Kinetics of Gelation and Thermal Sensitivity of N-Isobutyryl Chitosan Hydrogels†

Leticia Félix,‡ Javier Hernández,‡ Waldo M. Argüelles-Monal,§ and Francisco M. Goycoolea*,§

Centro de Investigación en Alimentación y Desarrollo, Laboratory of Biopolymers, P.O. Box 1735, Hermosillo, Sonora, 83000 Mexico, and Centro de Investigación en Alimentación y Desarrollo, Unidad Guaymas, Carr. Varadero Nac. Km 6.6, Guaymas, Sonora 85400, Mexico

N-Acylation of chitosan with carboxylic anhydrides in dilute acetic acid/methanol has been a well documented strategy to selectively modify chitosan. Although this reaction is known to lead to irreversible gel formation, the kinetics and mechanism of this process have not so far been addressed. To this purpose, gel formation during the N-isobutyrylation of chitosan was investigated as a function of the reaction stoichiometry (*R*), chitosan concentration, and temperature by small deformation oscillatory rheology. Gel formation follows closely the chemical reaction and it proceeds predominantly under second-order kinetics as established from the dependence of critical gel time, t_{gel}, on *R* and concentration. The activation energy value derived from t_{gel} vs $1/T$ data ($E_a = 68.29 \pm 1.80$ kJ/mol) was almost identical to values reported for the chitosan *N*-acetylation reaction in previous studies. An excess isobutyric anhydride is suggested to be necessary for nucleation and hydrophobic association. The potential application of N-isobutyrylchitosan (NIBC) hydrogels in the design of thermally sensitive materials is also demonstrated.

Introduction

Over the past years, a great deal of interest has been given to the development of "smart" materials including physiologically sensitive hydrogels made from nontoxic polymers, namely for biomedicine, biotechnology, and other fields of application. Chitosan has been among the key biological macromolecules in this regard. However, in many instances, it needs to be chemically modified in order to give rise to three-dimensional aqueous gel networks.[1]

Acylation of chitosan with acyl anhydrides in dilute acidic solution in methanol is a well-known and effective strategy to reacetylate chitosan and to prepare chitosan derivatives at low temperature and under homogeneous controlled conditions.[2−4] The method of N-acylation under homogeneous conditions was pioneered by the late Shigegiro Hirano, and much of the subsequent work was carried out by his group. Already from the early studies, it was shown that, depending on the structure and the amount of the carboxylic anhydride, this reaction may lead to the formation of a thermally nonreversible gel network.[4,5] For instance, it was observed that gels were obtained by treating a 2% w/v solution of chitosan (degree of acetylation 0%) in 10% v/v aqueous acetic acid with acetic anhydride, under a volume ratio of chitosan solution:acetic anhydride being 2:1. Rigid and transparent gels were obtained within 30 min at room temperature and after dialysis against distilled water. These gels were soluble in formic acid but insoluble in water, dilute acetic acid, alcohols, and acetone. Analysis of the gel dehydrated residue showed that there was 2.36 acetyl groups per anhydro-D-glucosamine residue, the presence of both N- and O-acetyl groups being confirmed by IR spectroscopy and the substitution level by NMR spectroscopy in deuterated formic acid. Subsequent studies addressed the preparation of *N*-acetyl-, *N*-propionyl-, and *N*-butyrylchitosan in 10% v/v aqueous acetic, propionic, and butyric acid as solvents for treatment with the appropriate acyl anhydride. In all cases, O-acylation accompanied N-acylation but the extent depended on the degree of substitution.[6]

Selective N-acylation was further achieved using a mixture of 10% v/v acetic acid:methanol as solvent and a series of acyl anhydrides up to dodecanoic anhydride.[7] The effects of the variation in the acetic anhydride:-NH_2 group mole ratio was also investigated by Hirano and Yamaguchi using acetic acid:methanol as solvents.[8] In this solvent, rigid gels were obtained for mole ratios of 1.7:1 and syneresis occurred for mole ratios 13:1 or greater, but did not occur with gels formed at mole ratios of up to 50:1 in the absence of methanol. The importance of other variables on the rate of gelation has also been addressed using the increase of viscosity as the main criterion to determine the time to onset of gelation.[9] Indeed, the rate of gelation was found to increase with chitosan concentration, acyl anhydride concentration and temperature, and with decrease in the molecular weight of the acyl anhydride. It was also suggested that the rate of gelation depends directly upon the rate of the N-acylation reaction. However, so far the interdependence of the involved

† This paper was presented at the III Iberoamerican Symposium on Chitin (III SIAQ), held in Córdoba, Spain, September 27−29.

* To whom correspondence should be addressed. Tel: +52-662-289-2400 (ext. 248). Fax: +52-662-280-0055. E-mail: fgoyco@cascabel.ciad.mx.

‡ Laboratory of Biopolymers.

§ Unidad Guaymas.

variables has not been addressed quantitatively nor attending to the various proposed gelation kinetics models available, so as to deepen the understanding of this class of gel materials.

In this work, we aimed to investigate the kinetics of gel formation during the N-acylation of chitosan with isobutyric anhydride in 0.1M acetic acid-methanol (1:2.3) to produce N-isobutyryl chitosan (NIBC). This modification is hypothesized here to be a strategy to the engineering and design of hydrogel materials with enhanced thermal sensitivity, similar to those obtained from other well estabilised "smart" polymers such as poly-*N*-isopropylacrylamide.[10] Indeed, there is a structural and steric similarity of $-NH-COCH(CH_3)_2$ functional groups born at C2 in NIBC, with those pendant of poly-*N*-isopropylacrylamide (poly-NIPA), $-CO-NHCH-(CH_3)_2$. Poly-NIPA is a synthetic polymer with firmly established thermal sensitivity and a sharp discontinuous volume phase transition at ∼33.8 °C with increasing temperature.[11] The driving force of the volume phase transition in NIPA gels has been suggested to be due to the hydrophobic interaction of the isopropyl groups in the side chains. As a result of this, hydrogels of poly-NIPA exhibit lower critical solution temperature (LCST) and hence negative thermal response (i.e., the degree of swelling of the gel network levels with decreasing temperature). In preliminary studies in our laboratories, it has been demonstrated that physical hydrogels of chitosan prepared from alkali chitin themselves and cross-linked with glutaraldehyde at pH ∼ 7.6 exhibit a negative thermal response.[12,13] Therefore, N-isobutyrylation of chitosan is expected to enhance this behavior.

