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Rheological Properties of Cartilage Glycosaminoglycans and Proteoglycans

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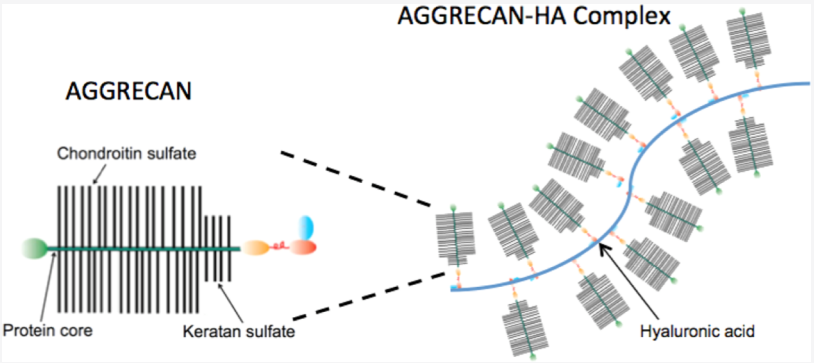

ABSTRACT: Glycosaminoglycans (GAGs) are molecules that govern the load-bearing and frictional properties of cartilage and the lubricating properties of synovial fluid of joints. Most GAGs in the body form proteoglycans (PGs), and the dominant PG in cartilage is the bottlebrush-shaped aggrecan, comprised of a protein core decorated by GAG side chains consisting mainly of chondroitin sulfate (CS) and keratan sulfate. Additionally, hyaluronic acid (HA) induces aggrecan to self-assemble into complexes having up to 100 aggrecan molecules attached to each HA chain "backbone". Here, we report the rheological properties of CS, HA, aggrecan, and aggrecan−HA complexes in water and salt solutions. We find that CS solutions are viscous liquids, while HA solutions exhibit viscoelasticity and shear thinning. Specifically, in the case of HA, the storage modulus G' exceeds the loss modulus G'' at high frequencies (ω) while the reverse is true at low ω. Thus, the rheologies of CS and HA exhibit somewhat distinct viscoelastic properties. Aggrecan, on the other hand, shows a rheology consistent with an incipient "weak gel" in which G' and G'' cross at a specific ω and follow a power-law scaling over a wide ω range. Moreover, aggrecan samples do not attain an observable plateau in the viscosity under steady shear and low shear rates. Finally, the complexation of aggrecan and HA over a period of 48 h results in a slow evolution ("aging") in measured rheological properties, with G' at low ω progressively increases in time, while G'' remains essentially unchanged. This suggests that aggrecan and HA gradually form a macroscopic network in which molecular complexes act as physical cross-links.

INTRODUCTION

Glycosaminoglycans (GAGs) are the most abundant polysaccharides in the body. These highly negatively charged biopolymers exhibit an extended configuration in solution, which makes their solutions moderately viscous at low GAG concentrations. There are several GAGs of great physiological significance, such as chondroitin sulfate (CS), keratan sulfate (KS), heparan sulfate (HS), dermatan sulfate (DS), and hyaluronic acid (HA).[1,2] GAGs are key components of synovial fluid and cartilage.[3−8] Most of the GAGs are attached to a protein core, forming proteoglycans (PGs). The dominant PG in the extracellular matrix (ECM) of cartilage is the bottlebrush-shaped aggrecan (see Scheme 1).[9,10] Aggrecan has a core protein with a contour length of 300−400 nm, with more than 100 GAG side chains (30−40 nm) extending outward from the core. The complex has a large molecular mass (>1000 kDa). Distinct regions of CS and KS are present along the aggrecan bottlebrush. Aggrecan bottlebrushes self-assemble in solution forming microgel-like assemblies.[7] These microgel regions are expected to be connected into a network by free aggrecan molecules. HA is unique among the GAGs in that it does not contain sulfate groups. In the extracellular matrix, aggrecan molecules interact with HA and link protein and form stable ternary complexes, which also have a bottlebrush morphology, as shown in Scheme 1. We note that complexation between aggrecan and HA also takes place

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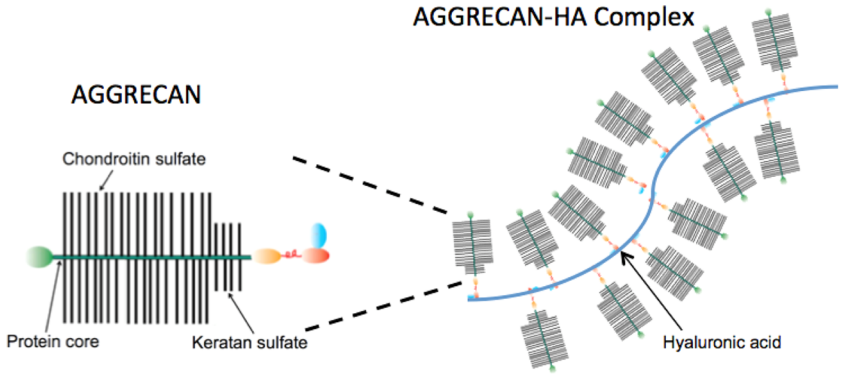
Scheme 1. Schematic Drawing of the Structure of Aggrecan (Left) and Aggrecan−HA Complex (Right)



in the absence of link protein as in the case of the present study. In cartilage, aggrecan−HA complexes are interspersed in a matrix of collagen fibers.[9,10]

Understanding the rheology of HA and aggrecan is essential to understanding their biological function. Synovial fluid, which lubricates joints, is rich in HA. The function of synovial fluid is much more complex than simply forming a thin layer between contacting surfaces.[13] It acts as a viscous liquid over long timescales (corresponding to slow joint movement) and as an elastic material at short timescales (corresponding to rapid joint movement). In cartilage, under mechanical loads, water and low-molecular-mass solutes are driven out from the aggrecan−HA−collagen network. This increases the polymer concentration, and the system becomes "gel-like", thus protecting cartilage surfaces from frictional damage.[14] In the gel-like state, the material does not flow over relatively long timescales. We can further distinguish between "weak" and "strong" gels. Strong gels have an infinite viscosity and exhibit a finite shear modulus at low frequencies (ω), i.e., at long timescales. Weak gels also have an infinite viscosity but may not have a finite shear modulus at low ω. A basic characteristic of weak gels is that the elastic (G') and viscous (G'') moduli obtained from the real and imaginary parts of the Fourier transform of the shear stress relaxation function, respectively, exhibit near-identical power laws at low oscillatory frequencies, ω. Many natural viscoelastic materials are "weak gels" in this precisely defined sense, existing at the boundary between ordinary "fluid" and "solid" rheological states based on Newton's definition of the shear viscosity η of fluids and Hooke's definition of solids as materials having a finite shear modulus G,[14] respectively.

