

# Hierarchy and Organization of Hyaluronic Acid in Physiological Salt Solutions

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## INTRODUCTION

Hyaluronic acid (HA) is a high molecular weight anionic polysaccharide that is widely distributed throughout the body.<sup>1</sup> HA is a primary constituent of the extracellular matrix and participates in various cell-to-cell interactions. Among its many functions, HA plays a critical role in cartilage, where large HA-aggregates that provide the resistance to compressive load enmesh the collagen network. In the vitreous humor of the eye a transparent HA gel fills the space between the lens and the retina. HA is also a major component of the synovial fluid in diarthrodial joints and, owing to its remarkable viscoelastic properties, is believed to contribute to the biolubrication of articular cartilage.<sup>2,3</sup>

Although numerous biophysical studies have been made on HA solutions, these investigations do not address the relation between structure and interactions that govern the thermodynamic properties at physiologically relevant ion concentrations.<sup>4-7</sup>

The aim of this study is to explore the structure and dynamic behavior of nearly physiological HA solutions at length scales intermediate to the macroscopic and the short-range atomic structure of the molecule, i.e., between 1  $\mu\text{m}$  and 1 nm. To investigate structures in this spatial range, small angle neutron scattering (SANS), and dynamic light scattering (DLS) are ideally suited.

## EXPERIMENTAL

**Sample Preparation.** Solutions of sodium hyaluronate (HA, Sigma  $M_w = 1.2 \cdot 10^6$ ) were prepared in  $\text{D}_2\text{O}$  containing 100 mM NaCl. The concentration of the HA was varied in the range 1 – 3% w/w. The sodium chloride concentration (= 100 mM) and pH (= 7) were identical in all samples.

**Small Angle Neutron Scattering Measurements.** SANS measurements were made on the NG3 instrument at NIST, Gaithersburg, MD. Solutions were placed in sample cells with quartz windows and 2 mm path length. The measurements were performed at three sample-detector distances, 1.35 m, 4 m and 13.1 m, using an incident wavelength of 8 Å. This configuration allowed us to cover the transfer wave vector range  $2.8 \cdot 10^{-3} \text{ \AA}^{-1} < q < 0.35 \text{ \AA}^{-1}$ . After azimuthal averaging, corrections for incoherent background, detector response and cell window scattering were applied.<sup>8</sup>

**Light Scattering.** DLS measurements were made with an ALV DLS/SLS 5022F goniometer equipped with fiber optic coupling and an avalanche diode, combined with a 22 mW HeNe laser and an ALV 5000E multi-tau correlator. Measurements were carried out in the angular range  $30^\circ$  to  $150^\circ$  with accumulation times of 200 s. To avoid shear degradation of the high molecular weight HA molecule, the solutions were measured without filtration.

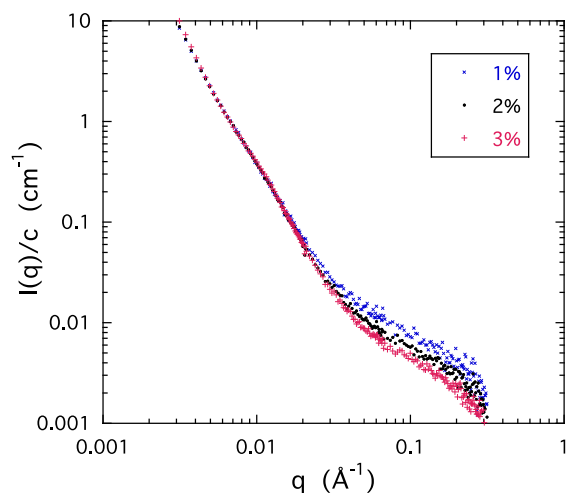
All measurements were made at  $25 \pm 0.1^\circ\text{C}$ .

## RESULTS AND DISCUSSION

In solutions containing extended objects such as macromolecular assemblies, scattering measurements provide information both on the size and dynamic properties of the assemblies.