Even though gelling during the N-acylation of chitosan has been firmly demonstrated,[14] in the present study, we aim to glean further understanding of the mechanisms and kinetics of these processes as well as to characterize the temperature dependence of the mechanical properties of this system using high sensitive oscillatory small-deformation rheology as the main investigative technique. The relevance of the presented findings in the development of a thermosensitive hydrogel material is also discussed.

Experimental Section

Materials. Chitosan was a sample obtained from a previously isolated batch of chitin from shrimp (*Lithopenaeus stilyrostris*) head waste deacetylated at ca. 100 °C in NaOH 50% (w/w) during 2 h in a pilot plant facility and had a degree of acetylation of 14.7% and a viscosimetric molecular mass (M_v) of ∼180 000 as determined in 0.3 M acetic acid/0.2 M sodium acetate at 25 °C ($[\eta] \sim 768.1$ mL/g with $K = 0.069$ g/mL and $a = 0.77$).[15]

Reagents were all analytical grade supplied as follows: Isobutyric anhydride was from Aldrich Chemical Co. Inc. (Milwaukee, WI); methanol from J. T. Baker, México (a division of Mallinckrodt Baker S. A. de C. V.); agar-agar was from Sigma (Mexico), and acetic acid from Merck-México S. A. (Mexico City). Distilled water was used throughout.

Table 1. Experimental Conditions of the Various Chitosan N-Acylation Tests

experimental test	final chitosan conc. (g/L)	R[a]	temp. (°C)
I. varying R	2.2	2.0	25
	2.2	3.0	25
	2.2	4.0	25
	2.2	6.0	25
	2.2	8.0	25
	2.2	10.0	25
	2.2	20.0	25
	1.0	6.57	25
	1.0	9.89	25
	1.0	16.42	25
	1.0	21.93	25
	1.0	31.0	25
	1.0	43.9	25
II. varying temperature	2.2	4	15
	2.2	4	20
	2.2	4	30
	2.2	4	35
III. varying chitosan concentration	1.4	4	25
	1.7	4	25
	2.2	4	25
	3.4	4	25
	4.6	4	25
	5.6	4	25
	7.0	4	25
IV. stepwise change in temperature	3.5	2	0−50−0−50...

[a] mol isobutyric anhydride/mol glucosamine.

N-Acylation Studies. The various N-acylation studies were conducted according to the following series of experimental strategies which are summarized in Table 1.

I. Varying Mole Ratio of Isobutyric Anhydride to Glucosamine (*R*). To 3.03 mL of a solution of 6.4 g/L of chitosan in acetic acid 0.1 M was added 3.94 mL of methanol, and the solution was stirred until a homogeneous mixture was obtained. To this solution were added varying amounts of isobutyric anhydride freshly dissolved in 3.03 mL of methanol so as to yield varying R as described in Table 1. The final concentration of chitosan in these gels was 2.2 g/L. In a second series of similar experiments, the final target chitosan concentration was 1.0 g/L and R values were in the range 6.5−43.9. Immediately after being prepared, the reacting solutions were loaded into the plate of the rheometer previously equilibrated at 25 °C. An aliquot of 7 mL of the remaining solutions of the first series of gels of $R = 6$, 8, 10, and 20 was poured in Petri dishes and left to react quiescently during 2 h at 25 °C before washing them thoroughly in water at 60 °C to neutrality (i.e., until no change in pH was detected at the gel surface from pH strips). These hydrogels were left to dry under vacuum at room temperature and were used for FTIR spectroscopy measurements.

II. Varying Temperature. The concentration of chitosan and R values were both fixed to 2.2 g/L and $R = 4.0$, respectively, and the gels were prepared as outlined above. These gels were loaded to the plate of the rheometer previously set at 15, 20, 30, or 35 °C.

III. Varying Chitosan Concentration at Fixed *R* (=4.0). A stock solution of 20 g/L of chitosan in 0.1 M acetic acid was diluted to yield solutions of 4.0, 5.0, 6.4, 9.8, 13.0, 16.0, or 20 g/L. To 3.02 mL of each solution were added 3.9 mL of methanol and varying amounts of isobutyric anhydride freshly dissolved in 3.03 mL of methanol so as to yield varying chitosan concentrations as in Table 1. Immediately after being prepared, the reacting solutions were loaded into the plate of the rheometer previously equilibrated at 25 °C.

IV. Preparation of a Washed N-Isobutyryl Chitosan Hydrogel for Rheological Determinations. A freshly prepared NIBC hydrogel of $R = 2.0$ and chitosan final concentration of 3.5 g/L was prepared in a Petri dish and thoroughly washed using the strategy outlined above in step I so as to obtain a gel slab ∼1.5 mm in height. The gel was loaded on the bottom element of the rheometer, and the upper plate was gently displaced down ensuring complete contact with the surface of both elements as the precise gap was registered with a micrometer. As a control to this experiment, a gel of agar-agar was prepared in water (*c*. 11 g/L) at 90 °C and left to set in a Petri dish exactly as for the NIBC gel. Normal compression was detectable when the upper tool made contact with the gel surface as monitored from the normal force transducer, but this compression gradually relaxed.

FTIR Studies. Thoroughly washed (i.e., neutral) hydrogels were dried under vacuum at room temperature for 48 h and subsequently finely ground in order to allow proper mixing with KBr (ratio sample to KBr 1:100). Pellets were formed at 6000 psi pressure delivered from a manually operated hydraulic press (International Crystal Laboratories, 12 Ton E−Z Press). Spectra were recorded in a Nicolet Protégé 460 ESP (Madison, WI) instrument in transmission mode.

