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New amphiphilic grafted copolymers based on polysaccharides

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ABSTRACT

Original amphiphilic copolymers based on methylcellulose grafted on chitosan and hyaluronan are prepared and characterized. It is shown that the reductive amination is successful and may be realized in mild conditions such as to graft neutral methylcellulose chains randomly along the ionic polysaccharide backbone. The equilibrium between electrostatic repulsion between charged chains and hydrophobic attraction due to methylcellulose grafted chains controls the physical properties in solution. Especially, the presence of external salt or/and temperature increase favours a 3D-network formation or reinforce the gel-like behaviour of these systems. The new copolymers prepared are still biocompatible and may find application in a wide range of domains where thermo-sensitive physical gel formation is needed.

1. Introduction

Chemical modification of polysaccharides is a wide range of research; it may allow us to produce new materials with new physical properties and open to new developments. Especially, our work consists to introduce new substituents on pseudo-natural polymers in a controlled way and in mild conditions (i.e. aqueous solution, slightly acidic pH). Under these conditions, we are able to modify polysaccharides randomly along the backbone such as to get reproducible physical properties. Additionally, it is important to perform the chemical modifications on specific positions when usually polysaccharides are full of non-specific –OH positions; it is the reason to select specific polysaccharides having specific functions (such as $-NH_2$).

Then, an interesting reaction consists to react $-NH_2$ groups with aldehyde using a reductive-amination mechanism in aqueous solution at pH around 6. Many reactions were performed on chitosan (Chit) due to the available free $-NH_2$ function on the C-2 position of β(1–4) D-glucosamine units (usually partly N-acetylated in chitosan). Reductive amination was previously developed on chitosan (Hall & Holme, 1986; Yalpani & Hall, 1984) to graft different carbohydrate moieties allowing control of the solubility. Alkylation of chitosan was extensively studied in our laboratory using aldehyde with different alkyl chain lengths. Interesting amphiphilic behaviour was observed in dilute solution especially with C12 alkyl chains and a substitution degree around 0.04 (Desbrieres, Martinez, & Rinaudo, 1996; Rinaudo, Auzely, Vallin, & Mullagaliev, 2005); large increase of bulk viscosity is obtained going to physical gel formation in presence of small amount of external salt. The association of alkylated grafted chain depends on pH, polymer concentration, salt concentration and temperature (Desbrieres, Rinaudo, & Chtcheglova, 1997; Rinaudo et al., 2005). Additionally, these chitosan derivatives have surface activity at much lower polymer concentration than the corresponding low molecular weight surfactant (Babak, Lukina, Vikhoreva, Desbrieres, & Rinaudo, 1999; Desbrieres et al., 1997; Desbrieres, Rinaudo, Babak, & Vikhoreva, 1997). Alkylation was also performed on a hyaluronan derivative (HA–ADH) with aldehydes having different alkyl chain lengths and a degree of substitution around 0.05 per repeat unit (Creuzet, Kadi, Rinaudo, & Auzely Velty, 2006a). In this case, a preliminary reaction is needed which consists in a selective modification of HA with adipic dihydrazide (ADH) at a degree of substitution around 0.08 per repeat unit allowing to introduce $-NH_2$ groups along the HA chain. Nearly same main physical properties are found for alkylated HA than for alkylated chitosan with same alkylated chain.

Thermo-thickening chitosan derivatives were also obtained by reductive amination involving a POEP thermo-sensitive copolymer (named Jeffamine; Mn = 2500 g/mol)) modified in a first step with a reducing monosaccharide (POEP–galacturonamide) before reaction on chitosan (Creuzet, Auzely-Velty, & Rinaudo, 2006b).

More recently, the reductive amination was used to graft a fluorescent label (2-aminobenzamide) on oxidized alginate (oxidation performed with periodate) (Kristiansen, Ballance, Potthast, & Christiansen, 2009) and to crosslink chitosan with oxidized neutral

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polysaccharides (galactomannan, maltodextrin and methylcellulose) (Rinaudo, 2010).

In this paper, we intend to prepare new amphiphilic polysaccharide derivatives by grafting a small amount of low molecular weight methylcellulose on chitosan and on HA–ADH. The preparation and characterization of these new copolymers will be described.

Methylcellulose is a thermo-sensitive cellulose derivative used in food or pharmaceutical applications. It is an amphiphilic polymer with original physical properties as it forms a physical gel when temperature increases up to around 60 °C (Hirrien, Chevillard, Desbrieres, Axelos, & Rinaudo, 1998). In our work, methylcellulose is chosen for its hemiacetalic end group able to react with the $-NH_2$ groups of chitosan or that introduced in HA–ADH in direct reductive amination process in the presence of sodium cyanoborohydride used as reducing agent (Hutchins & Natale, 1979; Smith & March, 2001). Chitosan (Chit) is a pseudo natural linear polysaccharide prepared from chitin extracted mainly from crustaceous shells. It becomes water soluble under acidic conditions as soon as pH is lower than 6.5 when its average degree of acetylation (DA) is equal or lower than 0.5. A review on its characterization and properties was recently published (Rinaudo, 2006). Its biocompatibility and biodegradability are important advantages allowing many biomedical and pharmaceutical applications such as artificial skin or drug release (Muzzarelli & Muzzarelli, 2002; Rinaudo, 2006, 2008). HA is a very important linear stereoregular alternated copolymer $(AB)n$; it is well known for its biological properties (Kennedy, Phillips, & Williams, 2002a, 2002b). In addition, it is also biocompatible and biodegradable and used in many biomedical applications (Rinaudo,2008). Main characteristics of HA were review in different publications (Berriaud, Milas, & Rinaudo, 1998; Kennedy et al., 2002a; Milas & Rinaudo, 2005).

