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ORIGINAL CONTRIBUTION

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Linear viscoelasticity of thermoassociative chitosan-g-poly(*N*-isopropylacrylamide) copolymer

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Abstract Chitosan-g-poly(*N*-isopropylacrylamide) (chitosan-g-PNIPAM) was synthesized and characterized rheologically in aqueous solutions. The copolymer solution exhibits a thermoassociative behavior in which its elastic response dramatically increases when temperature is above the critical temperature or the association temperature, T_{assoc}. The copolymer at low concentration shows typical solution property. When the temperature is increased up to the critical temperature, the copolymer exhibits a gel-like characteristic due to the formation of physical cross-links between chitosan backbones through the self-aggregation of PNIPAM side chains. At high concentration, the system exhibits a weak elastic response due to the entanglement of the copolymer at 25°C. As temperature is raised above T_{assoc}, the system shows a strong elastic behavior due to the formation of additional physical cross-links via the aggregation of PNIPAM side chains. Chitosan-g-PNIPAM offers an attractive associating behavior in aqueous solution at temperature close to the body temperature, thus providing potential applications in pharmaceutical and medical industries.

Keywords Linear viscoelasticity · Thermoassociative · Chitosan · Poly(*N*-isopropylacrylamide)

Introduction

Water-soluble associative polymers exhibiting thermothickening properties draw specific attention lately from industrial, biomedical, and pharmaceutical researchers due to their unique rheological properties (Aubry et al. 2003; Bokias et al. 2001; Durand and Hourdet 2000a). Their behaviors change from those of solutions to gels as temperature is increased above a critical temperature due to the presence of reversible intermolecular associations. A common way to obtain these associative polymers is by grafting polymers exhibiting a lower critical solution temperature (LCST) onto a hydrophilic polymer backbone (Aubry et al. 2003; Bokias et al. 2001; Durand and Hourdet 1999, 2000a). The gel formation is associated with system turbidity, indicating phase separation. Temperature where system turbidity is observed is called a cloud point. This phase separation is affected by LCST.

Copolymers of hydrophilic polymers and polymers exhibiting LCST are often investigated and developed to obtain thermothickening materials. These polymers are generally constituted of a hydrophilic backbone such as polyacrylic acid, carboxymethylcellulose with polymers exhibiting LCST distributed along the polymer backbone in the form of side chains or terminal groups (Aubry et al. 2003; Bokias et al. 2001; Hourdet et al. 1997 ; Durand and Hourdet 1999, 2000a,b). In a semidilute aqueous solution, when temperature is increased to the LCST of side chains or terminal groups, their thermothickening ability arises through reversible intermolecular associations of LCST chains into hydrophobic microdomains which physically act as cross-link junctions between hydrophilic backbones, leading to an increase in solution viscosity (Hourdet et al. 1997; Durand and Hourdet 2000b; Durand et al. 2000).

The temperature at the onset of an increase in viscosity is defined as the association temperature, T_{assoc} (Aubry et al.

2003; Durand and Hourdet 2000a,b). The value of T_{assoc} is normally close to the LCST of grafting molecules (Hourdet et al. 1998). However, in the case of stiff hydrophilic backbones, a slight difference between T_{assoc} and LCST may be observed, inducing topological constraints on the grafting chains and disturbing their phase separation (Bokias et al. 2001). As temperature is increased beyond T_{assoc}, the hydrophilic character of main chains does not allow the macroscopic phase separation of the side chains. Hence, the side chains are forced to be arranged in hydrophobic microdomains which can be identified with the small-angle neutron scattering technique (Hourdet et al. 1998).

