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Temperature and pH-sensitive chitosan hydrogels: DSC, rheological and swelling evidence of a volume phase transition

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Summary

On heating, alkali chitin solutions undergo phase separation describing a characteristic "U-shaped" cloud point curve with a lower critical solution temperature (LCST) centered at ~30 °C. The process is accompanied by gelation of the polymer-rich phase. A different strategy to induce alkali chitin phase separation/gelation is by applying vacuum to the solution at room temperature during aprox. 72 h. Once washed to neutrality, chitin gels had a degree of acetylation of ~30-40 % (i.e. they were converted into chitosan). On cooling, these gels exhibit an exothermic peak in micro-DSC and a depression in G'' and tan δ traces, evidencing a volume phase transition centered at ~20 °C. This transition is observed only within a narrow range of pH ~7.3 – 7.6. Variation in the mechanical response as a result of cyclic stepwise changes in temperature between 50 and 0 °C at pH values from 7.3-7.6, revealed that the G' modulus of the gels increases on heating and decreases on cooling, a behavior that persists over at least four cycles of temperature change. Only marginal changes in G' at pH 8.0 and not at all at pH 12.0 are observed. By contrast, the variation of G'' persists throughout the range of pH. This behavior is rationalized in terms of the existence of a fine balance between hydrophobic and hydrophilic interactions at varying temperature and pH, thus effectively controlling swelling and shrinking states of the gel network. The degree of swelling at pH 7.6 reaches a minimum at ~22-25 °C.

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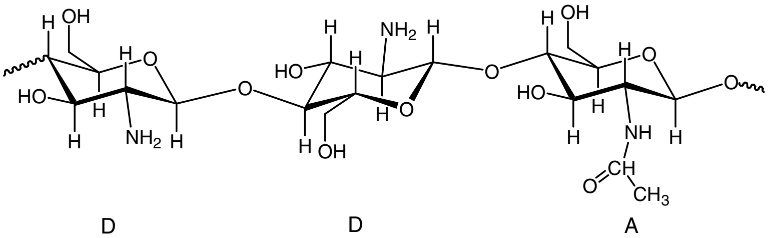
Introduction

'Smart', 'responsive', or 'intelligent' gels can be tuned to change their physical properties, namely swelling behavior, permeability or mechanical strength, in predictable and pronounced ways. These polymer systems are currently the subject of intensive study due to the great potential they bear for biomedical and bioengineering applications, namely, pulsatile drug release, molecular separation processes, diagnostics, cell culture and bioreactions [1-3] Often, temperature sensitive hydrogels exhibit lower critical solution temperature (LCST). Below the LCST, the gel is swollen, hydrated and hydrophilic, and above it, the gel becomes collapsed, dehydrated and hydrophobic. Furthermore, its volume phase transition can be controlled by incorporating more hydrophilic or hydrophobic functional groups or monomers in their structure [4]. This principle has been used to prepare copolymers that exhibit temperature-sensitive swelling-deswelling changes over a limited pH range [5]. Well known gel systems exhibiting LCST, include synthetic polymers such as poly-N-isopropylacrylamide (poly-NIPA) (LCST at ~32.0 °C) [6] its copolymers and interpenetrated networks, N,N-diethylacrylamide, poly(ethylene oxide)/poly(propylene oxide) block copolymers, among others, which undergo a discrete volume phase transition from swollen to shrunken state [7]. Such transitions were interpreted in terms of a 'volume phase transition' [7, 8, 9] and their mechanism was successively clarified [10]. Most such polymer systems are of synthetic origin and only little work has been conducted on hydrogels prepared from natural polymers. Special attention in this regard has been focused on employing natural polymers such as cellulose derivatives [11] to compose hydrogels with a specific response to a physicochemical or biological environment. Chitosan (Scheme 1) is particularly attractive due to its excellent biocompatibility and mechanical properties and has been used as a biomedical material. However, in many instances, it needs to be chemically modified in order to give rise to three dimensional aqueous gel networks. Chitosan hydrogels can be obtained by various mechanisms of chemical or physical crosslinking such as covalent [12, 13], ionic [14, 15], hydrogen-bonding [16, 17] or hydrophobic association [18, 19]. Recently, physical chitosan hydrogels prepared from alkali chitin [20], have been demonstrated to form as a result of a phase separation process dominated by hydrophobic association describing LCST behavior [21, 22]. Alkali chitin has LCST at ~30 °C [21]. This work accounts for the swelling, thermal and mechanical behavior of chitosan hydrogels at varying pH in the range 7.3 to 12.0 and we provide evidence of the existence of a phase transition centered at 22 °C.