The original synthesis which associates a low molecular weight neutral thermo-thickening polysaccharide with high molecular weight water soluble ionic polysaccharides is proposed in this paper. From our knowledge, it is the first time that such polymers are prepared.

2. Experimental conditions

Chitosan Kitomer is provided by Marinard (Canada); it is characterized by a degree of acetylation DA ~ 0.2 and Mw = 500,000. Methylcellulose Methocel A15 Premium LV is produced by Dow Chemical (Mv ~ 33,500; DS ~ 1.8). HA–ADH is a gift of ARD company (Pomacle, France); it is prepared following the method described previously (Rinaudo, Auzely, Kadi, Bresin, & Kubik, 2007). The synthesis is performed in the presence of EDC at ambient temperature and pH 4.75; after reaction, the product is purified by diafiltration in dilute solution (1 g/L) and used directly; the initial weight-average molecular weight of HA is 300,000 g/mol. A sample of HA with a weight-average molecular weight Mw = 800,000 provided by ARD Cy is used to test the additivity of physical properties on mixtures prepared with methylcellulose.

For synthesis of graft copolymers, chitosan is dissolved in water in presence of the stoichiometric amount of HCl to obtain a 10 g/L solution at pH ~3.5. HA–ADH is soluble in water and used directly after synthesis and purification. Then, a given weight of methylcellulose is added into the polysaccharide solution under stirring up to complete dissolution. An excess of reducing agent ($NaBH_3CN$ at a molar concentration 5 times the $-NH_2$ content) is added to reduce the intermediate imine, as shown previously, to obtain a stable linkage $-NH-CH_2-R$ ($R-CH{=}O$ represents the methylcellulose hemiacetal). After addition of the reducing agent, the pH is controlled and stabilized around pH ~6. After 24 h reaction, the solution of grafted HA is diluted with water and ultrafiltrated on porous membrane with a cut-off around 100,000 to separate salts and unreacted methylcelluloses before freeze drying. For copolymers of chitosan, a gel-like mass is formed after dialysis against water using a cellulosic membrane (Spectra/Por; exclusion limit $M = 6000–8000$) to eliminate the small salts; the supernatant consists of methylcelluloses (as identify by ^1H NMR) which may be easily separated from the gel phase by centrifugation. The supernatant is freeze dried and the gel phase is dried in presence of acetone. It is clear that the same method for purification of chitosan copolymers as used for HA copolymer may be adopted if preferred. Elemental analysis used to determine the nitrogen content is realized by the 'Service Central d'Analyse' from CNRS (Solaize, France).

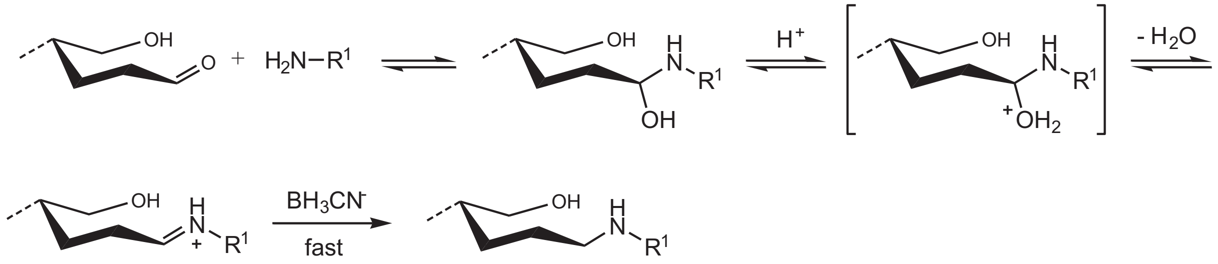
^1H NMR experiments are performed using a Bruker DRX400 spectrometer operating at 400 MHz. 1D NMR spectra are collected at 30 °C (to avoid too much aggregation) using 32K data points and 32 scans. Deuterium oxide (99.90% D) was obtained from Euriso-top (France).

The rheological behaviour of chitosan, hyaluronan, and their copolymers was determined using an AR 2000 rheometer from TA Instruments (USA) at 25 °C using a cone and plate geometry (the cone has 4 cm diameter and 3.59° angle). For unmodified polysaccharides and their derivatives in solution (or loosely cross-linked system), dynamic experiments were performed in the linear viscoelastic region where G' and G'' are independent on the stress applied. Dynamic moduli (storage modulus G' and loss modulus G'' in Pa) as well as complex viscosity $|\eta^*|$ were determined as a function of the angular frequency (ω) expressed in Hz. Temperature variation of 3°/min is imposed when necessary and controlled by a Peltier plate.

