Rheology of Sodium Hyaluronate under Physiological Conditions

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Sodium hyaluronate NaHA in phosphate-buffered saline behaves as a typical polyelectrolyte in the high-salt limit, as Newtonian viscosities are observed over a wide range of shear rates. There is no evidence of intermolecule hydrogen bonding causing gel formation in NaHA solutions without protein present. The concentration dependences of viscosity, relaxation time, and terminal modulus are consistent with observations on flexible, neutral polymers in good solvents, which are known to be in the same universality class as flexible polyelectrolytes in the presence of excess salt.

I. Introduction

Sodium hyaluronate (NaHA) is the sodium salt of hyaluronic acid (HA or hyaluronan, see Figure 1). HA is a high molecular weight biopolysaccharide (for a review see ref 1 and the references therein) discovered by Meyer and Palmer in 1934 in the vitreous humor of cattle eyes.[2] HA consists of repeating disaccharide units composed of *N*-acetyl-D-glucosamine and D-glucuronic acid linked by a β 1−4 glucosidic bond while the disaccharides are linked by β 1−3 bonds. HA is produced by almost all members of the animal kingdom as well as by streptococci.[1] HA is a simple and lightly charged member of the glycosaminoglycans, which also include chondroitin sulfate, dermatan sulfate, keratan sulfate, heparan sulfate, and heparin.

HA occurs not only in the vitreous humor but also in many living substrata such as the brain, the extracellular matrix, and synovial fluids.[1] HA's important role in both the vitreous humor and synovial fluid has led to basic research on the conformation, interactions, and rheology of HA in solution. Rheology of synovial fluid plays a vital role in the lubrication properties that protect joint tissue from damage during joint motion. Although interactions with proteins alter the rheology of synovial fluid,[3] in this paper the simpler rheology of pure NaHA in phosphate-buffered saline is discussed. Future publications from our group will explore the interactions between NaHA and the plasma proteins albumin and γ-globulins.

Despite the simple, well-defined structure of HA and over 60 years of research on the properties of HA solutions, the conformation of HA in solution is still controversial. This controversy was recently summarized by De Smedt et al.[4] Scott and co-workers, as well as other researchers (see refs 5−12), have studied the conformation of HA in solution via nuclear magnetic resonance (NMR), space-filling molecular models, and computer simulations.[1,13−21] The conformation

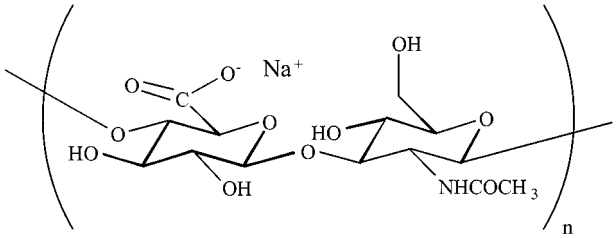

Figure 1. Structure of sodium hyaluronate.

of HA in solution was proposed by Scott and co-workers as an ordered structure incorporating up to five hydrogen bonds per disaccharide unit. A 2-fold, "tape-like" single helix was proposed. Scott and co-workers suggest that the two sides of the HA "tape" are identical but antiparallel. On alternate sides of the single HA helices, hydrophobic patches of eight or nine CH units stretching along three neighboring sugar units are proposed. Scott and co-workers suggest this feature helps explain HA's ability to interact with lipids and membranes and suggest that it might interact with itself in water. The hydrophobic patches were postulated to promote network formation and lateral aggregation. In Scott and Heatley's recent paper, they reaffirm their hypothesis that there is "a series of overlapping interactions in which each HA molecule associates with and binds to antiparallel molecules ahead and behind. Each HA molecule thus links to other HA molecules on either side of the HA ambidexteran by secondary valencies".[13] Scott and Heatley go on to point out that this structure is formally equivalent to a β-sheet.[13]

Scott and co-workers' hypothesis suggests that HA solutions are in effect reversible gels with a temporary network structure. Rheology is a powerful tool in determining whether such a reversible gel exists. The rheology of weak reversible gels shows pronounced viscoelasticity and is strongly dependent upon prior shear history. Neither characteristic has been observed in our laboratory or discussed in the literature for NaHA solutions. A number of rheological studies of NaHA solutions have been presented in the literature[22−40] (for reviews see refs 1 and 4). These studies are often

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inconsistent and are difficult to compare when different sources of HA are used. The rheology of HA solutions is extremely sensitive to protein contamination. This study and those by Milas and co-workers[22−25] have used similar molecular weight NaHA from bacterial sources to minimize protein-mediated aggregation of dissolved NaHA chains, which might occur if NaHA from an animal source were used. This study confirms Milas and co-workers' viscosity results, thereby demonstrating that NaHA from bacterial sources provide consistent information. Protein-free solutions of NaHA from bacterial sources in phosphate-buffered saline are viscoelastic liquids with a concentration-dependent viscosity typical of polyelectrolytes with excess salt. Additionally, the relaxation time and terminal modulus were determined, and the results were compared with the expectations of simple theories.

