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Thermoresponsive hydrogels from alginate-based graft copolymers

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ABSTRACT

Three samples of sodium alginate grafted by amino-terminated PNIPAM (low and high molecular weight poly(N-isopropylacrylamide)) and a random P(NIPAM-co-NtBAM) (NtBAM: N-tertiary butyl acrylamide) copolymer, were synthesized via the carbodiimide chemistry and their temperature-induced hydrogelation capability was evaluated by rheology. All the samples showed thermothickening behaviour depending on concentration, ionic strength and hydrophobic comonomer content, incorporated to the PNIPAM thermo-sensitive pendant chains. The main result of this study was that a slight hydrophobic enrichment (just 15 mol% NtBAM) of PNIPAM was sufficient to shift the gelation temperature well below the physiological temperature at 32 °C and at the lowest polymer concentration studied (i.e. 10 wt%). The consequence of that was that the ALG-g-P(NIPAM-co-NtBAM) graft copolymer hydrogel exhibited the best rheological thermoresponsive properties and might be used as injectable hydrogel for potential applications in biomedicine.

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1. Introduction

Thermo-responsive hydrogels based on a three dimensional, non-covalently formed, macromolecular structure, that undergo significant changes in their physical features and properties in response to temperature have attracted intensive interest because of their potential application in the field of biomedicine as injectable hydrogels [1]. The strategy usually adopted to design such kind of hydrogels is to prepare double hydrophilic block copolymers constituted of a permanent hydrophilic block bearing, either at both ends (telechelic) [2], or along the chain (grafted) [3], short hydrophilic blocks (potential stickers) that exhibit lower critical solution temperature (LCST) phase transition in water. Thus, upon increasing temperature in aqueous environments, these blocks are transformed to hydrophobic above their LCST, driving the system to self-assemble forming a 3D network above a percolation threshold [4].

One of the most important features of these potential stickers is that their LCST should be close and below the physiological temperature such as the polymer/water system to behave as sol at room temperature and as gel inside the human body. One of the polymers that fulfil these requirements is poly(N-isopropylacrylamide) (PNIPAM) and for this reason it has been widely used as thermoresponsive building block to prepare block copolymers, exhibiting thermothickening behaviour in aqueous media [5]. The value of LCST for high molar mass PNIPAM is around 32 °C. However, this critical transition temperature depends on molar mass, polymer concentration, and ionic strength (salinity). More importantly, the critical temperature of interest is in fact the sol to gel transition which is also affected from the nature of the partner block (ionic

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versus non-ionic), the hydrophobic/hydrophilic balance and the macromolecular topology (telechelic versus graft) of the copolymer. All these factors have to be taken into account in order to regulate the thermo-sensitivity of the final hydrogel.

As far as LCST is concerned, it can be further tuned by the incorporation of comonomers using random copolymers instead of homopolymers [6]. In this way, the LCST can be tuned to a desired temperature range by copolymerization with a more hydrophobic comonomer which shifts the LCST to lower values, or a more hydrophilic one, resulting in the opposite effect [7–11].

Another significant factor is biocompatibility and biodegradability which is required in targeted bioapplications. To this end, polysaccharides grafted with a thermosensitive synthetic polymer have been designed; the polysaccharide backbone can supply biodegradability and non-toxicity to the matrix while, the synthetic polymer provides the thermal sensitivity [12]. In the seminal paper of Hourdet and coworkers it was shown that poly(ethylene oxide-co-propylene oxide) (PEPO) grafted onto three different polysaccharides exhibited strong thermothickening behaviour (sol to gel transition induced by heating) which depends on various parameters like grafting extend, polymer concentration and ionic strength, while each kind of systems has its own thermothickening signature [12].

Among others, alginate is a natural polysaccharide that has been used as the backbone polymer chain for the preparation of biodegradable thickeners [13–15]. Alginates are the salts of alginic acid, a lineal polysaccharide obtained from brown algae constituted of two uronic acids as repetitive units, mannuronic acid (M) and guluronic acid (*G*), in the form of homopolymeric (MM- or GG-blocks) and heteropolymeric sequences (MG- or GM-blocks) [16]. The mannuronic acid forms β (1–4) linkages, so that the M-block segments show linear and flexible conformations while the guluronic acid, its C5-epimer, gives rise to α (1–4) linkages, introducing in this way a steric hindrance around the carboxyl groups. For this reason the G-block segments provide folded and rigid structural conformations, responsible of a pronounced stiffness of the molecular chains [17,18].

Alginatés importance mainly lies in its hydrocolloid properties, e.g., the ability to hydrate in hot or cold water to form viscous solutions, dispersions, or gels. Alginate has been regarded as an excellent polysaccharide for gel systems because of its unique features such as biocompatibility, biodegradability, immunogenicity, and non-toxicity [19]. Alginates are, in this way, unique in terms of their properties such as emulsifiers, thickeners, stabilizers, gelling and film forming, resulting in several applications for the food and pharmaceutical industries [14,20].

In this work, biodegradable thermo-responsive hydrogels formed by self-assembly of alginate-based graft copolymers has been investigated. The main motivation of the present work was to explore the influence of hydrophobic enrichment of PNIPAM grafts on the sol to gel transition and the viscoelastic properties of the resulted hydrogels in salt free and under ionic strength. For this purpose, an amino-terminated statistical copolymer comprising NIPAM and the hydrophobic monomer N-tert-butylacrylamide (NtBAM) was synthesized and grafted onto the alginate backbone. For the sake of comparison, alginates grafted with pure PNIPAM were also synthesized and explored. As it will be shown, a small amount of hydrophobic NtBAM comonomer (e.g. 15%mol) generates significant effects to the alginate hydrogel properties.

2. Experimental

2.1. Materials

The monomers N-isopropylacrylamide (NIPAM) and N-tert-butylacrylamide (NtBAM) were used as provided by Aldrich and Alfa Aesar, respectively. Ammonium persulfate (APS), provided by Aldrich, was used as initiator and2-aminoethanethiol hydrochloride (AET, HCl), obtained from Alfa Aesar, was used as chain transfer agent. 1-ethyl-3-(3′-(dimethylamino) propyl) carbodiimide hydrochloride (EDC) was obtained from Alfa Aesar and used as received as coupling agent. A low viscosity sodium alginate was provided by Alfa Aesar. The mannuronic/guluronic ratio (M/G) was estimated in 2.2 by ^{1}H NMR according to the literature [16,21]. Deuterium oxide (D_2O) was obtained from Aldrich. Ultra-pure water was obtained by means of a SG apparatus water purification unit.

2.2. Synthesis of amino-terminated polymers and graft copolymers

2.2.1. Synthesis of PNIPAM-NH_2

The polymerization reaction was accomblished following the method proposed by Durand and Hourdet with some slight modifications [22]. An aqueous solution containing 10 g (0.088 mol) of NIPAM were degassed with Argon. Appropriate amounts of APS and AET, HCl were separately dissolved in water and added to the NIPAM solution. The concentration of NIPAM in the final volume was 1 M in all reactions. The solutions were left under stirring and under Argon at room temperature for 24 h. The final homopolymers were recovered by dialysis against pure water (membrane cutoff: 12 kDa) and lyophilization. Two PNIPAM-NH_2 homopolymers with different molar mass were synthesized (Table 1).