Rheological Determinations. The rheological properties of the various studied systems were investigated using a strain-controlled rheometer (Rheometrics Mod. RFSII Fluids Spectrometer, Piscattaway, NJ), fitted with either a truncated cone-plate (cone angle: 0.0397 rad, diameter: 50 mm) or a parallel plate tool (diameter: 25 mm) and a circulating environmental system for temperature control. To prevent drying of the samples during experiments, a glass ring of ∼60 mm was placed around the measuring geometry, and the annulus was filled with silicone oil of low viscosity. The evolution of the gelation process was monitored by measurements of storage, $G'(t)$, and loss, $G''(t)$, moduli ($\omega = 2.51$ rad/s), recorded at strain values, γ, in the range of 0.15−0.30 over a period of up to 172 min. Nonlinear regression analysis of data recorded for t_{gel} as a function of R and chitosan concentration were conducted using the nonlinear curve fit procedure of Origin 6.2.

Results

FTIR spectra recorded in NIBC hydrogels once thoroughly washed are in Figure 1. The spectra show that even for too high a stoichiometric excess of isobutyric anhydride (i.e., $R = 6$, 8, 10, and 20) the reaction is highly selective toward N-acylation, as it can be confirmed by the strong amide I and II bands at ∼1652 (ν HNC═O) and 1549 cm^{-1} (ν_s HNC═O) and by the absence of a band present at ∼1750 cm^{-1}, diagnostic of the presence of O-acyl ester groups, even at *R* values as high as 20, in good keeping with the early work by Hirano.[16] Three bands centered in the region 2840−3000 cm^{-1} (ν −C−H), along with those at ∼1470 (δ_{as} −CH_3), at ∼1375 (δ_s −$CH(CH_3)_2$), and at 1155 cm^{-1} (γ −CH_3) were also unequivocal probes of the presence of methyl groups in the molecule.

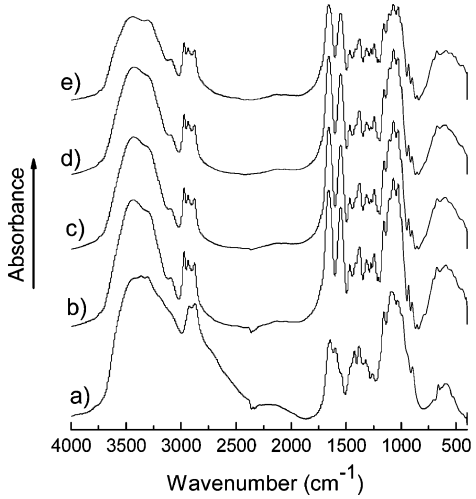


Figure 1. FTIR spectra of washed and dried chitosan hydrogels of varying isobutyric anhydride to glucosamine molar ratios, R, of: (a) 0 (i.e., unmodified chitosan); (b) 6, (c) 8, (d) 10, and (e) 20.

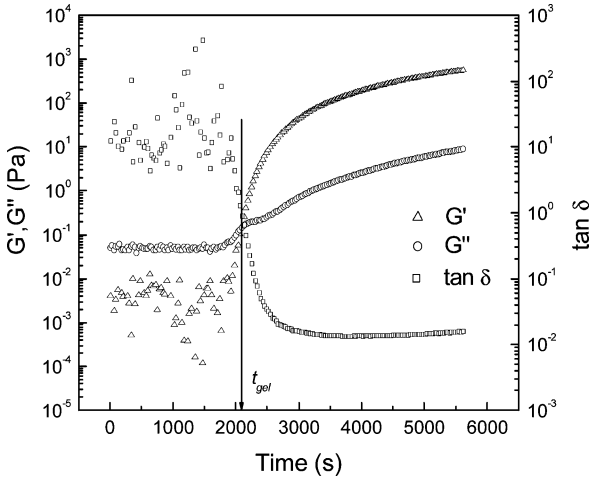


Figure 2. Variation of the viscoelastic moduli, G′ and G″ and tan δ during N-acylation reaction of chitosan with isobutyric anhydride in dilute acetic acid/methanol at 25 °C (4 mol of isobutyric anhydride per mol of glucosamine; chitosan concentration = 2.2 g/L; $\omega = 2.511$ rad/s; $\gamma = 0.30$).

Figure 2 shows an example of results obtained from the rheometer for the evolution of G', G'', and tan δ with time during the N-acylation reaction of chitosan. The general shape of the curves was the same for all of the samples. Initially the system behaves as a purely viscous fluid (i.e., $G'' > G'$, noisy G' and high tan δ values). After a certain time under continuous oscillation (∼ 2000 s) the storage modulus, G', suddenly rises exceeding the loss modulus, G'' and tan δ drops by several orders of magnitude. At longer times, G' continues to increase and exceeds G'' by about 2 orders of magnitude. A slight wave in the G'' trace can also be appreciated at the early stages of these changes. This so-

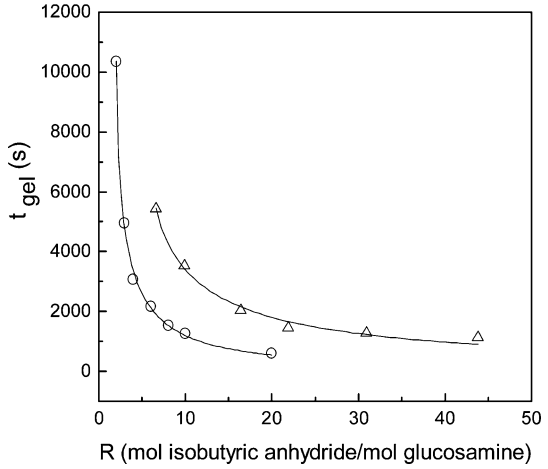


Figure 3. Critical gel time, t_{gel}, dependence on stoichoimetry, R (mol of isobutyric anhydride/mol of glucosamine) for N-isobutyryl chitosan at two different chitosan concentrations of (g/L): 1.0 (triangles) or 2.2 (circles) at 25 °C. Solid lines represent best-fit curves of eq 1.

called "loss peak" in G'' is diagnostic of relaxation processes and loose dangling chain ends of the polymer and has been observed in gelation processes of both covalent and physical networks.[17] The observed behavior of the viscoelastic moduli and tan δ is characteristic of a sol−gel transition. At the end of each run, strain dependence was tested and invariably linear viscoelasticity was confirmed (data not shown).

