed previously (Gori, 2002). The kinetics of the gelation process can be characterized as first order using the Eq. (Nishinari, 1997)

$$G'(t) = G_{sat}(1 - \exp - k(t - t_0)$$

where t_0 is a latency time, k is a rate constant and G_{sat} is the saturated value of the storage modulus after long times. Closer inspection of Fig. 2 (not shown here) also shows the viscous modulus slightly increasing with time and then stabilizing. This observation is attributed to the formation of initial chitosan clusters, which render the solution more viscous than individual molecules. Very quickly, these clusters form networks, thereby producing a more significant increase of elastic modulus (G').

Fig. 3(a) compares the rheological behaviors of chitosan solutions containing urea in presence versus the absence of urease. The complex viscosity (η^*) of chitosan/urea solution in the absence of urease is nearly frequency-independent, indicating Newtonian behavior, while the complex viscosity obtained in the presence of urease is much higher and decreases as the frequency increases, revealing a thixotropic behavior. The latter suggests the presence of associative polymer network that break down as the frequency dependent shear forces increase.

The frequency dependence of both G' and G'' has also been investigated for chitosan/urea solutions incubated at 37 °C for about 30 min both with and without addition of urease providing additional clear evidence of the difference in

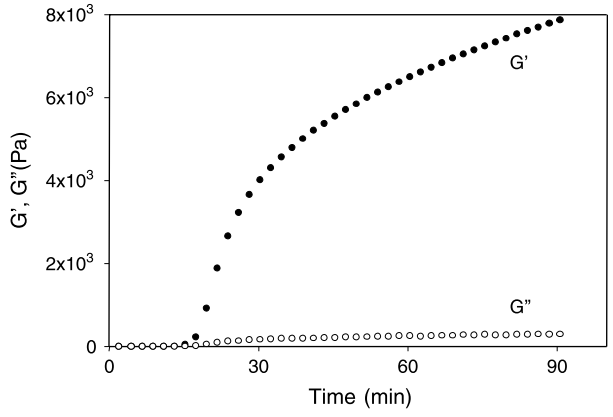

Fig. 2. Time evolution of G' and G'' at 37 °C for chitosan/urea solution in presence of urease.

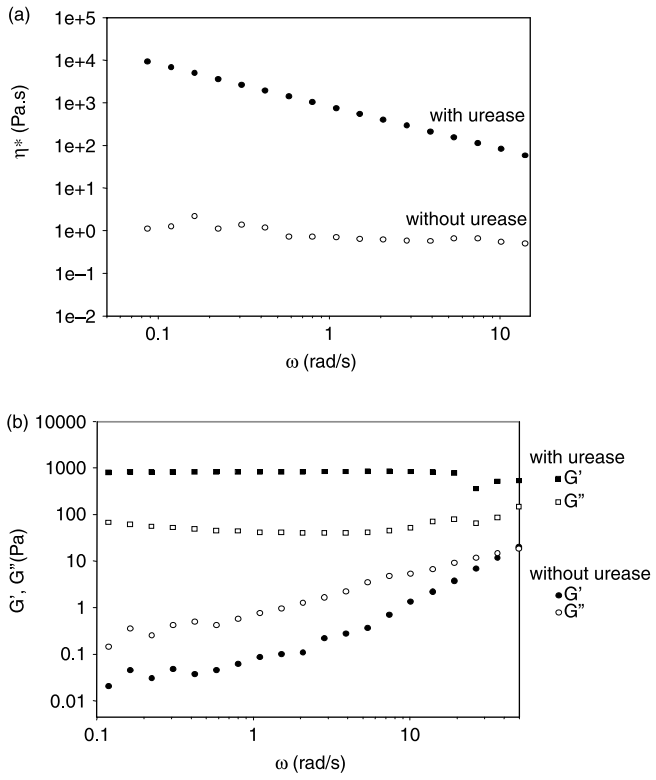

Fig. 3. Frequency-dependence of (a) complex viscosity and (b) elastic (G') and viscous (G'') moduli for chitosan/urea solutions incubated at 37 °C for 30 min with and without urease.