**Small Angle Neutron Scattering of HA Solutions.** Figure 1 shows the normalized SANS intensity  $I(q)/c$  from HA solutions at three

different concentrations,  $c = 1, 2$  and 3% w/w. Three distinct regions can be identified. First, for  $q < 0.006 \text{ \AA}^{-1}$  power-law behavior with a slope slightly greater than -3 is observed, corresponding to surface scattering. We attribute the cause of this scattering to loose domains of size exceeding several hundred nanometers similar to those observed in previous studies on other polyelectrolyte solutions.<sup>9</sup> In many cases scattering from these features can be expressed as a power law of the form  $I_{ag}(q) = Aq^{-m}$  where  $A$  is a constant and the exponent  $m$  is defined by  $3 \leq m < 4$  for surface scattering and  $m < 3$  for volume fractal behavior. In the present system the surfaces are so rough ( $D_s = 6 - m$ , where  $D_s$  is the surface fractal dimension) that they are at the limit of being volume fractals. Second, at  $q \approx 0.008 \text{ \AA}^{-1}$ , a deviation from the power law behavior in the form of a weak shoulder can be distinguished. This feature maybe related to the well-known “polyelectrolyte peak” observed in polyelectrolyte solutions at low salt concentration, although the scale is much larger in the present system where the salt concentration is relatively high. Third, in the upper  $q$ -range of Figure 1 the slope of the  $I(q)$  plots is approximately -1, which is the signature of rod-like elements, and is consistent with the stiff structure of the HA chain. The high- $q$  end of the curves is terminated by a downturn. A Guinier plot of this part of the spectrum yields for the chain cross-sectional radius approximately 0.25 nm, corresponding to a single HA strand.



**Figure 1.** SANS spectra of HA solutions, normalized by the concentration  $c$ , at three different concentrations, 1, 2 and 3 % w/w in 100 mM NaCl solutions with  $\text{D}_2\text{O}$ .

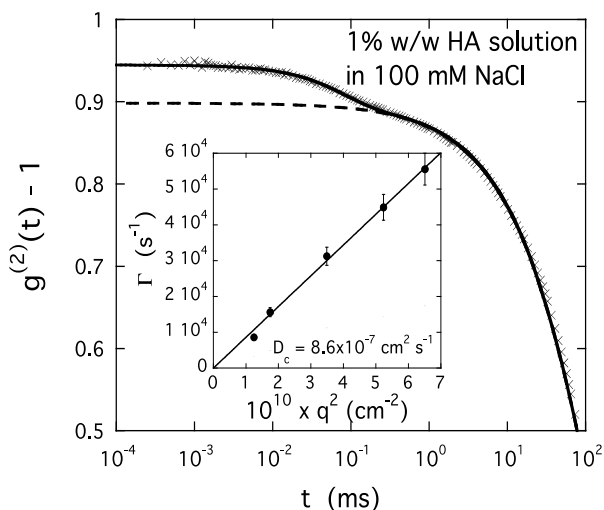
In the low- $q$  region of Figure 1 the data points fall on the same curve, i.e., the intensity is proportional to the polymer concentration. This indicates that the large clusters are distinct and approximately non-interacting and also that their structure is independent of the HA concentration. However, the concentration dependence of the intensity in the high  $q$ -region ( $q > 0.03 \text{ \AA}^{-1}$ ) is different. In this region the scattering intensity is primarily governed by the osmotic concentration fluctuations and is thus sensitive to the polymer concentration.  $I(q)$  exhibits a weaker than linear dependence on concentration, varying approximately as  $c^{0.5}$ . This behavior is different from the thermodynamic response of semi-dilute solutions of flexible polymers. In polymer solutions in general, the scattering intensity in this region is governed by the thermal fluctuations associated with the macroscopic osmotic pressure. The corresponding scattering intensity,  $I_{os}(q)$ , depends on the concentration and the structure according to:

$$I_{os}(q) \propto c^{\frac{3-2D_f}{3-D_f}} \quad (1)$$

$$\Gamma = D_c q^2 = \frac{k_B T}{6\pi\eta\xi_H} q^2 \quad (3)$$

where the fractal dimension  $D_f = 5/3$  applies in excluded volume conditions.<sup>10</sup> In that situation,  $I_{os}(q)$  is expected to decrease with concentration as  $c^{0.25}$ . For rigid rods  $D_f = 1$ , and hence  $I_{os}(q)$  should vary as  $c^{0.5}$ , which is consistent with the present observation. This exponent is also consistent with the theoretical expectation for stiff polymer molecules.<sup>11</sup> We conclude that the scattering from the HA solutions in the high  $q$ -region is due to thermodynamic concentration fluctuations involving rod-like chain segments.