A number of methods based on mechanical properties have been proposed for determining the gelation point for chemically and physically cross-linked systems.[18−20] The criterion adopted in the present study to mark the onset of incipient formation of the gel network or the percolation threshold (i.e., where the system is assumed to have formed the first cluster of infinite molecular mass), namely, the rheological critical gel time (t_{gel}), was given by the time of crossover of G' and G''.[20] Critical gel times, t_{gel}, calculated under this condition are known to be dependent on frequency as demonstrated in the rigorous theoretical argument by Winter and Chambon.[18] However, in many instances, the application of the Winter−Chambon criterion is either not experimentally feasible, nor rheological measurements at the critical gel point can be guaranteed to be at the linear viscoelastic region. Indeed, there is evidence that suggests that at the gelation point linear strain is at its minimum.[21] In the present study, oscillatory measurements using "multiwave" harmonic frequencies so as to monitor the gel formation process in real time ("on-the-flight") at varying frequency were not experimentally available, thus effectively making it impossible to apply correctly the Winter and Chambon criterion in the calculation of t_{gel}. Hence, t_{gel} was identified with the instant of the incipient prevalence of G' over G'' (i.e., when tan δ becomes just less than 1.00).[20] This empirical approach has been applied to polysaccharide gels such as pectin−calcium systems.[22] Moreover, close inspection of Figure 2 shows that t_{gel} coincides with the maximum in G'' peak in agreement with results for gels of β-lactoglobulin.[23]

Figure 3 shows results of the dependence of t_{gel} on R at two experimental chitosan concentrations, 1.0 and 2.0 g/L. In the plot are also shown the nonlinear best fit curves of a

Table 2. Best-Fit Parameters of the Kinetic Gelation Model by Ross-Murphy[24] (eq 1) for Gels of N-Isobutyryl Chitosan of Varying Concentration as a Function of R

	chitosan concentration (g/L)	
	1.00	2.20
$n' = 1.00$ correlation coefficient	0.99	1.00
k (min)	6188	5913
R_c	3.00	1.32
p	0.72	0.83

kinetic model in the form of the equation of Ross Murphy (eq 1)[24]

$$t_{gel} = \frac{k}{((R/Rc)^{n'} - 1)^p} \quad (1)$$

This model was originally proposed to account for the dependence of t_{gel} on polymer concentration, C, as it is shown below. Although the replacement of concentration by reaction stoichiometry, R, in eq 1 was made purely on an empirical basis, we thought that it would be informative and of practical relevance to know whether R is related in any form with the degree of conversion of the gel, as well as to estimate precisely the critical stoichiomety (R_c) of the process and whether such values depended or not on polymer concentration. Table 2 shows the estimated best-fit parameters derived from the nonlinear regression of eq 1 for $n' = 1.00$. Parameters k, R_c, and p were floated throughout the fitting procedure. It was interesting to notice that the estimated critical stoichiometric R_c values decrease from 3.00 to 1.32, whereas p exponents vary from 0.72 to 0.83, as chitosan concentration doubles from 1.0 to 2.2 g/L, respectively. The estimated R_c values of this study are within the limits of those previously reported under similar reaction conditions where carboxylic anhydride:glucosamine mole ratios as high as 13 have been documented for gel formation.[8] However, the best-fit values of the p exponent estimated for both concentrations are lower than those expected for a percolation process that should fall in the (albeit broad) range 2.5 ± 1.[25] Values of the n' exponents greater than 1.0 were also tested, yet the p parameter values obtained decreased to even lower values than those presented in Table 2.

Yet another important aspect to investigate was the dependence of t_{gel} on polymer concentration, C, at fixed stoichiometry ($R = 4.0$) such that $R > R_c$. Figure 4 shows the experimental data corresponding to the dependence of t_{gel} on concentration along with the best fit curve of Ross-Murphy's model (eq 2). The inset shows a plot of log $1/t_{gel}$ vs log C.

$$t_{gel} = \frac{k}{((C/C_0)^{n'} - 1)^p} \quad (2)$$

In eq 2, C and C_0, are concentration and minimal concentration needed for gel network formation at $R = 4.00$, respectively. The best fit parameters estimated when n' was fixed at integer values from 1.00 to 4.00 are presented in Table 3. Very high correlation coefficients were obtained for all of the tested n' values, though only for $n' = 1.00$ the

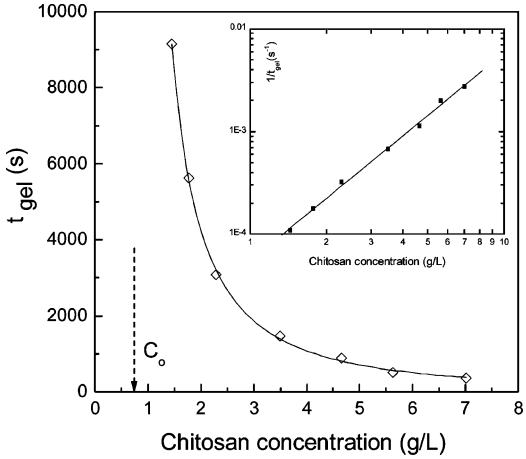


Figure 4. Critical gel time, t_{gel}, dependence on polymer concentration for N-isobutyryl chitosan gels for $R = 4.0$ at 25 °C. Solid line represent best-fit curve of the kinetic model of Ross-Murphy[24] (eq 2). Inset shows the same experimental data and best-fit curve plotted in the form of Oakenfull treatment.[27]

Table 3. Results of Calculations of the Parameters of the Kinetic Model by Ross-Murphy[24] (eq 2) for Gels of N-Isobutyryl Chitosan of Varying Concentration at Fixed Molar Ratio ($R = 4.00$)

	n' parameter			
	1.00	2.00	3.00	4.00
correlation coefficient	1.00	1.00	1.00	1.00
k	23616	20462	16788	14588
C_0	0.52	0.78	0.94	1.05
p	1.62	0.91	0.64	0.49

p exponent assumes a value that falls in the expected range.[25] Based on this evidence, it seems reasonable to argue that the reaction kinetics of the system is approximately second order. Note that C_0 values obtained for $n' < 3.0$ are consistently lower than 1.0 g/L. It is worth pointing out that these concentration values as well as those used for the experiments at varying R (1.0 and 2.0 g/L) are below the expected critical overlap concentration for the native chitosan ~1.9 g/L (i.e., considering critical coil overlap at $c[\eta] = 1.45$[26]). This may reflect a change in the overall dimensions of the polymer due to the chemical modification process, thus effectively causing a reduction on the critical overlap concentration for gelation. Alternative to the use of eq 2, a log−log plot of t_{gel} vs concentration (the so-called Oakenfull plot[27]) gives a straight line (inset of Figure 4), indicating a simple power law relationship. A linear regression gives a slope 2.02 ± 0.05, a value that has been related with the size or molecularity in a junction zone via the reaction order.[27] However, using this approach has led to unrealistic slope values as high as 12.[28,29] This and absence of a critical concentration parameter (C_0) prompted to develop the model proposed by Ross-Murphy. It was perhaps only fortuitous that the slope of the Oakenfull plot is also in agreement with second-order kinetics.