Scheme 1. Chemical structure of a chitosan chain showing its constituent monosaccharide residues: 2-amino-2-deoxy-β-D-glucose (D units) and 2-acetamido-2-deoxy-β-D-glucose residue (A unit) (D>>A).

Experimental

Materials

Chitin (degree of acetylation, DA ~79%) was a sample previously isolated from prawn (*Ploeticus mülleri*) shell waste and it was from the same processing batch as that used in previous studies [23]. Chemicals were from Sigma-Aldrich (Mexico City) of analytical grade. Bi-distilled water was used throughout. Buffer solutions for the various experiments at pH 7.3 to 8.0 were prepared with citric acid/Na_2HPO_4, whereas at pH 12 they were prepared with NaOH. In all cases, the ionic strength was adjusted to $I = 0.5$ with KCl.

Gels preparation

Alkali chitin solution (1.6 % w/w) was prepared in cold (~0°C) aqueous NaOH (16 % w/w) according with the protocol of Sannan *et al.*[24], and was poured into either a Teflon® plate (70 × 70 mm) perforated with cylinders ($\phi = 3$ mm, $l = 8.7$ mm) or into Petri dishes and left to stand under vacuum at ~0.125 µmHg at 25°C during 72 h, a process that led to setting of turbid hydrogels of well-defined dimensions ($\phi = 50$ mm, l=2mm). NaOH was removed by transferring the set gels from the plates into an excess volume of water at ~65 °C under gentle stirring. The gels were further washed exhaustively with water at room temperature by changing the water of the beaker until complete removal of NaOH was ensured from pH measurements at the gel surface. For swelling experiments the cylinder gels were freeze-dried, while for rheological and calorimetric determinations the gels made in Petri dishes were kept fresh. The polymer concentration of fresh hydrogels was 2.61 % (w/w).

Physicochemical characterization

The degree of acetylation of the chitosan hydrogels was 27.6 % as determined by UV first derivative spectroscopy [25]. The viscosity-average molecular weight, M_v, was ~158,300 as determined in 0.3M acetic acid/0.2 M sodium acetate at 25°C ([η]~695.7 mL/g with K=0.069 g/mL and a = 0.77) [26].

Microcalorimetric determinations

The thermal transitions in chitosan hydrogels were monitored by micro-DSC in a Setaram equipment (µDSC-IIIa, Caliure, France) by loading a piece of gel into the calorimeter pan and buffer solution was added. The corresponding buffer was used in the reference pan.

Rheological determinations

Dynamic viscoelastic measurements were conducted in a sensitive strain-controlled fluids rheometer (RFS-II, Rheometrics, Piscattaway, NJ) fitted with a parallel plate-plate tool (ϕ: 25 mm) and a circulating environmental system for temperature control. The strain was fixed in such a way to avoid nonlinear viscoelastic deformations (generally between 10 and 30%). In a typical experiment a gel slab formed in a Petri dish 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 value was registered with a micrometer. 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. A glass ring of $\phi = 60$ mm was placed around the measuring geometry, and the annulus was filled with the appropriate buffer.

Swelling tests

The degree of swelling of freeze dried gels was examined at pH 7.6. The swelling coefficient (S) was determined gravimetrically and represented the gel water uptake:

$$S = \frac{w_h - w_s}{w_s} \qquad \mathbf{(1)}$$

where w_{h} is the weight of the hydrated gel and w_{s} is the weight of the dried gel.