For characterization of physical properties, chitosan and chitosan–methylcellulose copolymers are dissolved in 0.3 M acetic acid (AcOH) or in 0.3 M acetic acid in the presence of small amount of sodium acetate (AcONa) to screen the electrostatic repulsion between the positively charged chains. Hyaluronan and the HA–methylcellulose copolymer are dissolved in water in absence or presence of different NaCl amounts.

3. Results and discussion

Our purpose is to graft a low molecular weight methylcellulose onto linear ionic polysaccharide based on the well-known direct reaction of reductive amination in which $-NH_2$ groups react with the aldehydic (or hemiacetalic group) chain end of methylcellulose



Scheme 1. Reaction scheme between hemiacetalic group and $-NH_2$ in the presence of cyanoborohydride.

at pH around 6 and in the presence of an excess of reducing agent $NaBH_3CN$ (see Scheme 1).

In these conditions, reduction of iminium ions is rapid compared to that of carbonyl groups and allows the *in situ* reaction of amine with carbonyl compound in the presence of cyanoborohydride (Hutchins & Natale, 1979; Smith & March, 2001). The advantage of such a reaction is that it is performed in mild conditions (slightly acid aqueous solution to preserve the solubility of polymers) and that *via* this reaction a stable linkage is formed avoiding possible hydrolysis in dependence with local pH.

3.1. Characterization of grafted polysaccharides

3.1.1. Grafting of chitosan

Taking into account the experimental conditions (aqueous solution, slightly acid medium, and ambient temperature), the grafting may be considered as random along the chitosan backbone. Our objective is to prepare amphiphilic copolymers still soluble; for that purpose, two different molar ratios ($-NH_2$/hemiacetal) are used and the composition of synthesised copolymers as well as their rheological behaviour is compared with initial chitosan properties. The main conditions used are given in Table 1. The supernatant isolated during purification of copolymers is shown to contain a large amount of methylcellulose (no signal for the methyl of acetylated units of chitosan in the NMR spectrum); it was disregarded for the study of physical properties of the isolated copolymers corresponding to the gel phase (see the protocol).

The two copolymers prepared are characterized by different techniques. Firstly, elemental analysis allows us to determine the nitrogen content in comparison with the yield of nitrogen of initial chitosan; this allows to directly determine the number of methylcellulose chains grafted on chitosan; for that purpose the number average molecular weight of methylcellulose value Mn = 31,500 is adopted to calculate the number of methylcellulose chains grafted on the chitosan backbone. The degree of grafting is expressed in terms of number of methylcellulose chains grafted per a given number of monomeric units (usually 100 acetylated and deacetylated units). The main results are given in Table 1. From the gel like fraction formed at end of reaction, it is estimated that around 50% (w/w) of the chitosan involved in the reaction is recovered in the copolymers isolated after purification.

From ^1H NMR, it is clearly shown that chitosan is associated with methylcellulose in the copolymer (data not shown). The signal at 2.04 ppm is characteristic of the acetyl group from acetylated unit in chitosan; the integral of the signals between 2.9 and 4.2 ppm is attributed to all protons (except H-1) of methylcellulose units. From these integrals, an apparent degree of grafting is determined which gives a much larger value than elemental analysis. Our interpretation is that the mobility of high molecular weight chitosan chains is reduced due to the hydrophobic interactions which cause the decrease (even at 30 °C, temperature adopted for NMR experiments) of the signal of $-CH_3$ from chitosan acetyl groups. Another hypothesis should be that some methylcelluloses remain adsorbed on the copolymers (giving a too large integral compared with that of chitosan) allowing to justify the difference between composition obtained by elemental analysis and NMR. Nevertheless this last hypothesis is not valid: estimation of the chitosan content in the copolymers from NMR composition should give 15.9 and 4.5% w/w, respectively, for the two copolymers; these values are much lower than the values obtained by nitrogen determination (these values are 48.5 and 35%, respectively) (see Table 1).

From these data, it is clear that the reactivity remains low as only 0.6 and 1 methylcellulose chains per 100 chitosan monomeric units are found from elemental analysis (the yield of reaction is nearly 1/8 of the amount introduced in the reactor); certainly, the reaction was not optimized; especially, the hemiacetalic group may be reduced in competition with the iminium formed transitory, even if from literature it is prevented at such pH (Hutchins & Natale, 1979). Interchain interactions perturb the composition determination obtained from NMR and it is considered that elemental analysis is the more valid technique.

Due to hydrophobic interactions and aggregation existing in aqueous solutions, it is not possible to determine the molecular weight of the copolymers by SEC analysis. In first approximation, based on the degree of grafting obtained by elemental analysis, it is estimated around 970,000 and 1,450,000 g/mol, respectively, assuming that, due to mild conditions used, the molecular weight of chitosan backbone remains unchanged.

Our conclusion is that the reaction adopted allows us to graft, on chitosan backbone, a small amount of methylcellulose chains with a low molecular weight which are able to modify the behaviour of chitosan in aqueous solutions. This aspect is examined in the following.

