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New associative systems based on alkylated hyaluronic acid. Synthesis and aqueous solution properties

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Abstract

This paper concerns the preparation of new water-soluble alkylated derivatives of hyaluronan (HA); these derivatives were synthesized under mild aqueous and well controlled conditions. The first step was to prepare a HA derivative selectively modified with adipic dihydrazide (HA-ADH) with a substitution degree of 0.08; then, alkyl chains with different chain lengths were introduced with a substitution degree around 0.05. These polymers being still water-soluble exhibit associating properties in the presence of external salt. Formation of hydrophobic domains is demonstrated from fluorescence measurements in the presence of pyrene as a probe. Then, it is shown that the viscosity of polymer solutions in 0.1 M NaCl becomes non-Newtonian for HA grafted with C-10 and C-12 chains and increases rapidly with the polymer concentration over 1 g/L. When the polymer concentration increases, a gel-like behaviour was observed from rheological measurements with the G' modulus larger than G'' in all the frequency range covered. The dependence of the shear stress on shear rate applied according to a continuous linear ramp provided evidence of the deformation or rupture of aggregates. The latter were shown to reform slowly. At the end, the influence of temperature was examined: first a slight increase in the modulus was observed followed by a decrease corresponding to reinforcement of the hydrophobic interactions.

Keywords: Amphiphilic hyaluronate; Autoassociative; Rheological properties

1. Introduction

Hydrophobically modified water soluble polymers that associate in solution via physical interactions are often efficient rheology modifiers [1–3]. These are used as thickening agents in many fields of applications such as paints, cosmetics, foods, oil recovery. The main microstructural feature of such polymers is their ability to give rise to weak intra and intermolecular hydrophobic interactions in aqueous solutions. In the semi-dilute regime, these intermolecular associations are predominant. Very viscous solutions or physical gels exhibiting a shear thinning behaviour can thus be obtained. With hydrophobically modified polyelectrolytes, the viscosity of the solution can also be enhanced by several orders of magnitude upon addition of salt unlike to non-associating polyelectrolytes with which a decrease in the viscosity is usually observed. Numerous studies have been devoted to synthetic associating polyelectrolytes. However, in spite of the numerous applications which may be offered by naturally occurring polyelectrolytes with associative groups in the fields of food industry, pharmacy, cosmetology, and medicine, few types of such polymers have been proposed [4–9]. For some time now, we performed alkylation of chitosan, a cationic naturally occurring polysaccharide giving interesting properties in the bulk. In particular, the role of the alkyl chain length and density of grafting on the association was examined under various environment conditions (temperature, salt concentration) [10]. In this work, we focused on the synthesis and aqueous solution behavior of new associative systems based on alkylated derivatives of hyaluronic acid. Hyaluronic acid (HA) is a linear polysaccharide composed of repeating disaccharide units of *N*-acetyl-D-glucosamine and D-glucuronic acid, belonging to the glycosaminoglycan family. It is a component of the synovial fluid, cartilage, vitreous humour and extracellular matrices, where it plays important structural and biological roles. This polysaccharide has become an attractive building block for the development of new biocompatible materials with many applications in viscosupplementation, tissue engineering and drug delivery [11,12]. Water-soluble hydrophobically modified HA derivatives have been prepared by Dellacherie

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et al. [6] by covalent grafting of long alkyl chains (C-12 and C-18 chains) on HA through ester bonds in organic media. The influence of the length of alkyl chain, its content on HA, and of polymer concentration was well identified and it was shown that some derivatives were potential materials useful for cartilage repair. In order to better understand the nature of associations exhibited by such HA derivatives in aqueous solution, we were interested in the role of hydrophobicity, temperature and addition of salt. For this purpose, we synthesized a series of new alkylated HA derivatives having C-8, C-10, C-12, C-14 and C-16 chains using a strategy different from that reported in the literature [6]. The alkylation reaction was performed under homogeneous aqueous conditions from a HA derivative selectively modified with adipic dihydrazide (HA-ADH). The aqueous behavior of these hydrophobic HA was investigated using fluorescent probe and rheology experiments.

2. Experimental section

2.1. Materials

Bacterial sodium hyaluronate samples were produced by ARD (Pomacle, France). The protein content was determined to be less than 0.1% by weight. The molecular weight distribution and the weight-average molecular weight were determined by size exclusion chromatography using a Waters GPCV Alliance 2000 chromatograph (USA) equipped with three on line detectors: a differential refractometer, a viscometer and a light scattering detector (MALLS) from Wyatt (USA); the solutions were injected at a concentration of 5×10^{-4} g/mL in 0.1 M $NaNO_3$. The polydispersity of the samples is $M_w/M_n\sim1.5$. The weight-average molecular weights were determined to be 1.5×10^6 and 3×10^5 g/mol. In the following, these samples are referred to HA 1500 and HA 300, respectively. The aldehydic chains, 1-octanal, 1-decanal, 1-dodecanal and 1-tetradecanal, and all other chemicals were purchased from Fluka (Buchs, Switzerland). 1-Hexadecanal was prepared from the corresponding alcohol according to a literature procedure based on the use of Dess–Martin periodinane (DMP) as an oxidizing agent [13].

2.2. NMR spectroscopy

^{1}H NMR experiments were performed using a Bruker DRX400 spectrometer operating at 400 MHz. 1D NMR spectra were collected using 16K data points. Deuterium oxide was obtained from SDS (Vitry, France). Details concerning experimental conditions are given in the figure captions.