Results and Discussion

Chitosan hydrogels addressed in this and in previously related studies [22, 23, 27] were invariably set by quiescent standing of alkali chitin under vacuum at ~25°C for a prolonged period of time (typically ~72 h). Up to now, however, we have offered no mechanism to why do alkali chitin phase separates and gels while left standing under vacuum at room temperature, as heating was inexorably required for the construction of the phase diagram at atmospheric pressure [22]. A possible interpretation can be offered by establishing an analogy to the effects of high pressure on hydrophobic poly-NIPA and on weakly-charged poly-NIPA-co-acrylic acid solutions and gels recently addressed using SANS [28,29]. Indeed, the shape of binodal curve of the temperature-pressure phase diagram for crosslinked gels of these polymers, describes a concave function with increasing pressure from atmospheric up to ~400 MPa. A maximum in temperature is reached at critical pressure above which the temperature for phase separation decreases again with a change in the sign of the slope of the dT/dP curve from positive to negative. This has been explained in thermodynamic grounds as the change in volume and enthalpy when going from the sol to the gel phase associated with the formation of "iceberg" water around hydrophobic groups in poly-NIPA. At pressures close to atmospheric the slope for the binodal curve on dT/dP (= $T\Delta V/\Delta Q$) is positive (i.e. as ΔV and ΔQ are both negative), hence, under vacuum it would be expected that the temperature for phase separation decreases with respect to the expected temperature from the binodal curve of alkali chitin solution of 1.6% w/w (~45°C) at atmospheric pressure [22].

Figure 1 below shows micro-DSC traces during heating of fresh chitosan hydrogels equilibrated in various buffer systems at varying pH from 7.3 to 12.0. It becomes clear that only at pH 7.3 and 7.6 an exothermic thermal transition centered at ~20°C is clearly observed, while no such process was found at pH neither 8.0 nor 12.0. The calculated enthalpy values of the exothermic transitions for the gels at pH 7.3 and 7.6, were -3.496 and -26.935 J/g of dry gel. Notice that there is almost an order of magnitude difference between the ΔH of the thermal processes at pH 7.3 and 7.6. This disparity can be attributed to the slightly greater charge density in chitosan at pH 7.3 than at 7.6, hence tipping the balance between hydrophobic and hydrophilic interaction. The interplay between these two effects involving directly the ionization state of -NH_2 groups seems necessary for the systems to exhibit its transition within

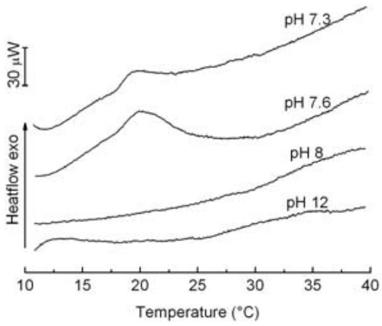


Figure 1. Micro-DSC traces during heating (1°C/min) of chitosan hydrogels equilibrated at various pH values (as shown in labels).

such narrow range of pH. Indeed, the fully neutral gel at pH 8.0 gives no discernable DSC peak from the background noise of the thermogram. Neither does it at pH 12, where one can expect that hydroxyl groups become ionized. Hence, the phase transition in chitosan gels seems to be closely related to the subtle balance between the ability of the polymer to form hydrogen bonds with water and the inter- and intra-molecular hydrophobic forces [30], a condition that seems to be maximized in the vicinity of pH 7.6.

Figures 2a and 2b show representative mechanical spectrum and a strain sweep, respectively, of a chitosan hydrogel equilibrated at pH 7.6 at 5°C. Notice in Figure 2a that the frequency dependence of *G'*, *G"* and η^* is that characteristic of a permanent gel network, as *G'* values remain independent on frequency and η^* has a negative slope of -1. Figure 2b illustrates the variation of *G'* and *G''* viscoelastic moduli showing that the rheological properties remain constant with increasing amplitude of oscillation up to a strain of ~4 %.

The strain dependence behavior confirmed that measurements in Figure 2a, were registered within the linear viscoelastic region ($\gamma = 2$ %). At strains greater than 4 %,

