Structure and Interactions in Hyaluronic Acid Solutions

Ferenc Horkay[1], Peter J. Basser[1], Anne-Marie Hecht[2] and Erik Geissler[2]

[1]Section on Tissue Biophysics and Biomimetics, Program in Pediatric Imaging and Tissue Sciences, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda MD 20892

[2]Laboratoire Interdisciplinaire de Physique CNRS UMR 5588, Université J. Fourier de Grenoble, B.P. 87, 38402 St Martin d'Hères cedex, France.

INTRODUCTION

Hyaluronic acid (HA) is a major constituent of the extracellular matrix.[1,2] Among its many biological functions, HA plays a critical role in cartilage, where large aggrecan-HA complexes that provide the resistance to compressive load enmesh the collagen network.[3,4] The carboxyl groups of the HA molecule in physiological conditions become charged and contribute to the osmotic pressure of the tissue. This effective charge is compensated by the mobile counterions in the surrounding solution, as required by the condition of electroneutrality. The osmotic pressure of the solution depends on the ion valency and the fixed charge density of the polymer. Increasing the salt concentration reduces the osmotic pressure, owing to the reduction in the repulsive interactions among the polyelectrolyte chains. We investigate the structure and dynamic properties of HA in near physiological salt solution by scattering techniques. We discuss the scattering results for HA in 100 mM NaCl solutions and examine the structural effects induced by calcium-sodium ion exchange.

EXPERIMENTAL

Solution Preparation. HA solutions (Sigma M_w = 1.2 10^6) were made in D_2O containing 100 mM NaCl or 100 mM NaCl + 100 mM $CaCl_2$. The concentration of the HA was varied in the range 1 – 4 % w/w. The sodium chloride concentration (100 mM) and pH (= 7) were identical in all samples. The samples were allowed to homogenize for 2-3 days.

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 (path length 2 mm). The measurements were made at three sample-detector distances, 1.35 m, 4 m and 13.1 m, using an incident neutron wavelength of 8 Å. This configuration allowed us to cover the transfer wave vector range 2.8×10^{-3} Å^{-1} < q < 0.35 Å^{-1}, where $q = (4\pi/\lambda)\sin(\theta/2)$, and λ and θ is the wavelength of the incident radiation and the scattering angle, respectively. After azimuthal averaging, corrections for incoherent background, detector response and cell window scattering were applied.[4] The measurements were made at 25° ± 0.1°C.

Small Angle X-ray Scattering Measurements. SAXS measurements were made at the BM2 beam line at the ESRF, Grenoble, France. Solutions were held in sample cells between 20 μm thick mica windows separated by a 2 mm Teflon spacer. Measurements were made at 15 keV incident energy with sample-detector distances of 1.56 m and 0.40 m. The wave vector range explored in these measurements was 8×10^{-3} Å^{-1} < q < 0.5 Å^{-1}. To reduce radiation damage effects, exposure times were limited to 20 s. The 2-dimensional scattering patterns were azimuthally averaged to yield the intensity curves $I(q)$, Corrections were made for grid distortion, dark current, sample transmission and background scattering from the solvent. The $I(q)$ data were normalized against a standard sample of semi-crystalline polyethylene (lupolen) of known cross-section.

Light scattering. Dynamic light scattering (DLS) measurements were made with an ALV DLS/SLS 5022F goniometer equipped with fibre optic coupling and an avalanche diode, combined with a 22 mW HeNe laser working at 6328 Å and an ALV 5000E multi-tau correlator. The temperature of the refractive index matching toluene bath was 25.0 °C. Measurements were made in the angular range 30° to 150° with accumulation times of 200 s.[5] To avoid shear degradation of the high molecular weight HA molecule, the solutions were measured without filtration.