2.2.2. Synthesis of poly(NIPAM-co-NtBAM)-NH_2

The same procedure as described for PNIPAM-NH_2 was used for the synthesis of the P(NIPAM-*co*-NtBAM)-NH_2 random copolymer. In this case, an aqueous solution containing both monomers NIPAM and NtBAM was prepared. The final concentration of monomers was 10 wt% and the feed composition used was 85%mol NIPAM, 15%mol NtBAM (Table 1).

2.2.3. Synthesis of graft copolymers

A condensation reaction was used to synthesize the alginate-*g*-PNIPAM or alginate-*g*-P(NIPAM-*co*-NtBAM) copolymers. The graft copolymers were obtained by coupling reaction between the carboxyl groups of sodium alginate and the terminal amine groups of PNIPAM or

Table 1
Reaction conditions and characterization of PNIPAM-NH_2 and P(NIPAM-*co*-NtBAM)-NH_2 polymers.

Polymer	NIPAM (g)	NtBAM (g)	AET (% mol)	APS (% mol)	Mn (g/mol)[c]	Composition (%mol in NIPAM)
PNIPAM-L	10	–	2.5[a]	2[a]	4200	100
PNIPAM-H	10	–	1.0[a]	2[a]	15,800	100
P(NIPAM-co-NtBAM)	5	1	1.0[b]	2[b]	11,200	85[d]

[a] Relative to the moles of NIPAM.
[b] Relative to the moles of NIPAM and NtBAM.
[c] From acid-base titration.
[d] From ^{1}H NMR.

P(NIPAM-*co*-NtBAM) chains, using EDC as coupling agent. For the alginate-*g*-PNIPAM copolymer, an aqueous solution of alginate (1.5% *w*/*w*) was prepared. Then, a PNIPAM-NH_2 solution was added and the mixture was left under stirring. The amount of alginate and PNIPAM-NH_2 was 4 g and 1 g, respectively (molar percentage of repetitive units 69.5/30.5). 0.1 g of EDC previously dissolved in water, was added in three steps in the reaction mixture. The reaction was left under stirring for 4 days at room temperature. Then, the mixture was lyophilized and washed in soxhlet with methanol for at least 30 h in order to remove the unreacted PNIPAM-NH_2.

For the alginate-*g*-P(NIPAM-co-NtBAM) copolymer, a mixture of solvents was needed in order to improve the solubility of the thermosensitive polymer. Thus, a water/dioxane mixture 65/35 *v*/*v* was used. The amount of alginate and copolymer in the reaction mixture was 70/30, based on molar percentage of repeating units.

2.3. Characterization techniques

2.3.1. Potentiometric titration

The number average molar mass of the thermosensitive precursor polymers PNIPAM-NH_2 and P(NIPAM-co-NtBAM)-NH_2 was determined by acid-base potentiometric titration of end group. 0.25 g of polymer were dissolved in 10 mL of water containing an additional amount of NaOH solution 0.1 M, to ensure that pH of the initial solution is high enough (pH > 11). HCl 0.005 M was used as a tritant.

2.3.2. ^{1}H NMR

^{1}H NMR characterization of precursor polymers and graft copolymers was performed in D_2O, using a BRUKER AVANCE DPX 400 MHz spectrometer. Note that the alginate was studied at 80 °C, while the P(NIPAM-*co*-NtBAM)-NH_2 precursor and the corresponding graft copolymer were studied at 15 °C. All the other polymers were studied at room temperature.

2.3.3. FTIR

FTIR spectra were recorded at a Nicolet 520 spectrophotometer by accumulation of 30 scans, with a resolution of 4 cm^{-1}, using KBr pellets.

2.3.4. Thermogravimetric analysis (TGA)

TGA was carried out using a Discovery TGA™ equipment under nitrogen atmosphere, with a flow rate of 40 mL/min. Temperature varied from 30 to 800 °C and the heating rate used was 10 °C/min.

2.3.5. Cloud point measurements

The phase transition of polymer aqueous solutions (0.1 wt%) was monitored by turbidimetry at 490 nm using a HITACHI U-2001 spectrophotometer. The heating rate was regulated at 1 °C/min and the cloud point was defined as the temperature of the initial transmittance decrease, reflecting the onset of turbidity (T_{cp}).

2.3.6. Static light scattering (SLS)

SLS was performed using a He—Ne laser (632.8 nm) with a thermally regulated (0.1 °C) BI-200SM goniometer provided by Brookhaven Instruments Corporation (USA).

2.3.7. Rheological measurements

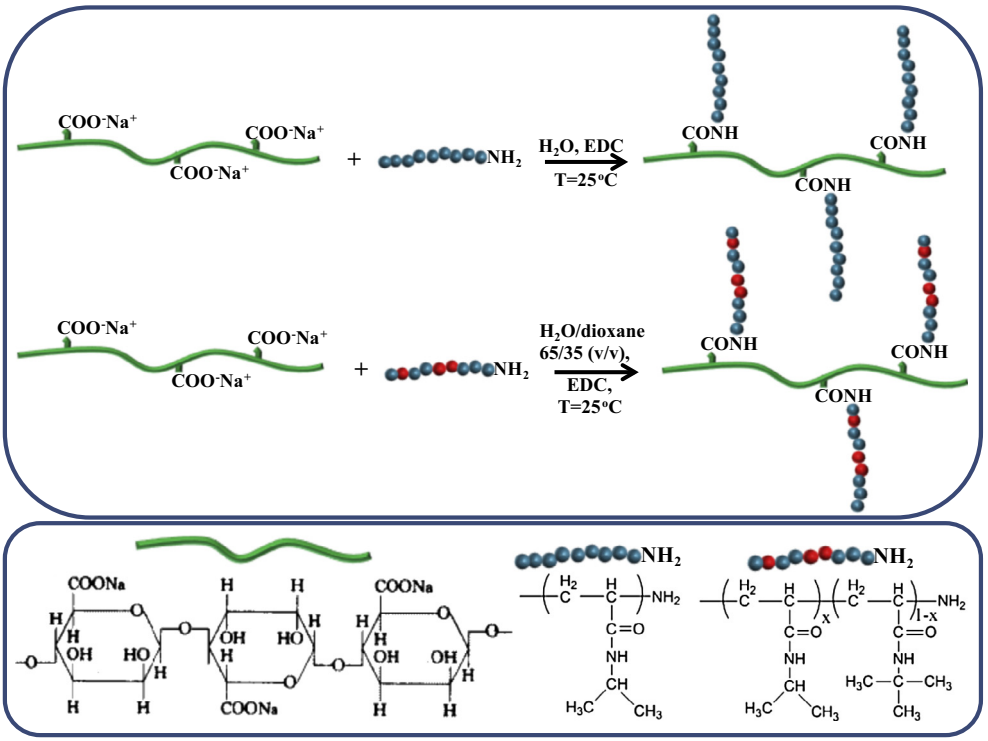
Rheological studies were performed in polymer solutions either in pure water or with different concentrations of Na_2SO_4 in the aqueous media. Polymer concentration is expressed in wt% while salt concentration is expressed in M. Rheological properties of the polymers were studied using a stress-controlled AR-2000ex (TA Instruments) rheometer equipped with a cone/plate geometry (diameter 20 mm, angle 3°59′49″, truncation 111 μm). The experiments were performed in the linear viscoelastic regime, which was established for each sample by a stress sweep at frequencies between 0.1 and 1 Hz. The rheometer was equipped with a Peltier control system that allows accurate control of temperature (±0.1 °C). A solvent trap was used to minimize changes in concentrations due to water evaporation.