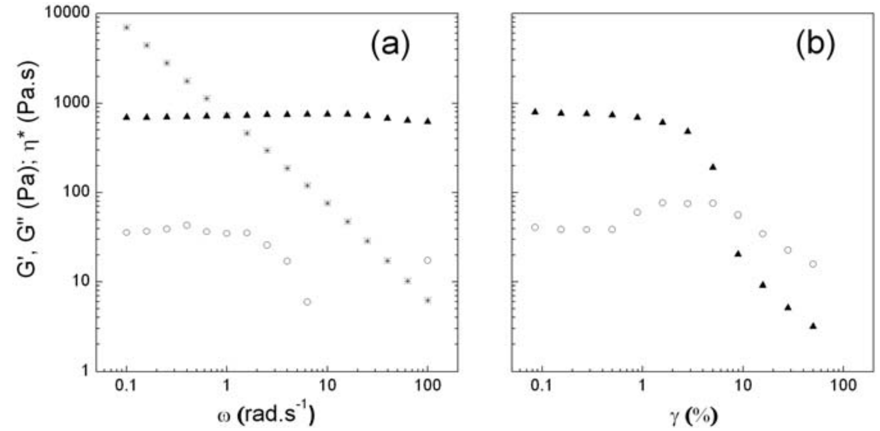


Figure 2. (a) Frequency dependence of the storage *G'* (▲), and loss, *G''* (○), shear moduli and of the complex viscosity, η^* (∗) ($\gamma = 2$ %, 5 °C) and (b) Strain dependence of *G'* (▲), and loss, *G''* (○) moduli ($\omega = 4.6$ rad/s, 5 °C) of chitosan hydrogel at pH 7.6.

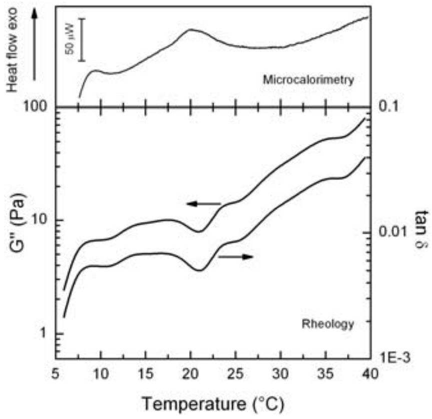


Figure 3. Temperature dependence of the loss modulus, G'', and tan δ (ω=4.6 rad/s, γ= 2 %) during heating (1 °C/min) of chitosan hydrogels equilibrated at pH 7.6. In the upper frame is reproduced the corresponding micro-DSC exotherm registered under identical conditions.

the solid character drops off sharply, presumably due to the breakdown of the polymer network. The loss modulus values, however, show an initial increase and subsequent decrease; this can be attributed to fragments of disordered chains disengaged from the gel network, conferring a viscosity contribution which decreases as they are broken down further. Similar effects have been observed in the strain dependence of *G"* of soy protein gels [31].

Once confident that the rheological measurements were registered well within the linear viscoelastic region, a series of experiments were conducted in order to investigate the temperature dependence of the mechanical properties of the system under small-deformation oscillatory shear rheology. Figure 3 shows the temperature dependence of the shear loss modulus, *G"*, and of tan δ during heating from 5 to 40 °C a chitosan gel at pH 7.6. Also in the plot, for comparison, the corresponding heating thermogram for the same gel (the same one as in Figure 1) is included. Inspection of the *G"* and tan δ full heating traces reveals at first glance that heating results in an overall increase in *G"* by an order of magnitude.

However, this increase is not monotonic but reveals several distinct regions. From ~7 to ~13 °C, *G"* values tend to increase with temperature, while on further heating to ~17 °C attain a steady value followed by a depression with a minimum at ~21 °C before they increase again but under a greater gradient as heating proceeds up to 40 °C. Notice that the minimum in the depression event coincides closely with the maximum in the exothermic process observed by micro-DSC. Interestingly, no such process was observed in the storage modulus, *G'*, trace (not shown).

A further experiment was devised in order to explore the response of the system to oscillating changes in temperature. Figure 4 shows the response of the system to stepwise changes in temperature between 5 and 40 °C. Inspection of the behavior of the gel system at pH 7.3, 7.6 and to a less extent also at 8.0, shows that initially as the temperature increases, *G'* values do so too, while upon cooling to 0 °C, they descend.

This pulsatile mechanical response is reversible and persists to at least four cyclic stepwise changes in temperature. Although the gel at pH 7.6 could only be tested for two cycles, its overall response was essentially identical to that at pH 7.3, though it attained slightly greater values of *G'* on each heating cycle. At pH 8.0, the variation in