The most often investigated polymers exhibiting LCST are polyethylene oxide (PEO), poly(*N*-isopropylacrylamide) (PNIPAM), and ethylene oxide–propylene oxide copolymers (Aubry et al. 2003; Bokias et al. 2001; Durand and Hourdet 2000a; Hasan et al. 2002; Molyneux 1987; Schild 1992). Among these polymers, PNIPAM is of great interest for uses in biomedical applications, such as drug delivery, tissue engineering, and enzyme or protein modification since LCST of PNIPAM aqueous solution is about 33°C, which is close to the body temperature (37°C) (Aubry et al. 2003; Schild 1992; Wu 1998; Okano 1998). Below the LCST, PNIPAM can dissolve in water due to the formation of hydrogen bonding between polar groups of polymer and water molecules. Above the LCST, the hydrogen bonding is broken, which can be attributed to the dehydration of the hydrophobic isopropyl groups during the coil-to-globule transition, resulting in the precipitation of polymer in water. Copolymerization of PNIPAM with other monomers having different hydrophobicity yields copolymers with different LCST. The more hydrophobic the comonomer, the lower the LCST becomes. In addition, LCST is generally altered by the addition of additives such as electrolytes, organic solvents, and surfactants (Hourdet et al. 1997; Lessard et al. 2003).

The graft copolymer studied in this present paper consists of a low molecular weight chitosan backbone and PNIPAM side chains. Chitosan, a copolymer of glucosamine (Glu) and *N*-acetylglucosamine, is derived from the *N*-deacetylation of chitin, the second most abundant naturally occurring polysaccharide (Muzzarelli 1977). Chitosan is a biopolymer with biodegradable (Struszczyk et al. 1992), biocompatible (Chandy and Sharma 1990; Hirano et al. 1990), and mucoadhesive (Struszczyk et al. 1992; Chandy and Sharma 1990; Hirano et al. 1990; He et al. 1998; Henriksen et al. 1996; Lehr et al. 1992) properties which play many important roles in biomedical applications (Felt et al. 1998; Illum 1998; Malette et al. 1983; Sanford 1989). The objective of grafting of PNIPAM side chains onto the hydrophilic low molecular weight chitosan backbone is to synthesize a material with thermosensitive properties in aqueous solution. Since the phase transition of PNIPAM occurs at temperature of about 33°C, which is close to the body temperature, chitosan-g-PNIPAM can provide potential uses in pharmaceutical and medical applications.

In the present paper, low molecular weight chitosan-g-PNIPAM was synthesized. The association phenomenon of the graft copolymer in aqueous solution was rheologically investigated as a function of temperature and polymer concentration.

Experimental

Materials

N-isopropylacrylamide (NIPAM) (Sigma-Aldrich Co., USA) was recrystallized from hexane–toluene solution before use. Ceric ammonium nitrate (CAN), without further purification, was used as an initiator. A commercial chitosan (Seafresh Chitosan (Lab) Co., Ltd., Thailand) with degree of deacetylation of 95% [as determined by Fourier transform infrared (FTIR) spectroscopy] and molecular weight of 4.5×10^4 [as determined by gel permeation chromatography (GPC)] was used in this study. Sodium borohydride ($NaBH_4$) and sodium nitrite ($NaNO_2$) were purchased from Sigma-Aldrich Chemical Co. (Singapore). Isopropanol, hydrochloric acid (HCl), acetone, acetic acid, methanol, hexane, toluene, and tetrahydrofuran (THF) were provided by Labscan (Thailand). Sodium hydroxide (NaOH) was obtained from BDH Chemical (Thailand), and *N*,*N*′-dicyclohexylcarbodiimide (DCC) was supplied by Fluka (Switzerland). All reagents and solvents were used as received unless otherwise specified.

Synthesis and characterization

To improve solubility of the obtained product, low molecular weight chitosan was used to prepare chitosan-g-PNIPAM copolymer. The commercial chitosan was depolymerized via nitrous acid, followed by graft polymerization of NIPAM monomers onto depolymerized chitosan backbone. The products obtained from each step were characterized by FTIR spectroscopy and proton nuclear magnetic resonance, ^{1}H NMR, spectroscopy. FTIR spectra were obtained in the wavelength range of 400–3600 cm^{-1} using a Perkin Elmer System 2000R FTIR spectrometer. The spectrum of each sample was collected from 100 scans with a resolution of 4 cm^{-1}. ^{1}H NMR spectra were obtained from a 300-MHz Bruker DPX-300 spectrometer using 1.5 vol% acetic-d_3 acid in D_2O as a solvent.

