Novel Hyaluronic Acid Based Supramolecular Assemblies Stabilized by Multivalent Specific Interactions: Rheological Behavior in Aqueous Solution

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ABSTRACT: The behavior in aqueous medium of new hyaluronic acid (HA) based supramolecular assemblies was fully investigated by rheological measurements. The physical networks studied are stabilized by specific interactions between (i) monomeric β-cyclodextrin (CD) and adamantane (AD) molecules or (ii) dimeric CD and AD molecules, each randomly grafted along the polysaccharide chain. The viscoelastic properties of the resulting "mono- and bi-sticker systems", in which inclusion complexes play the role of sticky point, were analyzed as a function of polymer concentration, temperature and addition of host competitive molecules. These networks were shown to exhibit a marked non-Maxwellian behavior. The mono- and bi-interchain complexes considerably slow down the global dynamic and are believed to involve unusual viscoelastic properties that clearly differ from those of alkylated HA-based systems. These rheological features could be explained by a special mechanism of association leading to the formation of networks constituted by double-chain strands connected by fourfold junction points.

Introduction

Controlling interactions between macromolecules is essential for the development of new functional materials. In the case of biocompatible and biodegradable polysaccharides, specific interactions can be advantageously used to produce highly viscous solutions or hydrogels with promising applications in the biomedical, pharmaceutical and cosmetic fields. In this context, various hydrophobically modified polysaccharides, such as dextran,[1−2] chitosan,[3,4] hyaluronic acid,[5,6] pullulan,[7] and alginate,[8] have been synthesized by the covalent grafting of alkyl chains or of other hydrophobic moieties. These derivatives have been shown to be efficient rheology modifiers, some of them giving rise to well-defined nanostructured hydrogels.[9]

In the continuing challenge to develop original physical networks based on polysaccharides, our approach was to use specific recognition between β-cyclodextrin and a hydrophobic adamantane derivative, each grafted on a carbohydrate polymer, i.e., chitosan.[10,11] Amiel et al.[12] had previously reported associating synthetic polymeric systems resulting from the complexation between a branched β-CD polymer and different molecular architectures containing AD moieties but to the best of our knowledge, the combination of polysaccharides with β-CD/AD inclusion complexes had not been reported in the literature before. The detailed rheological study[11] of the mixed AD-grafted chitosan and CD-grafted chitosan, in the semidilute regime of partially overlapping chains, demonstrated the existence of temporary networks. The analysis of the dynamic rheological data indicated that the relaxation processes involve the chain relaxation but also the CD/AD complexes (stickers) which, acting as dissipative friction centers, quench drastically the motions of the chain. The sticky points were shown to dominate the long time dynamics. Therefore, the latter was closely related to the number of stickers per chain and their lifetime.[11]

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We then extended these chemical modifications to hyaluronic acid (HA). For some time now, we have been indeed interested in the chemical functionalization of HA, with the purpose to obtain highly viscous solutions or physical hydrogels, in particular. HA is a linear polysaccharide composed of repeating disaccharide units of *N*-acetyl-D-glucosamine and D-glucuronic acid. It is the only non-sulfated glycosaminoglycan found in the extracellular matrix. Because of its unique viscoelastic properties and biological performance, HA has become an attractive building block for the development of new biocompatible materials with many applications in viscosupplementation, tissue engineering and drug delivery.[13,14] Such an approach, based on the formation of β-CD/adamantane complexes playing the role of junction points between the polysaccharide chains, was thus expected to provide original temporary networks of which properties could be interestingly compared with those based on chitosan derivatives. The synthesis of such networks stabilized not only by single CD/AD complexes but also by pairs of CD/AD complexes have been recently reported.[15,16] The use of divalent complexes, characterized by a much higher association constant compared to the monovalent ones ($K_{CD/AD}$ = 75 000 L/mol and $K_{CD2/AD2} = 1.14 \times 10^6$ L/mol), was envisaged in order to evaluate the effect of the energy of the junction on the viscoelastic properties of the networks. The preparation of the assemblies involved the controlled synthesis of suitable monomeric and dimeric CD and AD molecules in order to be selectively grafted on a HA derivative possessing pendant reactive hydrazide groups along the chain. The resulting "host" and "guest" polymers, i.e., HA(CD), HA$(CD)_2$, HA(AD), and HA$(AD)_2$, are shown schematically in Figure 1.

Dynamic rheological measurements performed on the HA(AD)/HA(CD) and HA$(AD)_2$/HA$(CD)_2$ mixtures, i.e., the "mono-sticker" and "bi-sticker" systems, demonstrated a viscoelastic behavior which markedly differs from the solution behavior of initial HA. In addition, the dynamics for the "bi-sticker" system was shown to be slower than that for the

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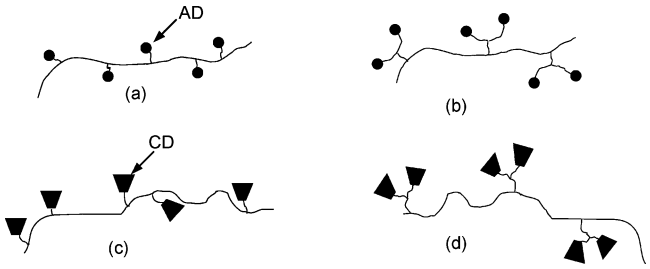

Figure 1. Schematic representation of associating HA derivatives bearing monomeric or dimeric adamantane or cyclodextrin molecules. (a) HA(AD); (b) $HA(AD)_2$; (c) HA(CD); (d) $HA(CD)_2$. The average degree of substitution (DS, number of mole of substituent per mole of repeating disaccharide unit) of polymers in parts a and b is 0.06 ± 0.01 and 0.03 ± 0.005, respectively. The DS of parts c and d was found to be 0.05 ± 0.01 and 0.025 ± 0.005, respectively.

corresponding "mono-sticker" system. These supramolecular assemblies represent a new class of temporary networks stabilized by well-defined junction points and constitute a relevant model involving punctual multivalent interactions.

The present study seeks to examine the viscoelastic behavior of these networks as a function of various external parameters such as concentration of the polymers, temperature and addition of a competitive host molecule. Our aim was to elucidate the key parameters governing the dynamics and stability of the networks. First, the behavior in aqueous solution of the initial hyaluronic acid sample and effect of grafting monomeric and dimeric host and guest molecules on the polysaccharide are examined by viscometric measurements. Then, the influence of external parameters on the viscoelastic properties of the network is analyzed by dynamic rheological measurements.