2.3. Fluorescence spectroscopy

Pyrene emission spectra were measured on a Perkin–Elmer luminescence LS 50B spectrometer between 360 and 500 nm. Pyrene solubilized in ethanol was added up to a concentration of 10^{-7} M in the polymer solution and excited at 334 nm. The I_1/I_3 ratio of the intensities of the first and the third peaks of fluorescence spectrum of pyrene was used to study the formation of hydrophobic microdomains resulting from the association of amphiphilic molecules [14].

2.4. Dilute solution viscometry

The intrinsic viscosities were determined by measuring viscosity of polymer solutions at low concentrations (<1 g/L) with an Ubbelohde capillary viscometer ($\phi=0.58$ mm) and extrapolating to infinite dilution using the Huggins equation [15] as described below:

$$\frac{\eta_{sp}}{C}=[\eta]+k'[\eta]^2C \quad (1)$$

In this relation, (η_{sp} is the specific viscosity, C is the polymer concentration (g/mL) and k', the Huggins constant. The intrinsic viscosity $[\eta]$ for HA 300 and HA 1500 in 0.1 M NaCl at 25 °C was found to be equal to 780 and 1915 mL/g, respectively.

A low shear viscometer (LS30 from Contraves) was used to test polymer solutions in the range of polymer concentration from 1 to 3 g/L in 0.1 M NaCl.

2.5. Rheological experiments

Oscillatory experiments were performed with a cone-plate rheometer (AR1000 from TA Instruments). All the dynamic rheological data were checked as a function of strain amplitude to ensure that the measurements were performed in the linear viscoelastic region. Steady shear flow experiments for polymer concentrations higher than 3 g/L and continuous flow tests were carried out with the same cone-plate rheometer. The cones used have a diameter of 4 cm and an angle of 3°59′ or a diameter of 6 cm and an angle of 1° depending on the viscosity to be measured. Most of experiments were carried out at 25 °C, with a film of silicone to avoid solvent evaporation. Temperature ramps were imposed at a given rate (2 or 3 °C/min) using the Peltier plate. Solutions of alkylated HA derivatives were prepared by dissolving them in the aqueous solvent (0.1 or 0.025 M NaCl). After stirring overnight, the samples were kept in the refrigerator for 1 day prior to characterization; the solution was let on the rheometer plate during 30 min before starting the experiment.

2.6. Synthesis

All the derivatives presented in this work were prepared from the HA 300 sample.

2.6.1. HA-ADH **3**

HA 300 (4 g, 9.97 mmol) was dissolved in water to a concentration of 4 g/L. Adipic dihydrazide (17.3 g, 99.7 mmol) was added to this solution. The pH of the reaction was then adjusted to 4.75 using 0.1 N HCl. Next, an aqueous solution of 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide (EDC) (0.287 g, 1.49 mmol) was added slowly to the mixture. The pH of the reaction mixture was maintained at 4.75 by addition

of 0.1 N HCl. The reaction was allowed to proceed at room temperature until no further change in pH was observed (i.e. 4 h). The pH of the reaction was then adjusted to 7.5 with 0.1 N NaOH. After addition of NaCl at a concentration of 0.5 M, the modified HA was precipitated with EtOH in the proportion EtOH/H_2O 3/2 (v/v). The precipitate was successively washed with different mixtures of EtOH/H_2O (7/3, 7.5/2.5, 8/2, 9/1) and then, was filtered to give HA-ADH (3.48 g, 85%). The chemical integrity and purity of the final product were checked by ^{1}H NMR. Digital integration of the NMR signals arising from the anomeric protons of HA and methylene protons of ADH gave a substitution degree of 0.08 per disaccharide repeat unit.

*2.6.2. Alkylated HA **5a***

To a solution of HA-ADH (0.6 g, 1.46 mmol) in water (144 mL), EtOH (82 mL) was added. The pH of the solution was then adjusted to 5.1 by the dropwise addition of a 0.1 M aqueous HCl solution. The aldehydic chain (1-decanal) (0.015 g, 0.096 mmol) was then added, followed by a solution of $NaCNBH_3$ (0.21 g, 2.84 mmol) in water (2 mL). After stirring for 24 h at room temperature, the pH of the reaction was then adjusted to 7.5 with aqueous 0.1 N NaOH. After addition of NaCl at a concentration of 0.5 M, the modified HA was precipitated with EtOH in the proportion EtOH/H_2O 3/2 (v/v). The precipitate was successively washed with different mixtures of EtOH/H_2O (7/3, 7.5/2.5, 8/2, 9/1) and then, was filtered to give the HA derivative named HA-5C10.