3.1.2. Grafting of hyaluronan

The same reaction as performed on chitosan is realized on hyaluronan–ADH derivative dissolved in water to demonstrate the general reactivity of polyamine with hemiacetalic functions. The anomeric position of methylcellulose reacts with the free $-NH_2$ introduced at a rate around 8% of the hyaluronan repeat units to allow reductive amination and to prepare a grafted copolymer with amphiphilic properties when HA is very hydrophilic. The HA–methylcellulose copolymer is recovered by ultrafiltration and freeze dried. The main experimental conditions are recalled in Table 2. The composition of this copolymer as well as its rheological behaviour is discussed in the following.

The determination of nitrogen content allows us to determine the composition of the purified copolymer; it is found that the yield in HA is 19.3% (w/w) from which we calculate that there are 5.5 methylcellulose chains per 100 (AB) repeat units when the experimental conditions were 16 methylcellulose chains per 100 repeat units introduced in the reactor (the yield of reaction is around 0.35, i.e. larger than the ratio obtained with chitosan). From ^1H NMR analysis, the integral of the signal at 2.04 ppm attributed to the N-acetylated position in HA is compared with the integral from methylcellulose to get the composition. The calculation gives 17.2

Table 1
Experimental conditions and characteristic of the composition of the chitosan based copolymers.

	Amount of Mecell (g)[a]	Fraction of gel (w/w %)[b]	Experimental conditions	Nitrogen % w/w	Chitosan % in the copolymer[c]	Degree of grafting from N%	Degree of grafting from NMR
Initial chitosan			–	7.56	–		
Copolymer 1	0.81	11.0	4.34 Mecell chains/100 units	3.67	48.5	0.6 Mecell/100 monomericunits	2.8 Mecell /100 momeric units
Copolymer 2	1.45	10.3	7.77 Mecell chains/100 units	2.63	35	1Mecell/100 monomeric units	11.3Mecell /100 monomeric units

[a] Added in 20 mL of a 5 g/L chitosan solution (i.e. 100 mg solubilised by stoichiometric amount of HCl).

[b] Estimated on the basis of total amount of polysaccharides engaged in the reaction.

[c] Determined from nitrogen content.

Table 2
Synthesis and characterization of hyaluronan–methylcellulose copolymer.

	Experimental conditions[a]	Nitrogen content (w/w %)	HA in the copolymer (w/w %)	Degree of grafting from N%	Degree of grafting from NMR
HA–ADH	–	4.45[b]			
HA–Methylcellulose copolymer	16 Mecell chains /100 units AB[c]	0.86	19.3	5.5 Mecell chains/100 units AB[c]	17.2 Mecell chains/100 units AB[c]

[a] *Experimental conditions*: 20 mL of HA–ADH solution (1 g/L) + 0.246 g of methylcellulose. Diafiltration on cut-off membrane of $M \sim 100{,}000$.
[b] Calculated from the chemical composition of HA–ADH.
[c] AB is the repeat unit of hyaluronan (with or without ADH substitution).

methylcellulose chains per 100 repeat units and content in HA of 7% w/w which is too low compared to experimental elemental analysis data (19.3%) (see Table 2). This estimation of the content in methylcellulose is much larger when determined from NMR than from elemental analysis as previously found for chitosan copolymers. Clearly as previously found for chitosan derivatives, the mobility of HA chains is decreased due to interactions between methylcellulose hydrophobic chains.

3.2. *Rheological behaviour of initial polymers in dependence of the temperature*

3.2.1. *Unmodified chitosan*

The moduli obtained by dynamic rheology performed on chitosan solution show that in absence or presence of external salt all the solutions are characterized by $G'' > G'$ whatever is the frequency. All the values are low and nearly not modified by salt nor after a thermal treatment (heating from 25 to 65 °C followed by cooling from 65 down to 25 °C). With polyelectrolyte, the external salt is often used to screen the electrostatic repulsions and favours inter-chain interactions (if any attractive force exists). These series of data (not shown) allow us to compare chitosan behaviour with the behaviour of copolymers in solution prepared exactly in the same conditions. It is important to point out that when temperature increases, all the rheological parameters for initial chitosan are decreasing as shown in Fig. 1 and that there is only a slight difference between increase and decrease of temperature.

3.2.2. *Methylcellulose: influence of solvent and temperature*

Methylcelluloses are water soluble and have a viscoelastic character at low temperature in dependence with the polymer concentration and molecular weight; they present a LCST-type phase separation on temperature increase and the LCST is located at 29 ±2 °C. In the conditions tested in this work at 7 g/L, one gets $G' > G''$ at 25 °C which corresponds to the "clear gel" domain as predicted from the phase diagram (Hirrien et al., 1998). The behaviour is also tested in 0.3 M acetic acid used as solvent for chitosan. In this case, all the rheological parameters are lower at 25 °C which seems to indicate that acetic acid is a worst solvent than water for methylcellulose. Nevertheless, as shown in Fig. 1, when temperature increases, large increase of G' is obtained in the two solvents going nearly to the same G' and G'' values at 65 °C. At 65 °C, G'/G'' at 1 Hz equals 12 ± 1 in the two solvents; after a temperature cycle, the loose gel-like behaviour at 25 °C is reinforced and one gets $G'/G'' = 3.8$ and 1.8 at 1 Hz in water and in acetic acid, respectively. A large hysteresis is obtained as usually with methylcellulose; this point was discussed previously (Desbrieres, Hirrien, & Rinaudo, 1998; Hirrien et al., 1998). The gelation of methylcellulose is connected with hydrophobic interaction and two steps were recognized: the clear gel involving highly substituted zones in methylcellulose and the turbid gel occurring generally around 60 °C (corresponding to the phase separation) (Hirrien et al., 1998).