While our viscosity data agree nicely with those of Milas et al.,[22−25] we do not agree with their interpretation. Polyelectrolytes with excess salt are analogous to uncharged polymers in good solvent, for which the concentration dependence of viscosity is understood quite well. In dilute solution, where the coils do not overlap each other, the Huggins equation describes viscosity.[41]

$$\eta_{sp}(c) \equiv \frac{\eta(c) - \eta_s}{\eta_s} = [\eta]c + k_H([\eta]c)^2 + ... \qquad (1)$$

The polymer contribution to viscosity is given by the specific viscosity η_{sp}, η is the measured viscosity, η_s is the solvent viscosity, $[\eta]$ is the intrinsic viscosity, c is the polymer concentration, and k_H is the Huggins coefficient. The Huggins equation only applies in dilute solution, where the viscosity of the solvent is only slightly altered by the presence of the polymer. The intrinsic viscosity is the initial slope of the concentration dependence of specific viscosity and is related to the root-mean-square end-to-end distance R of the linear polymer chain of N monomers through the Fox−Flory relation.[42]

$$[\eta] \cong R^3/N \qquad (2)$$

The overlap concentration c^* is the border between the dilute and semidilute concentration ranges and is the highest concentration for which the Huggins equation should be applied. The overlap concentration is determined as the point where the concentration inside a single coil equals the solution concentration.

$$c^* \cong \frac{N}{R^3} \cong \frac{1}{[\eta]} \qquad (3)$$

These considerations suggest two criteria for proper choice of the overlap concentration: $c^*[\eta] \cong 1$ and $\eta_{sp}(c^*) \cong 1$. In this paper we find quite typical values of $c^*[\eta] = 1.5$ and $\eta_{sp}(c^*) = 2.0$, whereas Milas et al. have chosen a significantly larger overlap concentration, resulting in unreasonable values of $c^*[\eta] = 6$ and $\eta_{sp}(c^*) = 30$.

II. Experimental

A. Materials. The sodium hyaluronate (NaHA) was obtained from a microbial preparation in order to minimize any protein contamination and was purchased from Genzyme Pharmaceuticals (product number 998476, batch number B207997). It had a reported molecular weight of 1.6×10^6 and a protein content of <0.1%. The reported molecular weight was verified as 1.5×10^6 by determining the intrinsic viscosity $[\eta]$ (2.49 mL/mg) and using the Mark−Houwink relation ($[\eta] = KM^a$) where $K = 0.0336$ mL/g and $a = 0.79$ at 25 °C in 0.1 M NaCl (aq).[25] All solutions were studied in a phosphate-buffered saline mix, purchased from Sigma (pH 7.4, 0.138 M NaCl, 0.0027 M KCl).

B. Rheological Measurements. A computerized Contraves Low Shear 30 viscometer (LS 30) was used to measure the apparent viscosity of the lower viscosity samples ($\eta <$ 200 cP) at low shear rates in the range $0.02 \le \dot{\gamma} \le 100$ s^{-1}. A stainless steel concentric cylinder fixture of outer diameter 12.0 mm and inner diameter 11.1 mm was used under steady shear. The sample temperature was maintained at 25.0 ± 0.2 °C by adding a temperature-controlled water bath surrounding the sample cell in addition to the standard circulating water within the cup assembly.[43]

A Rheometrics SR-2000 stress-controlled rheometer (SR-2000) with a cone and plate geometry (40.0 mm diameter plastic cone with a 0.0397 rad cone angle and a stainless steel plate) was used with steady and oscillatory stress. Viscosity measured as a function of steady stress extended by over a decade the shear rate range accessed by the LS 30.