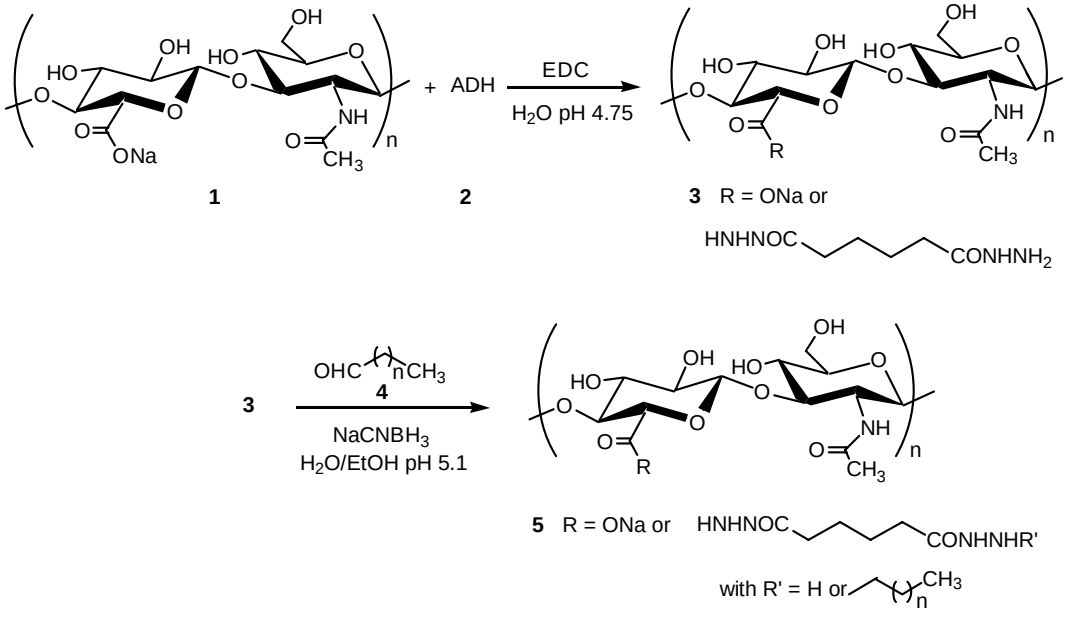
The synthesis of the other alkylated HA samples with different chain length of the alkylated moiety was performed using the same procedure; the quantity of the aldehydic chain was adapted to the desired degree of substitution.

The different polymers obtained are designated as HA-*x*C*y* where *x* reflected the degree of substitution (DS; $x = 100$ DS) and *y* the number of carbon of the pendant alkyl chain. The DS is controlled by the stoichiometric conditions and is able to vary up to 0.08.

3. Results and discussion

3.1. Synthesis and characterization of alkylated HA derivatives

Recently, we developed a new synthetic route to β-cyclodextrin-conjugated HA [16]. This was based on the selective functionalization of HA by reactive dihydrazide groups followed by a coupling reaction with a β-CD derivative possessing an aldehyde group using reductive amination conditions. Although this synthetic strategy requires two steps, it has the advantage of producing selectively modified polymers under mild and homogeneous conditions, i.e. allowing a random substitution without degradation of the HA chains. Consequently, we used the same approach for the synthesis of alkylated HA derivatives. Reaction of HA **1** with 10 molar equivalents of adipic dihydrazide (ADH) **2** and 0.15 molar equivalent of 1-ethyl-3-[3-(dimethylamino)-propyl]-carbodiimide (EDC) in water at pH 4.75 allowed us to obtain a highly pure HA-ADH derivative **3** (Scheme 1). The degree of substitution (DS) determined from ^{1}H NMR was found equal to 0.08. We targeted a low DS value for HA-ADH ($DS \leq 0.1$) in order to preserve the unique viscoelastic properties observed with the native polymer and also to maintain a high charge density on the HA backbone, which is at the origin of the water-solubility of the polymer. Then, HA-ADH **3** was reacted with different aldehydic chains **4** with 8, 10, 12, 14 and 16 carbon atoms in the presence of sodium cyanoborohydride in a water/ethanol mixture at pH 5.1 (Scheme 1). Although the addition of a reducing agent should not be necessary, in the present case, it allowed to significantly increase the yield as reported elsewhere [16]. Indeed, under such conditions, the coupling reaction was shown to be quasi-



Scheme 1. Synthesis of alkylated HA derivatives **5a** ($n = 6$), **5b** ($n = 8$), **5c** ($n = 10$), **5d** ($n = 12$) and **5e** ($n = 14$).

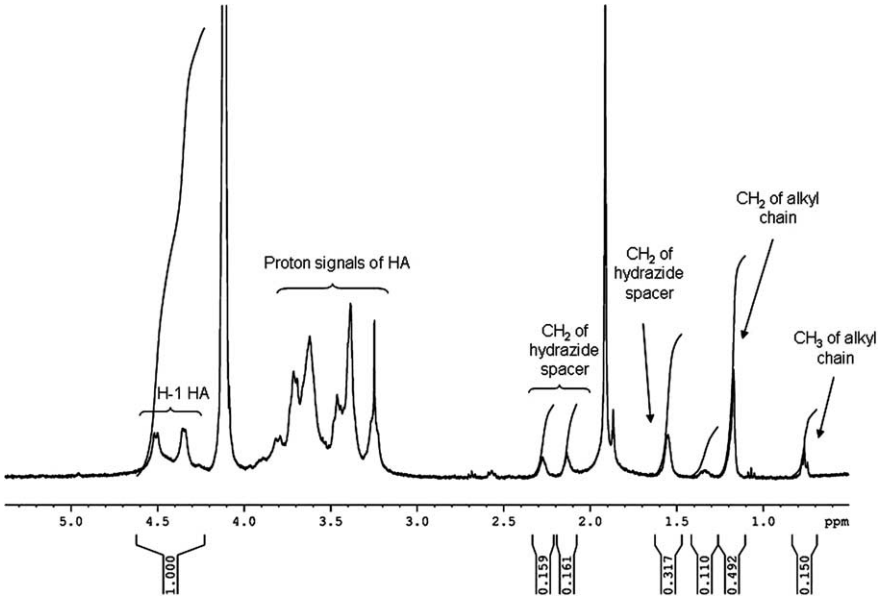


Fig. 1. ^{1}H NMR spectrum (400 MHz, 80 °C, 6 mg/mL in D_2O) of a HA sample with C-10 alkyl chains (DS = 0.05).