The molecular weight distribution of chemically depolymerized chitosan was determined using GPC technique. A PL-GPC 110 system (Polymer Laboratories) was equipped with an ultrahydrogel linear column and a reflective index detector. The flow rate was 0.6 ml/min, using 0.5 M acetate buffer as the eluent.

Depolymerization of chitosan via nitrous acid Chitosan (20 g, HCl form) was dissolved in 400 ml of 1% v/v HCl. $NaNO_2$ (0.6 g) was added, and the depolymerization was allowed to proceed for 3 h at room temperature. Prior to purification, the solution was neutralized by aqueous NaOH solution, and the end groups of chitosan were reduced with $NaBH_4$. At the end of the reaction, the mixture was precipitated in acetone and filtered through a nylon membrane. The precipitate was vacuum-dried at room temperature overnight to yield 14.9 g (75%) of the chitosan with average molecular weights of M_n~8,800 and M_w~32,000 as determined by GPC.

Grafting reaction NIPAM was grafted onto the depolymerized chitosan backbone (1:3 by mole) using a modification of the method published by Kim et al. (2000). Graft polymerization was carried out using CAN as an initiator under a nitrogen atmosphere. Depolymerized chitosan (0.5 g, 3 mol) and NIPAM monomer (1.02 g, 9 mmol) were dissolved in 10% v/v aqueous acetic acid solution (5 ml). While bubbling nitrogen gas, CAN (47 mg, 0.086 mmol) was added, and the reaction mixture was stirred at 25°C for 2 h. The grafting polymerization was then terminated by precipitating the reaction solution in an excess acetone and separated by filtration. The NIPAM homopolymer formed during the reaction was removed from the grafted polymer by a Soxhlet extraction with methanol for 48 h; the obtained product was then vacuum-dried until a constant weight was attained.

Rheological measurement

Aqueous solutions of chitosan-g-PNIPAM at desired concentrations were prepared by dissolving the synthesized low molecular weight chitosan-g-PNIPAM in deionized water. Rheological experiments were performed with a strain-controlled rheometer (Rheometric Scientific, Inc., ARES) equipped with a solvent trap to prevent water evaporation during measurement. Two sizes of stainless-steel cone and plate geometry, i.e., 50- and 25 mm diameters with 0.04 rad cone angle, were employed depending on solution viscosity. The operating temperature ranging from 25 to 43°C was set and controlled by a circulating water bath.

Cloud-point measurement

The determination of the cloud point of the copolymer solutions was performed visually by following the variation of the turbidity with temperature. The solution in a test tube was immersed in a thermostated bath heated to a desired temperature. The cloud point was defined as the temperature at which the solution started to turn cloudy.

Results and discussion

Synthesis and characterization

The changes in chemical structure of the chitosan-g-PNIPAM copolymer were confirmed by FTIR and ^{1}H NMR spectroscopy techniques. The FTIR result was similar to that previously published by Kim et al. (2000; data not shown).

The NMR spectrum shows proton peaks derived from the chitosan backbone and the PNIPAM side chains, qualitatively demonstrating that the desired grafting composition of the chitosan-g-PNIPAM was achieved. Table 1 lists the experimentally estimated compositions for the graft copolymer studied, based on integration of ^{1}H NMR spectrum. Characteristic peaks of protons in chitosan and PNIPAM were found at 3.0–3.1 and 0.8–2.1 ppm, respectively. The integrals of these two characteristic peaks were used to measure the molar ratio of Glu (repeating unit of chitosan backbone) to NIPAM in the grafted copolymer.