3. Results and discussion

3.1. Synthesis and characterization

Three sodium alginate-based graft copolymers were synthesized by a two step synthetic route. In the first step, two amino-terminated PNIPAM (low and high molecular weight) and a random P(NIPAM-co-NtBAM) copolymer precursors were prepared by free radical polymerization. In the second step, a "grafting onto" method was used to attach the amino-terminated precursors on the alginate backbone through carbodiimide chemistry (Scheme 1).

The number average molar mass of the precursors were calculated from the two equivalent points observed in the acid-base titration curves: the first equivalent point



Scheme 1. Synthetic method for the preparation of alginate-based graft copolymers.

corresponds to the neutralization of the excess of NaOH, while the second equivalent point corresponds to the total basic groups (NaOH and -NH_2 end polymer groups) (Table 1). The composition of P(NIPAM-*co*-NtBAM) was calculated from the integration area of characteristic peaks of ^{1}H NMR spectrum (Fig. 1), as reported in the literature [10]. The NIPAM content in the copolymer was determined from the intensity ratio of the NIPAM monomer (N—C—H resonance at $\delta_H \sim 3.78$–4 ppm) and the total amount of monomers in the chain (NIPAM, 9 H, and NtBAM, 12 H, with resonances at $\delta_H \sim 0.78$–2.18 ppm). On the other hand, the composition of the alginate-*g*-PNIPAM copolymers, named ALG-*g*-PNIPAM-L (alginate-*g*-PNIPAM-L) and ALG-*g*-PNIPAM-H (alginate-*g*-PNIPAM-H), was estimated using the integrated area at δ = 4.9–5.2 ppm corresponding to the anomeric hydrogen of guluronate unit and the M/G ratio of the alginate and the integrated areas of the peaks at δ_H = 1.12 ppm (6 H); 1.55 ppm (1 H), and 1.99 ppm (2 H) corresponding to methyl and methylene groups of NIPAM (Fig. 1). The composition of the ALG-*g*-P(NIPAM-*co*-NtBAM) (alginate-*g*-P(NIPAM-*co*-NtBAM)) copolymer was calculated using the integrated area at δ = 4.9–5.2 ppm corresponding to the anomeric hydrogen of the polysaccharide, as mentioned previously, and the integrated area of the peaks at δ = 2.18–0.78 ppm representing 9.453 hydrogen regarding the molar composition of P(NIPAM-*co*-NtBAM) already calculated. All composition values obtained by ^{1}H NMR are shown in Table 2.

The chemical structure of the synthesized polymers was verified through FTIR spectroscopy. A comparison of the graft copolymers spectra with those of the respective precursors (alginate, PNIPAM-L, PNIPAM-H and P(NIPAM-co-NtBAM)) confirms the success of the grafting reaction procedures. In Fig. 2 the FTIR spectra of alginate, the side chain precursors, as well as the alginate-based graft copolymers are presented.

Characteristic bands of alginate have been widely described in literature [23–25]. In the present spectrum, principal signals have been lighted out. At 3400 cm^{-1} a broad band corresponding to stretch vibration of hydroxyl groups (ν_{O-H}) is observed. The vibration of Csp^3-H bond appears at 2928 cm^{-1}. Two strong peaks at 1609 and 1413 cm^{-1} are attributed to the asymmetric and symmetric stretching of carboxyl groups (ν^{as}_{COO-}; ν_{COO-}), respectively. The signals observed in the range of ~1200 cm^{-1} to 1050 cm^{-1} correspond to symmetric and asymmetric vibration bands of C—O—C bonds (ν_{C-O-C}; ν^{as}_{C-O-C}) typical of the polysaccharide rings. At 1300 cm^{-1} a weak band appears representing the vibration of C—O bond (ν_{C-O}). PNIPAM spectrum displays absorption bands at 2972 cm^{-1} and 2870 cm^{-1} corresponding to symmetric and asymmetric vibration bands of methyl groups (ν_{CH3}; ν^{as}_{CH3}), respectively. As it can be seen, in the spectrum of PNIPAM a band due to Csp^3-H bond vibration appears at 2933 cm^{-1}. Typical amide I band consisting of C=O stretching ($\nu_{C=O}$) at 1657 cm^{-1} and amide II band representing the N—H vibration bond (δ_{N-H}) at 1547 cm^{-1}, are clearly shown. Asymmetric and symmetric vibration bands of geminal methyl groups are observed as a weak signal at 1460 cm^{-1} (δ^{as}_{CH3}) and as a double peak band at 1377 cm^{-1} (δ^{s}_{CH3}) [26,27].

For the ALG-*g*-PNIPAM-L graft copolymer, the characteristic absorption bands of alginate can be easily

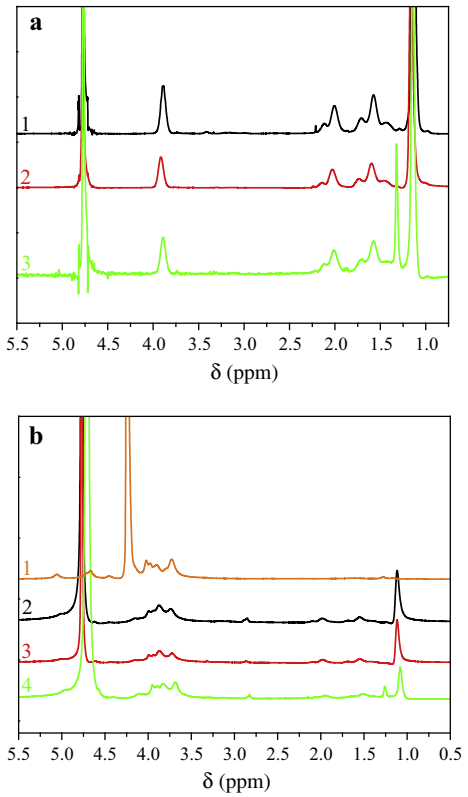


Fig. 1. ^{1}H-NMR spectra of (a) the synthesized side chains: 1. PNIPAM-L, 2. PNIPAM-H, and 3. P(NIPAM-*co*-NtBAM) and (b) of the 1. alginate 2. ALG-g-PNIPAM-L, 3. ALG-g-PNIPAM-H and 4. ALG-g-P(NIPAM-*co*-NtBAM).

observed, while the typical bands of PNIPAM are less pronounced. For example, the amide II band at 1535 cm^{-1} appears as a shoulder of the amide I signal which is overlapped to carboxylic group band of alginate. The peak at 2969 cm^{-1} due to methyl stretching vibration (ν_{CH3}) is observed as an individual peak, adjacent to C_{sp3}-H stretching band. Similar results were obtained from the spectra of ALG-*g*-PNIPAM-H and ALG-*g*-P(NIPAM-*co*-NtBAM).