quantitative. The chemical integrity and purity of the final products **5** were checked by high-resolution ^{1}H NMR. The NMR analysis was performed in D_2O and demonstrated that the alkylated HA derivatives was free of any by-product. As an example, Fig. 1 shows the NMR spectrum of a HA sample with C-10 chains where digital integration of the NMR signals arising from the anomeric protons of HA and the protons of the CH_3 group of the chain gives a substitution degree (DS) of 0.05. Table 1 gives the DS calculated from ^{1}H NMR integration for the different synthesized alkylated HA derivatives together with the qualitative observation of their behavior in 0.1 M NaCl at a concentration of 10 g/L. From this table, it can be first assumed that the optimal alkyl chain length for the obtention of associative systems is C-10 or C-12 since no real thickening is observed with the C-8 alkylated HA solution at least for the polymer concentration used, whereas C-14 and C-16 HA are insoluble in water. These limits are in fact nearly the same as those observed with alkylated chitosan [10]. The ionic concentration of the solvent and temperature have some effect on this behavior as it will be discussed later.

3.2. Behavior in solution

Fig. 2 displays the log–log variations of the specific viscosity determined at zero shear rate for the two initial HA samples (HA 300 and HA 1500) used versus the overlap parameter $C[\eta]$, where $[\eta]$ is the intrinsic viscosity. In this representation, the data obtained for the two polymers lie in the same curve within experimental accuracy. The higher molar mass HA sample (HA 1500) was tested to be able to extend the domain of $C[\eta]$ covered and draw the general behaviour of HA in the experimental conditions used. In the dilute regime, i.e. for $C[\eta] < 1$ assuming that $C^*[\eta]$ is about unity [17] with C^* the overlap concentration of the polymer chains, η_0 increases moderately with the concentration. For $C[\eta] > 6$, η_0 follows a power law with an exponent of ~ 4 for the polymer concentration dependence. This value is close to the theoretical prediction ($\eta_0 \sim C^{15/4}$) for polyelectrolytes in saline media [18] and neutral polymers [19,20] in the semi-dilute entangled regime. Thus, it can be considered that for $C[\eta] > 6$, entanglements become elastically effective which corresponds for HA 1500 and HA 300 to a polymer concentration C of ~ 3 and ~ 7 g/L, respectively. In this work, the aqueous solution behavior of the alkylated HA derivatives was examined in ranges of concentrations from 0 to 7 g/L which corresponds to the dilute regime ($C^* \sim 1.3$ g/L) and semi-dilute loosely entangled regime for HA 300.

3.2.1. Role of the hydrophobicity and polymer concentration

Information about association of alkyl chains on a molecular level was obtained from fluorescence measurements using pyrene as a probe. The I_1/I_3 intensity ratio of the first and third vibronic peaks in the fluorescence emission spectrum of pyrene, which is sensitive to the polarity of the microenvironment of the probe, was measured as a function of polymer concentration for the alkylated HA derivatives, HA-ADH and initial HA300 (Fig. 3). As can be seen from Fig. 3, the I_1/I_3 ratio decreases when polymer concentration increases for the HA-5C10 and HA-4C12 derivatives, reflecting the presence of hydrophobic microdomains where pyrene is preferably

Table 1
Degree of substitution determined from ^{1}H NMR of the different synthesized alkylated HA 300 derivatives together with the qualitative observation of their behavior in aqueous solution

Alkylated HA derivative	n	DS	Behavior in 0.1 M NaCl (C = 10 g/L)
HA-7C8 **5a**	6	0.07	Transparent solution
HA-5C10 **5b**	8	0.05	Transparent soft gel
HA-4C12 **5c**	10	0.04	Bluish soft gel
HA-C14 **5d**	12	Not determined	Water-insoluble
HA-C16 **5e**	14	Not determined	Water-insoluble

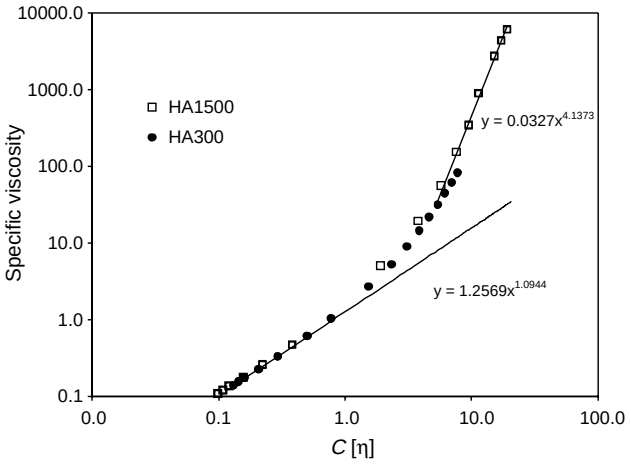


Fig. 2. Variation of the specific viscosity with the polymer concentration of solutions of two initial HA samples (HA 300 and HA 1500) in 0.1 M NaCl at 25 °C.