Rheological behavior

The rheological behavior of chitosan-g-PNIPAM was studied at solution concentrations of 10, 20, and 30 mg/ml, which are above the overlap concentration of the copolymers. The overlap concentration (c^*) is defined as the concentration where the monomer density inside the coil is equal to the overall monomer density in the solution. It is a crossover between dilute and semidilute solutions. Empirically, the c^* is usually at a polymer concentration where its zero-shear viscosity (η_o) is about twice as high as that of the solvent ($\eta_o \sim 2\eta_s$; Boris and Colby 1998). In our study, the zero-shear viscosity of water (η_s), which was used as solvent, at 25°C is 0.89 mPa s. Thus, every concentration reported in this study is well above the c^*.

Table 1 Experimentally determined compositions for the chitosan-g-PNIPAM

Glu/NIPAM ratio	Molar fed ratio	1:3
	Molar measured ratio	1:5.1
Composition (mol%)	Glu repeating units	16.4
	NIPAM repeating units	83.6
Composition (wt%)	Glu repeating units	22.5
	NIPAM repeating units	77.5
Estimated number of groups per copolymer molecule[a]	Glu repeating units	52
	NIPAM repeating units	265

PNIPAM Poly(*N*-isopropylacrylamide), *Glu* glucosamine
[a]Calculated based on the molar compositions and 52 Glu groups per chitosan molecule, estimated from molecular weight of chitosan

Determination of linear viscoelasticity from strain sweep measurement

To determine the linear viscoelastic subrange of the chitosan-g-PNIPAM aqueous solution, a strain sweep experiment was carried out at a fixed angular frequency (ω=10 rad/s) at the copolymer concentration of 10 mg/ml. Figure 1 shows the plot of the storage, G', and the loss, G'', moduli as functions of strain amplitude. The data indicate that at 25°C, the system shows solution behavior as $G''>G'$, and all loss tangents (tanδ) are higher than 1 for all strains studied. When the temperature is raised to 35°C, the sample becomes elastic as $G'>G''$, and all loss tangents are lower than 1 for all strains studied. At 35°C, the observed moduli are strain independent and display linear viscoelasticity (for all of the strain amplitudes studied), while at 25°C, the moduli are strain independent when the applied strain is lower than 25%. Therefore, results reported below were obtained from experiments at strain amplitude within the linear range.

Characterization of the association process by rheological measurement

Figure 2 shows plots of storage and loss moduli as functions of angular frequency of 10 mg/ml chitosan-g-PNIPAM aqueous solution at different temperatures. At 25°C, the copolymer solution behaves as most fluids, where G'' is higher than G'. The transition from solution at low temperature (25°C) to gel at high temperature is observed. This transition point where the system behavior changes from solution to gel is called a gel point. Beyond the gel point, elastic property dominates due to the network ability to store energy as can be seen from the increase in G'.

To determine the gel point, a method proposed by Winter and Chambon (1986) and Winter (1987) has been used to

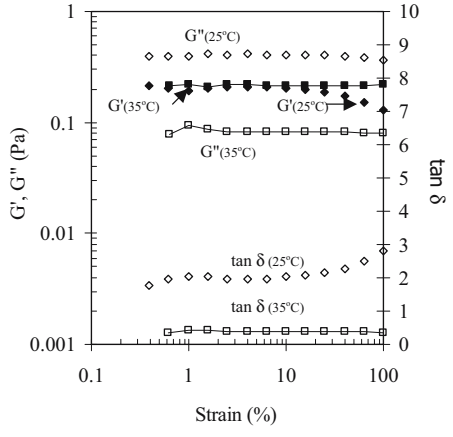

Fig. 1 Storage and loss moduli as well as tanδ as functions of 10 rad/s of 10 mg/ml chitosan-g-poly(*N*-isopropylacrylamide) (chitosan-g-PNIPAM) in aqueous solution at 25 and 35°C