Below 20−200 cP depending on molecular weight, the SR-2000 cone and plate viscosity data are systematically lower than the LS 30 concentric cylinder viscosity data by 10−25%. The same shift has been noted in the previous work on polyelectrolyte rheology.[44,45] We have shifted the SR-2000 data on the viscosity scale to match the LS 30 data. This mild geometry effect is not yet fully understood and occurs in aqueous polyelectrolyte solutions in both the high- and low-salt limits, and its magnitude is affected by the material used to make the rheometer fixtures. This geometry effect is tentatively attributed to wetting, since the effect is also seen for deionized, distilled water but not seen for silicone oil based viscosity standards.

III. Results and Discussion

For each solution of NaHA, a plot of the shear rate dependence of apparent viscosity was analyzed, as shown in Figure 2. The plots contain data from both the Contraves LS 30 and the Rheometrics SR-2000 if the zero-shear rate viscosity η_0 is in the range of 60 cP < η_0 < 200 cP. Below 60 cP only the LS 30 was used, and above 200 cP only the SR-2000 was used. The solution containing 3 mg/mL (7.5×10^{-3} M) NaHA is highlighted in Figure 2 because this is the relevant concentration found in human synovial fluid.

The zero-shear rate viscosity, η_0, and the relaxation time from steady shear τ were determined from the plots in Figure 2 using established procedures.[44,45] The high shear rate apparent viscosity data were fit to a power law in shear rate, and extrapolation of this power law to η_0 gives our definition of the relaxation time τ as the reciprocal of this crossover shear rate. These solutions are in the "high-salt limit" where

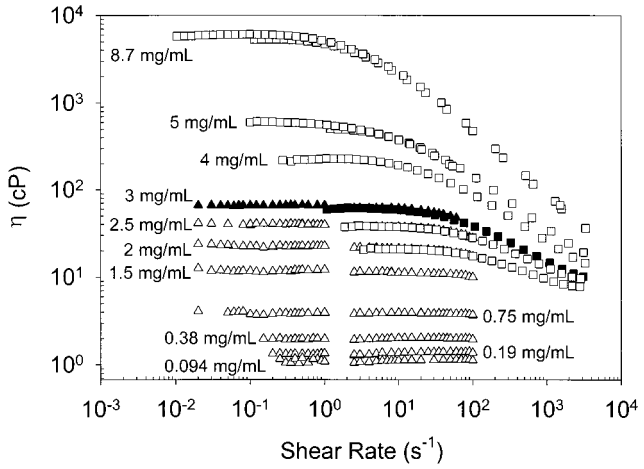

Figure 2. Shear rate dependence of apparent viscosity of NaHA in phosphate-buffered saline at 25 °C. The triangles are Contraves LS 30 data and the squares are Rheometrics SR-2000 data.

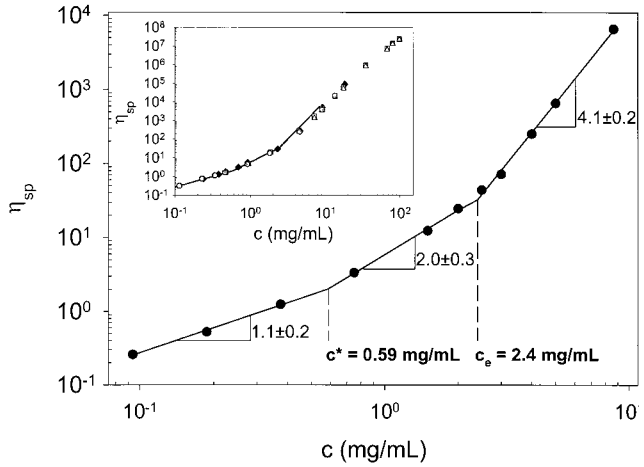

Figure 3. Concentration dependence of the specific viscosity of NaHA in phosphate-buffered saline at 25 °C. Inset: Literature data (open squares, $M_w = 1.5 \times 10^6$ at 20 °C;[22] open triangles, $M_w = 1.5 \times 10^6$ at 30 °C;[22] filled diamonds, $M_w = 1.435 \times 10^6$ at 25 °C;[23] shaded circles, $M_w = 1.35 \times 10^6$ at 25 °C,[24] all in 0.1 M NaCl; solid lines, this work).