Experimental Section

Materials. Bacterial hyaluronic acid under the sodium salt form was produced by ARD (Pomacle, France). 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 polymolecularity of the sample is $M_w/M_n \sim 1.5$. The weight-average molecular weight was determined to be 3×10^5 g/mol. The overlap concentration C^* for this HA sample in 0.1 M NaCl at 25 °C is around 1.3 g/L. This value was derived from the intrinsic viscosity[5] assuming that $C^*[\eta]$ is about unity.[17] The HA(AD), $HA(AD)_2$, HA(CD) and $HA(CD)_2$ derivatives were synthesized in the laboratory as described previously.[16] The grafting degree of β-CD for HA(CD) and $HA(CD)_2$, determined by ^{1}H NMR spectroscopy, was found to be 0.05 ± 0.01. The grafting degree of AD for HA(AD) and $HA(AD)_2$, also determined by ^{1}H NMR spectroscopy, is equal to 0.06 ± 0.01. All other chemical products and reagents were purchased from Fluka (Buchs, Switzerland). Polymer concentrations are expressed in g/L or monomol/L (number of moles of modified or unmodified repeating disaccharide units/L). The concentrations of the functional groups grafted on the polymers (cyclodextrin or adamantane) are expressed in mol/L.

Rheological Experiments. Flow experiments were carried out with a cone−plate rheometer (AR1000 from TA Instruments) or with a Contraves LS30 low-shear rheometer, depending on the sample viscosity. Oscillatory experiments were performed with an AR1000 rheometer. 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. The cone used has a diameter of 4 cm and an angle of 3° 59′. Experiments were carried out at 25 °C, with a film of silicone to avoid solvent evaporation. HA-(CD), $HA(CD)_2$, HA(AD), and $HA(AD)_2$ were dissolved in aqueous 0.025 M NaCl. Salt was added in order to partially screen the long range electrostatic repulsions between negatively charged chains. The dissolution time was at least 12 h at room temperature. The solutions of CD- and AD-grafted HA samples were then mixed. Gel-like samples were vigorously stirred and allowed to rest for at least 1 h at room temperature. We checked that the rheological properties of the samples did not change with time (from 1 to 24 h).

Results and Discussion

1. Properties of the Host and Guest Derivatives of Hyaluronic Acid in Aqueous Solution Alone. Because of chemical modification, the host and guest HA derivatives are expected to exhibit solution properties different from those of the HA precursor. Indeed, secondary interactions due to the presence of CD and AD groups on the negatively charged HA backbone, that could disturb complex formation, may be expected. In the case of adamantane-grafted chitosan for example, the hydrophobic adamantane groups were shown to promote a self-associating behavior.[11] Such a behavior is however dependent on the balance between electrostatic repulsions between the positively charged chitosan chains and hydrophobic attractive interactions between adamantanes. Therefore, it appeared to us important to compare the behavior of the host and guest HA derivatives alone together with initial HA in aqueous solution by varying the ionic strength. Salt concentration not only may have an effect on the macromolecular intra- and interchain interactions but also on the properties of the CD/AD complexes. Indeed, it has been shown previously that the presence of electrostatic interactions between charged host and guest molecules and/or macromolecules may influence the complexation thermodynamics.[15,16,18] In the case of the complexation between negatively charged HA bearing pendant cyclodextrin dimers ($HA(CD)_2$) and a free negatively charged AD dimer, the enthalpy and entropy values appeared to increase when the concentration of NaCl is increased from 0.025 to 0.2 M. This resulted in a higher value of the association constant in 0.2 M NaCl, where electrostatic repulsions can be considered to be completely screened.

Figure 2 compares the variation of the zero-shear viscosity as a function of polymer concentration for solutions of HA-(AD), HA(CD) and initial HA in (A) 0.025 M and, (B) 0.1 M NaCl. The steady shear viscosities were measured in the range of polymer concentration from 0.2 to 10 g/L. It can be seen from this figure that the viscosity of the different HA samples are close to each other until a critical concentration, approximately equal to 1.8 g/L, that we will refer to as C_η,. Above C_η, which is $1.4C^*$, C^* being the overlap concentration of initial HA, viscosities become different. The large increase of the viscosity observed for the HA-AD derivative in 0.1 M NaCl can be attributed to hydrophobic interactions between the grafted AD groups, which promote a self-associating behavior. However, given the bulky structure of the AD groups, the AD/AD auto-associations are weaker than those observed for alkylated hyaluronic acid derivatives with similar degrees of substitution.[5] In 0.025 M NaCl, the hydrophobic AD/AD interactions are not favored as reflected by the similar viscosity values obtained for the solutions of HA(AD) and initial HA. Contrary to HA-(AD), solutions of HA(CD) in 0.1 M NaCl exhibit lower viscosities compared to those of initial HA. This might be related to hydrogen bonding between the grafted CD cavities. This phenomenon was also observed by size exclusion chromatography analysis of HA(CD) (data not shown). The behavior of HA(CD) in 0.025 M NaCl, is close to that of initial HA.

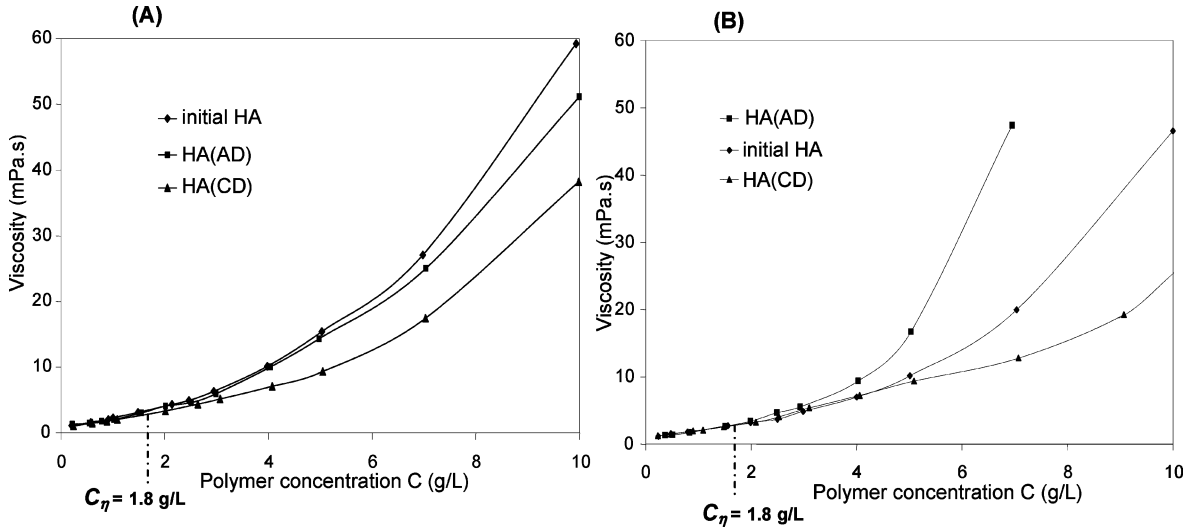

Figure 2. Variation of the zero shear viscosity with the polymer concentration of HA(CD), HA(AD) and initial HA solutions in (A) 0.025 M and (B) 0.1 M NaCl at 25 °C.