The thermal behaviour of the homopolymers of alginate, PNIPAM-L and PNIPAM-H, the random copolymer P(NIPAM-*co*-NtBAM) and the graft copolymers ALG-*g*-PNIPAM-L, ALG-*g*-PNIPAM-H and ALG-*g*-P(NIPAM-*co*-NtBAM) was investigated by thermogravimetric analysis. The thermogravimetric curves of alginate, PNIPAM and the P(NIPAM-*co*-NtBAM) copolymer are shown in Fig. 3a. Both alginate and PNIPAM homopolymers exhibit an initial mass loss at 150 °C due to the dehydration of the samples.

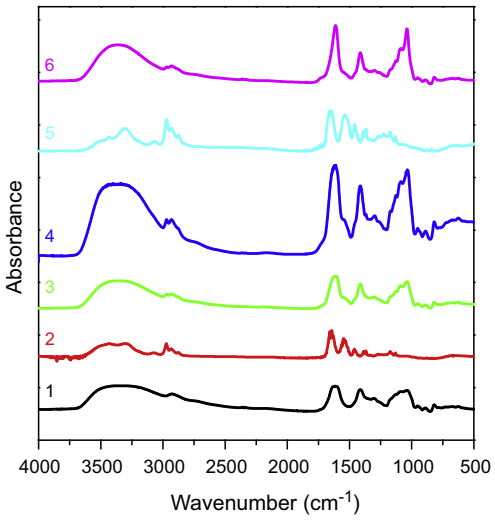


Fig. 2. FTIR spectra of 1. alginate, 2. PNIPAM-L, 3. ALG-g-PNIPAM-L, 4. ALG-g-PNIPAM-H, 5. P(NIPAM-*co*-NtBAM) and 6. ALG-g-P(NIPAM-*co*-NtBAM).

Alginates degradation curve after dehydration exhibits three steps: the first one with an important mass loss of approximately 30% in the temperature range from 200 to 300 °C, a second mass loss of approximately 15% in the range of 300–500 °C and finally, a mass loss of approximately 15% at temperatures from 500 to 600 °C. After 700 °C, a residual of approximately 25% of the initial mass was obtained. On the other hand, PNIPAM presents only one degradation in the range of 300–450 °C that involves almost all its mass, leaving a residue of less than 2.5% of the initial mass [28]. As expected, TGA curves of P(NIPAM-*co*-NtBAM) copolymer exhibited the same degradation profile as PNIPAM, taking into account the chemical structure similarity. However, mass loss in this copolymer was shifted to lower temperatures. Fig. 3b shows the TGA curves for the three graft copolymers. Comparing the thermogravimetric spectra ALG-*g*-PNIPAM-L, ALG-*g*-PNIPAM-H and ALG-*g*-P(NIPAM-*co*-NtBAM) with the spectra of the homopolymers of alginate and PNIPAM in Fig. 3a, it can be observed that the graft copolymers exhibit a similar thermal behaviour as that of alginate. All three graft copolymers present firstly an important mass loss of approximately 30% in the temperature range from 200 to 300 °C. Secondly, a mass loss of approximately 15% in the range of 300–500 °C and finally, a mass loss of approximately 15% at temperatures from 500 to 600 °C were observed. Moreover, TGA can provide the composition of

Table 2
Graft copolymers characterization.

Polymer	Feed Composition	^{1}H NMR (%mol of side chains)	TGA (%mol of side chains)	TGA (wt%)
ALG-*g*-PNIPAM-L	69.6% mol alginate 30.4% mol PNIPAM-L	28.0	28.8	18.8
ALG-*g*-PNIPAM-H	69.5% mol alginate 30.5% mol PNIPAM-H	32.7	27.8	18.0
ALG-*g*-P(NIPAM-*co*-NtBAM)	69.9% mol alginate 30.1% mol P(NIPAM-co-NtBAM)	26.9	27.1	17.8

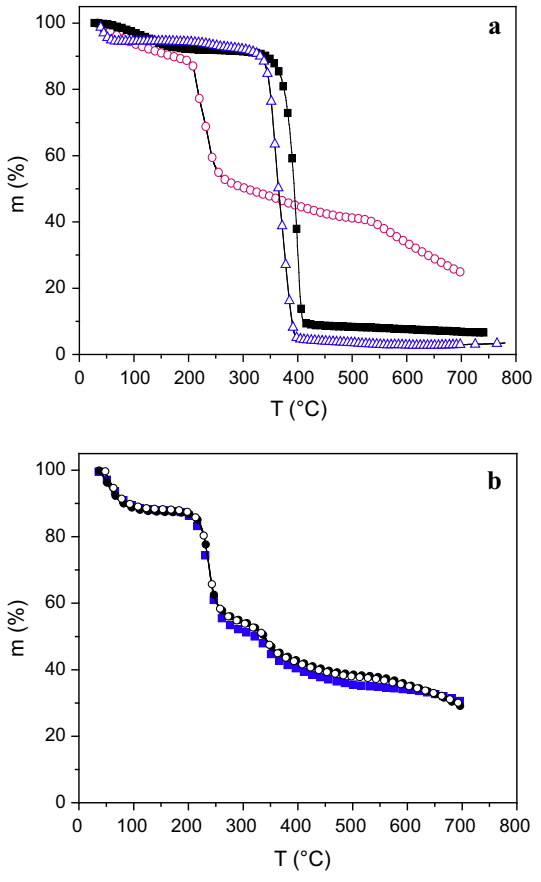


Fig. 3. Percentage of mass loss as a function of temperature for (a) the homopolymers (○) alginate, (■) PNIPAM and (△) P(NIPAM-*co*-NtBAM) and (b) the alginate-based graft copolymers (●) ALG-*g*-PNIPAM-L, (○) ALG-*g*-PNIPAM-H and (■) ALG-*g*-P(NIPAM-*co*-NtBAM).

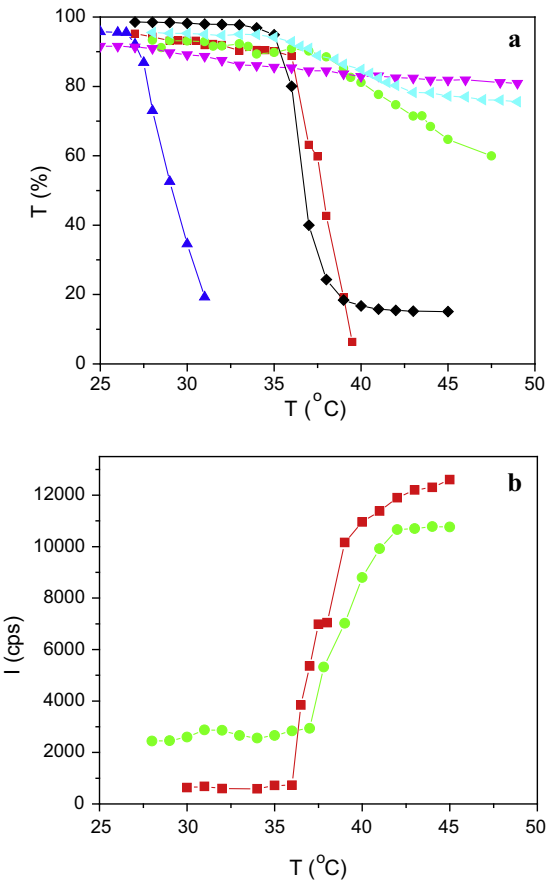


Fig. 4. Transmittance at 490 nm (a) and SLS intensity (b) as a function of temperature for aqueous solutions (0.1 wt%) of (■) PNIPAM-L, (♦) PNIPAM-H (▲) P(NIPAM-*co*-NtBAM), (●) ALG-g-PNIPAM-L, (◀) ALG-g-PNIPAM-H and (▼) ALG-g-P(NIPAM-*co*-NtBAM).

the alginate-based graft copolymers which can be extracted from the first derivative of the curves. The calculated values are presented in Table 2 and are in very good agreement with those calculated from ^{1}H NMR.