there are more salt ions present than counterions ($fc \ll 2c_s$, where c is the monomer concentration, c_s is the salt concentration, and f is the fraction of monomers bearing effective charge). The scaling predictions for the specific viscosity η_{sp} and relaxation time τ for polyelectrolytes in the high-salt limit are shown below for both semidilute entangled and unentangled solution.[46] These predictions are identical with the scaling predictions for uncharged polymers in good solvent[47] because the electrostatic interactions are thought to be analogous to excluded volume.[48]

$$\eta_{sp} \sim \begin{cases} c^{5/4} & \text{semidilute unentangled} \\ c^{15/4} & \text{semidilute entangled} \end{cases} \quad (4)$$

$$\tau \sim \begin{cases} c^{1/4} & \text{semidilute unentangled} \\ c^{3/2} & \text{semidilute entangled} \end{cases} \quad (5)$$

The specific viscosity ($\eta_{sp} = (\eta_0 - \eta_s)/\eta_s$, where η_s is the solvent viscosity) data are plotted in Figure 3. Our data span 2 decades in concentration and crossovers from the dilute to the semidilute unentangled to the semidilute entangled regime are observed as changes in slope. In dilute solution, viscosity should be proportional to concentration ($\eta_{sp} \sim c$) for polyelectrolytes in the high salt limit (as well as neutral polymers in good solvent).[46] This study found $\eta_{sp} \sim c^{1.1\pm0.2}$ in dilute solution, in excellent agreement with the scaling prediction.

The overlap concentration was determined to be $c^* = 0.59$ mg/mL, and the entanglement concentration c_e which characterizes the end of the semidilute unentangled regime and the beginning of the entangled regime was determined to be $c_e = 2.4$ mg/mL, as shown in Figure 3. The overlap concentration, c^*, can be confirmed by determining whether the overlap criterion ($c^*[\eta]$) is of order unity. We find $c^*[\eta] = 1.5$, where $[\eta] = 2.49$ mL/mg. Also, the viscosity at c^* should be roughly twice the solvent viscosity; we found $\eta_{sp} = 2.0$ at c^*. We determined the Huggins coefficient $k_H = 0.34$, typical for nonassociating neutral polymers in good solvent.

In semidilute unentangled solution, we find $\eta_{sp} \sim c^{2.0\pm0.3}$. This concentration dependence is much stronger than predicted ($\eta_{sp} \sim c^{5/4}$).[46] In semidilute entangled solution, this study found $\eta_{sp} \sim c^{4.1\pm0.2}$. The exponent is similar to those found for neutral polymers,[49−51] although it is slightly higher than the reptation prediction ($\eta_{sp} \sim c^{15/4}$) for semidilute entangled solutions of polyelectrolytes in the high salt limit[46] and semidilute entangled solutions of neutral polymers in good solvent.[47,52]

These data are in excellent agreement with the work done by Milas and co-workers,[22−25] whose data are shown in the inset of Figure 3. In semidilute entangled solution, Milas et al. found $\eta_{sp} \sim c^{4.0\pm0.2}$.[24] Milas et al. analyze their data from the dilute solution regime and semidilute unentangled solution regime as one. They find $\eta_{sp} \sim c^{1.20\pm0.5}$,[24] which is also in agreement with this study ($\eta_{sp} \sim c^{1.2}$) if the data below 2 mg/mL are analyzed in the same manner. Milas et al. interpreted the largest change in slope in Figure 3 at $c_e = 2.4$ mg/mL to be the overlap concentration even though they recognized that the viscosity is ca. 30 times larger than the solvent viscosity.[24] On the basis of previous studies of polyelectrolyte solutions in the high salt limit, this critical concentration clearly corresponds to the onset of entanglement effects.[44,46]

For the most concentrated solutions, relaxation times τ were determined from the onset of shear thinning and are plotted in Figure 4, along with literature data on NaHA in similar salt conditions and varying chain lengths. The chain length variability is accounted for nicely by plotting τ/M^3 against concentration, as expected from reptation scaling. However, with the very limited range of chain lengths for which the shear thinning onset is reported, $\tau/M^{3.4}$ would reduce the data as well. We find $\tau \sim c^{2.0\pm0.3}$ for all data with $c > c_e$, which has a stronger concentration dependence than the semidilute entangled prediction $\tau \sim c^{3/2}$.[46] This disagreement between theory and experiment is not entirely unexpected, because in the low salt-limit the theory also fails in the entangled regime.[44,45] Also uncharged polymers in good solvent, which theory expects to have the same concentration dependences as polyelectrolytes with excess salt in entangled