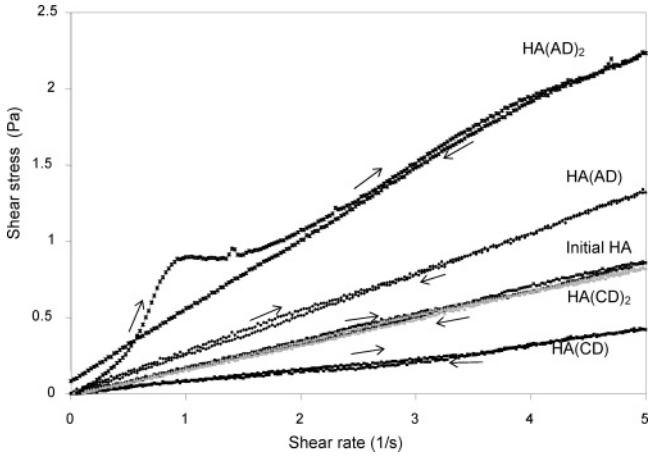

Figure 3. Shear stress as a function of shear rate (from 0 to 5 s^{-1} in 1 min) of initial HA, HA(AD)$_2$, HA(CD)$_2$, HA(CD), and HA(AD) solutions at 7 g/L in 0.025 M NaCl at 25 °C.

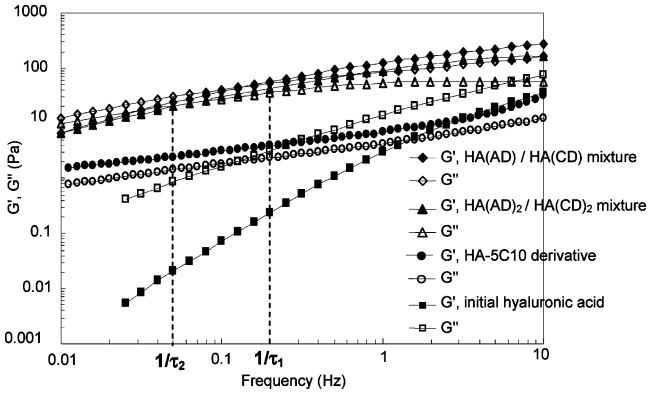

Figure 4. Comparison of the storage and loss moduli as a function of frequency for a HA(AD) (DS = 0.06)/HA(CD) (DS = 0.05) mixture (C_p = 10 g/L), a HA(AD)$_2$ (DS = 0.03)/HA(CD)$_2$ (DS = 0.025) mixture (C_p = 10 g/L), a HA-5C10 solution (C_p = 10 g/L) and for initial HA (C_p = 30 g/L), in 0.025 M NaCl (temperature: 25 °C).

As the secondary interactions which may impair the recognition phenomenon are limited in 0.025 M NaCl, we selected this solvent for the preparation of the networks.

In order to get additional information about the loose intra- and interchain interactions due to chemical modification, the response of solutions of HA(AD), HA(CD), HA(AD)$_2$ and HA-(CD)$_2$ in the 0.025 M NaCl solvent (C_p = 7 g/L) 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 physical aggregates following the application of stress and the rate of rebuilding of these structures following removal of the stress.[5] The data obtained from such experiments are given in Figure 3. For the HA(AD), HA(CD), and HA(CD)$_2$ derivatives and for initial HA, the shear stress increases linearly when the shear rate is increased from 0 to 5 s^{-1} in 1 min, reflecting a Newtonian behavior. In the case of the HA(AD)$_2$ derivative, a critical stress (~1.2 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. These large structures can be attributed to intra- or/and interchain hydrophobic interactions between the grafted AD moieties as previously suspected by calorimetric titration experiments.[16] 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 ~3 h, it recovers the initial behavior (data not shown).

The viscosity measurements performed on the different HA samples thus provided interesting information about the properties of polymers in solution. These indicated that loose inter- and intrachain may be minimized in 0.025 M NaCl, especially for the HA(AD), HA(CD), and HA(CD)$_2$ derivatives. This solvent was thus selected for the preparation of the networks to be examined as a function of their environment.

2. Formation of the Two Supramolecular Assemblies and Effects of External Parameters on the Rheological Properties. Formation of the Two Physical Networks. As reported previously,[16] when a solution of HA(CD) or HA(CD)$_2$ is added to a solution of HA(AD) or HA(AD)$_2$, respectively, at a total polymer concentration 1.5 times higher than the critical overlap concentration C^* (~1.3 g/L) of initial HA, the rapid formation of transparent "gels" can be observed macroscopically. The formation of such networks results from the simultaneous formation of many complexes between the monomeric or dimeric CD and AD molecules grafted along the HA chain. Figure 4 compares the frequency dependence of the dynamic rheological moduli, G' and G'', of HA(AD)/HA(CD) and HA-(AD)$_2$/HA(CD)$_2$ mixtures (total polymer concentration C_p = 10 g/L) and a solution of an alkylated HA derivative (HA-5C10)