solubilized. For both systems, the I_1/I_3 transition occurs over a broad concentration range of more than one decade. The end of the plateau of the I_1/I_3 curve usually obtained at high concentration for small associating molecules could not be reached because of the high viscosity of the solutions. In the case of HA-4C12 which has longer chains, one can notice a more pronounced decrease in the I_1/I_3 ratio; the hydrophobic aggregation starts at a polymer concentration of $\sim$0.01 g/L and the midpoint of the transition occurs at $C \sim 0.5$ g/L (or 6.6×10^{-5} mol/L, if calculated with respect to the concentration of C12 alkyl chains). We can thus conclude that for HA-4C12, hydrophobic aggregates already exist at $C \leq 1$ g/L, i.e. at concentrations below the overlap concentration C^* of the parent polymer HA 300. One can assume it is also the case for HA-5C10. The existence of such hydrophobic associations in dilute solutions has previously been observed for alkylated

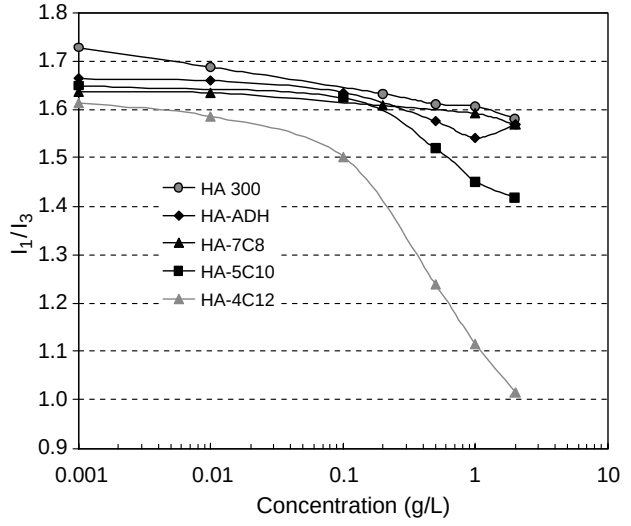


Fig. 3. Variation of the I_1/I_3 intensity ratio in the fluorescence emission spectrum of pyrene as a function of the concentration of initial HA 300, HA-ADH and alkylated HA samples in 0.1 M NaCl at 25 °C.

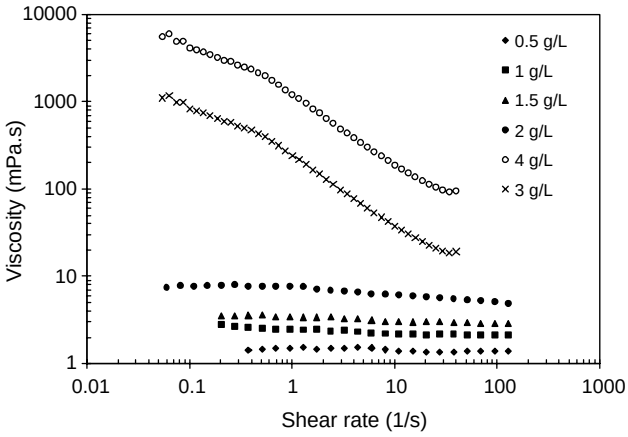


Fig. 4. Viscosity dependence on shear rate for HA-4C12 solutions (0.1 M NaCl, 25 °C) in the range of concentration from 1 to 4 g/L.

chitosans [21]. On the other hand, fluorescence data do not really demonstrate a hydrophobic character for HA-7C8 which shows a behaviour similar to those of initial HA and HA-ADH.

The steady shear viscosities of solutions of the different HA samples in the low concentration regime and at low shear rates were also investigated. In Fig. 4, it is shown that for the C-12 alkylated HA derivative, the solutions have a Newtonian plateau up to 2 g/L whereas above this concentration, which is close to the critical overlap concentration of the parent polymer, there is no Newtonian plateau any more. A similar behaviour was observed for HA-5C10. On the opposite, with C-8 substitution, the solutions have a Newtonian plateau up to 10 g/L.

From these flow curves, the viscosity values in the Newtonian plateau when it exists, or at the lower shear rate available were extracted and plotted as a function of the polymer concentration (Fig. 5). For HA-5C10 and HA-4C12, a sharp increase in the viscosity can be observed at polymer concentrations slightly lower than the overlap concentration C^* found for initial HA. The results from viscosity measurements together with those of fluorescence thus indicate that the aggregation of C-10 and C-12 alkyl chains appears in the dilute

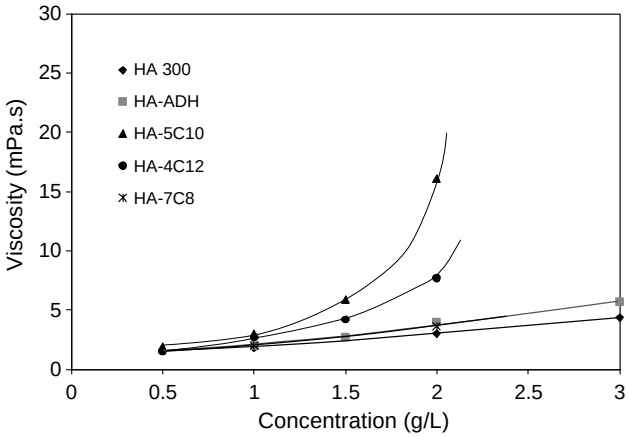


Fig. 5. Viscosity at low-shear rate as a function of polymer concentration in 0.1 M NaCl at 25 °C for HA 300, HA-ADH and alkylated HA samples.