The rheology of cartilage polymers has received considerable attention during the last decades. Among these polymers, HA has been studied most extensively. Gibbs et al.[11] investigated the effects of temperature, concentration, and ionic strength on the dynamic rheology of HA solutions. HA solutions are viscoelastic, exhibiting a transition from viscous to elastic behavior with increasing frequency ω. The response becomes more elastic with increasing HA concentration and molecular mass. Time-temperature, time-concentration, and time-ionic strength superpositions are applicable to HA rheology, suggesting that these variables do not modify the essential nature of the relaxation process but rather the relaxation timescale.[12] In comparison to HA, only a few studies have been done on CS solutions. CS is significantly different from HA with regard to both its structure and dynamics.[20,21] Interestingly, adding divalent cations to CS solutions promotes its assembly into extended complexes with linearly aligned regions, as inferred from neutron scattering experiments.[20] However, the effect of ion valence (e.g., monovalent vs divalent cations) on the rheology of cartilage biopolymers remains unexplored.[7,18−21,27] Divalent calcium (Ca^{2+}) ions are especially important because cartilage plays an essential role in mineralized bone tissue formation.

A few rheological studies have been conducted on aggrecan−HA complexes, and the studies have found the samples to be examples of weak gels, as mentioned above. Jamieson et al.[15,16] demonstrated that aggrecan−HA complexes are elastic and exhibit a yield stress at physiological conditions. A sol−gel transition occurs as the concentration of the complexes exceeds the overlap concentration. When the ionic strength is decreased below the physiological range, this class of gels is transformed into viscous fluids, evidently because of electrostatic repulsions between the complexes. However, little is known about how the rheology varies in the course of complexation between aggrecan and HA. In particular, the impact of aggrecan/HA ratio on the rheology is important to study because it plays a critical role in cartilage biological function. For instance, the fixed charge density (FCD) of articular cartilage is known to vary depending on age, health condition, and anatomical site.[22,23]

Previous studies have significantly advanced our understanding of the behavior of GAGs and PGs.[7,18−21,27] However, several critically important questions were not addressed in these former studies. One of the outstanding questions is the effect of ion valence on the dynamic properties. In earlier works made on PG solutions, the ionic strength was varied but only the effect of monovalent ions was systematically investigated. In the physiological milieu, however, divalent ions are also present. Specifically, calcium ions are important because cartilage plays an essential role in mineralized bone tissue formation, as noted above.

Here, we systematically investigate the rheology of CS, HA, aggrecan, and aggrecan−HA complexes in solution. We probe the dynamic response of these solutions over the broad frequency range relevant to the biological function of cartilage. This paper is organized as follows. First, the rheology of CS solutions is presented over a wide concentration range from 1% m/m (dilute solution) to 30% m/m (concentration of CS in the aggrecan bottlebrush), where concentrations in m/m refer to the mass of additive divided by the mass of solution. The effects of both mono- and divalent salts (NaCl and $CaCl_2$) on the rheology of CS solutions are determined. We compare the rheology of CS with that of HA solutions. Then, the

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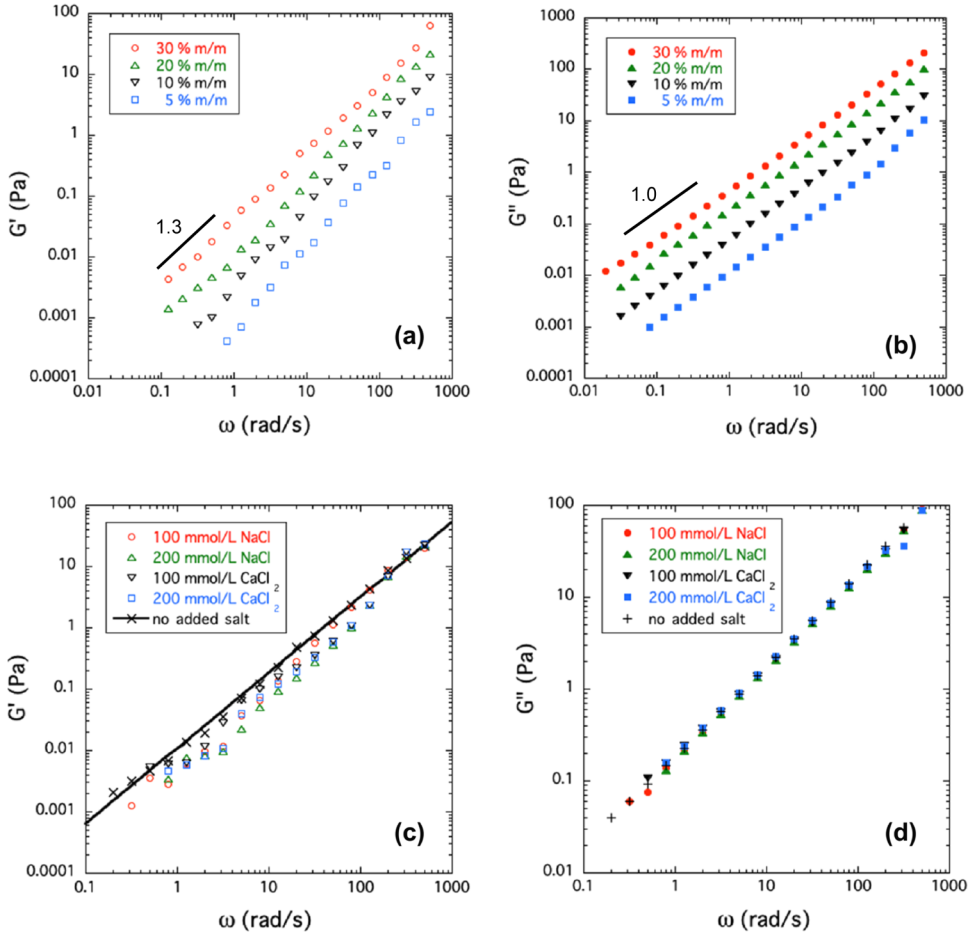