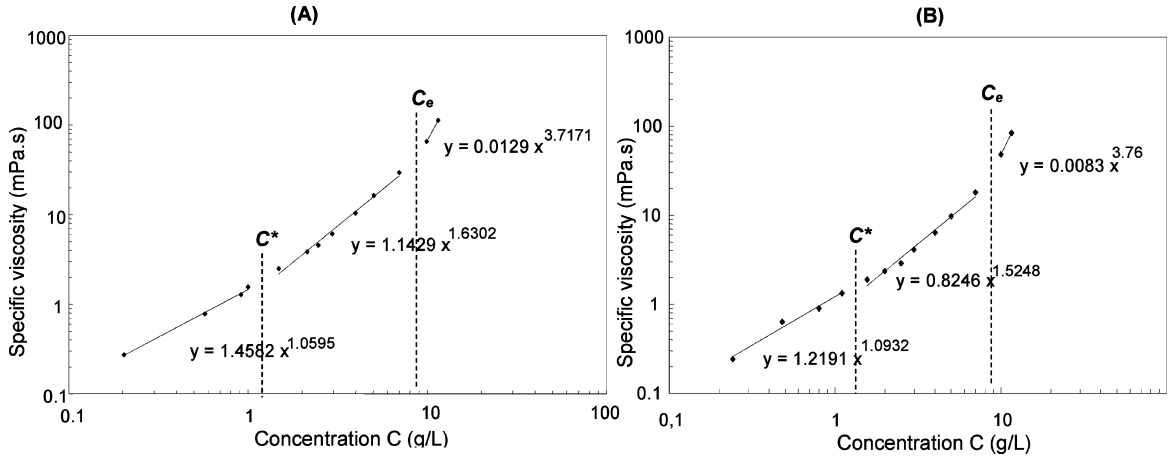

Figure 5. Variation of the specific viscosity with the concentration of initial HA solution in (A) 0.025 M NaCl and (B) 0.1 M NaCl at 25 °C.

prepared in our laboratory (C_p = 10 g/L) with that of initial HA in 0.025 M NaCl. Such alkylated derivatives, referred to as HA-*x*C*y* (where *x* is the degree of substitution (DS) and *y* the number of carbon of the pendant alkyl chain), have been shown to exhibit a remarkable thickening effect in aqueous solution, giving rise in some cases to physical hydrogels.[5]

Contrary to the solution of initial HA which exhibits a viscous character, G' is larger than G'' within a large range of frequency for the HA(AD)/HA(CD) and HA$(AD)_2$/HA$(CD)_2$ mixtures and, within the whole range of frequency covered for the alkylated HA solution. Moreover, the host−guest assemblies exhibit higher values for the storage and loss moduli compared to those obtained for the solution of initial HA, although its concentration is 3 times higher. This reflects the associative character for these different systems. However, though the HA(AD)/HA(CD), HA-$(AD)_2$/HA$(CD)_2$ mixtures and the alkylated HA derivative in aqueous solution give rise macroscopically to transparent "gels", the oscillatory shear data clearly show that the dynamics of the systems are completely different. This suggests different mechanisms for the formation of the transient networks.

If we compare now the frequency dependence of the G' and G'' moduli for the HA(AD)/HA(CD) and HA$(AD)_2$/HA$(CD)_2$ mixtures, we can observe interesting differences related to the nature of the interchain junctions. It can be noted here that the HA-AD and HA-$(AD)_2$ derivatives on the one hand, and HA-CD and HA-$(CD)_2$ derivatives on the other, possess the same average number of AD molecules and CD cavities per HA chain, respectively. The characteristic relaxation time (τ_c), obtained from the inverse of the angular frequency f_c obtained from the crossover point of G' and G'' which characterizes the slowing down of the dynamics of the system, is longer whereas the values of the G' and G'' moduli are lower for the HA$(AD)_2$/HA$(CD)_2$ system. Since this system is stabilized by pairs of complexes resulting in a higher binding constant compared to that of the single CD/AD complex as demonstrated by ITC,[16] and a likely slower exchange rate, the HA chains, on which divalent complexes play the role of sticky points, will require a longer time for relaxation. Moreover, the lower values found for the G' and G'' moduli may reflect the smaller density of effective interchain junction as the content in AD and CD is similar for both mixtures, but they are distributed by pairs along the polymer chain in the case of the "bi-sticker" system. Given the exceptional increase of the association constant found by ITC for the complex between the free dimers of CD and AD, we could have expected a much slower dynamics for the system with bi-stickers than that for the corresponding mono-sticker system. Gain resulting from divalent interactions is limited probably due to significant elastic energy penalty for the formation of the $(CD)_2$/$(AD)_2$ complex which requires certainly a considerable frustration of the short spacer connecting the AD and CD molecules and by the presence of hydrophobic AD/AD interactions, which compete with the formation of inclusion complexes between the grafted dimers.

Effect of Polymer Concentration. Figure 5 depicts the main features of the rheological behavior of initial HA in (A) 0.025 M and (B) 0.1 M. By plotting the log−log variation of the zero-shear specific viscosity as a function of polymer concentration, three different concentration regimes can be distinguished in both solvents:

(i) The dilute regime $C < C^*$, with C^* = 1.3 g/L (0.00323 monomol/L).[5]

(ii) The semidilute disentangled regime $C^* < C < C_e$, where C_e is the concentration at which entanglements become elastically effective.

Above C^*, the dependence of the viscosity follows a power law at 1.5−1.6. At $C_e \sim 9$ g/L (0.022 monomol/L), a break in the variation of the viscosity with the concentration is observed and the power rises to 3.7. The scaling behavior of viscosity with concentration is in good agreement with theoretical predictions for polyelectrolytes in saline media[17] and neutral polymers.[19,20] The scaling predicted is $\eta \sim C^{5/4}$ for $C^* < C < C_e$ and $\eta \sim C^{15/4}$ for $C_e < C < C^{**}$. In this regime, the viscoelastic properties of the solution are controlled by the Rouse dynamics.[21]

(iii) The semidilute entangled regime with $C_e < C < C^{**}$, where C^{**} corresponds to the transition to the concentrated domain. In this concentration domain, the viscoelasticity of the solution is described by the reptation model.[19]

As can be seen from Figure 5, HA exhibits similar behaviors in 0.025 M NaCl and 0.1 M NaCl. Therefore, it could be speculated that in 0.025 M NaCl, the long range electrostatic interactions seem screened enough and that the behavior of the semirigid polysaccharide could be comparable to the one of a neutral polymer.