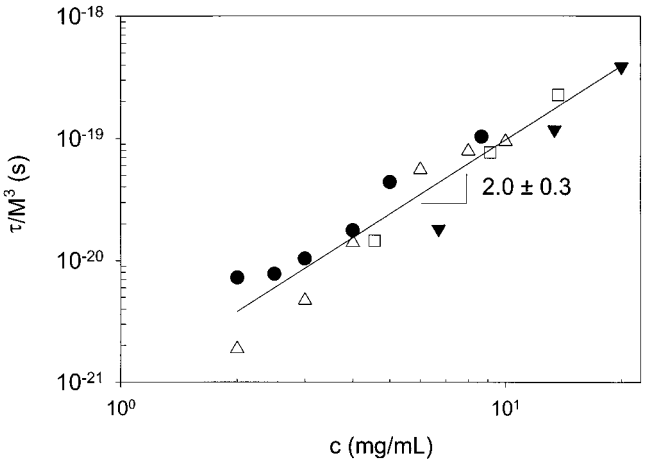

Figure 4. Concentration dependence of the longest relaxation time of various entangled NaHA chains at 25 °C in roughly 0.1 M salt, scaled for chain length differences using reptation theory. The filled circles are our data for $M_w = 1.5 \times 10^6$ in phosphate-buffered saline. Literature data (open triangles, $M_w = 2.2 \times 10^6$; open squares, $M_w = 1.35 \times 10^6$; filled inverted triangles, $M_w = 1.0 \times 10^6$),[24] all in 0.1 M NaCl.

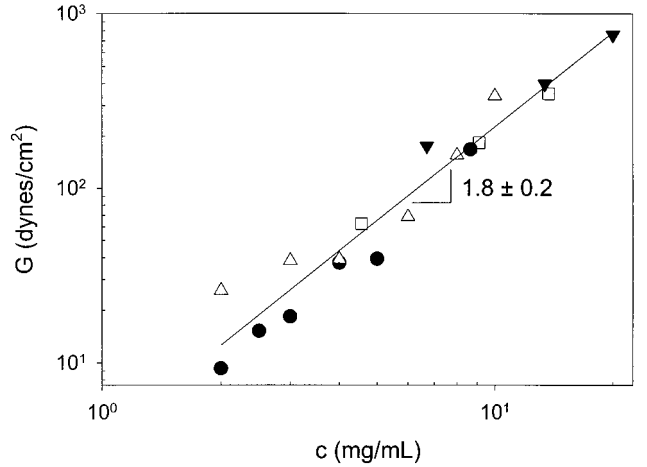

Figure 5. Concentration dependence of the modulus at the longest relaxation time for entangled NaHA chains at 25 °C in roughly 0.1 M salt. Symbols are the same as those given in Figure 4.

solution, have slightly larger exponents than those predicted by reptation.[49,51]

Figure 5 shows the terminal modulus as a function of concentration, for our data and literature data. The terminal modulus was calculated from the ratio of zero-shear-rate viscosity and the longest relaxation time of the chain ($G \approx \eta/\tau$). In semidilute entangled solution, the modulus is predicted to scale as $c^{9/4}$ for polyelectrolytes in the high-salt limit[46] (as well as neutral polymers in good solvent).[47,52] We find $G \sim c^{1.8\pm0.2}$ for NaHA in phosphate-buffered saline. While scaling predictions underestimate the concentration dependence of both viscosity and relaxation time, the prediction of $G \sim c^{9/4}$ somewhat overestimates the experimentally observed concentration dependence.

IV. Conclusions

The rheology of NaHA in phosphate-buffered saline is typical of flexible polyelectrolytes in the high-salt limit. There is no evidence that it forms a reversible gel in solution, as Newtonian viscosities are observed over a wide range of shear rates and all rheology results are independent of shear history. These results, combined with the weak temperature dependence of viscosity previously reported for NaHA solutions,[22] prove there are no strong associations between NaHA chains under physiological conditions. The reversible gel nature of synovial fluid is thus believed to be caused by interactions between NaHA and proteins.

The concentration dependences of viscosity, relaxation time, and terminal modulus are quite consistent with observations on neutral polymers in good solvents, as predicted by scaling models for polyelectrolytes with excess salt.[46,48] The results from this study are in excellent agreement with Milas and co-workers.[22−25] The fact that these measurements agree from different labs with different bacterial sources of NaHA suggests that the inconsistencies in the rheology of NaHA reported in the literature are most likely due to different levels of protein contamination. Future papers from our group will address the interactions between NaHA and the plasma proteins, albumin and γ-globulins, and their effect on rheology and osmotic pressure of synovial fluid.

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