regime. On the other hand, no increase in viscosity can be seen for HA-7C8 which suggests that no interchain alkyl/alkyl interactions exist for this polymer at the polymer concentration tested as also indicated by fluorescence experiments in the same range of concentration. Finally, one can notice that the curves of the viscosity dependence on polymer concentration for initial HA 300, HA-ADH and HA-7C8 are nearly superimposed which shows similar behaviours for these three polymers. From these experiments, it can be concluded that the minimum chain length allowing the formation of alkyl/alkyl interchain interactions in HA is C-10 (Fig. 5). These results are in contrast to alkylated chitosan for which the optimum alkyl chain length for associative properties is C-12 [10]. This may be related to the lower charge density of the HA chain. Hence, in the following, we focused on the rheological behaviour of HA-5C10 and HA-4C12 at higher polymer concentrations in the semi-dilute regime.

3.2.2. Rheological behavior in the semi-dilute regime

The variation in steady shear viscosity as a function of shear rate for solutions of HA-5C10 in 0.1 M NaCl is shown in Fig. 6. The most noticeable feature in the steady shear response of HA-5C10 is a shear thinning effect in the entire shear rate region with the absence of Newtonian plateau at low shear rates. This suggests the presence of an apparent yield stress. In order to get additional information about such a behavior, the response of the system to successive continuous flow experiments in a short range of shear rates was examined. Such studies can provide useful information about the rate of destruction of the network structures following the application of stress and the rate of rebuilding of these structures following removal of the stress. The data obtained from such experiments are given in Fig. 7. When the shear rate is increased from 0 to 5 s^{-1} in 1 min, a critical stress ($\sim$55 Pa) is observed around 0.8 s^{-1} which can be attributed to the existence of large structures that are deformed and/or broken under the applied stress. On the other hand, such a behavior is not observed for initial HA 300 in the same experimental conditions. Moreover, a pronounced hysteresis can be seen by comparing the increasing and decreasing stress curves, indicating that the global relaxation of the structure occurs for longer time than that of the experiment. In fact, it was shown that when the solution is left at rest for $\sim$30 min, it recovers the initial behavior (Fig. 7).

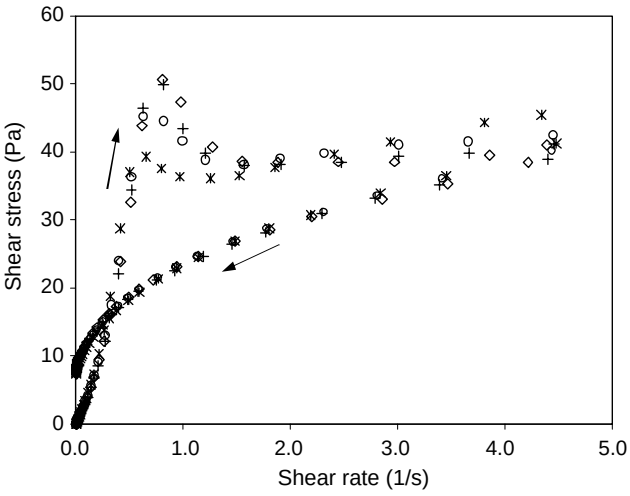


Fig. 7. Shear stress as a function of shear rate (from 0 to 5 s^{-1} in 1 min) for a solution of HA-5C10 (7 g/L in 0.1 M NaCl) at 25 °C. (◇) initial solution after 30 min on the rheometer plate; (+) after 30 min at rest after shearing; (○) after 20 min at rest; (*) after 10 min at rest.

The structured nature of the C-10 alkylated HA derivative was also clearly shown by the oscillatory shear data. The frequency dependences of the storage G' and loss G'' moduli plotted in Fig. 8 demonstrate that the solutions behave like a highly elastic physical gel ($G' > G''$ over the entire range of frequencies covered) whereas in the same range of concentrations, initial HA behaves like a viscous solution ($G'' > G'$ in all the range of examined frequencies; data not shown). Furthermore, it can be noticed that this elastic behavior is not observed any more for polymer concentrations below 4 g/L. The C-12 alkylated HA derivative also behaves like a highly elastic gel with G' higher than G'' by about one order of magnitude over the entire range of examined frequencies

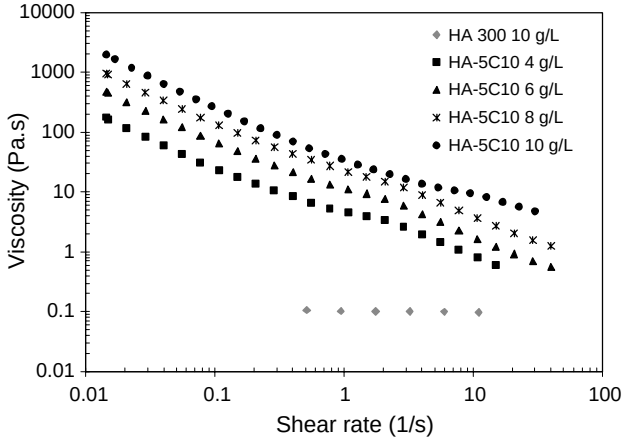


Fig. 6. Steady-state shear viscosity as a function of shear rate for solutions of initial HA 300 and HA-5C10 in the range of concentration from 4 to 10 g/L in 0.1 M NaCl at 25 °C.