For polymer concentrations corresponding to the dilute regime of initial HA, the two networks lead to heterogeneous solutions made of "microgels" dispersed in the aqueous solvent. We considered that for these concentrations, we were below the gelation threshold. From a polymer concentration of approximately 1.7 g/L (or 0.004 monomol/L, which is about 1.3 times the overlap concentration of initial HA), "homogeneous gels" were obtained from both HA(AD)/HA(CD) and HA$(AD)_2$/HA$(CD)_2$ mixtures. We thus examined the viscoelastic properties of the assemblies from a total polymer concentration higher than

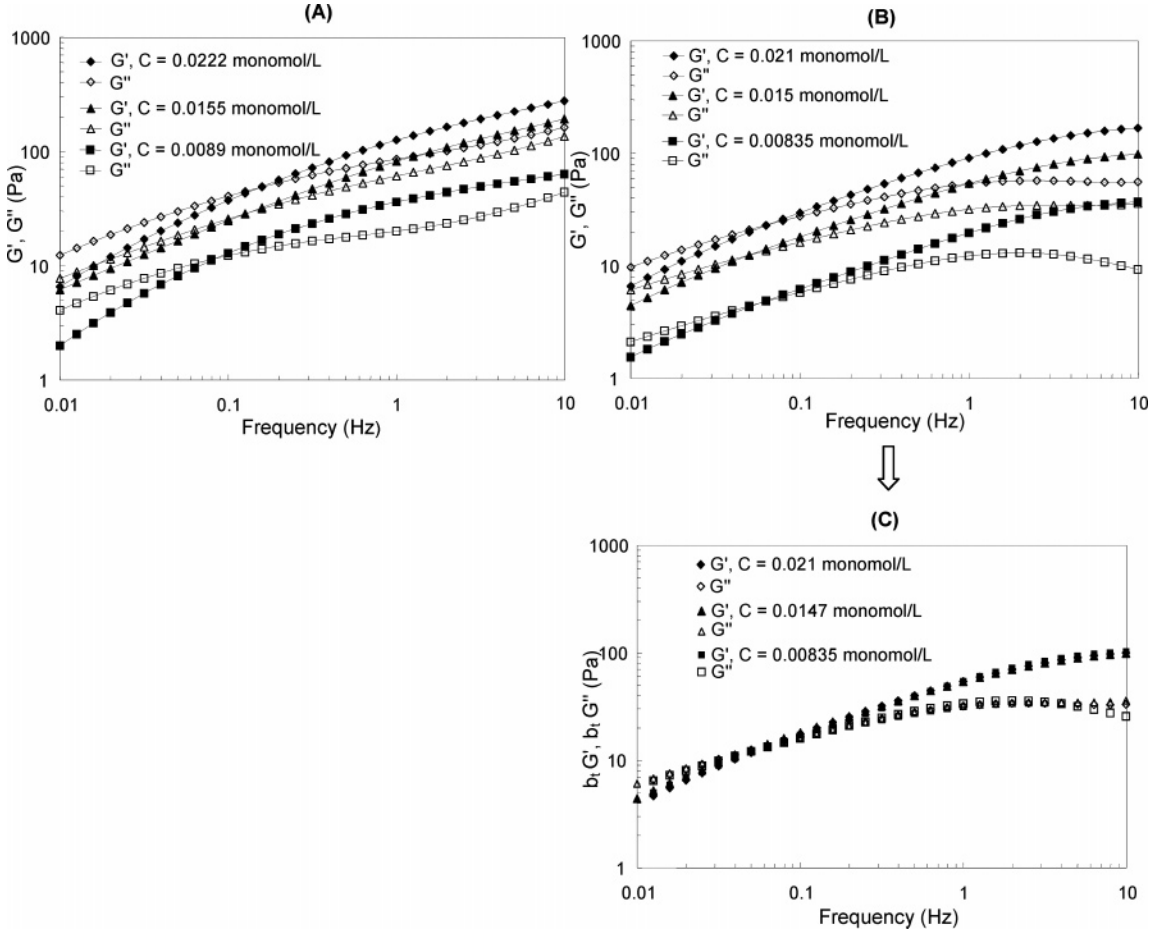

Figure 6. Storage and loss moduli dependence on frequency for (A) the HA(AD)/HA(CD) and, (B) the $HA(AD)_2/HA(CD)_2$ mixtures ([CD] = [AD]) at various polymer chain concentrations in 0.025 M NaCl at 25 °C. (C) Master curve of the G' and G'' moduli for the $HA(AD)_2/HA(CD)_2$ mixture, obtained after superposition. The reference curve, for the $HA(AD)_2/HA(CD)_2$ mixture is the G' curve corresponding to the concentration of 0.021 monomol/L.

this critical concentration (C > 1.7 g/L or 0.004 monomol/L) up to a concentration to 10 g/L, 1.1 times C_e ($C_e \sim 9$ g/L or 0.0235 monomol/L).

Figure 6 shows effects of polymer concentration on the G' and G'' curves as a function of frequency. It can be seen that the storage and loss moduli increase with the polymer concentration for both mixtures. However, the characteristic relaxation time τ_c, which characterizes the slowing down of the dynamics of the system, does not seem to be significantly affected by the variation of polymer concentration. These results were also obtained for the supramolecular assemblies based on host and guest chitosan derivatives.[11] We tried to form a master curve by shifting the moduli-frequency spectra along the horizontal and vertical directions as for time−temperature superposition. The G' curves and G'' curves could be superposed on the reference curves ($C = 7$ g/L) only by a vertical shifting in both cases (see Figure 6C). The same shift factor was used to obtain master curves for both storage and loss moduli, which is an indication of the validity of the superposition. As mentioned above, the relaxation mechanism does not seem to be affected by a modification of polymer concentration since only a vertical shifting is necessary. Given that the polymer chain concentrations used for the two mixtures belong to the semidilute disentangled regime of initial HA, the entanglements are not yet predominant. Thus, the dynamics of the systems is mainly governed by the inclusion complexes which form interchain labile junctions and by their characteristic lifetime.

Effect of Temperature. The behavior of G' and G'' for the two assemblies ($C_p = 10$ g/L) at various temperatures is shown in Figure 7, parts A and B. Owing to the enthalpic nature of the β-CD-adamantane inclusion complexes, their number decreases as the temperature increases. Also, their exchange rate likely depends on the temperature. From Figure 7, parts A and B, it is clear that raising the temperature leads to a shortening of the longest interaction time scale. Time−temperature superposition[22,23] allowed us to obtain master curves for the storage and loss moduli (Figure 7, parts C and D). Here, 25 °C was the reference temperature. For both G' and G'', the same horizontal shift factors could be applied with a negligible vertical shifting. The fact that only a horizontal shift is performed indicates that only the dynamics of the chain is modified by temperature.