In Figure 5 is represented the variation of G' with C for gels of $R = 4.0$. As a first approach to test for the form of the concentration dependence of G', shear moduli values at varying concentration were considered not at equilibrium but at $t/t_{gel} = 1.72$. Notice that at concentrations ≥~5 g/L, G' decreases with concentration rather than describing the expected ascending monotonic trend. In many biopolymer systems, an exponential relationship has been observed. This dependence has been treated theoretically by the cascade formalism approach for a range of biopolymer gels, developed by Clark and Ross-Murphy to describe the basic features of biopolymer gelation.[30,31] Under this treatment, the equilibrium G' (G_e) or only G' moduli values are plotted as a function reduced concentration, C/C_0, and at the high concentration, both moduli are linear functions of C^2. Clearly, no linear dependence is observed in the data for NIBC gels, thus effectively reflecting that the values of the elastic modulus are not dependent only on the number of elastically active network chains but on other more complex processes that must be operative leading to the collapse of the gel network.

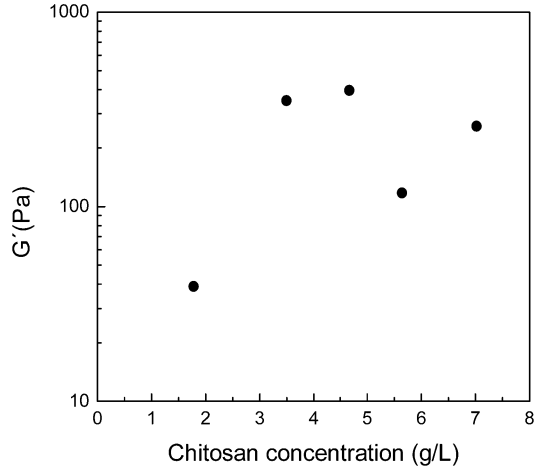


Figure 5. Variation in storage modulus, G', with chitosan concentration for N-isobutyryl chitosan gels for $R = 4.0$ at $t/t_{gel} = 1.72$ (ω = 2.512 rad/s; $\gamma = 0.20$; 25°C).

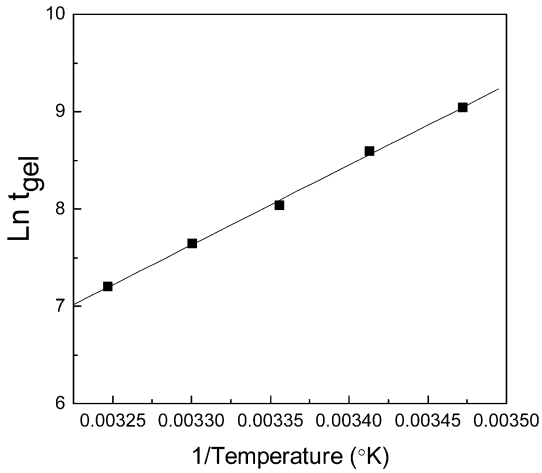


Figure 6. Arrhenius plot of ln t_{gel} vs. reciprocal absolute temperature data for N-isobutyryl chitosan gels of $R = 4.0$ and chitosan concentration of 2.2 g/L formed at varying temperature.

The dependence of t_{gel} with temperature is shown in Figure 6 in the form of an Arrhenius plot for gels of $R = 4.00$ and a chitosan concentration of 2.2 g/L. Clearly, there was a linear dependence of ln t_{gel} with reciprocal absolute temperature. From the value of the slope of the linear best-fit regression (= 8214.355), the activation energy, E_a, of the reaction process was estimated to be $E_a = 68.29 \pm 1.80$ kJ/mol, a value that is in excellent agreement with E_a values

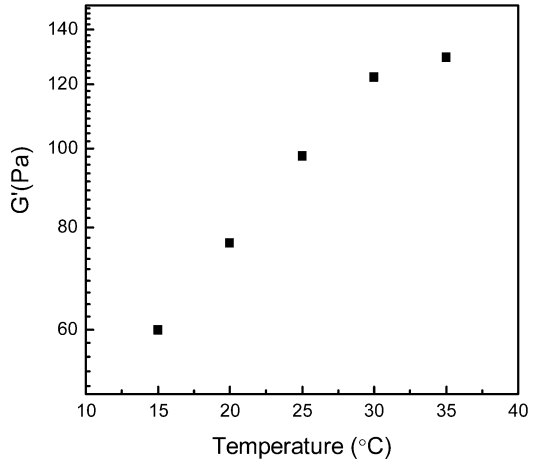


Figure 7. Increase in storage modulus, G', with temperature at t/t_{gel} = 1.72 for N-isobutyryl chitosan gels of R = 4.0 and chitosan concentration of 2.2 g/L (ω = 2.512 rad/s; γ = 0.25).

previously reported for the *N*-acetylation reaction of chitosan using the same reaction strategy (68 ± 7 kJ/mol).[32]

In another series of experiments conducted at fixed values of both R = 4.00 and polymer concentration (2.2 g/L), it was also possible to assess the temperature dependence of the shear storage modulus, G', during gel formation at t/t_{gel} = 1.72 depicted in Figure 7. Clearly, G' values experience a monotonic increase trend as the temperature rises up to ∼30 °C, beyond which only little further increase is observed. The increase in storage shear modulus as temperature rises up to ∼30 °C can be interpreted as the consequence of the gradual break down of the entropy-driven hydrophobic hydration effect whereby structured water around isobutyryl substituents at the chitosan chain sheds its sheath of highly constrained structured "iceberg" or "cage-like" water molecules,[33] thus effectively favoring the self-association of the hydrophobic isobutyryl groups. However, at temperature >∼30 °C a different mechanism may take place as it is likely that the gel has surpassed its LCST, and hence, it undergoes a discontinuous volume phase transition into a collapsed state. This may lead to formation of cross-linking junctions of lower connectivity (i.e., cyclization) and/or other processes leading to cross-link dissipation[34] that do not seem to operate to the same extent at temperatures below the LCST.