Figure 1. Dynamic rheology of CS solutions. In all cases, the storage modulus G' and the loss modulus G'' are plotted as a function of frequency ω. (a, b) Data for different CS concentrations. (c, d) Data for solutions containing either NaCl or $CaCl_2$ at a constant CS concentration of 20% m/m.

rheology of aggrecan and the effect of salt on it are discussed. Finally, we report the rheology of aggrecan−HA complexes at different stages of complexation. Rheological studies of these cartilage polymers are important to make progress in understanding arthritic and other medical conditions.

We remind the reader that a basic feature of cartilage biopolymers is that they are generally highly charged macromolecules. The viscoelasticity of charged polyelectrolyte solutions is affected by many factors, such as the concentration and valence of counterions, the intrinsic properties of the polymer (e.g., chain rigidity), polymer conformation, molecular mass, etc., as well as inter- and intramolecular interactions (e.g., ion condensation, hydration, hydrogen bonding).[17] Because of the complexity of these interactions, the modeling of polyelectrolyte solutions is inherently difficult. To date, no satisfactory theoretical framework embodying the relationship between molecular/supramolecular structure, macroscopic properties, and biological function of either synthetic or natural polyelectrolyte brush molecules has been developed, making experimental studies of these molecules important for future progress in understanding the arthritic and other medical conditions related to the dysfunction of bottlebrush molecules.

MATERIALS AND METHODS

Sample Preparation. Chondroitin sulfate sodium salt (molecular mass, M_n = 40 kDa), hyaluronic acid sodium salt (M = 1.3 MDa), and aggrecan were purchased from Sigma-Aldrich. Each polymer was dissolved in deionized (DI) water, with the pH fixed at 7 for all samples. The concentration of CS in solution was varied between 5 and 30% m/m, the concentration of HA between 1 and 10% m/m, and that of aggrecan between 1 and 6% m/m. Salt concentrations (NaCl and $CaCl_2$) between 0 and 200 mmol/L were studied. Solutions were centrifuged at 100 rad/s for 5 min to get rid of air bubbles before making the rheological measurements.

Rheological Measurements. Rheological experiments were conducted on an AR2000 stress-controlled rheometer (TA Instruments). Experiments were done on a cone-and-plate geometry (40 mm diameter, 2° cone angle), and a solvent trap was used to prevent evaporation of the sample during the tests.[24] The temperature was controlled by a Peltier assembly on the rheometer, and all experiments were made at 25 °C. Dynamic frequency sweep experiments were conducted in the linear viscoelastic regime for each sample, which was first determined from strain−sweep experiments. The angular frequency ω in these experiments was varied between 0.1 and 500 rad/s. In the case of steady-shear rheology, the apparent viscosity was measured as a function of shear rate over the range of 0.01−100 s^{-1}.

RESULTS AND DISCUSSION

Chondroitin Sulfate (CS). Figure 1a−d show plots of the storage or elastic modulus (G') and loss or viscous modulus (G'') against frequency ω for different concentrations of CS in salt-free solutions (1a and b), and in solutions containing either NaCl or $CaCl_2$ (1c and d). Both G' and G'' increase with increasing CS concentration. The addition of salt causes G' to slightly decrease, but G'' remains practically unchanged. No qualitative difference can be observed between the data for the

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monovalent and divalent salts (Na^+ and Ca^{2+}), implying that no significant contribution to the rheology arises from hypothetical bridge formation between the negatively charged CS chains and Ca^{2+} ions. Such an association should increase the value of G'.

Since all curves are similar in shape, we shifted the data relative to the lowest CS (salt-free) sample until the data superposed. Figure 2 illustrates that both G' and G'' follow

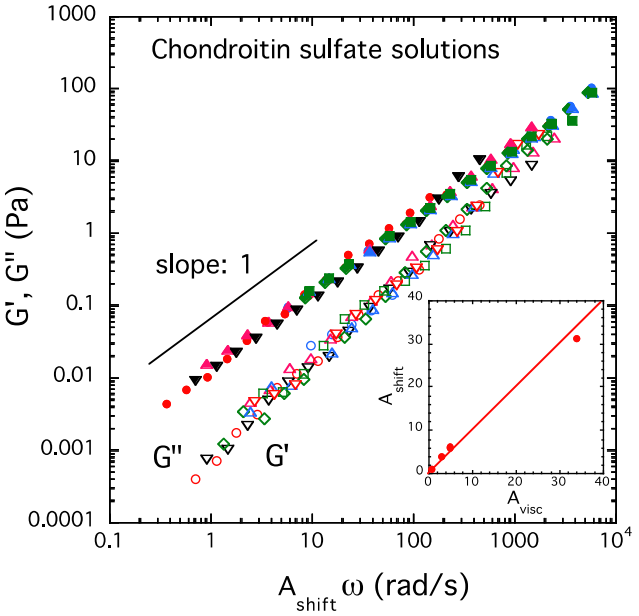

Figure 2. Master curves for the dynamic rheology of CS solutions. Data shown in Figure 1a–d for G' (open symbols) and G'' (closed symbols) are replotted after shifting. The slope of 1 implies that G'' increases linearly with the frequency. In the inset, we show the rheological shift factor A_{shift}, obtained based on the assumption of time-concentration superposition of the data in Figure 1. We interpret A_{shift} to arise from the change of solution shear viscosity η with polymer concentration, c. In particular, we compare the empirical A_{shift} with the ratio, $A_{visc} \equiv \eta(c)/\eta(c_o)$, where c_o is the concentration of a reference polymer solution.