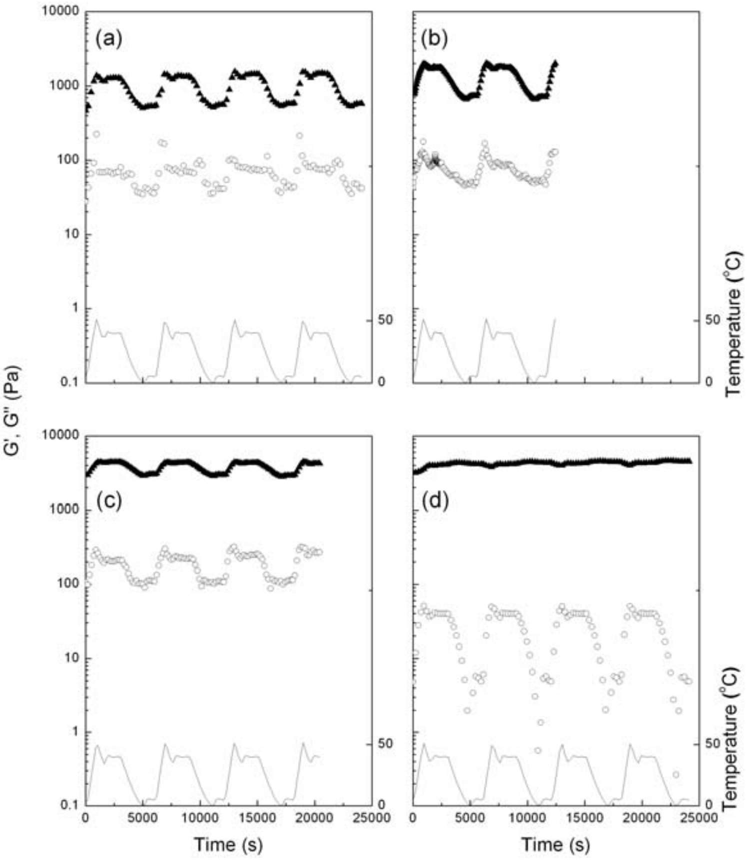


Figure 4. Variations in storage, *G’* (▲), and loss, *G’’* (○), moduli, with stepwise periodic changes in temperature (continuous line) between ~0 and 50 °C for chitosan hydrogels at pH: a) 7.3; b) 7.6; c) 8.0 or d) 12.0 ($\omega = 4.6$ rad/s, $\gamma = 2$ %).

G’ with temperature is less pronounced than at pH 7.6, while virtually no changes are seen at pH 12.0. In turn, the variation of *G”* values persists with the cyclic change in temperature but by contrast with the behavior of *G’*, the magnitude of the difference between the end of the cooling and heating cycles becomes more pronounced as the pH increases. This rheological response is consistent with the notion that chitosan hydrogels behave as a hydrophobic system in which as the temperature increases, the abrupt increase in shear modulus is predominantly associated with the collapse of the gel network mainly due to the contribution of the association of residual N-acetyl groups. At the same time, the higher free energy may be a consequence of the entropic contribution of the water phase. This is in sharp contrast with other biopolymer gel systems including a large series of polysaccharide and proteins dominated by hydrophilic type interactions [32]. In a recently published article [33], we have shown that hydrogels of a hydrophobic derivative of chitosan, N-isobutyryl chitosan, behave essentially in the same manner as found here for chitosan at pH 7.3-8.0 when subjected to exactly the same experiment. By contrast, in hydrogels of agar-agar, heating leads to a reduction of the shear modulus, and cooling to an increase [33]. It is important to mention that the mechanical behavior of chitosan hydrogels shown in Figures 3 and 4 is affected not only by the change in temperature that controls the degree of connectivity in the network but also by the change in the degree of swelling (see below); a reduction in the elastic modulus as the swelling degree increases is predicted theoretically in vulcanized rubber, which is in agreement with our results.

The observed thermal and rheological behavior of chitosan gels here seems to be consistent with the proposal that two network phases can co-exist in the system, namely a rigid network whose connectivity determines the shear storage modulus, and a second viscous phase that experiences a collapsing transition [34]. The rigid network is thought to involve cooperative junctions zones mediated by hydrogen bonding between -NH_2 groups, hence the thermally dependent response of *G'* is highly sensitive to pH, while the second network involves coil or globular aggregated regions mediated by hydrophobic interactions, and therefore they are not sensitive to pH. Difference in the behavior of *G'* and *G"*, may reflect the contributions of each of these two phases.