**Dynamic Light Scattering of HA Solutions.** In Figure 2 is shown the DLS correlation function for a 1% HA solution measured at scattering angle  $120^\circ$ .



**Figure 2.** Intensity correlation function  $g^{(2)}(t)-1$  for a 1% w/w HA solution in 100 mM NaCl measured at  $\theta = 120^\circ$ . The continuous line shows the fast component and the dashed line the slow component. Inset: variation of the relaxation rate  $\Gamma$  of the fast mode as a function of  $q^2$ .

Two distinct relaxation processes are distinguishable in the figure. The first process, which appears around 0.1 ms, is due to the relaxation of the HA molecules and exhibits approximately a simple exponential decay. The slower process, which is more complex as it contains a broad range of decay times, corresponds to the translational motion of large polydisperse clusters through the viscous medium. It can be described by a stretched exponential decay. The normalized intensity correlation function is thus

$$g^{(2)}(\square) - 1 = \beta \{ a \exp(-\Gamma \square) + (1-a) \exp[-(\square/\tau \square^\mu)] \}^2 \quad (2)$$

where  $a$  and  $\Gamma$  are the relative amplitude and relaxation rate of the fast process, while  $(1-a)$  and  $1/\tau$  are the corresponding quantities for the HA clusters, and  $\beta$  is the coherence factor. Figure 2 shows the least squares fit of eq. 2 to the curve. To illustrate the relative amplitude of the fast component, the stretched exponential component (dashed line) is also displayed. The value of the exponent  $\mu$  is found to be 0.65.

For diffusive processes, the relaxation rate  $\Gamma$  is proportional to the square of the wave vector, as defined by<sup>10</sup>

where  $D_c$  is the collective diffusion coefficient,  $\eta$  is the viscosity of the pure solvent,  $\xi_H$  is the hydrodynamic correlation length,  $k_B$  is the Boltzmann constant, and  $T$  is the absolute temperature.

It should be noted that  $D_c$  is defined for strongly overlapping polymer solutions where collective motions dominate, i.e., its value differs from the translational diffusion coefficient that characterizes molecular transport properties in dilute solutions.  $D_c$  is measured by DLS that probes the relaxation rate of the concentration fluctuations in the solution and hence the characteristic diffusion rate of the solvent through the polymer network.

The inset in Figure 2 shows the relaxation rate  $\Gamma$  of the fast component as a function of  $q^2$ . The least squares fits through the data points indicate that eq. 3 is obeyed. The value found for  $D_c$ , namely  $8.6 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$  is similar to that reported for solutions of flexible polymer chains. This finding implies that the rigid HA chains possess sufficient local flexibility to allow fully developed thermodynamic fluctuations. The hydrodynamic correlation length  $\xi_H$  corresponding to the osmotic fluctuations, deduced from eq. 3 is 2.8 nm.

## CONCLUSIONS

We investigated the organization and the dynamic scattering properties of hyaluronic acid solutions in near physiological salt solutions. SANS reveals a hierarchical structure composed of clusters greater than several hundred nanometers. Dynamic light scattering is used to gain information on the diffusion processes that govern the transport of solvent molecules within the HA matrix. In HA solutions the autocorrelation function exhibits two components, a fast exponential decay and a slow complex motion. The fast component, which is a small fraction of the total scattered intensity, is diffusive. The slow component indicates that the observed intensity is due to compositional fluctuations in large clusters. At higher values of wave vectors, the SANS response corresponds to rod-like structures, the length of which is in reasonable agreement with the hydrodynamic correlation length associated with the fast diffusion coefficient determined by DLS.

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