master curves. The slope of the G'' vs $(A_{shift}\,\omega)$ plot is close to 1, corresponding to primarily viscous behavior, while that of the G' vs $A_{shift}\omega$ plot is greater, by a factor ≈ 1.3. It is evident that $G'' > G'$ over the whole frequency range, i.e., the solutions behave as viscous liquids even at a 30% m/m CS concentration. The lack of a plateau region for G' is taken to imply that the CS chains are not entangled or strongly associated. In our discussion below, we show that HA exhibits rheological characteristics of associated polymer solutions and approximate linear scaling of G' with ω such that the fluid viscoelesticity is roughly consistent with the Maxwell model of viscoelasticity.

Although rescaling of the frequency dependence of G' and G'' is a purely empirical procedure, this procedure is consistent with the common procedure of introducing empirical "shift factors" A_{shift} when varying temperature, salt concentration, or other variables influencing the relaxation of the polymer solution.[11] In the present context, the shift factor A_{shift} is determined by empirically shifting our viscoelastic data to achieve a "best" reduced variable reduction of our viscoelastic data and, in the inset of Figure 2, we compare the estimated A_{shift} with the ratio, $A_{visc} = \eta(c)/\eta(c_o)$, where c_o denotes a reference concentration. This estimate of A_{shift} in terms of the viscosity ratio assumes the existence of an average τ for the solution, which can be estimated from the Maxwell relation ($\eta \sim G\tau$), where G is a concentration-dependent high-frequency shear modulus. The quality of the reduction of the viscoelastic relaxation data through the introduction of a c-dependent frequency shift factor and the near linear relation between A_{shift} and A_{visc} provides some justification for these assumptions.

In Figure 3a, the viscosity η of CS solutions from steady-shear measurements is plotted as a function of shear rate $\dot{\gamma}$. All of the CS solutions are Newtonian fluids, i.e., they exhibit a nearly constant η over the range of $\dot{\gamma}$. The value of η increases with increasing polymer concentration, as expected. Figure 3b shows that the addition of NaCl or $CaCl_2$ gradually decreases η. The decrease in viscosity could possibly arise from a reduction of the chain stiffness (i.e., the persistence length, and thus the radius of gyration) with the addition of salt.[28,29]

Hyaluronic Acid (HA). We now describe the rheology of HA solutions. The HA studied here has a molecular mass of 1300 kDa, and its concentration was varied between 1 and 10% m/m. HA samples were prepared in water (pH = 7) and in different salt solutions. Figure 4 shows master curves of G' and G'' for all HA concentrations. (The original data are shown for

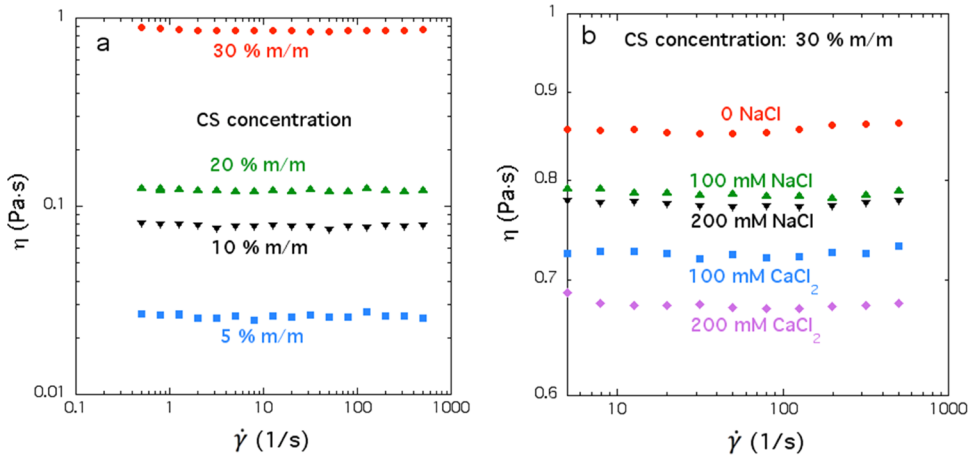

Figure 3. Steady-shear rheology of CS solutions. In all cases, the apparent viscosity η is plotted as a function of shear rate $\dot{\gamma}$. (a) Data for CS at different concentrations; (b) data for different concentrations of NaCl and $CaCl_2$ at a constant CS concentration of 30% m/m.

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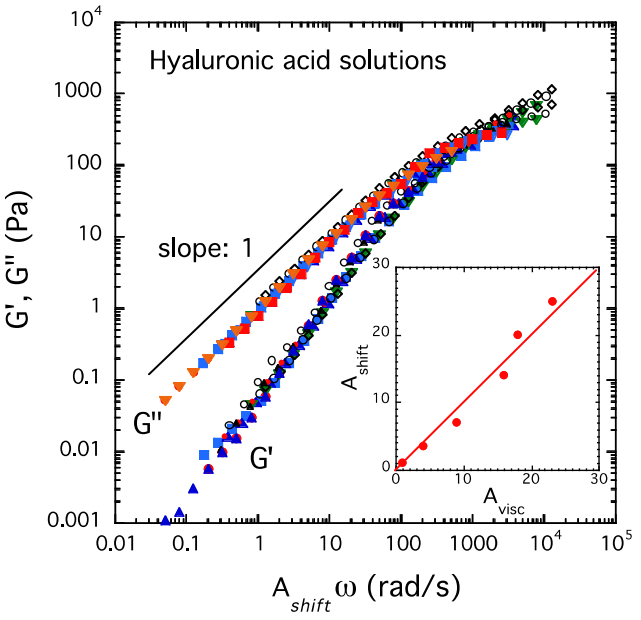