To study the thermal response properties of NIBC hydrogels once they were thoroughly washed in water in order to get rid of methanol, acetic acid, excess isobutyric anhydride, and formed isobutyric acid, a gel of R = 2.00 and *c*. 3.5 g/L was washed thoroughly in distilled water and tested for their mechanical properties as a function of stepwise changes in temperature between 5 and 40 °C. Figure 8a shows the results obtained for a gel of NIBC. In the NIBC hydrogel it is evident that as the temperature decreases, so does the storage modulus, G′, while heating to 50 °C, leads to an increase in G′. This pulsatile mechanical response is reversible and persists to at least 4 cyclic stepwise changes in temperature. A gel of agar-agar known to melt at much greater temperature (∼85 °C) was used as a control to this experiment. Figure 8b shows the behavior of this gel studied under identical conditions. In contrast with the response observed for the

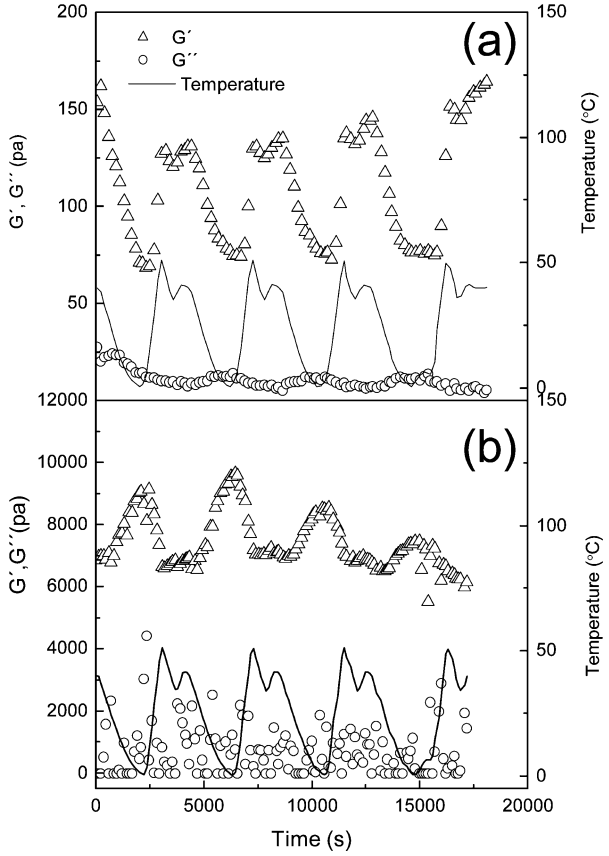


Figure 8. Variations in storage, G', and loss, G'', moduli with stepwise periodic changes in temperature between ∼50 and 0 °C for (a) freshly washed N-isobutyryl chitosan gels of R = 2.0 and a chitosan final concentration of 3.5 g/L (ω = 6.31 rad/s; γ = 0.01) or (b) agar-agar 11.0 g/L (ω = 2.0 rad/s; γ = 0.005).

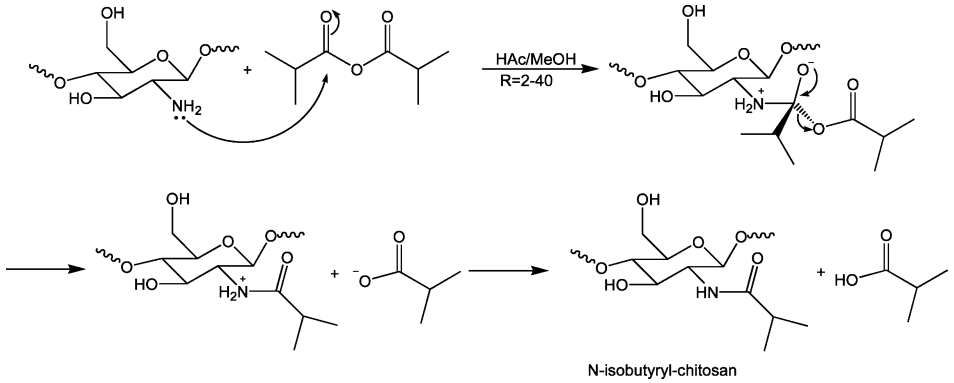
NIBC hydrogel, that of agar-agar becomes softer as the temperature increases and stronger as the temperature decreases.

Discussion

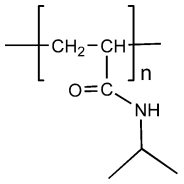
The general mechanism of the N-acylation reaction of chitosan with isobutyric anhydride in dilute acetic acid/methanol is shown in Scheme 1. It is a nucleophilic substitution, SN_2 type reaction. Comparison of the structure of NIBC with that of synthetic poly-NIPA (Scheme 2) allows the similarities between the pendant functional groups in both structures to be clearly appreciated. This functionality has been suggested to impart hydrophobic character to poly-NIPA, to which its LCST behavior is attributed.[35] Hence, it was anticipated that NIBC gels would also show thermal sensitivity comparable to that of NIPA gels.

The N-acylation of chitosan in acetic acid/methanol reaction is highly selective as previously suggested. Absence of bands at ∼1760−1700 cm^{-1} and at 3650−3590 cm^{-1} in the FTIR spectra indicated that no residual free isobutyric nor acetic acids, or methanol, respectively, remained in the hydrogel after the exhaustive washing was applied. In general, washed NIBC hydrogels were transparent and insoluble in water. On storage for several days the gels of all tested stoichiometry and chitosan concentration experienced substantial syneresis. They swelled in 90% v/v and were fully soluble in formic acid 100% v/v.