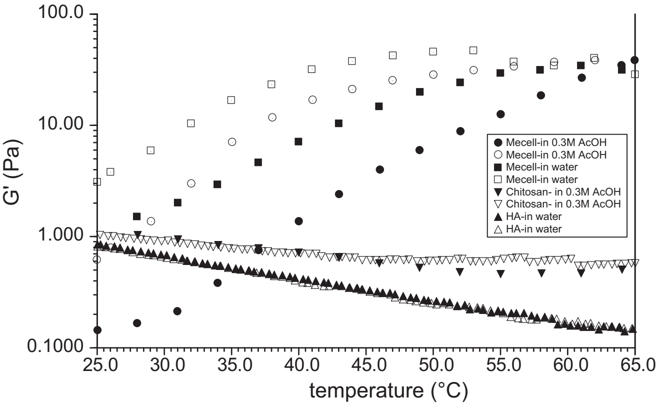


Fig. 1. Storage modulus (G' in Pa) as a function of temperature for methylcellulose (Mecell in water and in 0.3 M AcOH), chitosan in 0.3 M AcOH and hyaluronan (HA) in water. Filled symbols are used for increase of temperature and open symbols for decrease of temperature.

3.2.3. *Behaviour of hyaluronan*

Hyaluronan is a highly water soluble polysaccharide around neutral pH, under its sodium salt form. Its behaviour was investigated in the past (Berriaud et al., 1998; Milas & Rinaudo, 2005). In Fig. 1, it is shown that the G' modulus as well as the other rheological parameters decreases reversibly when temperature increases in water. This thermal behaviour looks like that of chitosan.

3.3. *Rheological characterization of copolymers*

3.3.1. *Chitosan–methylcellulose grafted copolymers*

A completely different behaviour is obtained for the copolymers compared with the parent high molecular weight polymer. An example is given in Fig. 2 for a 7 g/L copolymer concentration (i.e. 3.4 g/L in chitosan for copolymer 1 when the composition is taken into account) in 0.3 M AcOH. In the study of influence of temperature, temperature increases from 25 °C up to 65 °C and returns

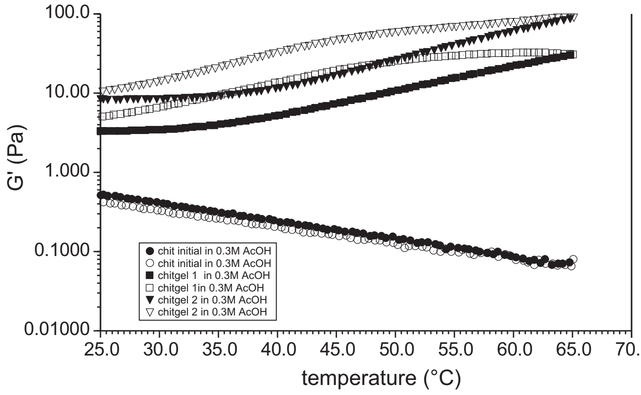


Fig. 2. Storage modulus G' at 1 Hz as a function of temperature for solutions of chitosan and copolymers 1 and 2 in 0.3 M acetic acid at 7 g/L. Filled symbols are used for temperature increase and open symbols for decreasing temperature.

to 25 °C at a constant rate of 3°/min. On contrary to chitosan, for the amphiphilic copolymers, G' which is much larger than that of chitosan at 25 °C increases when temperature increases. The copolymer 2 being richer in methylcellulose gives larger G' values. A hysteresis is obtained when temperature decreases just as it is observed for methylcellulose alone as shown in Fig. 1. It is clear that the stronger gel is formed with copolymer 2 (containing a larger weight fraction of methylcellulose) in absence of salt, acetic acid being a good solvent of chitosan and causing a high degree of protonation. In presence of external salt (0.033 M AcONa) the same type of behaviour is obtained but the difference between the two copolymers is less important. But, when salt increases (0.05 M AcONa), the rheological behaviour of copolymer 2 is lower than that of copolymer 1. This ionic concentration may correspond to a starting point of phase separation for copolymer 2 as observed also previously for alkylchitosans (Desbrieres et al., 1997).