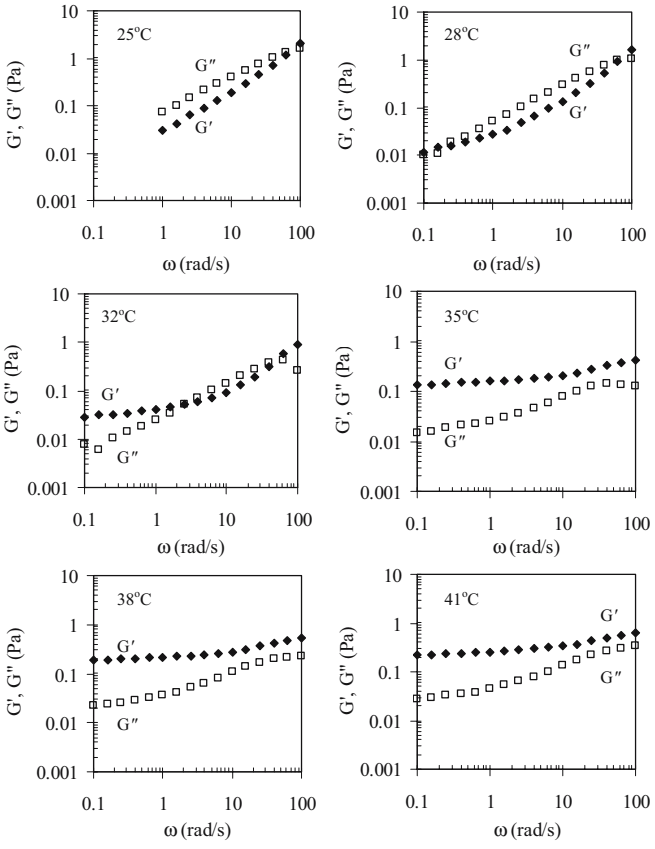

Fig. 2 Storage and loss moduli vs angular frequency at a strain of 10% of 10 mg/ml chitosan-g-PNIPAM in aqueous solution at various temperatures

describe the rheological behavior of a critical gel with a power law where the dynamic moduli are related as

$$G'(\omega) = \frac{S\pi\omega^n}{2\Gamma(n)\sin(\pi n/2)} \quad (1)$$

$$G''(\omega) = \frac{S\pi\omega^n}{2\Gamma(n)\cos(\pi n/2)} \quad (2)$$

where $\Gamma(n)$ is the Legendre gamma function, n is the relaxation exponent, and S is the gel strength parameter (Winter and Chambon 1986; Winter 1987) depending on the cross-linking density and the molecular chain flexibility. At the gelation temperature, G' and G'' have the same power law frequency dependence (n), or equivalently, where the loss tangent becomes independent of oscillatory frequency:

$$G'_c(\omega) \;\alpha\; G''_c(\omega) \;\alpha\; \omega^n \quad (3)$$

$$\tan\delta_c = \frac{G_c''(\omega)}{G_c'(\omega)} = \tan\left(\frac{n\pi}{2}\right) \quad (4)$$

where $G'(\omega)$ and $G''(\omega)$ are the frequency-dependent storage and loss moduli, respectively. Subscript c denotes the critical point. In the case that the scaling exponent is equal to 0.5 (n=0.5) at the gel point, the loss tangent is equal to 1 ($\tan\delta_c$=1) and independent of frequency. Thus, the gel point can be detected by the crossover of G' and G''. Detection of gel point is also possible when the relaxation exponent is not equal to 0.5 ($n\neq0.5$). The dynamic moduli of the system at the gel point follow the same power law, along with different prefactors. They do not necessarily coincide at the gel point, but they are parallel, $G'=G''/\tan(n\pi/2)$. In this case, the gel point can be detected from $\tan\delta=G''/G'$, which is independent of the frequency (Winter 1987).