Scheme 1. General Mechanism of N-acylation of Chitosan



Scheme 2. Structure of Poly-N-isopropyl Acrylamide



poly-NIPA

Central to the aim of this investigation was to glean understanding on the mechanism of gel formation during the N-acylation reaction of chitosan with isobutyric anhydride in acetic acid/methanol. This implied to address the question whether the underlying macromolecular association processes in this type of gelling systems are a direct consequence of the kinetics of chemical derivatization of chitosan itself or is the result of more complex thermodynamic phenomena concomitant to the occurring reaction leading to the percolation of the gel network. It also implied the elucidation of the nature of the underlying forces responsible for the interpolymer interaction, whether cross-linking points or extended junctions, which altogether govern the mechanical and thermal properties of the system. To this end, the kinetics of gel formation was studied as a function of the stoichiometry (isobutyric anhydride to glucosamine moles), chitosan concentration, and temperature, using small-amplitude oscillatory rheology.

Up to equimolar conditions of isobutyric anhydride to glucosamine, the kinetics of N-isobutyrylation of chitosan should give rise to second-order kinetics. However, no gel was formed unless a stoichiometric excess of isobutyric anhydride was added, in experimental agreement with previous studies.[8] Hence, the chemical reaction order itself may change as R and concentration do, thus effectively reflecting that kinetic wastage effects are clearly involved in this system.

The critical gel time has been defined as the time at which the gel point conversion is reached during a kinetic gelation process. For chemically cross-linked gels, when critical conversion can be estimated reasonably well from the stoichiometry and branching functionality of the precursor species, then gel time can be estimated reasonably well, by investigating the kinetic order of the cross-linking process. For physical gels, the situation is more complex.[36]

Regardless of the method used to estimate the critical stoichiometry for gel formation, R_c, its values were found to be dependent on chitosan concentration reflecting a process governed by law of mass action. The fact that the predicted R_c values are greater than 1.00 can be considered as evidence that the gel kinetics are not solely governed by the chemical reaction of chitosan itself but by other rate limiting processes of greater complexity in which excess of isobutyric anhydride in the sol fraction must also play a role. Such processes are believed to be of a physical nature, as it is not plausible to invoke interchain covalent cross-linking (Scheme 1). Indeed, in previous studies in chemically cross-linked pectin gels with 1,6-dibromohexane (DBH), critical stoichiometric values were close to 0.084,[37] much lower (by ~50 times) than those estimated in this study. Yet another process operating in this system, still waiting for experimental demonstration (e.g., by static light scattering), is the possibility that the macromolecular dimensions of the derivatized polymer increases with respect to the original chitosan as a consequence of the chemical modification. This phenomenon would have a direct effect on C_0 and also account for the complex gelling behavior of this system.

Analysis of the parameters obtained by fitting independently t_{gel} vs. R and t_{gel} vs. C using Ross-Murphy's model, pointed to second-order kinetics. In this regards, it is well-known that, for the simplest second-order kinetic (irreversible) gelation process, the time required to reach a given degree of chemical conversion is proportional to $(1/C)$.[38] This is in good agreement with the predictions of Ross-Murphy's model (Tables 2 and 3). However, the reaction order in this system cannot be considered as a true order as it seems to be concentration dependent.

The concentration dependence of G' (Figure 5) did not agree at all with the $G'\,C^2$ generalized behavior but showed a decrease in G' at $C \geq \sim 5.00$ g/L. This can also be rationalized in terms of the collapse of the gel structure due to concentration-driven network inhomogeneities (i.e., microphase separation) or microcrystallization processes, occurring once the gel is formed. Such phenomena can be the consequence of unequal solvent partition effects of the gel phase in the water/methanol mixed solvent.[39] Future X-ray diffraction studies at the structural level to probe this system would be highly informative in this regards.

A very interesting property of NIBC hydrogels was that the activation energy calculated using the Arrhenius dependence of t_{gel} on temperature yielded a value in extremely close agreement with previous chemical studies. It is

therefore reasonable to conclude that the kinetics of gel formation are directly related to that of the N-acylation reaction itself.

In turn, the variation of the G' modulus with temperature for a series of NIBC gels of fixed R and C during gel formation, allowed us to establish that the polymer behaves as an hydrophobic system in which as the temperature increases, predominant contributions to the increase in shear modulus are due to the association of the N-isobutyryl groups in the polymer and the higher free energy state is due to the entropic contribution of the water phase. In cellulose derivatives, it is well accepted that the hydrophobic effect drives thermogelation.[40−42] This is in sharp contrast with other biopolymer gel systems including a large series of polysaccharide and proteins.[43] The role of excess isobutyric anhydride in these effects is not fully understood but it may serve as nucleation sites for hydrophobic junctions.

As a final remark, it can be postulated that cross-linking in N-isobutyryl chitosan and similar types of derivatives obtained under large stoichiometric ratios of acyl anhydrdides gelling is governed by hydrophobic association, where the system is under the kinetic control of the N-acylation reaction. In our system, an excess of isobutyric anhydride seems to play a role in acting as a nucleation point for the self-association of the modified NIBC species, thus effectively lowering the free energy of network formation.

Once the NIBC gel is freed from excess of disparate species present (acetic, acid, methanol, isobutyric acid, isobutyric anhydride), the system remains in a highly associated state and behaves as a thermally sensitive hydrogel material similar to other thermally synthetic systems such as poly-NIPA. This was demonstrated by the response of the mechanical properties of the system to stepwise cyclic changes in temperature (Figure 8), a behavior which was in contrast with an agar-agar gel tested under identical conditions. This behavior has been documented for poly-NIPA and other hydrogels and bears great potential in applications including the engineering of thermosensitive devices such as sensors, actuators, controllable membrane for separations, and modulators for delivery of drugs for use in medicine, biotechnology, and other fields.[44,45]

Acknowledgment. An international research grant from the Royal Society of Chemistry (United Kingdom) to conduct this study is gratefully acknowledged.