Figure 4. Master curves for the dynamic rheology of HA solutions. Data for G' and G'' on samples with 1−10% m/m HA are replotted after shifting. At low frequencies (long timescales), the loss modulus G'' dominates and G'' is linearly proportional to the frequency. The inset shows the empirically estimated viscoelastic shift factor A_{shift} in comparison with A_{visc} obtained from the change in the solution viscosity with the polymer concentration c, as shown in Figure 2.

reference in the Supporting Information section.) Here, the superposed G' and G'' curves exhibit a crossover point as a function of ω, such a crossover point is expected in the CS solutions at higher ω than we investigate. Below this crossover frequency, i.e., in the terminal regime, $G'' > G'$ (implying that the behavior is viscous at longer timescales); conversely, above this frequency, $G' > G''$ (implying that the behavior is elastic at shorter timescales). Such crossing of G' and G'' is the characteristic of polymer chains exhibiting molecular association into networklike structures, where the crossover frequency may be identified with the average network lifetime.[30,31] Physically this means that at timescales over which $G'' > G'$, polymer chains have sufficient time to escape from the constraints of surrounding chains, and thus show viscous behavior, while at shorter timescales, the chains are localized by surrounding chains, and the material exhibits elastic behavior. A similar viscoelastic response is observed in "entangled" polymer solutions, along with the same trend in the crossover frequency with polymer concentration.[25,26]

The tube model[32−34] has been introduced as a mean-field model of the topological interactions, leading chain to transient chain localization in long, flexible neutral polymers in solution. The reptation model[35,36] was introduced to model the resulting constrained motion of a representative polymer chain in a static hypothetical tube describing the average caging effect of surrounding polymer chains.

It is important to emphasize that the present macromolecules are highly charged and such polymers have a propensity to exhibit supramolecular assembly in solution.[37,38] Their interactions are clearly rather distinct from neutral flexible polymers, and the dynamic clustering in these solutions can be expected to depend on several factors such as ion and polymer solvation, the presence of salt, hydrophobic and hydrophilic groups on the polymer chains, etc., rather than just chain length as in solutions of neutral flexible polymers. We thus attribute the concentration dependence of the viscoelastic spectra to derive from dynamic clustering of the polymer chains. Chains within these clusters are localized so that some elements of the tube and reputation models may apply, but the polymer solution is populated by polydisperse large-scale chain clusters. Strong shear thinning and other nonlinear rheological effects may be expected to play important roles when these systems are deformed or otherwise perturbed.[14]

Figure 5a illustrates the effect of polymer concentration on the steady-shear viscosity of HA solutions. η increases with polymer concentration, as expected. The samples show a plateau in η at low $\dot{\gamma}$ and then a decrease in η at higher $\dot{\gamma}$ (i.e., shear thinning). We interpret this behavior to arise from the disruption of chain clusters in the HA solution by the application of shear.[14] There may also be a contribution from chain alignment under shear to the observed shear thinning. Figure 5b shows η vs $\dot{\gamma}$ plots for a 4% m/m HA solution containing different amounts of $CaCl_2$. All samples show shear thinning, but the viscosity in the plateau region decreases by nearly 20% as the $CaCl_2$ concentration increases from 0 to 200 mmol/L. No evidence for precipitation was seen in the

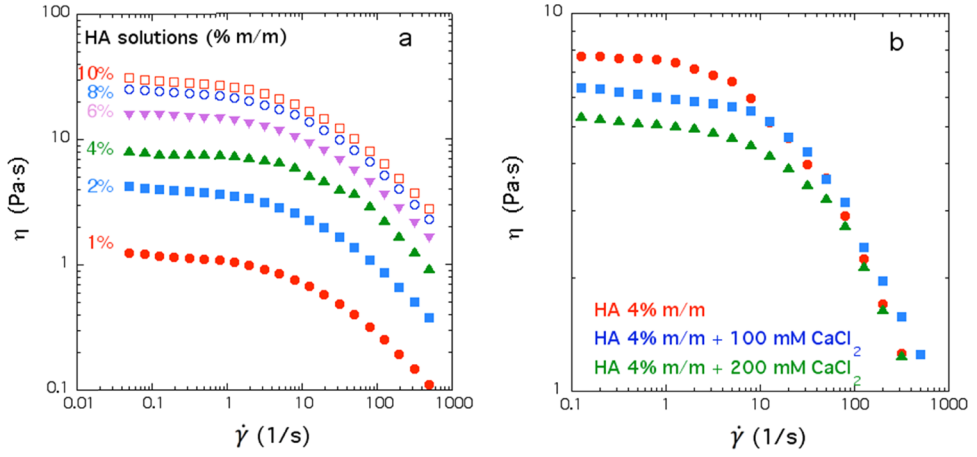

Figure 5. Steady-shear rheology of HA solutions. In all cases, the apparent viscosity η is plotted as a function of shear rate $\dot{\gamma}$. (a) Data for HA at different concentrations; (b) data for a fixed 4% m/m HA concentration and with different concentrations of $CaCl_2$.