For our 10 mg/ml chitosan-g-PNIPAM aqueous solution, we determined the scaling exponent (n) from the slope of plots of storage and loss moduli vs frequency (Fig. 2), respectively, as shown in Table 2. The scaling exponents of both moduli decrease as temperature increases. The scaling exponent of G' approaches 0 at high temperature, while the power law exponent of G'' appears to reach a nonzero constant value. This finding may reflect the fact that the characteristic of the copolymer network structure is sensitive and can be easily disrupted at high frequency since it is only a physical cross-linking of low molecular weight chitosan. At 28°C, the values of both n are nearly the same, suggesting that the gel point should be near this temperature according to the Winter–Chambon criteria. To quantify the gelation temperature according to the Winter–Chambon criterion for a critical gel, the gel point can be identified from a frequency-independent value of tanδ as obtained from a plot of tanδ vs temperature at different frequencies. As can be seen in Fig. 3, our system does not strictly obey this criterion. The plots of tanδ vs temperature at different frequencies of 10 mg/ml chitosan-g-PNIPAM in aqueous solution do not intersect at a single point for all frequencies investigated, suggesting the absence of a *single* self-similar, fractal structure at the gel point. The Winter–Chambon criterion has been found not to hold for a few previously reported systems where a single-point intersection of tanδ is not detected (Chiou et al. 2001; Richtering et al. 1992; Ilvaský et al. 1996, 1998, 1999; Izuka et al. 1997).

Table 2 The values of n obtained from the slope of plots between storage and loss moduli vs frequency, respectively, for 10 mg/ml chitosan-g-PNIPAM solution

Temperature (°C)	Power law (n) scaling exponent of G'	Power law (n) scaling exponent of G''
25	0.899	0.682
28	0.759	0.798
32	0.207	0.723
35	0.095	0.374
38	0.094	0.361
41	0.099	0.358

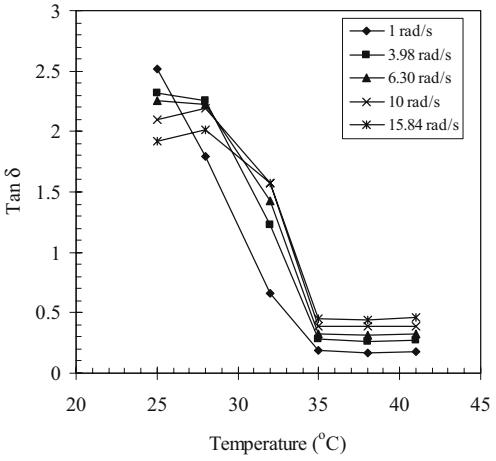

Fig. 3 Loss tangent (tanδ) vs temperature at various frequencies of 10 mg/ml chitosan-g-PNIPAM in aqueous solution

Since we cannot identify the gel point according to the Winter–Chambon criterion, we identified the gel point by the first appearance of a nonzero equilibrium modulus, G_e, or by the divergence of the viscosity (Chiou et al. 2001). From Fig. 2, G_e can be first seen at temperature between 28 and 32°C, meaning that the gel point of this system should be in between these two temperatures.

Dynamic viscosity of 10 mg/ml chitosan-g-PNIPAM aqueous solution was plotted as a function of temperature in Fig. 4. As temperature increases from 25 to 32°C, the viscosity of the system decreases. This behavior can be explained by the Arrhenius equation, $\eta\sim\exp(E_a/kT)$, where η is the viscosity, E_a is the activation energy, k is the Boltzmann's constant, and T is the absolute temperature (Rubinstein and Colby 2003), and has been observed in

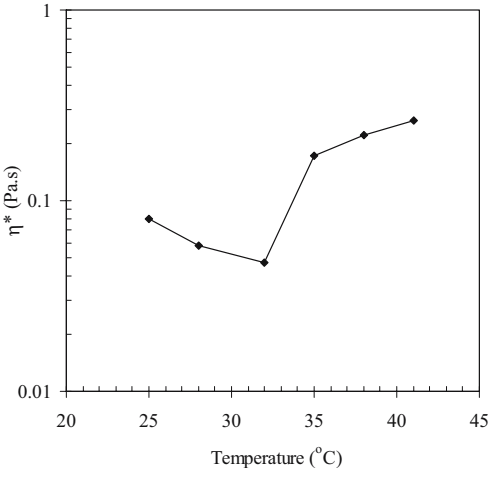

Fig. 4 Dynamic viscosity vs temperature of 10 mg/ml chitosan-g-PNIPAM aqueous solution at a frequency of 1 rad/s