The apparent activation energy ΔH_r could be estimated from the dependence of the horizontal shift factor on the absolute temperature. The slope of $\ln(a_T)$ vs $1/T$ gave $\Delta H_r = 62.5$ kJ/mol for the HA(AD)/HA(CD) mixture and 75 kJ/mol for the $HA(AD)_2/HA(CD)_2$ mixture, each assembly having the same total chain polymer concentration. ΔH_r can be considered as a potential barrier that a polymer chain must overcome to diffuse across the other chains.[22,24] The experimental ΔH_r values are much higher than that found for a solution of initial hyaluronic acid, at a same polymer concentration ($(\Delta H_r)_{max} = 22.5$ kJ/mol)[25] due to the labile interchain junctions slowing down the dynamics of the system. These apparent activation energies are also higher than the binding energies corresponding to the enthalpies of monovalent and divalent β-CD/AD complexes determined by calorimetric experiments (−25.8 and −43.1 kJ/mol, respectively).[15,16]

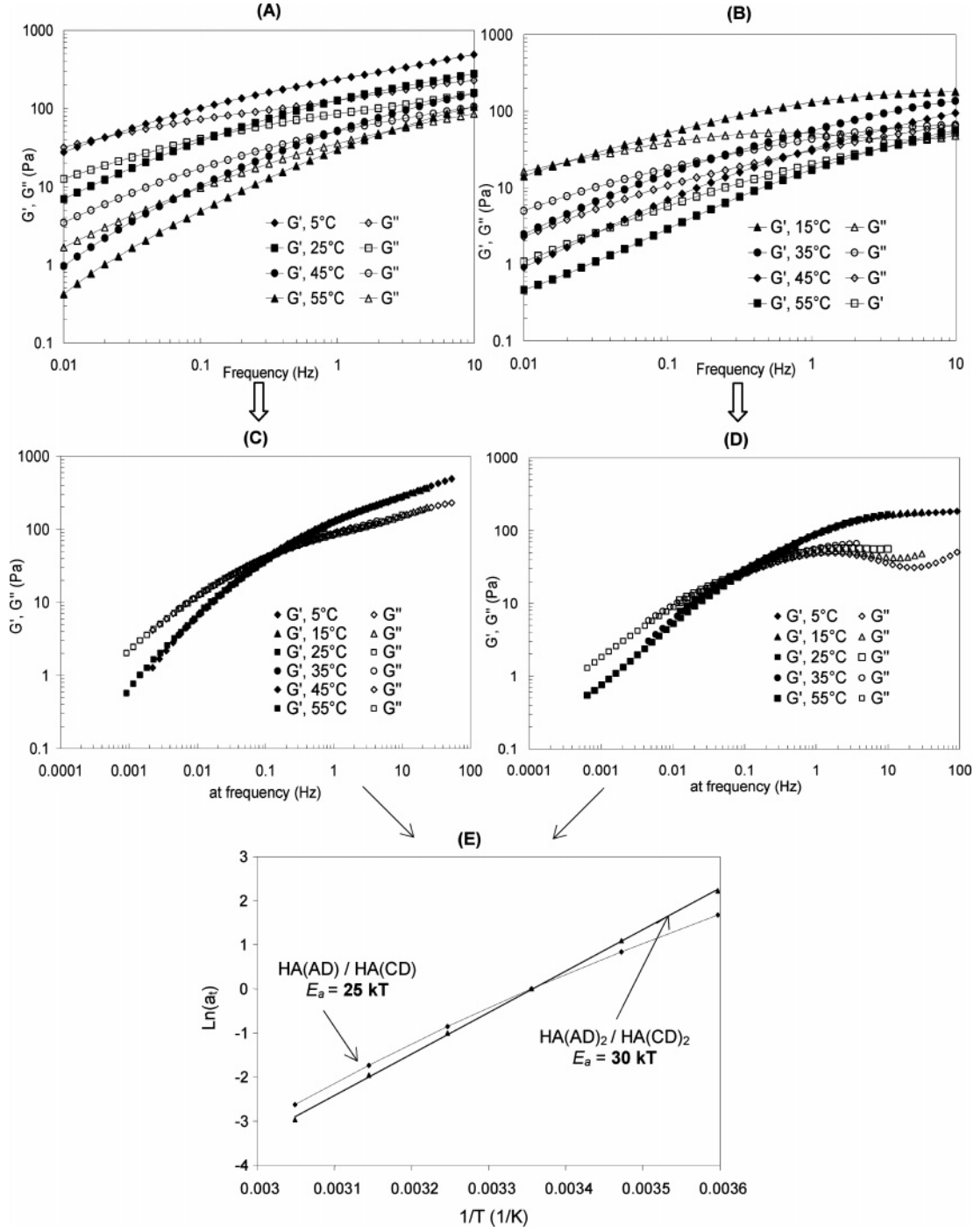

Figure 7. Storage and loss moduli dependence on frequency for (A) a HA(AD)/HA(CD) mixture (C_p = 10 g/L or 0.022 monomol/L, [AD] = [CD])) and (B) a $HA(AD)_2/HA(CD)_2$ mixture (C_p = 10 g/L or 0.021 monomol/L, [AD] = [CD])) in 0.025 M NaCl at different temperatures, varying from 5 to 55 °C. For the sack of clarity, four out of six measurements are shown. (C and D) Master curves of the G' and G'' moduli for the HA(AD)/HA(CD) and $HA(AD)_2/HA(CD)_2$ mixtures, respectively, obtained after horizontal shiftings. For the two systems, the reference curve is the G' curve corresponding to 25 °C. (E) Variation of $\mathrm{Ln}(a_t)$ as a function of $1/T$ for the two mixtures.

Competition Experiments. Since the two networks result from multivalent specific interactions between β-CD and AD derivatives, we can assume that the addition of competitive host and guest molecules should disrupt it. It was thus interesting to examine effects of addition of such molecules on the supramolecular assemblies.