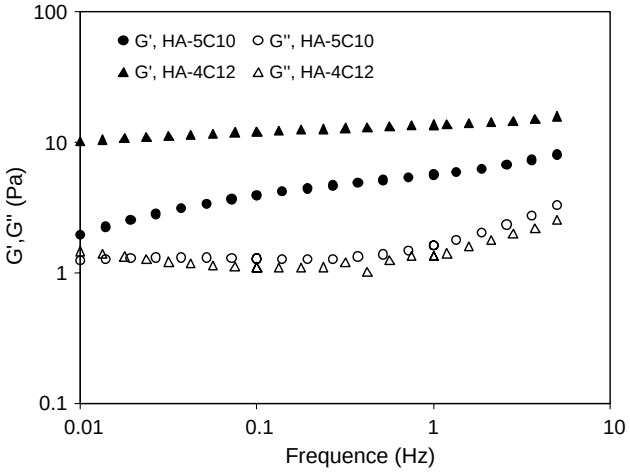


Fig. 8. Comparison of the storage and loss moduli as a function of frequency for solutions of HA-5C10 and HA-4C12 (7 g/L in 0.1 M NaCl) at 25 °C.

(Fig. 8). It can be noticed from Fig. 8 that the value of the G' modulus is much higher for the C-12 alkylated derivative compared to the C-10 one, which can be attributed to a larger stability of interchain junctions. Moreover, the characteristic relaxation time τ_c, obtained from the inverse of the frequency f_c corresponding to the crossover point of G' and G'', which characterizes the slowing down of the dynamics of the system, appears to be much higher for the C-12 HA than for the C-10 HA. The dependence of the G' and G'' moduli on frequency for this C-12 derivative differs remarkably from that reported in the literature [6]. Indeed, in the frequency range from 0.016 to 1.6 Hz, the C-12 derivative (DS = 0.05) reported in the literature exhibits a viscoelastic solution-type behavior at concentrations of 7 and 8 g/L in 0.15 M NaCl. For the solution of C-12 HA at 8 g/L, the crossover point is observed at a frequency of 0.16 Hz. In contrast, the C-12 HA described in this work shows a gel-like behavior at 7 g/L in 0.1 M NaCl in the investigated frequency range. This may be attributed to the presence of the adipic spacer arm in our case and/or the different experimental conditions used for the grafting of alkyl chains. The structured nature of the C-12 HA solutions was additionally evidenced by continuous flow experiments. The latter performed under the same conditions than those applied to C-10 HA indicated that the time required for the complete recovery of the global structure is around 1 h.

Since the associative properties of these HA derivatives are controlled by the complex balance between electrostatic repulsions and hydrophobic attractions associated with intra and interchain hydrogen bond interactions, the influence of temperature and added salt on these properties were investigated. Fig. 9 displays the dependence of the G' and G'' moduli on temperature for HA-5C10 in 0.1 M NaCl. Three successive heating and cooling cycles between 10 and 65 °C were applied at rates of 2 and 3 °C/min. A slight increase in both moduli around 30–40 °C followed by a pronounced decrease in the values can be observed during each heating cycle. This may be related to the strengthening of hydrophobic interactions and breakage of hydrogen bond interactions when the temperature is increased, leading first to the enhancement of viscoelastic properties of the network and then to its collapse.

It has been proposed that the major contribution to the stiffness of HA and the related non-Newtonian viscoelastic properties are due to intrachain hydrogen bonding between adjacent carbohydrate units [22]. The stiffness of HA characterized by the intrinsic persistence length was shown by molecular modeling to decrease when the temperature increases [23]. The same feature was also demonstrated by viscosity measurements and NMR experiments. In addition, on heating HA solutions from 24 to 80 °C, a considerable sharpening of the ^{13}C resonance of the acetamido C=O, involved in intrachain hydrogen bonding, can be observed [24]. Although this change in NMR properties was suggested to be the result of chain–chain association, the presence of such structures in solution remains unclear. In particular, analysis of the concentrated solution properties of HA by confocal-FRAP showed no evidence of such association [25]. These results thus suggest that heating solution of alkylated HA may lead to an important contraction of the polymer chain due to the cumulative effects of destiffening by the breakage of intrachain hydrogen bonds and enhancement of hydrophobic interaction between pendant alkyl chains. It may be not surprising to observe the pronounced decrease in both the storage and loss moduli at high temperature.

The recovery of the initial network structure, which is modified by heating, requires a rather long time (~30 min) as previously noted with continuous flow experiments. A similar dependence on temperature was observed (Fig. 10) for HA-4C12. However, the slight increase in the moduli around 30–40 °C does not appear clearly in that case. Moreover, as the number of heating and cooling cycles increases, the value of the G' modulus decreases. This may be the consequence of the longer time required for the recovery of the global structure of the network in the case of C-12 HA compared to C-10 HA. This decrease cannot be due to a chain degradation as was

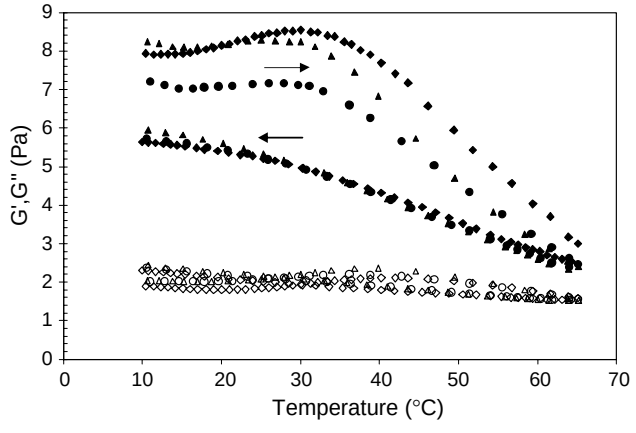


Fig. 9. Storage and loss moduli as a function of temperature for HA-5C10 (7 g/L in 0.1 M NaCl). Closed symbols: G' curves. Open symbols: G'' curves. First heating and cooling cycle (2 °C/min) after equilibration at 10 °C for 30 min (♦), (◇); second heating and cooling cycle (2 °C/min) after equilibration at 10 °C for 20 min (●), (○); third heating and cooling cycle (3 °C/min) after equilibration at 10 °C for 30 min (▲), (△).