As it is well known, PNIPAM is a thermo-responsive polymer exhibiting lower critical solution temperature behaviour in water which can be monitored through turbidity using a UV spectrophotometer. In Fig. 4a the temperature dependence of the transmittance at 490 nm of a 0.1 wt% aqueous solutions of the PNIPAM, P(NIPAM-*co*-NtBAM) precursors and the alginate-based graft copolymers are demonstrated.

From this plot, the cloud point of solutions were determined at the temperature where the transmittance starts to decrease abruptly, i.e. T_{cp} = 36 °C, and 37 °C for PNIPAM-L and ALG-g-PNIPAM-L, respectively. The T_{cp} of P(NIPAM-co-NtBAM) copolymer, was shifted about 10 °C at lower temperature (T_{cp} = 26 °C), as anticipated (Fig. 4a). It should be mentioned here that the hydrophobic content of the copolymer was chosen to be 15 mol%, as to avoid undesirable lowering of the LCST of the side grafting chains providing thus the targeting property of gel injectability (see below). In contrast to the side chain precursors, the alginate-based graft copolymers exhibited a less pronounced and more gradual transmittance decrease. As a consequence of their attachment onto the hydrophilic alginate backbone, the phase separation (formation of hydrophobic domains) of the PNIPAM-L grafted chains in water upon heating above T_{cp} is limited to the nanoscale regime [14a]. Therefore, the solution of the ALG-g-PNIPAM-L copolymer becomes less turbid, compared to PNIPAM-L. This can be verified by SLS which is a more sensitive technique as it can detect the formation of associates of much smaller size. As an example, Fig. 4b shows that at temperatures lower than T_{cp}, the light scattering intensity at 90°, I, is low for both polymers, since no polymer association occurs. Above T_{cp}, I raised dramatically for PNIPAM-L, in accordance with the turbidity results. This abrupt I augmentation is due to the hydrophobic association above T_{cp}. On the other hand, it increased less sharply in the case of alginate graft copolymer. Apparently, the hydrophilic alginate backbone perturbs the phase separation mechanism, leading to the formation of smaller micellar self-assemblies than PNIPAM aggregates [29].

The cloud points (T_{cp}) of 0.1 wt% aqueous solutions of the thermosensitive polymeric side chains as well as the alginate-based graft copolymers obtained from transmittance measurements are presented in Table 3.

Table 3
T_{cp} of polymer aqueous solutions obtained by turbidimetry.

Grafting polymer	T_{cp} (°C)	Alginate-based graft copolymer	T_{cp} (°C)
PNIPAM-L	36	ALG-g-PNIPAM-L	37
PNIPAM-H	35	ALG-g-PNIPAM-H	35
P(NIPAM-*co*-NtBAM)	26	ALG-*g*-P(NIPAM-*co*-NtBAM)	27

It is known that PNIPAM-based polymers are thermosensitive in aqueous solution presenting a lower T_{cp} as either the molar mass or the hydrophobic monomer content increases. T_{cp} is almost independent of concentration and chain length for PNIPAM based polymers with a molar mass higher than 50,000 g/mol [30]. On the other hand, low molar mass PNIPAM-based polymers show a T_{cp} which is a function of concentration and chain length [31]. In our case the T_{cp} of the PNIPAM-L and PNIPAM-H homopolymers were about the same, i.e. 36 and 35 °C respectively, showing that a decrease of molecular weight about 11.000 Da had negligible effect on T_{cp}. On the other hand, for the P(NIPAM-*co*-NtBAM) copolymer a remarkably decrease was observed, i.e. 10 °C for only 15 mol% hydrophobic enrichment of PNIPAM, showing that the tunability of T_{cp} is more effective by this way. Finally, the values of T_{cp} observed for the alginate-based graft copolymers are similar to the values of T_{cp} for the corresponding temperature sensitive polymer side chains grafted to alginate, within experimental error.

3.2. Rheological properties

3.2.1. ALG-g-PNIPAM-L

The non-modified sodium alginate aqueous solutions are viscous without any sign of viscoelasticity (no entanglements) and exhibited Arrenious behaviour with temperature in all concentrations explored, up to 12 wt%. In order to detect the appearance of thermothickening behaviour of the alginate grafted samples, oscillatory shear experiments were conducted as a function of temperature starting with ALG-g-PNIPAM-L. In Fig. 5a, temperature ramp (heating rate 1 °C/min) of the storage (G') and the loss modulus (G''), obtained in the linear viscoelastic regime at low frequency (0.1 Hz), are presented for several concentrations from 10 to 14 wt%. At concentrations equal or lower than 10 wt% (plots not shown) the ALG-g-PNIPAM-L copolymer solution exhibited a weak temperature dependence of its moduli and the G'' was higher than G' in all temperature range investigated. At higher concentrations, 12 and 14 wt%, a more complex response was observed characterized by an Arrhenious behaviour at low temperatures and a thermothickening effect at higher temperatures. In Fig. 5b, the temperature effect on the dynamic moduli for the 12 wt% solution is compared with that of pure sodium alginate in order to underline the effect of grafting. As can be observed, grafting of alginate with the thermo-sensitive PNIPAM short chains provokes remarkably different thermo-response at elevated concentrations.

Two critical temperatures can be distinguished. The first temperature, which will be designated as T_{ass}, divides

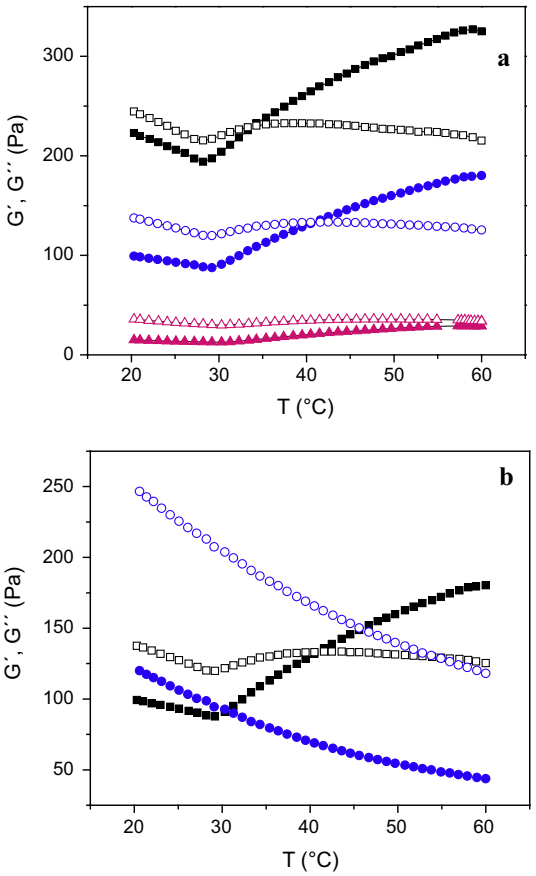


Fig. 5. (a) Viscoelastic properties G' (solid symbols) and G'' (open symbols) of ALG-g-PNIPAM-L aqueous solutions (▲) at 10 wt%, (●) at 12 wt%, and (■) at 14 wt% polymer concentrations as a function of temperature at 0.1 Hz, 0.38 Pa and a heating rate of 1 °C/min. (b) G' (solid symbols) and G'' (open symbols) of 12 wt% aqueous solutions of (■) ALG-g-PNIPAM-L and (●) sodium alginate as a function of temperature.