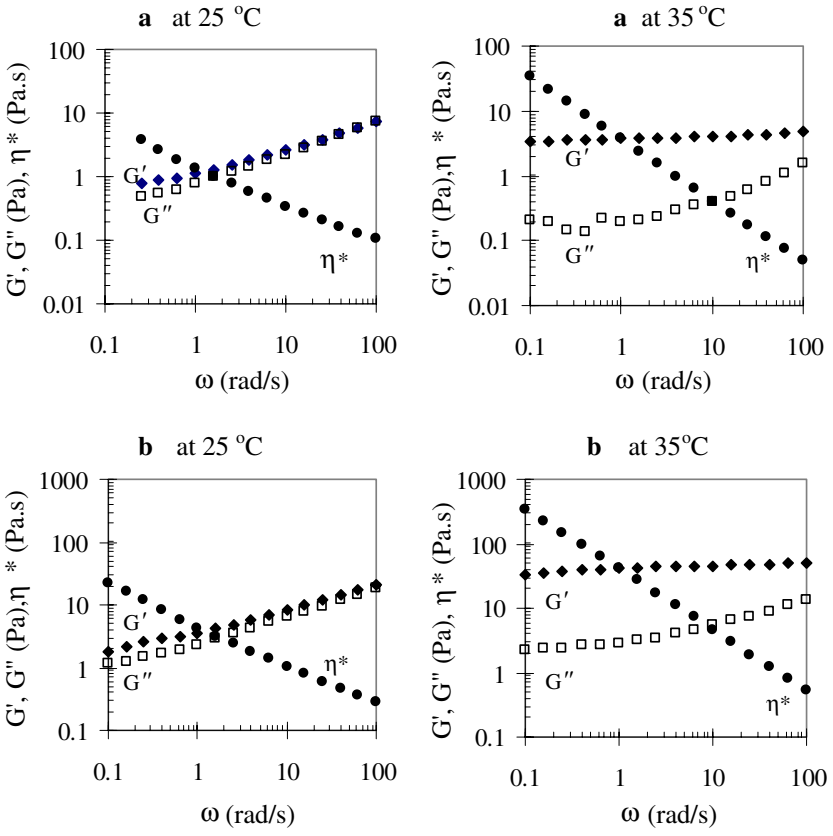

Fig. 5 Storage and loss moduli as well as dynamic viscosity vs angular frequency at 25 and 35°C of samples: **a** 20 mg/ml chitosan-g-PNIPAM in water at 10% strain; **b** 30 mg/ml chitosan-g-PNIPAM in water at 7% strain at 25 and 35°C

several thermoassociative systems as well (Bokias et al. 2001; Durand and Hourdet 1999, 2000a,b; Plucktaveesak et al. 2000). When the temperature is further increased to 35°C, the dynamic viscosity is abruptly increased. This is attributed to the self-aggregation of PNIPAM side chains to form hydrophobic microdomains which physically cross-link the chitosan backbones, leading to the gelation or the formation of a high-viscosity reversible network (Bokias et al. 2001). From these results, the gel point of this system should be in between 28 and 32°C as determined from the first appearance of G_e (Fig. 2) and by the divergence of the dynamic viscosity (Fig. 4). As the temperature is increased further to 35, 38, and 41°C, the sample shows a strong elastic response.