We selected natural β-CD as competitive host molecules. Figure 8 shows the results obtained by the addition of the CD molecules at different concentrations to the HA(AD)/HA(CD) and $HA(AD)_2/HA(CD)_2$ assemblies respectively. From this figure, it can be seen that the relaxation mechanism is affected by addition of the natural β-CD competitive host molecules. The addition of the free host β-CD molecules involves a decrease of the longer relaxation time. Using the same principle as for the time−temperature superposition and for the effect of the polymer chain concentration, we tried to obtain a master curve by shifting the moduli-frequency spectra along the horizontal and vertical directions. The reference curve was the one obtained without addition of competitive host molecule. For the two systems, the master curve was obtained by only a horizontal shift (Figure 8, parts C and D). The dynamics of the two systems and the exchange rate of the interchain inclusion complexes are totally modified since an AD molecule, as a monomer or a dimer, can choose to complex with a free or a

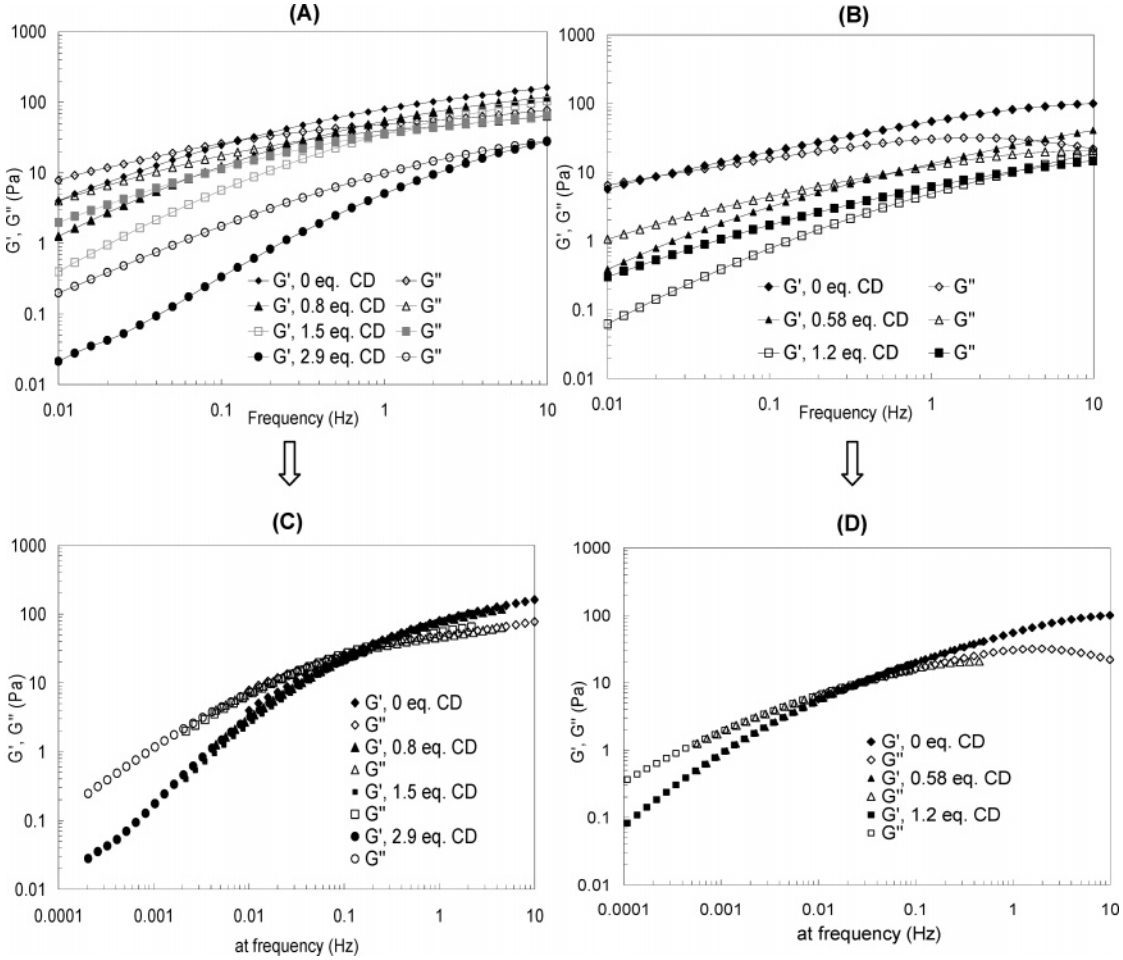

Figure 8. Storage and loss moduli as a function of frequency for (A) a HA(AD)/HA(CD) mixture and (B) a $HA(AD)_2/HA(CD)_2$ mixture (C_p = 7 g/L or 0.015 monomol/L, [AD] = [CD]) containing free natural β-CD at different concentrations, in 0.025 M NaCl at 25 °C. (C and D) Master curves of the G' and G'' moduli for the HA(AD)/HA(CD) and $HA(AD)_2/HA(CD)_2$ mixtures after horizontal shiftings. For the two systems, the reference curve is the G' curve corresponding to the experiment without addition of competitive host molecule.

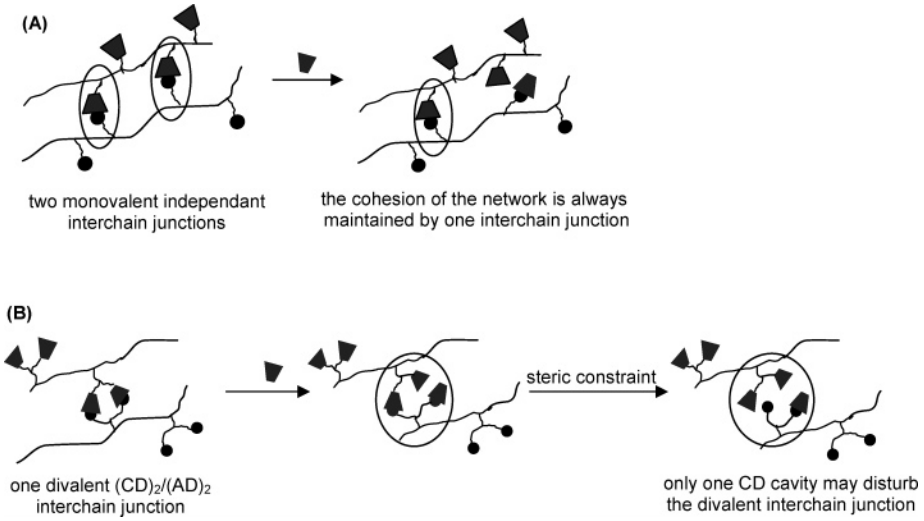

Figure 9. Schematic representation of the effect of the addition of natural β-CD on the stability of the (A) HA(AD)/HA(CD) and (B) $HA(AD)_2/HA(CD)_2$ mixtures.

grafted CD cavity. Moreover, Figure 8 indicates that disruption of the $HA(AD)_2/HA(CD)_2$ network requires a lower amount of β-CD molecules. This can be explained by the fact that this assembly is stabilized by twice less interchain junctions and that only one β-CD cavity may be sufficient to dissociate a divalent inclusion complex for steric reasons, as represented on Figure 9. We can thus assume that the complete destruction of the $HA(AD)_2/HA(CD)_2$ network could be achieved with twice less free β-CD molecules in comparison with the total dissociation of the HA(AD)/HA(CD)assembly.