Figure 5 shows the temperature dependence of equilibrium swelling, S_{eq}, for chitosan hydrogels at pH 7.6 at varying discrete temperatures in the range from 5 to 40 °C. Each point in the curve represents the swelling after equilibrium was reached (typically 3 h) of gels allowed to swell directly at each corresponding temperature. Notice that at 5 °C the largest swelling was observed. As the temperature increases up to ~25 °C a progressive decrease in S_{eq} is seen and a minimum in the curve is attained, followed by a rise as the temperature increases up to ~40 °C. The initial decrease in swelling S_{eq} can be ascribed to the collapsing of the gel network favored by the elevation in temperature. This behavior, however, is different to the one observed in other thermally sensitive polymers such as poly-NIPA or hydroxypropylcellulose, for which a sharp discontinuous decrease in S_{eq} appears at 32-33 °C as the gel undergoes its volume phase transition, thus effectively describing a sigmoidal curve [6, 7, 35]. This seems to argue again in favor of two operating mechanisms underlying the hydration of the gel network. On the one hand, an hydrophobically driven process that leads to collapse of the gel network as this is conducted at progressively greater temperatures, hence attaining its minimum swelling close to the volume phase transition temperature (~20 ºC). On the other, when the uptake of water is conducted directly at temperature greater than the volume phase transition, the diffusion of solvent into the polymer struts is obstructed and hence convection of solvent through the pores becomes dominant [11]. Hence, most of the water uptaken is conceived to be

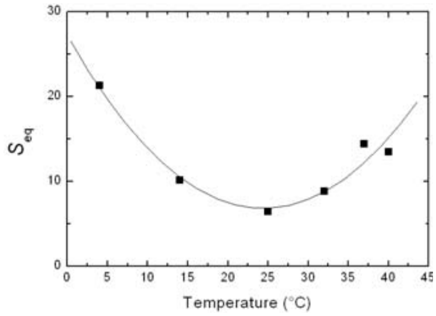


Figure 5. Temperature dependence of equilibrium swelling, S_{eq} of freeze-dried chitosan hydrogels at pH 7.6.

present as free water. In agreement with this suggestion, we have demonstrated that when chitosan hydrogels are swollen directly at 37 °C at varying pH from 6.9 to 12.0, there is no variation at all in S_{eq} [36]. The temperature for minimum in S_{eq} found here in chitosan (~20 ºC) lies well below the LCST found in alkali chitin (~30 °C). This may be the consequence of the difference in solvent used in each system (water *vs.* concentrated NaOH, respectively).

The equilibrium swelling data in the range from the minimum value at 25 ºC up to 40 ºC were adjusted to the Gibbs-Helmoltz equation [37]:

$$\frac{d \ln S_{\mathrm{eq}}}{d(1/T)} = -\frac{\Delta H_{\mathrm{m}}}{R} \qquad (\mathbf{2})$$

where ΔH_{m} is the mixing enthalpy between the dry gel and an infinite amount of water and R is the gas constant. Figure 7 shows the best fit resulting curve where a negative enthalpy evidencing an endothermic mixing process. The obtained value of ΔH_{m} was 42.8 kJ mol^{-1}. This is in direct contrast with the behavior of the system at temperatures below the polymer phase transition, for which an exothermic enthalpy of mixing can be readily foreseen. As the temperature increases progressive hydrophobic dehydration of chitosan may occur up to the transition temperature above which the creation of water ‘íceberg’ structures around hydrophobic groups is expected to occur.

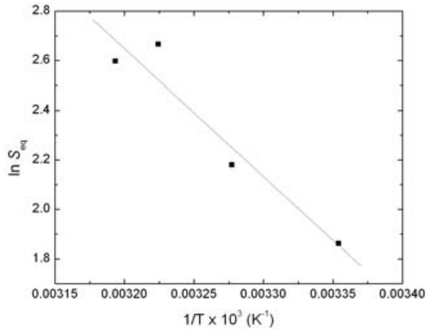


Figure 7. Gibbs-Helmoltz representation of the dependence of equilibrium swelling data on reciprocal absolute temperature for chitosan gels in the interval 25 to 40 °C (data from Figure 6).

Conclusion

We have demonstrated by DSC and oscillatory small-deformation rheology that chitosan hydrogels have a volume phase transition at ~21°C. The origin of the observed transitions in this system is consistent with the existence of a LCST. This is the result of the influence of temperature on polymer-polymer and polymer-water interactions such as hydrogen bonding and hydrophobic interactions. This transition is observed only within a narrow range of pH (7.3-7.6) and entails the ability to modify the mechanical shear modulus as a function of oscillating variation in temperature.

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