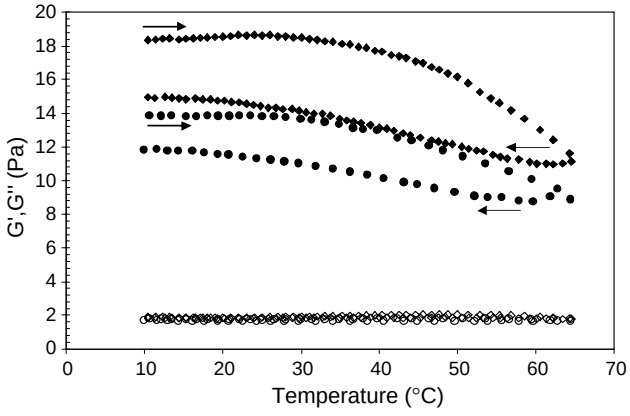


Fig. 10. Storage and loss moduli as a function of temperature for HA-4C12 (7 g/L in 0.1 M NaCl). Closed symbols: G' curves. Open symbols: G'' curves. First heating and cooling cycle (2 °C/min) after equilibration at 10 °C for 30 min (♦), (◇); second heating and cooling cycle (3 °C/min) after equilibration at 10 °C for 30 min (●), (○).

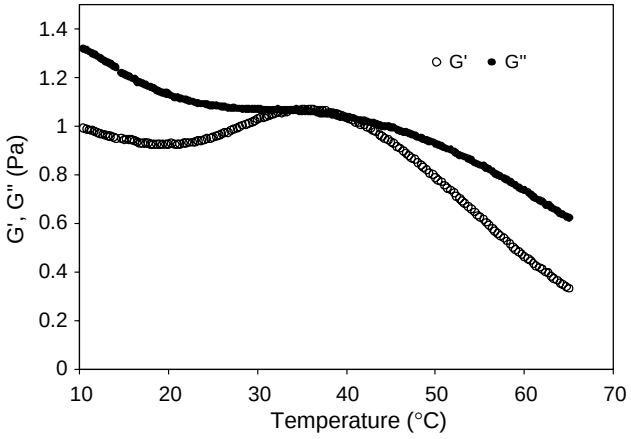


Fig. 11. Storage and loss moduli as a function of temperature for HA-5C10 (7 g/L in 0.025 M NaCl). Closed symbols: G' curve. Open symbols: G'' curve.

demonstrated in a separate experiment on HA 300 and HA 1500 under the same experimental conditions.

As shown by Fig. 11, salt concentration is very important to control the associative properties of alkylated HA in water. Indeed, it can be seen for HA-5C10 (C = 7 g/L) that in 0.025 M NaCl, $G'' > G'$ at 1 Hz whereas at the same concentration in 0.1 M NaCl, $G' \gg G''$ (Fig. 9). From these results, it is clear that interchain connections are promoted by the screening of the electrostatic chain repulsions and enhancement of hydrophobic interactions by salt addition. On the other hand, Fig. 11 tends to confirm the strengthening of hydrophobic interactions by heating at around 30–40 °C, the value of G' becomes equal to G''or slightly higher before decreasing at higher temperatures.

4. Conclusion

New amphiphilic derivatives of HA bearing pendant alkyl chains from 8 to 16 carbon atoms were synthesized under homogeneous aqueous conditions and their solution properties were investigated by fluorescence and rheology experiments. An important associating behavior was observed for the compounds having chains of 10 and 12 carbon atoms. With derivatives having chains with 8 carbon atoms, no thickening effect was obtained whereas compounds possessing chains of 14 or 16 carbon atoms were shown to be insoluble in aqueous solution. The formation of temporary networks was demonstrated for HA C-10 and C-12 derivatives above the overlap concentration C^* of initial HA in 0.1 M NaCl, by flow and dynamic experiments. The network structure obtained for the C-12 derivative appeared to be more stable than that made of HA C-10 as a result of a larger stability of interchain junction based on C-12 chains. The influence of salt concentration is important to control the balance between electrostatic repulsion and hydrophobic attractions. In the presence of salt, a gel like behaviour is observed with labile junctions depending on the temperature and on the stress applied.

The large increase in the viscosity of alkylated HA in the semi-dilute regime as well as the gel-like behaviour evidenced by dynamic measurements are remarkable and allow to envisage applications as thickeners or gelling polymers in cosmetic or biomedical domains in contolled drug delivery and/or encapsulation of fragile materials.

Especially, these C-10 and C-12 alkylated HA derivatives in aqueous solutions should find application in the field of viscosupplementation where the physical crosslinkage enhances the rheological performance of the polymers.

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