References and Notes

(1) Berger, J.; Reist, M.; Mayer, J. M.; Felt, O.; Gurny, R. *Eur. J. Pharm. Biopharm.* **2004**, *57*, 35.
(2) Moore, G. K.; Roberts, G. A. F. In *Proceedings of the First International Conference on Chitin/Chitosan*; Muzzarelli, R. A. A., Pariser, E. R., Eds.; MIT Sea Grant Report: Massachussets, 1977; p 421.
(3) Hirano, S.; Ohe, Y. *Carbohydr. Res.* **1975**, *41*, C1−C2.
(4) Roberts, G. A. F. *Chitin Chemistry*; The Macmillan Press: Ltd.: London, 1992.
(5) Otake, K.; Inomata, H. *Macromolecules* **1990**, *23*, 283.
(6) Hirano, S.; Ohe, Y. *Agric. Biol. Chem.* **1975**, *39*, 1337.
(7) Hirano, S.; Ohe Y.; Ono, H. *Carbohydr. Res.* **1976**, *47*, 315.
(8) Hirano, S.; Yamaguchi, R. *Biopolymers* **1976**, *15*, 1685.
(9) Moore, G. K.; Roberts, G. A. F. *Int. J. Biol. Macromol.* **1980**, *2*, 73.
(10) Hoffman, A. S. *J. Controlled Release* **1987**, *6*, 297
(11) Hirokawa, Y.; Tanaka, T. J. *Chem Phys.* **1984**, *81*, 6379.
(12) Goycoolea, F. M.; Heras, A.; Aranaz, I.; Galed, G.; Fernández-Valle, M. E.; Argüelles-Monal, W. *Macromol. Biosci.* **2003**, *3*, 612.
(13) Goycoolea, F. M.; Heras, A.; Aranaz, I.; Galed, G.; Fernández-Valle, M. E.; Argüelles-Monal, W. In *Advances in Chitin Science*; Varum, K. M., Domard, A., Smidsrod, O., Eds.; Norges Teknisk-Naturvitenskapelige Universitet: Trondheim, Norway, 2003; Vol VI, p 169.
(14) Hirano, S.; Yamaguchi, Y.; Kamiya, M. *Macromol. Biosci.* **2003**, *3*, 629.
(15) Rinaudo, M.; Milas, M.; Le Dung, P. *Int. J. Biol. Macromol.* **1993**, *15*, 281.
(16) Hirano, S.; Kondo, S.; Ohe, Y. *Polymer* **1975**, *16*, 622.
(17) Bibbo, M. A.; Valles, E. M. *Macromolecules* **1984**, *17*, 360.
(18) Winter, H. H.; Chambon, F. *J. Rheol.* **1986**, *30*, 367.
(19) Djabourov, M. *Polymer Int.* **1991**, *25*, 235.
(20) Tung, C. Y. M.; Dynes, P. J. *J. Appl. Polym. Sci.*, **1982**, *27*, 569
(21) Rodd, A. B.; Dunstan, D. E.; Ross-Murphy, S. B.; Boger, D. V. *Rheol. Acta* **2001**, *40*, 23
(22) Durand, D.; Bertrand, C.; Clark, A. H.; Lips, A. *Int. J. Biol. Macromol.* **1990**, *12*, 14
(23) Stading, M.; Hermansson, A. M. *Food Hydrocolloid* **1990**, *4*, 121
(24) Ross-Murphy, S. B. *Rheol. Acta* **1991**, *30*, 401.
(25) Stauffer, D.; Coniglio, A.; Adam, M. *Adv. Polym. Sci.* **1982**, *44*, 103.
(26) Argüelles-Monal, W.; Goycoolea, F. M.; Peniche-Covas, C.; Higuera-Ciapara, I. *Polym. Gels Networks* **1999**, *6*, 429.
(27) Oakenfull, D. G.; Scott, A. In *Gums and Stabilisers for the Food Industry*; Phillips, G. O., Wedlock, D. J., Williams, P. A., Eds.; Elsevier Applied Science: London, 1986; Vol. 3, p 465.
(28) Oakenfull, D. G. *Crit. Rev. Food Sci. Nutrit.* **1987**, *26*, 1.
(29) Oakenfull, D.; Scott, A. In: *Gums and Stabilisers for the Food Industry Vol. 4*; Phillips, G. O., Wedlock, D. J., Williams, P. A., Ed.; IRL Press: Oxford, 1988; p 127
(30) Clark, A. H.; Ross-Murphy, S. B. *Br. Polym.* **1985**, *17*, 164.
(31) Clark, A. H. In *Food Structure and Behaviour*; Blanshard, J. M. V., Lillford, P., Eds.; Academic Press: London, 1987; p 13.
(32) Moore, G. K.; Roberts, G. A. F. *Int. J. Biol. Macromol.* **1980**, *2*, 78.
(33) Franks, F. *Water*; Royal Society of Chemistry: London, 1983.
(34) Ross-Murphy, S. B.; Stepto, R. F. T. In *Cyclic Polymers*; Semlyen, J. A., Ed.; Elsevier Appl. Sci.: Barking, 1986.
(35) Shild, H. G. *Prog. Polym. Sci.* **1992**, *17*, 163.
(36) Ross-Murphy, S. B.; Tobitani, A. In *Hydrocolloids Part 1. Physical Chemistry and Industrial Application of Gels, Polysaccharides, and Proteins*; Nishinari, K., Ed.; Elsevier Science B. V.: Amsterdam, 2000; p 379.
(37) Matricardi, P.; Dentini, M.; Crescenzi, V.; Ross-Murphy, S. B. *Carbohydr. Polym.* **1995**, *27*, 215.
(38) Atkins, P. W. *Physical Chemistry*, 6th ed.; Oxford University Press: Oxford, 1998.
(39) Clark, A. H. *Faraday Discuss.* **1995**, *101*, 77.
(40) Sarkar, N. *Carbohydr. Polym.* **1995**, *26*, 195.
(41) Haque, A.; Morris, E. R. *Carbohydr. Polym.* **1993**, *22*, 161.
(42) Desbrières, J.; Hirrien, M.; Ross-Murphy, S. B. *Polymer* **2000**, *41*, 2451.
(43) Richardson, R. K.; Ross-Murphy, S. B. *Int. J. Biol. Macromol.* **1981**, *3*, 315.
(44) Kaneko, Y.; Sakai, K.; Okano, T. In *Biorelated Polymers and Gels. Controlled Release and Applications in Biomedical Engineering*; Okano, T., Ed.; Academic Press: San Diego, CA, 1998; p 29.
(45) Osada, Y. *Adv. Polym. Sci.* **1987**, *82*, 1.