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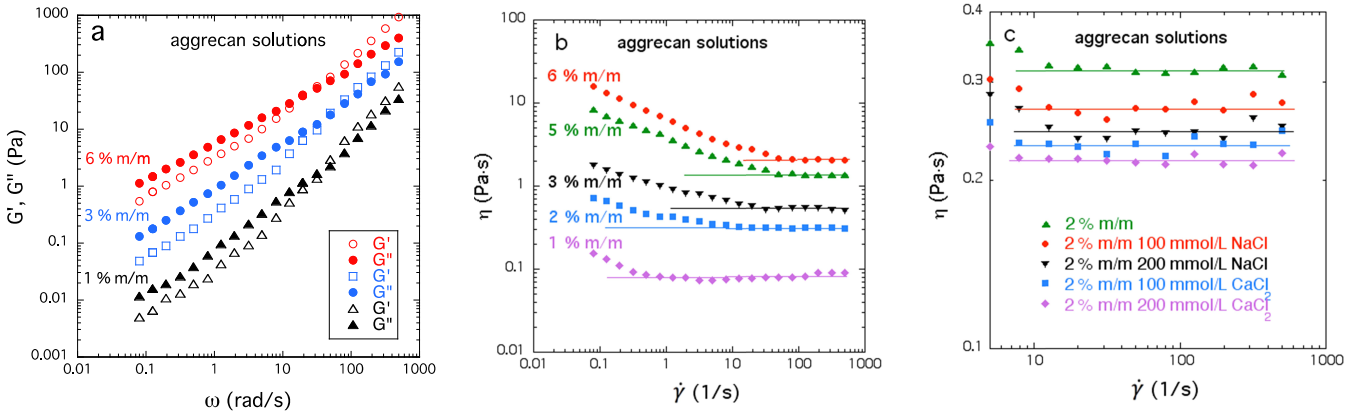

Figure 6. Rheology of aggrecan solutions. (a) Data from dynamic rheology for G' and G'' vs frequency ω for different aggrecan concentrations. (b) Data from steady-shear rheology for the viscosity η vs shear rate $\dot{\gamma}$ for different aggrecan concentrations. (c) η vs $\dot{\gamma}$ data for 2% m/m aggrecan at different concentrations of NaCl and $CaCl_2$.

samples due to hypothetical Ca^{2+} bridges between the HA chains.

Superposition of G' and G'' with a variation of the polymer concentration is achieved, indicating that the relaxation mechanism is remarkably insensitive to the polymer concentration, salt concentration, and ion valence over the wide ranges investigated. While CS solutions exhibit predominantly viscous rheology over the range of timescales probed, HA solutions are viscoelastic and exhibit elastic behavior at short and viscous behavior at long timescales. This implies that chains of HA form clusters at the concentrations studied, which is likely due to the higher molecular mass of HA. The additions of monovalent (NaCl) and divalent ($CaCl_2$) salts reduce the steady-shear viscosity of CS and HA solutions, but we observe no sign of salt-induced phase separation or Ca^{2+}-bridge formation with the negatively charged groups of the polymer chains. We do not exclude this phenomenon altogether in all polyelectrolytes, but this type of direct ion-bridging association between chains does not seem to exist in the polyelectrolytes studied in the present paper. The decrease in viscosity in the presence of salt suggests that ions screen intramolecular repulsions between these highly charged polymer chains and thereby reduce the chain persistence length and the overall polymer radius of gyration.[28,29] This is a commonly observed pattern of behavior in polyelectrolyte solutions, although the opposite trend or very little change of the solution viscosity is observed in polyelectrolytes in which the ions in the chain monomer are paired, i.e., polyzwitterions, an effect termed as "antipolyelectrolyte" effect.[39−41] Evidently, the charge distribution on the polymer chains can profoundly influence the sensitivity of these molecules to the presence of salt in the solution.

Aggrecan. In the aggrecan molecule, GAG chains (mainly CS and KS) are tethered to an extended protein core (Scheme 1). First, we investigate the rheology of aggrecan solutions and then study the complex formation between aggrecan and HA. Figure 6a shows data from dynamic rheology for G' and G'' vs ω at three aggrecan concentrations. All samples show a crossover at about 40 rad/s, with G' exceeding G'' above this frequency. This is somewhat similar to the viscoelastic rheology seen for HA (Figure 4), but with some important differences. First, the shapes of the G' and G'' curves for aggrecan are clearly different from those for CS and HA. In the latter system, the curves are typical for associated polymers in solution in which the moduli showing greater slopes in the terminal regime (below the crossover frequency) compared to their slopes above the crossover.[25] In contrast, aggrecan exhibits a power-law behavior for G' and G'' with approximately the same slope (approximately 0.8 at low ω) below and above the crossover frequency. Also, the curves for different aggrecan concentrations have different shapes and cannot be superposed, unlike the cases of CS and HA. In general, in solutions of linear polymers, the crossover frequency ω_c is shifted to lower frequencies with increasing polymer concentration.[25]

The approximate power-law scaling in the rheology of aggrecan is a signature of an incipient "weak gel", as described in the Introduction. Indeed, it has been proposed in the literature that in aggrecan solutions, free bottlebrushes coexist with microgel-like assemblies.[7,27] The term microgel in this context refers to microparticles or microdomains containing aggrecan chain clusters with a significant amount of water and ions associated with the chains, forming polymer-hydration−ion complexes. The driving force for microgel formation is expected to be the difference in water affinity between the N-terminal domain of the protein core in the aggrecan bottlebrush and the highly charged hydrophilic polysaccharide side chains. The weak-gel rheology may then correspond to the networking of aggrecan microgels by free aggrecan bottlebrushes.

The distinct response of aggrecan is also reflected in steady-shear rheology (Figure 6b,c). Here, we note a shear-thinning response at all polymer concentrations. That is, in all cases, there is no plateau in the viscosity η at low $\dot{\gamma}$; on the contrary, η does show a plateau at high $\dot{\gamma}$. Once again, it is worth contrasting the behavior of aggrecan with the more typical behavior seen for HA (Figure 5), which shows a plateau in η at low $\dot{\gamma}$ and then shear thinning at higher $\dot{\gamma}$. The lack of a low-shear plateau in viscosity for aggrecan again suggests a weak-gel-like microstructure. Other features in Figure 6b,c are consistent with those seen for CS and HA: the viscosity increases with aggrecan concentration and decreases as salt is added.