the thermogram into two temperature ranges: bellow T_{ass} both moduli decreases upon heating and under the same rate (Arrhenious behaviour). Above T_{ass}, the storage modulus increases more sharply than the loss modulus crossing it at a given temperature. This is the second critical temperature (at the G' G'' crossover) above which the elastic modulus prevails over the loss one which usually is related to the sol to gel transition and will be designated as T_{gel}. The first critical temperature T_{ass} is a few degrees lower than the association temperature observed by light scattering at C_P = 0.1 wt% (Fig. 4b), which is related to the LCST of PNIPAM, and it is weakly dependent on polymer concentration. Thus, provided that LCST decreases with C_P, it should reveal the onset of PNIPAM side-chain hydrophobic association, leading gradually to the formation of physical junctions of a reversible network.

T_{gel} appears well above the LCST of PNIPAM, exhibiting a more pronounced decrease with concentration, i.e. from 40.5 to 34 °C when C_P increases from 12 to 14 wt%. Moreover, the G'/G'' ratio is getting higher as temperature and C_p increase showing strengthening of the formed network, i.e. higher number of junctions as the hydrophobicity of the

PNIPAM stickers increases upon heating. Using this as criterion we observe that at physiological temperature (37 °C) only in the 14 wt% solution it is higher than unity, i.e. $G'/G'' = 1.1$, suggesting the formation of a weak gel.

Steady state shear viscosity measurements were also performed in the sol and gel state, well below and above T_{gel}. Fig. 6 displays the shear viscosity as a function of shear stress for the 12 and 14 wt% ALG-g-PNIPAM-L solutions at two different temperatures. As it can be seen, for both solutions the viscosity profile changes remarkably from 20 to 60 °C. At low temperature a Newtonian plateau, followed by a smooth shear thinning effect was observed resembling to those found in pure sodium alginate solutions of similar concentrations, which implies unassociated polymer chains with low degree of entanglements. Well above T_{gel}, the viscosity profiles exhibit the typical behaviour of amphiphilic associative polymers [32], comprising a pronounced shear thinning effect (several orders of magnitude viscosity drop upon shearing) initiated at low shear stresses. These viscosity profiles verify the formation of a physical network arisen from the association of the pendant PNIPAM hydrophobic stickers. Furthermore, the low-shear viscosity enhancement upon heating is about 300% and 500% for the 12 and 14 wt% solutions respectively. Moreover, at a given stress depending on concentration, the shear viscosity at 60 °C becomes lower than that at 20 °C, occurring at about 20 Pa for 12 wt% and at remarkable higher 100 Pa for the more concentrated solution (14 wt%). The abovementioned findings show that the ALG-g-PNIPAM-L graft copolymers exhibits thermothickening behaviour at concentrations above 10 wt% which is more pronounced at the higher concentration of 14 wt%.

Due to the well-known salting out effect that affects the LCST of thermo-sensitive polymers [22a,33] and provided that the polymer backbone exhibits polyelectrolyte character, it was anticipated that the viscoelastic behaviour of the system should be sensitive to ionic strength. Thus, oscillatory measurements were conducted in the presence of Na_2SO_4. In Fig. 7, the viscoelastic properties of 10 wt% ALG-g-PNIPAM-L are presented in salt free solutions as well as at solutions with increasing ionic strength. Several phenomena can be observed by comparing the three sets of data of Fig. 7. The salting out effect induced a smooth decrease of T_{ass}, about 10 °C upon increasing salt concentration up to 0.3 M in good agreement with the results of ALG-g-PEPO aqueous solutions reported previously [12]. A more marked effect was observed for the T_{gel}. Although it was not visible in the salt free solution, it appeared at 52 °C for the system in the presence of 0.1 M salt and decreased strongly at 25 °C upon further increasing the salt concentration to 0.3 M. This remarkable effect arises from the combination of two effects, i.e. salting out and screening of the repulsive electrostatic interactions of the sodium alginate backbone chain of the copolymer. Moreover, the interval between T_{ass} and T_{gel} was decreased significantly, from 24° to 4° upon increasing salt concentration from 0.1 to 0.3 M. This should be attributed to the stronger screening effect that permits more intensive hydrophobic intermolecular interactions among the pendant PNIPAM stickers resulting thus to a stronger network [34]. This is reflected also in the elastic modulus enhancement which is about 500% higher from the salt free up to 0.3 M Na_2SO_4 solution. Finally, it was observed that the elastic modulus decreased unexpectedly above 48 °C for the 0.3 M salt

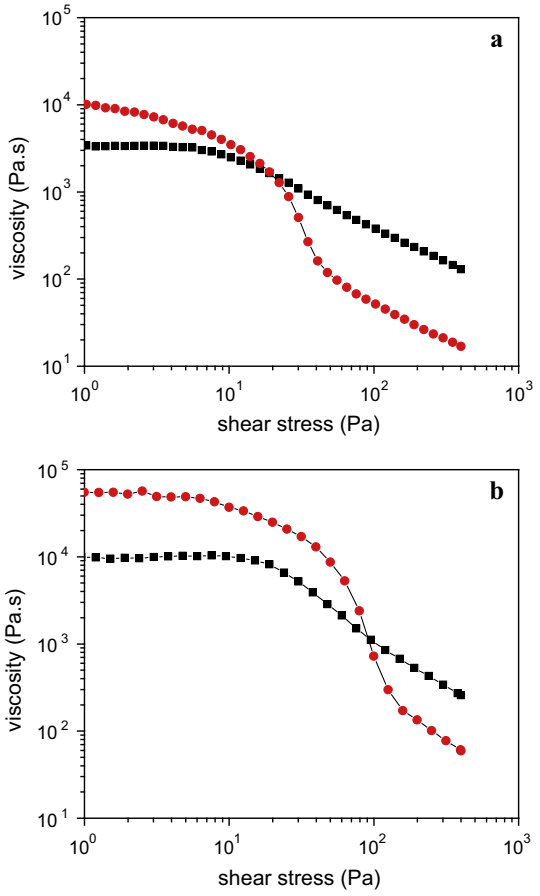


Fig. 6. Shear viscosity as a function of shear stress of (a) 12 wt% and (b) 14 wt% aqueous solutions of ALG-g-PNIPAM-L at (■) 20 °C and (●) 60 °C.