The results obtained for dynamic measurements by direct dissolution of copolymer 1 at 25 °C are given in Fig. 3a; it is shown the predominant influence of external salt: in 0.3 M AcOH, G' is nearly superposed with G'' in all the range of frequency; it indicates that it is limit for gelation from Winter and Chambon criteria with a slope of 0.52 (Winter & Chambon, 1986). In presence of low salt concentration, even if the polymer concentration is lower than in absence of external salt, it is found $G' > G''$ in all the range of frequency and, moreover, the G' modulus at 3.5 g/L in presence of 0.05 M AcONa nearly equals that of a 7 g/L solution in absence of salt. After a thermal cycle, there is a large modification of the moduli and the gel-like behaviour is reinforced at 25 °C as shown by the evolution of G' (see Fig. 3b) and of G'/G'' ratio. As an example, for copolymer 1, one gets for a 5 g/L solution in 0.3 M AcOH/0.033 M AcONa at 1 Hz, G' = 4.4 Pa, G'/G'' = 2.79 at 25 °C and G' = 21.5 Pa, G'/G'' = 8.5 at 65 °C; in the same conditions, for initial chitosan G' = 0.14 Pa, G''/G' = 0.23 and G' = 0.03 Pa and G'/G'' = 0.13, respectively.

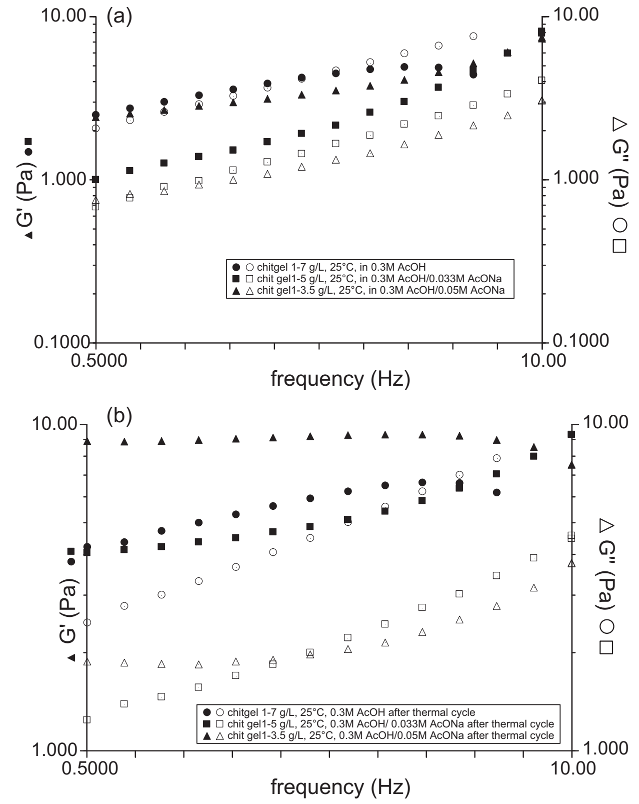


Fig. 3. Storage (G'; filled symbols) and loss (G''; open symbols) moduli of chitosan copolymer 1 solubilised in different conditions are given as a function of the frequency. (a) Direct measurement at 25 °C; (b) after a thermal cycle up to 65 °C.

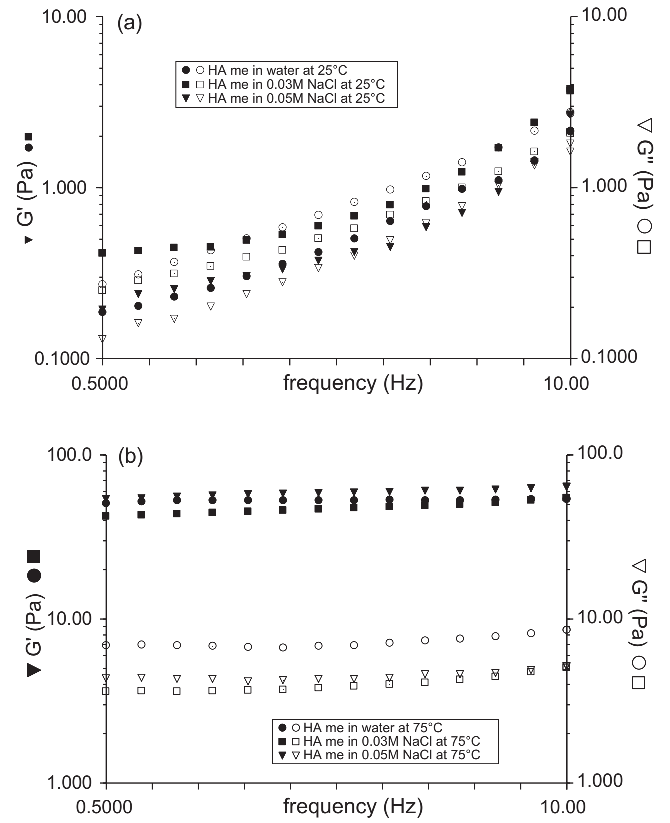


Fig. 4. Storage (G'; filled symbols) and loss (G''; open symbols) moduli as a function of the frequency for hyaluronan–methylcellulose copolymer in solution in different conditions (at 7 g/L in water, 0.03 M and 0.05 M NaCl) at 25 °C (a) and 75 °C (b).