Aggrecan−HA Complexes. In cartilage, aggrecan monomers complex with a linear HA chain and form a secondary bottlebrush (see Scheme 1). This complexation process is known to be quite slow, taking 48 h or more to complete. We attempted to follow the rheology of aggrecan−HA mixtures

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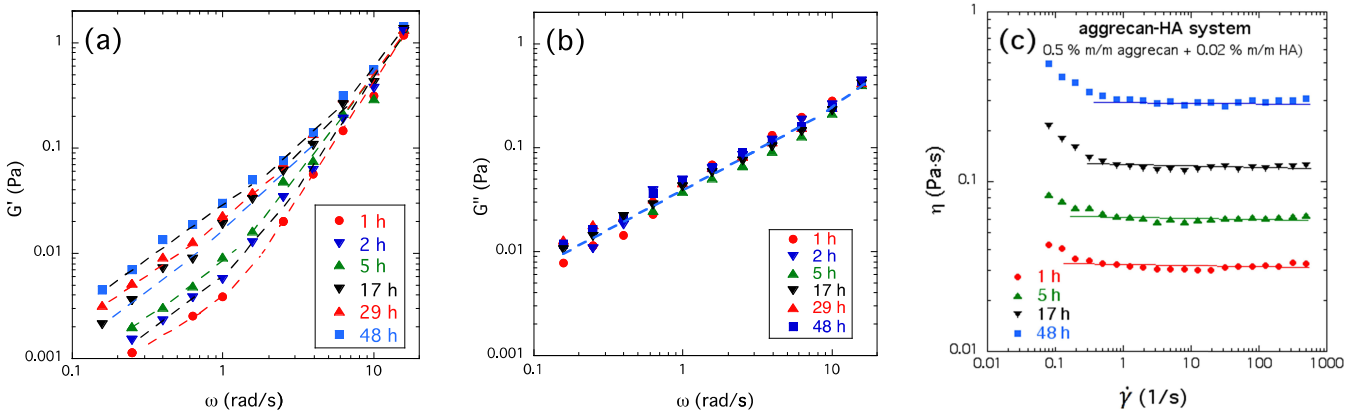

Figure 7. Changes in dynamic rheology during the complexation of aggrecan with HA. For this experiment, 0.5% m/m aggrecan was mixed with 0.02% m/m HA at $t = 0$. Plots of G' (a) and G'' (b) vs frequency ω are shown at different stages of complex formation. (c) Data from steady-shear rheology for the viscosity η vs shear rate $\dot{\gamma}$ for aggrecan−HA solutions at different times.

during the course of the complexation process. At time $t = 0$, we mixed 0.5% m/m aggrecan with 0.02% m/m HA; thus, the mass ratio of aggrecan to HA was 25:1. Thereafter, we probed the dynamic rheology of the system at various time points between $t = 1$ and 48 h. Data for G' vs ω at different times is presented in Figure 7a and similar data for G'' in Figure 7b. The rheology at the early stages ($t = 1$ h) reflects a complex influence of the components present (i.e., aggrecan and HA). At this stage, G'' significantly exceeds G' at the lowest ω probed. As time progresses, there is a sharp increase in G' at low ω (Figure 7a) whereas there is hardly any change in G''. After 48 h, G' and G'' have comparable magnitudes over the entire range of ω. Such an increase in G' alone is akin to that observed when polymers in solution undergo cross-linking into a volume-filling branched polymer network. The increase in G' reflects a transition to a more elastic system; also, the pseudo-plateau in G' at low ω means that the network can store an applied deformation without relaxing. We interpret this to mean that, when aggrecan molecules gradually attach to the HA scaffold, the resulting complexes are then able to further connect with each other across the sample volume. This may well lead to a permanent volume-filling network after long-aging time.

The steady-shear rheology of the aggrecan−HA solution is consistent with the rheological characteristics of a fully developed weak-gel; the data show that in the course of complexation the connections between the microgel-like aggrecan−HA aggregates are progressively enhanced. In both aggrecan and aggrecan−HA solutions, shear thinning is observed at low shear rates. The disruption of the aggregates at high shear rates provides a natural explanation of the largest contribution to this shear thinning. In particular, as the shear rate exceeds the inverse lifetime of the chain complexes, the shear viscosity of the solution decreases indicating that the polymer complexes are progressively broken down under flow.[14]

In certain biomedical applications of the polymers considered in the present work such as vehicles for gene therapy, nanoparticle drug delivery, tissue engineering, and as a material for boosting the viscoelastic properties of the synovial fluid of the joints of patients with arthritis, the molecules are "hydrophobically modified" by grafting alkanes and other relatively hydrophobic side groups (e.g., proteins) to these polymers. Kandadai et al.[30] investigated the rheology of HA modified by isotactic poly(L-leucine) and found that its behavior indicated a weak-gel rheological response. This effect was interpreted in terms of the supramolecular polymer assembly of the hydrophobically modified chains, similarly to our interpretation of the rheological properties of aggrecan−HA solutions.

As a final point, we mention the gel formation of "vitramers",[42] where the molecules exhibit strong associative interactions that similarly lead to complex formation as in aggrecan and HA and hydrophobically modified HA. High-precision rheological measurements on this vitramer material show the existence of weak-gel viscoelasticity over a wide temperature range and we expect that this same pattern of viscoelasticity to be prevalent in diverse biological hydrogels. Aggrecan-containing systems should be studied over a wide range of temperature, added salt, polymer concentrations, etc., to quantify the transition from a viscoelastic polymer solution to an elastic gel state.

CONCLUSIONS

This study probed the rheology of CS, HA, aggrecan, and aggrecan−HA complexes in solutions over a broad range of polymer and salt concentrations. CS and HA solutions exhibited rheology characteristics of viscoelastic polymer solutions. CS solutions were viscous approximately Maxwellian liquids, while HA solutions (due to the higher molecular mass of HA) displayed viscoelasticity and shear thinning consistent with a strongly associating fluid. The addition of monovalent (NaCl) and divalent ($CaCl_2$) salts caused a modest reduction in the low-shear viscosity of CS and HA solutions, but we observed no signs of either salt-induced phase separation or Ca^{+2} "salt-bridge" formation. Aggrecan, on the other hand, exhibited a different rheology that may be described as an incipient weak gel possibly due to the presence of supra-molecular assemblies (microgel particles) of aggrecan. When aggrecan was combined with HA, the rheological properties of the aggrecan-HA complex solution slowly evolved and became more elastic over a 48 h period, i.e., weak gel rheology fully developed after a rather long time. These findings suggest that aggrecan molecules gradually form complexes with the HA backbone and, in turn, a large-scale network of associated polymers is formed in solution, possibly with free aggrecan chains coexisting with this dynamic network.