The above features in the rheology of the HA-based assemblies bear a striking resemblance to the rheology of chitosan-based assemblies reported previously.[11] These similarities suggest a correspondence between the association mechanisms.

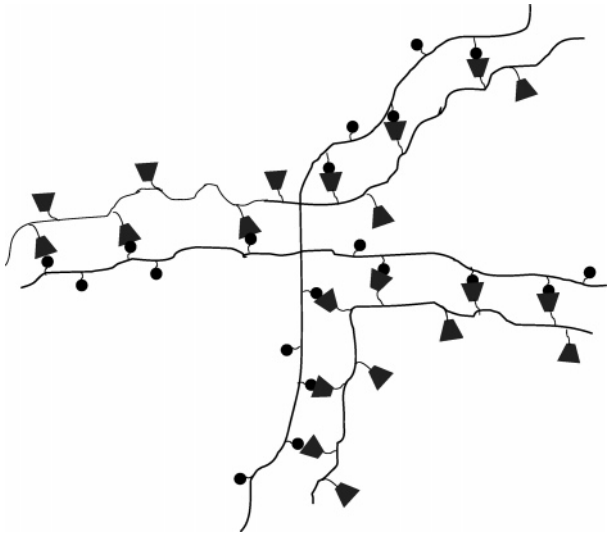

Figure 10. Schematic representation of double-chain strands connected by fourfold junction points constituting the reversible network made of HA(AD) and HA(CD).

On the other hand, considerable differences are observed between the binary solutions of associating HA derivatives and solution of HA-5C10, as discussed above. Although the latter behave like elastic physical hydrogel, it could be easily analyzed by NMR spectroscopy. On the other hand, no NMR signals could be detected in the case of the mixtures of host and guest HA derivatives, suggesting the "freezing" of the polymer chains. What could be the physical reason for the lack of chain mobility? As discussed in details in a recent paper,[26] in semidilute solution and in dilute solution as well, the mixtures of CD-grafted HA and AD-grafted HA may result in formation of double chains, which benefit from the high energy of the CD/AD complex. Above a given concentration which is around the HA polymer coil overlap concentration C^*, the latter form a reversible network in which the cross-links are junction points where the crossed double-chains interchange their partners (Figure 10). The relaxation of the network structure thus involves two types of motions: the Rouse-like chain relaxation but also topological relaxations due to slow diffusion of junction points along the chains. The latter quenches drastically the motions of the chain. In addition, it has been demonstrated that the chemical irregularity plays a major role on the dynamical properties. It has a slowing effect, which is controlled by the parameter $\beta = B \times (DS \times N)^{1/2}$, where N is the total number of unit per chain. The parameter B is related to the distribution of the stickers along the chain. B increases with the polydispersity of the main-chain spacers between the stickers. Indeed, this implies that matched double-chain fragments coexist with mismatched ones. The former are very stable, and consequently, contribute to significantly slow down the structural relaxation of the network. From these considerations, chain concentration may have a negligible effect on the dynamical properties, which can be observed experimentally.

On the other hand, increasing temperature results in a somewhat faster stress relaxation. The high experimental apparent activation energies for the associating polymers is related to the multisticker interaction stabilizing the assemblies. The theoretical model recently developed[26] for such HA-based assemblies, with flexible inter-sticker main-chain spacers ($L \gg L_p$ where l_p is the backbone persistence length and L is the length of the backbone spacer between neighboring stickers), predicts that the terminal stress relaxation time τ_r scales as $K_a^{3/2}$. Here, K_a is the association constant of the CD/AD complex. Thus, the apparent activation energy for relaxation $\Delta H_r = R(\partial(\ln \tau_r)/\partial(1/T))$ is related to the association enthalpy $\Delta H_a = R(\partial(\ln K)/\partial(1/T)) \approx 25.8$ or 43.1 kJ/mol, according to the monovalent of divalent nature of the complex.[16] $\Delta H_r \approx {}^3/_2\Delta H_a + \Delta H_0$, where ΔH_0 is a correction accounting for the temperature dependence of other factors defining τ_r. Assuming the value of the activation energy of the solution of initial HA at the same polymer concentration ($\Delta H_r = 22.5$ kJ/mol) for ΔH_0,[25] we find a "theoretical" value of ΔH_r of 61.2 and 87.1 kJ/mol, for the mono-sticker and bi-sticker systems, respectively. These values are in reasonable agreement with the experimental ones.

Adding competitive host molecules remarkably diminishes the characteristic time $\tau_c = 1/\omega_c$ corresponding to $G' = G''$. These molecules compete for pairing with grafted bond-forming stickers, thus increasing the probability of the dissociated state of a pair of grafted stickers. Comparison between the HA- and chitosan-based mono-sticker systems indicates that the effect is particularly dramatic in the latter case,[11] with stiff inter-sticker main-chain spacers ($L \ll l_p$) and a marked irregular distribution of stickers. Indeed, the relaxation time decreases by many orders of magnitude even when the number of added free stickers is less than the amount of grafted stickers. This has been related to the fact that the added free stickers contribute to decrease the high elastic energy penalty resulting from the double chain structure with all stickers forming bonds. On the other hand, the different effects observed upon addition of free stickers on the dynamics of the HA-based mono- and bi-sticker systems can be related to the fact that the latter system is stabilized by twice less interchain junctions and that only one β-CD cavity may be sufficient to dissociate a divalent inclusion complex for steric reasons.

In conclusion, this study shows that the novel hyaluronic acid-based supramolecular assemblies stabilized by multivalent specific interactions exhibit unusual viscoelastic properties in the semidilute regime. The effects of different external parameters such as polymer chain concentration, temperature, and addition of free competitive host molecules on the viscoelastic behavior were found to be similar for both systems and the previously reported chitosan-based assemblies,[11] as well. These results might be explained by a peculiar mechanism of association resulting in a network of double-chain strands connected by fourfold junction points. From a fundamental point of view, these supramolecular assemblies represent a new class of temporary networks that may constitute a relevant model involving punctual multivalent interactions like those occurring naturally in biological processes.

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