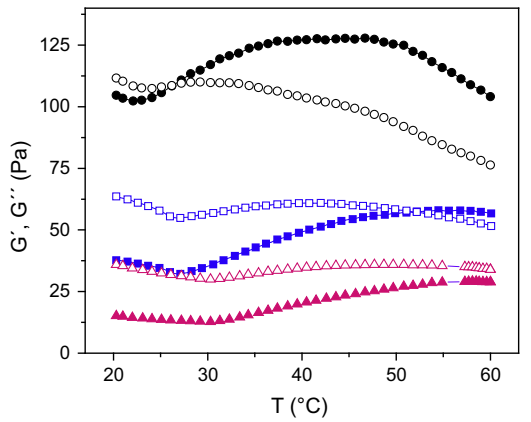


Fig. 7. Temperature dependence of the viscoelastic properties G' (solid symbols) and G'' (open symbols) of 10 wt% ALG-g-PNIPAM-L salt free (▲), 0.1 M Na_2SO_4 (■) and 0.3 M Na_2SO_4 (●) solutions at 0.1 Hz, 0.38 Pa and a temperature rate of 1 °C/min.

solution, which could be attributed to partial transformation of the inter- to intra- molecular hydrophobic association, resulting eventually to a decrease of the network connectivity.

3.2.2. ALG-g-PNIPAM-H

In the following, the ALG-g-PNIPAM-H copolymer bearing pendant PNIPAM grafts of higher molecular weight was explored. Yet, provided that ALG-g-PNIPAM-L and ALG-g-PNIPAM-H have the same PNIPAM wt%, the two samples differ also in the grafting density (average number of grafts per chain) which is about 3.7 times lower for the ALG-g-PNIPAM-H sample.

The viscoelastic properties of the salt free aqueous solutions of ALG-g-PNIPAM-H versus temperature for concentration 10, 12 and 14 wt% are demonstrated in Fig. 8. Besides the general trend of the effect of concentration on the transition temperatures (T_{ass} and T_{gel}), additional noticable differences can be observed between the two graft copolymers. Firstly, the thermothickening phenomenon appears at lower concentration, i.e. 10 wt%, followed with a slight T_{ass} shift to lower temperatures for the higher M_n grafted sample. More importantly, at the highest concentration studied, 14 wt%, no T_{gel} was observed since G' remained over G'' even below T_{ass} and in the entire temperature range studied. This behaviour implies that strong entanglements between the graft copolymer chains occur at this polymer concentration due to the significantly 3.7 times higher length of PNIPAM grafts. The thermothickening effect comes out as a slight increase of G' above T_{ass}, followed again with a G' decrease above 38 °C, which is now ~10° lower with respect to the ALG-g-PNIPAM-L sample. Thus, this temperature defining the onset of G' decrease, depends on the PNIPAM length. Finally at 12 wt% concentration, the T_{ass} to T_{gel} interval decreased significantly, from 13° to 5° upon increasing the length of PNIPAM grafts. This behaviour is equivalent to the salt effect (from 0.1 to 0.3 M) for the 10 wt% ALG-g-PNIPAM-L copolymer (Fig. 7) although the latter is more pronounced.

3.2.3. ALG-g-P(NIPAM-co-NtBAM)

In order to investigate the influence of the hydrophobic enrichment of the grafting chains on the various critical temperatures and the viscoelastic properties of the alginate-based graft copolymer hydrogels, a random copolymer of P(NIPAM-co-NtBAM), comprising 15%mol of NtBAM, was grafted onto sodium alginate, with grafting chains of intermediate length, with respect to the PNIPAM-based graft copolymers already presented. Fig. 9 presents the temperature dependence of the viscoelastic properties G' and G'' of a 10 wt% salt free ALG-g-P(NIPAM-co-NtBAM) aqueous solution. T_{ass} shifted about 9° to lower temperatures than those of the ALG-g-PNIPAMs, as expected from the parallel shift on the cloud points (Table 3) and the G', G'' cross over (T_{gel}) appeared at 32 °C, which is 6° lower and about the same with as those of 10 and 12 wt% ALG-g-PNIPAM-H respectively. Thus, although the length of P(NIPAM-co-NtBAM) is lower than that of PNIPAM-H, the hydrophobic impact on T_{gel} shift is stronger for the copolymer grafting chains. Moreover, G'/G'' at the physiological temperature is about 1.6, considerable higher than 1.1–1.2 corresponding to 12 wt% of the ALG-g-PNIPAM copolymers, implying stronger thermo-gelation efficiency for the ALG-g-P(NIPAM-co-NtBAM) manifested also at lower polymer concentration.

The better thermo-gelation efficiency for ALG-g-P(NIPAM-co-NtBAM) was also corroborated by steady state viscosity measurements. In Fig. 10, the viscosity profiles of a 10 wt% solution at two different temperatures, 19 °C < T_{gel} and 55 °C > T_{gel} are demonstrated. The general qualitative behaviour, i.e. higher zero-shear viscosity and pronounced shear thinning effect well above T_{gel}, is similar to that observed for the ALG-g-PNIPAM-L samples (Fig. 6). The quantitative differences however are remarkable. The zero shear viscosity rises about 50 times from 19 to 55 °C which is one order of magnitude higher than that found for the 14 wt% ALG-g-PNIPAM-L solution. Moreover, the critical stress above which the shear thinning effect occurs, is 10 (corresponding to γ 1 s^{-1}) and 5 Pa (0.1 s^{-1}) for ALG-g-P(NIPAM-co-NtBAM) and ALG-g-PNIPAM-L, respectively

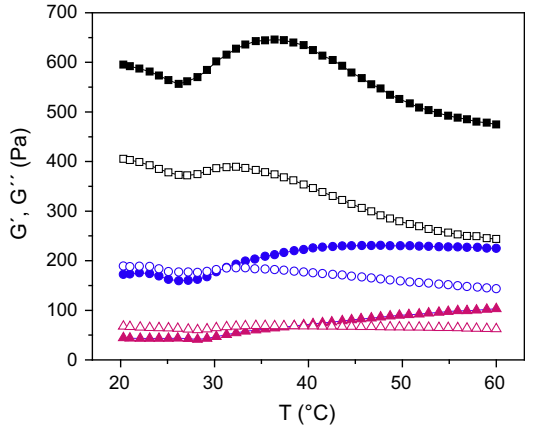


Fig. 8. Viscoelastic properties G' (solid symbols) and G'' (open symbols) of (▲) 10 wt%, (●) 12 wt%, and (■) 14 wt% aqueous solutions of ALG-g-PNIPAM-H as a function of temperature at 0.1 Hz and 0.38 Pa.

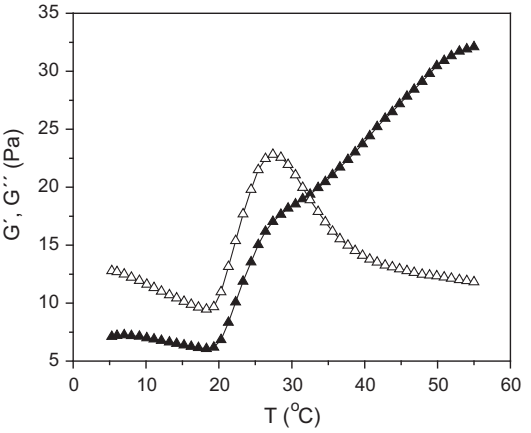


Fig. 9. Viscoelastic properties G' (▲) and G'' (△) of a 10 wt% ALG-g-P(NIPAM-co-NtBAM) aqueous solution as a function of temperature at 0.1 Hz and 0.38 Pa.

showing that the network structure for the former polymer exhibited higher resistance to shear, which could be attributed to the stronger network connectivity for ALG-g-P(NIPAM-co-NtBAM). On the other hand, the fact that the zero shear viscosity and modulus below T_{gel} are considerably lower for the ALG-g-P(NIPAM-co-NtBAM) with respect to the PNIPAM grafted copolymers in any concentration and/or ionic strength is beneficial for the hydrogel injectability.