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■ ASSOCIATED CONTENT

Supporting Information

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Storage (G') and loss modulus (G'') data for hyaluronic acid solutions at different concentrations at 25 °C (PDF)

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Notes

The authors declare no competing financial interest.

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SUPPORTING INFORMATION

Rheological Properties of Cartilage Glycosaminoglycans and Proteoglycans

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Storage (G') and loss modulus (G") data for hyaluronic acid solutions at different concentrations at 25 °C

1 % (m/m) Hyaluronic acid solution

ω (rad/s)	G' (Pa)	G'' (Pa)
499.90	155.81	153.94
315.40	113.37	124.73
199.00	70.865	95.573
125.60	43.027	71.712
79.240	29.938	51.797
50.010	19.634	36.979
31.550	10.436	25.373
19.910	5.2787	16.978
12.560	2.5498	11.156
7.9220	1.2779	7.2346
5.0010	0.58890	4.6526
3.1550	0.26042	2.9736
1.9910	0.12359	1.9037
1.2560	0.058788	1.2108
0.79280	0.030252	0.76939
0.50000	0.015120	0.48816
0.31550	0.0098280	0.31046
0.19910	0.0058320	0.19771
0.12560	0.0030480	0.12673
0.079270	0.0014160	0.081144
0.050010	0.0011040	0.051144

2 % (m/m) Hyaluronic acid solution

ω (rad/s)	G' (Pa)	G'' (Pa)
499.90	257.60	181.00
315.40	187.00	160.60
199.00	136.50	137.20
125.60	96.950	114.60
79.240	66.600	92.840
50.010	44.070	73.220
31.550	27.830	55.570
19.910	16.860	41.050
12.560	9.6430	29.030
7.9220	5.3560	20.330
5.0010	2.8180	13.720
3.1550	1.4350	9.1100
1.9910	0.72570	6.0120
1.2560	0.35280	3.8680
0.79280	0.17220	2.4490
0.50000	0.091400	1.5810
0.31550	0.052610	1.0220
0.19910	0.031640	0.64620
0.12560	0.020610	0.42470
0.079270	0.013210	0.27190
0.050010	0.0091200	0.17220

4 % (m/m) Hyaluronic acid solution

ω (rad/s)	G' (Pa)	G'' (Pa)
499.90	507.14	368.37
315.40	376.07	323.84
199.00	276.01	275.14
125.60	196.15	229.47
79.240	168.46	186.21
50.010	110.57	145.10
31.550	78.877	110.08
19.910	47.423	80.935
12.560	30.263	57.418
7.9220	19.036	39.506
5.0010	9.9099	26.565
3.1550	4.9683	17.533
1.9910	2.4081	11.370
1.2560	1.1631	7.3383
0.79280	0.57233	4.7172
0.50000	0.30492	3.0360
0.31550	0.17635	1.9494
0.19910	0.092190	1.2508
0.12560	0.049920	0.79872
0.079270	0.025130	0.51574
0.050010	0.016280	0.33839

6 % (m/m) Hyaluronic acid solution

ω (rad/s)	G' (Pa)	G'' (Pa)
499.90	686.90	427.70
315.40	545.30	392.00
199.00	428.50	349.70
125.60	327.70	305.80
79.240	243.40	261.20
50.010	174.40	216.50
31.550	120.40	174.10
19.910	79.890	135.60
12.560	51.000	102.50
7.9220	31.320	75.150
5.0010	18.540	53.650
3.1550	10.570	37.330
1.9910	5.8220	25.380
1.2560	3.1390	17.000
0.79280	1.6570	11.230
0.50000	0.86080	7.3310
0.31550	0.44290	4.7390
0.19910	0.22910	3.0390
0.12560	0.12250	1.9380
0.079270	0.070290	1.2240
0.050010	0.044730	0.77120

8 % (m/m) Hyaluronic acid solution

ω (rad/s)	G' (Pa)	G'' (Pa)
499.90	924.54	527.00
315.40	748.27	485.36
199.00	597.18	446.18
125.60	465.18	399.00
79.240	351.09	346.91
50.010	255.91	292.91
31.550	179.54	239.54
19.910	121.09	189.73
12.560	78.491	145.64
7.9220	48.927	108.46
5.0010	29.318	78.427
3.1550	16.918	55.255
1.9910	9.4270	38.009
1.2560	5.1000	25.664
0.79280	2.6880	17.073
0.50000	1.3850	11.200
0.31550	0.70700	7.3020
0.19910	0.36200	4.7420
0.12560	0.19000	3.0740
0.079270	0.10500	1.9800
0.050010	0.064000	1.2680

10 % (m/m) Hyaluronic acid solution

ω (rad/s)	G' (Pa)	G'' (Pa)
499.90	1185.0	706.20
315.40	958.00	645.30
199.00	763.30	586.00
125.60	590.60	521.00
79.240	442.60	450.80
50.010	320.40	378.90
31.550	223.10	308.50
19.910	149.20	243.00
12.560	95.750	185.30
7.9220	58.970	137.00
5.0010	34.980	98.470
3.1550	19.940	68.920
1.9910	11.140	47.430
1.2560	5.9630	31.870
0.79280	3.1150	21.050
0.50000	1.5980	13.720
0.31550	0.81880	8.8910
0.19910	0.42570	5.7330
0.12560	0.22900	3.6880
0.079270	0.12990	2.3780
0.050010	0.080120	1.5430