rheological behavior between these solutions (Fig. 3(b)). In the absence of urease, G' is below G'' at low frequencies and both moduli increase with the frequency. The increase of G' is greater so that it exceeds G'' at higher frequencies, as is characteristic of semi-diluted polymer solution. On the other hand, in the presence of urease, G' is much higher than G'' over the entire frequency range, and both moduli are essentially frequency independent, a behavior that is typical of hydrogels (Delben, Lapasin, & Pricl, 1990; Nishinari, 1997) and quite similar to that reported for chitosan/glycerophosphate thermosensitive hydrogels (Chenite, Buschmann, Wang, Chaput, & Kandani, 2001).

The effects of key parameters such as temperature, urea and urease concentrations on the auto-gelation of chitosan/urea solutions were investigated via their influence on the gelation time (t_g).

3.1.1. Influence of Temperature

Fig. 4(a) shows an exponential decrease of gelation time (t_g) when temperature increases from 15 to 45 °C. The acceleration of the network formation could be attributed to the enhancement of the diffusion of reactants (urea, urease and chitosan) and their interactions with the increase of the temperature as well as the attainment of maximum activity of urease at 37 °C. The linear relationship between $\ln(t_g)$ and $1/T(K)$, depicted in Fig. 4(b), suggests that the temperature-dependence of the gelation can be represented by the Arrhenius equation

$$\ln(t_g) = A + \frac{Ea}{RT}$$

where A is a constant, R the ideal gas constant, and T the temperature. Using this equation, an activation energy, Ea, of 59.8 kJ/mol, was calculated and is similar to the values of 68 and 57 kJ/mol, calculated for the gelation of chitosan induced by N-acetylation and N-hexanoylation respectively (Moore & Roberts, 1980).

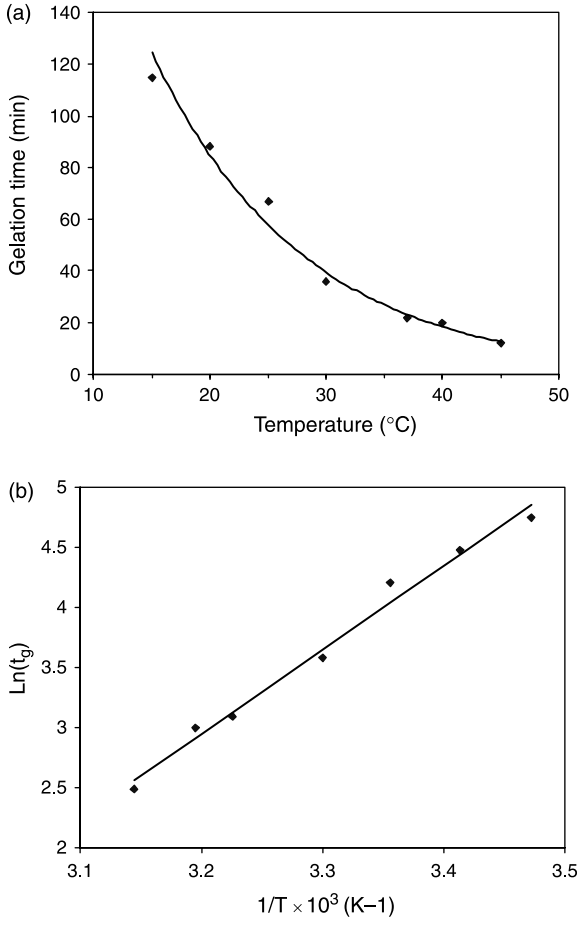

Fig. 4. Variation of gelation time (t_g) as function of temperature: (a) exponential decrease of t_g with the temperature, and (b) linear relationship of $\ln(t_g)$ versus $1/T$.