Finally, to make a direct comparison of the thermal behaviour presented by the aqueous solutions of the three different copolymers, the relative complex viscosity (η^*_{rel}) has been plotted against the reduced temperature (T/T_{ass}, in K) for 10 wt% aqueous solutions in Fig. 11, according to Karakasyan et.al. [12]. η^*_{rel} is defined as the ratio of the experimental complex viscosity η^*(T) and the complex viscosity without interaction $\eta^*_{dis}(T)$ (Eq. (1)):

$$\eta^*_{rel} = \frac{\eta^*}{\eta^*_{dis}} \tag{1}$$

η^*_{dis} values were calculated using an empirical equation known as Andrade equation (Eq. (2)) [35]:

$$\eta^* = A \exp\left(\frac{E_a}{RT}\right) \tag{2}$$

where A is a pre exponential constant, E_a is the flow activation energy, R is the universal gas constant, and T is the absolute temperature. The parameters A and E_a were obtained from the viscosity data at temperatures lower than T_{ass}. Then, the complex viscosity without interactions at a given temperature higher than T_{ass} was calculated by linear extrapolation.

Fig. 11 gives a global idea of the thermothickening effect arisen from the three graft copolymers studied. Provided that the grafting composition of alginate-based graft copolymers and the polymer concentration are the same, the differences in the rheological behaviour can rationally evaluate the thermothickening ability of the different types of the thermo-sensitive side chains. Fig. 11 clearly demonstrates that the ALG-g-P(NIPAM-co-NtBAM) graft copolymer exhibited the highest thermogelation ability which occurred also at the lowest concentration studied.

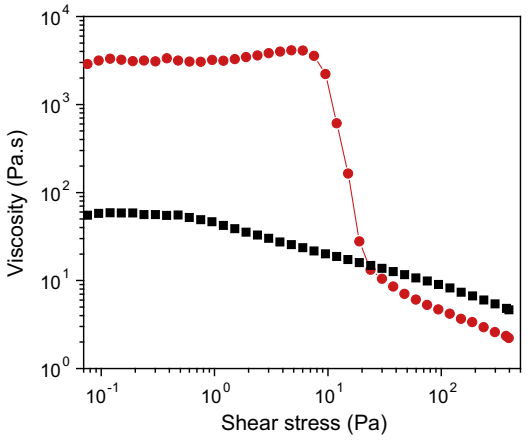


Fig. 10. Viscosity of 10 wt% ALG-g-P(NIPAM-co-NtBAM) aqueous solution as a function of shear stress at (■) 19 °C and (●) 55 °C.

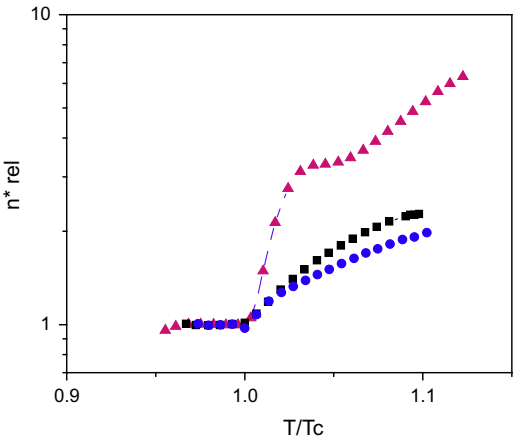


Fig. 11. Relative complex viscosity, η^*_{rel}, for 10 wt% aqueous solutions of ALG-g-PNIPAM-L (■), ALG-g-PNIPAM-H (●) and ALG-g-P(NIPAM-co-NtBAM) (▲) copolymers.

Evaluating the effect of hydrophobic enrichment of the pendant stickers, we found that the chosen amount of 15 mol% NtBAM proved quite successful for two reasons; desirable T_{gel} shift to 32 °C and stronger gel above it, which ensures the aimed gel injectability of the system. Thus, we did not extend our work to other copolymer compositions. Yet, it was shown that the hydrophobic enrichment proved more efficient to tune T_{gel} than playing with PNIPAM molecular weight.

4. Conclusions

In the present work, three sodium alginate graft copolymers, of the same grafting percentage, were synthesized by the carbodiimide chemistry, using two thermo-responsive amino-terminated PNIPAM homopolymers and a random P(NIPAM-co-NtBAM) (15%mol hydrophobic NtBAM) copolymer. The main goal of the work was to evaluate the thermo-gelation ability of these biodegradable copolymers and particularly the influence of hydrophobic enrichment of PNIPAM grafts on the sol to gel transition and the rheological properties of the resulted hydrogels. More importantly, the properties of the hydrogels were evaluated also targeting applications as injectable hydrogel, i.e. low viscosity liquid at room temperature and gel like behaviour at physiological temperature.

All the samples form hydrogels at elevated concentrations (entangled regime) upon heating, through hydrophobic interactions, that are developed well above the cloud points of the grafting chains, gradually forming the physical junctions of a transient 3D network. The viscoelastic response of the graft copolymer aqueous systems as a function of temperature, revealed two critical temperatures, named T_{ass} and T_{gel} defining the onset of hydrophobic association and the sol to gel transition respectively. Both temperatures were influenced remarkably from a number of factors, i.e. molecular characteristics of the grafting chains, polymer concentration and ionic strength.

The T_{ass} decreased smoothly with concentration and grafting chain length (ALG-g-PNIPAM) but more pronounced with ionic strength and hydrophobic enrichment (ALG-g-P(NIPAM-co-NtBAM) case). In salt free solutions and at the lowest concentration studied, the highest T_{ass} shift (just below 20 °C) was observed for the ALG-g-P(NIPAM-co-NtBAM) graft copolymer. The same trend, at least qualitatively, was observed for the T_{gel} which has to be tuned well below the physiological temperature.

The most interesting result was that the incorporation of a small amount of hydrophobic comonomer into the PNIPAM grafting chains gave a desired shift of the T_{gel} (32 °C) at 10 wt% polymer concentration, the lowest concentration studied. Moreover this slight hydrophobic enrichment (just 15%mol) was sufficient for the hydrogel to exhibit the highest G'/G'' ratio (1.6) at physiological temperature, implying stronger thermo-gelation efficiency for the ALG-g-P(NIPAM-co-NtBAM) graft copolymer. This was also verified by analysing the data using reduced coordinates, i.e. η^*_{rel} vs T/T_{ass}. Yet, the zero shear viscosity and modulus below T_{gel} (sol state) were considerably lower for the ALG-g-P(NIPAM-co-NtBAM) with respect to the PNIPAM grafted copolymers in any concentration and/or ionic strength exhibiting thermothickening which is advantageous for the hydrogel injectability. Therefore, aqueous solutions of the ALG-g-P(NIPAM-co-NtBAM) graft copolymer, easily synthesized by simple chemistry, could be considered as a good candidate for biodegradable thermo-responsive injectable hydrogels for potential applications in biomedicine.

Acknowledgments

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