From these results, it is clear, that even in absence of salt, hydrophobic interaction dominated the electrostatic repulsions between positively charged chitosan chains in acidic conditions. This conclusion confirms the behaviour observed in NMR determination.

Even when polymer concentration is decreased from 7 g/L to 3.5 g/L, the moduli become larger when the salt concentration increases. The screening of electrostatic repulsion between positively charged chitosan chains is dominated by the hydrophobic attraction causing a loose physical gelation (expressed directly by the G'/G'' ratio larger than 1).

3.3.2. *Rheology of HA–methylcellulose copolymer*

The dynamic behaviour of this copolymer is given in Fig. 4 where the G' and G'' moduli are shown as a function of the externam salt concentration and of the frequency at two temperatures (25 °C and 75 °C). At 25 °C, in water, HA copolymer has a normal solution behaviour with $G' < G''$; when salt is added, it turns to a loose gel-like behaviour with $G' > G''$ in all the range of frequency (Fig. 4a). At 75 °C, the behaviour is deeply modified and a gel like behaviour is obtained even in water: $G' > G''$; in addition, G' and G'' become independent on the frequency and the values are of the same range of magnitude whatever the external salt concentration (see Fig. 4b). It is pointed out that the polymer concentration is constant in this series of experiments (contrary to chitosan experiments). The rheological parameters obtained on HA copolymer are of the same order of magnitude than those obtained for chitosan copolymers. The thermal sensitivity of the HA–methylcellulose copolymer is shown in Fig. 5. In this figure, the behaviour of the copolymer is compared with that of HA (with a higher molecular weight than the HA backbone of the copolymer) at 7 g/L in water. The hysteresis is larger than that obtained with chitosan copolymers (compare with

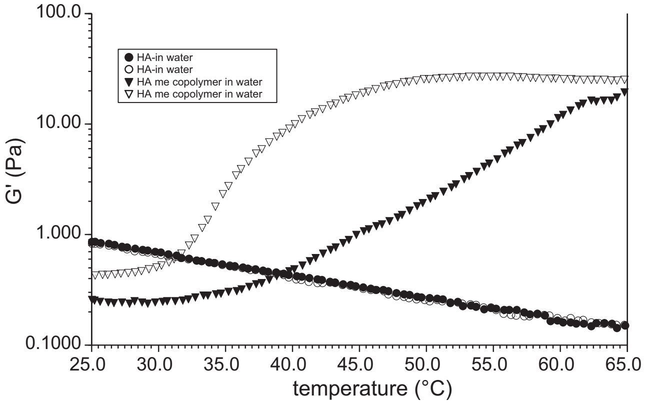


Fig. 5. Storage modulus G' at 1 Hz as a function of temperature for solutions of HA and of HA–methylcellulose copolymer at 7 g/L in water. Filled symbols are used for temperature increase and open symbols for decreasing temperature.

Fig. 2) and the gel-like behaviour is reinforced after the thermal cycle (as for pure methylcellulose and chitosan–methylcellulose copolymers; compare Figs. 1, 3 and 4). This behaviour is attributed to the larger hydrophilicity of HA, the larger chain flexibility of HA compared with chitosan and also the higher yield in methylcellulose. Nevertheless, the external salt screens the electrostatic repulsion between negatively charged HA chains and favoured the hydrophobic attraction which stabilizes a 3D-network.

3.3.3. *Comparison with direct mixtures of chitosan or hyaluronan with methylcellulose*

In a first experiment, a chitosan/methylcellulose mixture at 50/50% w/w was prepared in D_2O in the presence of DCl. The ^1H NMR signal integrals were compared as we did for the copolymers; it comes that the ratio between methylcellulose protons and chitosan $-CH_3$ corresponds to a weight ratio of 0.74 (and not 1 as prepared); this indicates again some disturbance of the chitosan relaxation in presence of methylcellulose.

Then, different blends of initial chitosan with methylcellulose were prepared at a total polymer concentration of 7 g/L, in acetic acid (0.3 M) such as to compare the behaviour of copolymers with that of mixtures at same polymer composition. The storage modulus is given at 25 and 65 °C as a function of the composition for the different mixtures (Fig. 6a). Interactions between polysaccharides promote the increase in G' modulus mainly at 65 °C when additivity (absence of interaction) should follow a linear dependency. This synergy indicates interaction between the two separated polymers whatever the temperature in the range tested. The mixture at 35/65% (w/w) corresponding to the copolymer 2 composition has a modulus lower than that of the copolymer 2: at 25 °C, 7 g/L in 0.3 M AcOH and 1 Hz, G' of the mixture equals 1.9 Pa and that of the copolymer 10.5 Pa; at 65 °C, it is found 46 Pa and 89.0 Pa, respectively.

So, the grafting of methylcellulose on chitosan backbone increases the rheological parameters (from which G') compared to the separated polymers. For copolymer 1, compared with the 50/50 mixture, the influence of interaction looks less efficient on the rheology: at 25 °C, $G' = 2.6$ Pa for the 50/50 mixture and 5 Pa for the copolymer; at 65 °C, $G' = 43$ Pa for the mixture and 30.7 Pa for the copolymer 1. These data show clearly the advantage to increase the yield in methylcellulose in the copolymer. Whatever the conditions, even at 25 °C after a first temperature cycle, copolymers have $G' > G''$ in all the range of frequency indicating loose interaction when chitosan alone has low viscosity and low G' at 1 Hz ($G' = 0.51$ Pa at 25 °C and 0.07 Pa at 65 °C) with $G' < G''$.