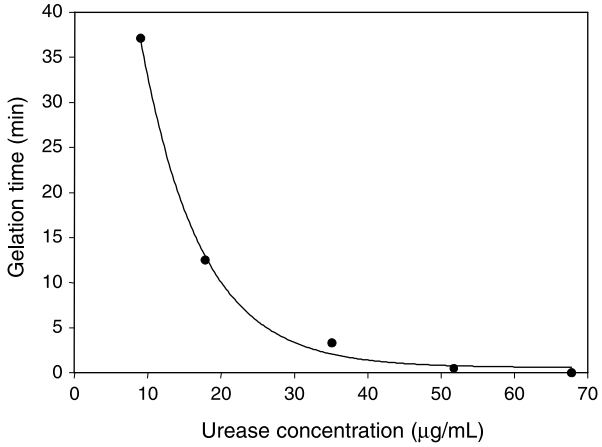

Fig. 5. Gelation time (t_g) versus urease concentration for chitosan/urea solution. Chitosan 2% w/v and urea (43 mM).

3.1.2. Influence of urease concentration

Gelation was also accelerated by increasing urease concentration (Fig. 5). A urease concentration of 40 µg/mL and higher produces essentially instantaneous gelation at 37 °C under the given experimental conditions.

3.1.3. Influence of urea concentration

Gelation time (t_g) also depended on the urea concentration (Fig. 6). However, the significant acceleration of the gelation

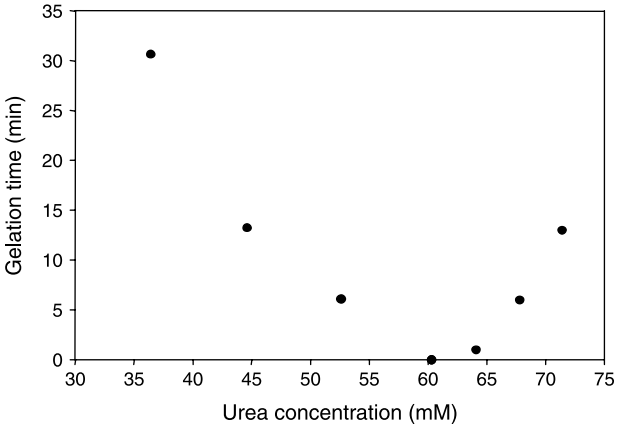

Fig. 6. Dependence of gelation time (t_g) as function of urea concentration for chitosan solution (2% w/v) in presence of urease (17.2 µg/mL).

observed when urea concentration was increased up to 60 mM, was followed by a slowing down of gelation at higher urea concentrations. This latter slowing down of gelation can be attributed to inhibition due to high substrate concentrations or to the ability of urea to reduce attractive interchain hydrogen bonding and hydrophobic forces, or possibly to a combination of both factors. However, the minimum urea concentration (36 mM in Fig. 6) to obtain gelation corresponds to a urea concentration that brings the pH of the chitosan solution just above 6.2 after 30 min of urea hydrolysis at 37 °C. This pH also corresponds approximately to the apparent pK_a of chitosan amino groups that is also known to be the limit above which block acetylated chitosan cannot be maintained in a solution state.

4. Conclusion

The present study demonstrates that pH-induced monolithic hydrogels can be produced via uniform neutralization of slightly acidic chitosan solutions with ammonia generated from enzymatic hydrolysis of urea. The resulting gels have a homogeneous 3D coherent structure. Rheology was used to characterize the gelation process and revealed the effects of various parameters such as temperature and concentrations of urea and urease. We found that gelation time decreases as temperature increases from 15 to 45 °C possibly due to a synergistic effect of increased reactants diffusion and increased urease activity (with a known maximum at 37 °C). In general, gelation is accelerated with increased urea concentration up to a certain limit after which a slowing down of gelation kinetics was observed possibly due to substrate inhibition and the ability of urea to reduce attractive interchain hydrogen bonding and hydrophobic forces.

Enzymatic hydrolysis of urea constitutes an alternative approach to create pH-induced chitosan-based hydrogels and may offer advantages in developing new in situ forming hydrogels. This approach also provides autogelling solutions of chitosan, which can be considered as injectable gels for tissue engineering and for drug delivery. Potential benefits of injectable gels are that liquid materials can easily fill and mould to any shape of an in vivo void or defect and may further contain various therapeutic agents (drugs, growth factors or cells). These gels may be implanted in vivo through a hypodermic needle, thus also avoiding the cost and complications associated with surgical procedures.

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