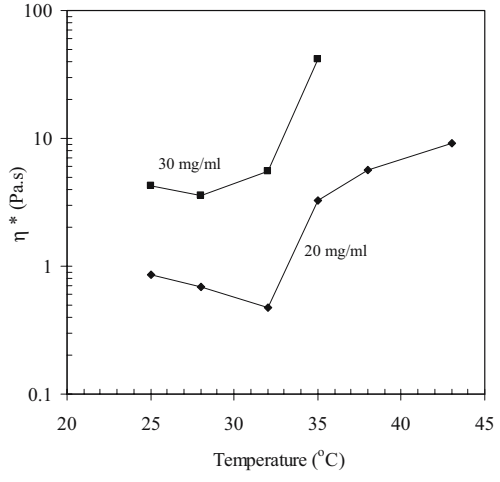

Fig. 6 Dynamic viscosity vs temperature of 20 and 30 mg/ml chitosan-g-PNIPAM aqueous solutions at a frequency of 1 rad/s

As previously mentioned, the association temperature (T_{assoc}) is defined as the temperature where PNIPAM side chains start to self-aggregate which physically cross-links the hydrophilic backbones, leading to the formation of a reversible associative network (Hourdet et al. 1998). Therefore, T_{assoc} can be determined from the abrupt increase in the dynamic viscosity of the system. The gelation at high temperature observed in 10 mg/ml chitosan-g-PNIPAM is due to the mechanism of self-aggregation of PNIPAM side chians.

Table 3 The association and the cloud-point temperatures of chitosan-g-PNIPAM aqueous solution

Concentration (mg/ml)	T_{assoc} (°C)	$T_{cloud\ point}$ (°C)
10	28–32	31–32
20	28–32	31–32
30	28–32	30–31

We also studied the system at higher concentrations of the copolymer (20 and 30 mg/ml). Fig. 5 shows the storage and loss moduli and the dynamic viscosity of 20 and 30 mg/ml aqueous solutions of chitosan-g-PNIPAM at 25 and 35°C. The solutions are weakly elastic at 25°C due to entanglement of the copolymer. However, as the temperature was increased, the solutions exhibited a predominantly elastic behavior due to the additional formation of physically cross-linked points through aggregation of PNIPAM side chains. The association temperatures of the copolymer solutions of 20 and 30 mg/ml were determined from a plot of dynamic viscosity vs temperature, as shown in Fig. 6. From the divergence of dynamic viscosity and the first appearance of G_e (data not shown), we determined T_{assoc} of both 20 and 30 mg/ml copolymer solutions to be in between 28 and 32°C.

Characterization of the association process by turbidity measurement

The association phenomenon of PNIPAM side chains is associated with the system turbidity. By observing the solution turbidity at various temperatures with naked eyes, the cloud points of solutions were determined. All solutions of various concentrations studied appear to be clear at 25°C. As temperature was increased to the critical point, systems changed from clear to cloudy. This critical point is referred as the cloud point where microscopic phase separation occurs. The system is thermoreversible as it turns from cloudy to clear when temperature is decreased below the cloud point. Table 3 shows the cloud-point temperature and the association temperature of chitosan-g-PNIPAM aqueous solutions. The cloud-point temperature is nearly the same as the association temperature obtained from the rheological measurement.

Conclusion

Chitosan-g-poly(*N*-isopropylacrylamide) copolymer was synthesized via radical copolymerization of PNIPAM onto backbone of low molecular weight chitosan. Grafting PNIPAM side chains onto the hydrophilic chitosan backbone does not permit a macroscopic phase separation of the side chains at high temperature. However, microscopic phase separation does occur when PNIPAM side chains start to self-aggregate when temperature is increased to the critical temperature, the so-called association temperature. The microscopic phase separation was observed as the system turned to cloudy. The aggregation of the side chains leads to the formation of reversible physical network of the copolymer which induces the thermothickening properties of the system.

The solution behavior of this copolymer in water was rheologically investigated in terms of temperature and concentration. T_{assoc} of the system was determined and was close to that of the cloud-point temperature. The synthesized chitosan-g-PNIPAM offers thermothickening behavior in aqueous solution at temperature close to the body temperature, providing potential applications in pharmaceutical and medical industries.

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