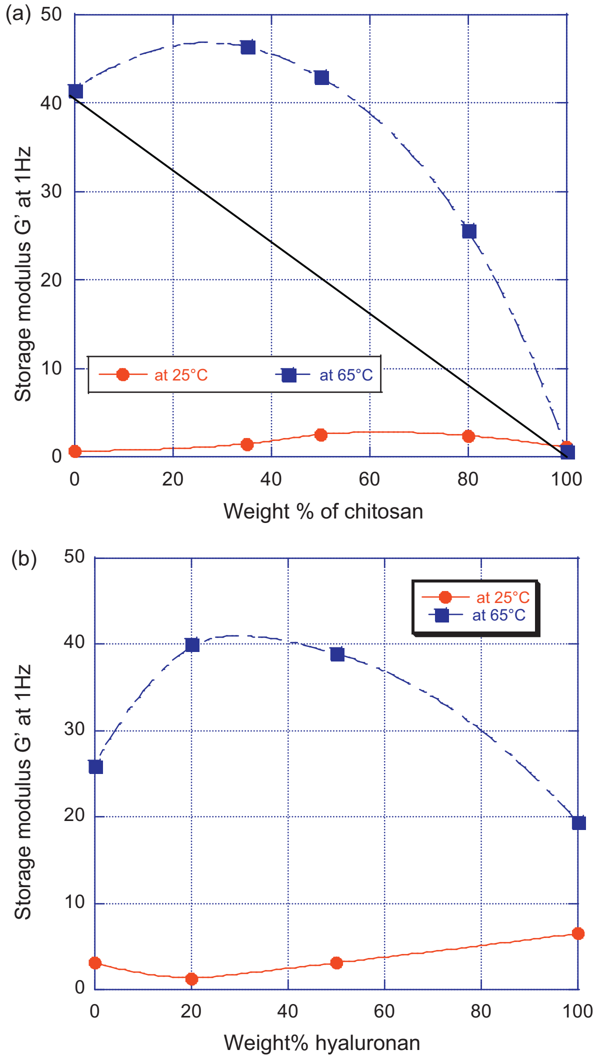


Fig. 6. Storage modulus (G' in Pa at 1 Hz) as a function of polysaccharide mixtures composition. Chitosan/methylcellulose samples (a) are dissolved in 0.3 M AcOH and HA/methylcellulose samples (b) are dissolved in water. Total polymer concentration is 7 g/L.

For the mixture between HA and methylcellulose, at 25 °C, incompatibility is shown in Fig. 6b as was previously obtained for xanthan/methylcellulose mixtures (Rinaudo & Moroni, 2009). But synergy between the two polymers is demonstrated at 65 °C. For HA, it is not possible to directly compare the G' values of the mixtures with that of the copolymer because the HA used to prepare the mixture have a higher Mw. Compared with chitosan copolymers, at 25 °C, even after a thermal cycle, one gets $G' < G''$ for the HA–methylcellulose copolymer in absence of salt.

4. Conclusion

Amphiphilic copolymers are obtained by direct reaction of low molecular weight methylcellulose with chitosan and hyaluronan–ADH. The reaction involved reductive amination between hemiacetalic end group of methylcellulose and the $-NH_2$ groups of chitosan and HA–ADH. The three initial polymers engaged are soluble in aqueous medium; in addition they are biocompatible. It is shown that few methylcellulose chains are grafted per 100 repeat units of the backbone; the chemical composition of the copolymers is determined by elemental analysis and ^1H NMR. It is demonstrated by rheology that interchain interactions in the copolymer systems disturb the relaxation time of $-CH_3$ groups of chitosan preventing chemical composition analysis by NMR.

The original copolymers prepared are thermo-sensitive in aqueous solution (in dependence of the external salt concentration) and have a loose gel-like behaviour (with $G' > G''$ in all the range of frequency) in the range of temperature covered ($T \geq 25\,^\circ\mathrm{C}$). The storage moduli are reinforced after a temperature cycle and increases over 35–40 °C (corresponding to the clear gel domain of methylcellulose). A large hysteresis exists between heating and cooling curves for the different copolymers as it is also observed for separated methylcellulose.

From the comparison between behaviour of copolymers and polymer mixtures with equivalent polymer composition, the advantages of grafting methylcellulose chains are established: the rheological behaviour is improved and a loose gel is formed even in dilute solution at 25 °C; strong thermo-thickening systems are created with physical properties in aqueous medium which depend on temperature and external salt addition. These properties depend on the electrostatic repulsions between charged backbone which counterbalance the hydrophobic attractions between the grafted side chains. These new polymers are biocompatible and their backbone are still biodegradable as shown previously on chitosan–methylcellulose co-gels (Rinaudo, 2010).

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