RESULTS AND DISCUSSION

Static Scattering. In Figure 1 the combined scattering response (SANS+SAXS) of two HA solutions is compared, one containing 100 mM NaCl and the other containing in addition 100 mM $CaCl_2$. At high values of q the two curves approach each other, indicating that calcium ions have no significant effect on the cross-section of the HA subunits. At intermediate q values calcium ions induce a modest increase in intensity, the difference being most pronounced in the range 0.01 < q < 0.1 Å^{-1}. This change corresponds to an increased spatial extent of the concentration fluctuations. Denser packing, as well as clustering, is facilitated by the reduced repulsive electrostatic interaction that occurs when calcium ions replace sodium ions. At low values of q the slope of the scattering curve remains practically unchanged, i.e., calcium ions do not modify the surface roughness of the largest clusters.

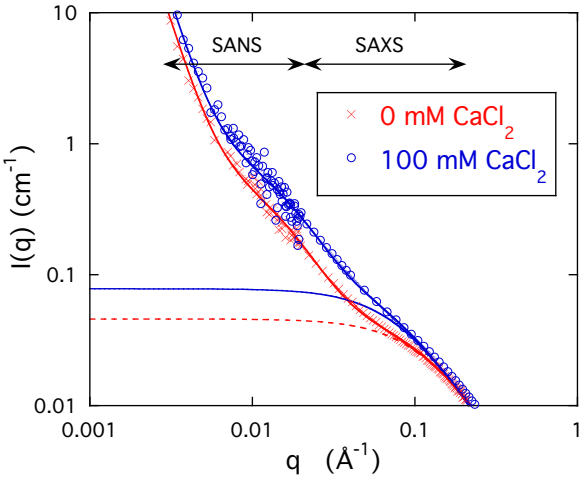

Figure 1. Combined SANS and SAXS spectra of 4% w/w HA solution in 100 mM NaCl, with (O) and without (×) 100 mM $CaCl_2$, normalized to the absolute intensity of the SAXS measurement. Dashed and continuous lines are the first term of eq. 2 for Ca-free and Ca-containing samples respectively.

It is generally found that the scattering intensity from such systems is described by a sum of a dynamic and a static component[6]

$$I(q) = I_{dyn}(q) + I_{stat}(q) \qquad (1)$$

We analyzed the scattering response of the HA solutions by the equation[7]

$$I(q) = \Delta\rho^2 \left[\frac{kT\varphi^2}{\kappa_{os}} \frac{1}{[1+(qL)^2]^{1/2}\left(1+q^2R^2\right)} \right] + Aq^{-m} \qquad (2)$$

where $\Delta\rho^2$ is the contrast factor between polymer and solvent, k is the Boltzmann constant, T is the absolute temperature, φ is the volume fraction of the polymer, L is the mesh size of the solution, R is the cross-sectional radius of polymer chain, κ_{os} is the osmotic modulus, and A and m are constants. As can be seen in Figure 1, eq. 2 provides an acceptable fit to the experimental data over the q-range explored by SANS and SAXS. The dashed curve in Fig. 1 shows the

first term of eq. 2. This is the term that contains the osmotic modulus κ_{os}. In cartilage, the osmotic modulus controls both the load bearing capacity and the rate at which the solution returns to equilibrium after load has been removed.

Dynamic Scattering. In solutions, spontaneous thermal fluctuations occur around the equilibrium value of the concentration φ. The underlying diffusive process by which the system returns to equilibrium is defined by the decay rate of the concentration excursions, as well as by their amplitude. The osmotic modulus governs both these quantities. In solutions of overlapping polymer molecules, the rate of diffusion of the solvent molecules is given by the collective diffusion coefficient[8]

$$D_c = \frac{\kappa_{os}}{\varphi f} \qquad (3)$$

where *f* is the friction coefficient between polymer and solvent. Eqs. 2 and 3 capture the relationship between the static quantity κ_{os} and the dynamic properties of the solution. It is important to note that D_c differs from the translational diffusion coefficient that characterizes molecular transport properties in dilute polymer solutions. D_c is defined for more concentrated solutions, where collective motions dominate.[8] D_c can be measured by dynamic light scattering that probes the relaxation rate of the concentration fluctuations in the polymer solution and hence the characteristic diffusion rate of the solvent through the network.

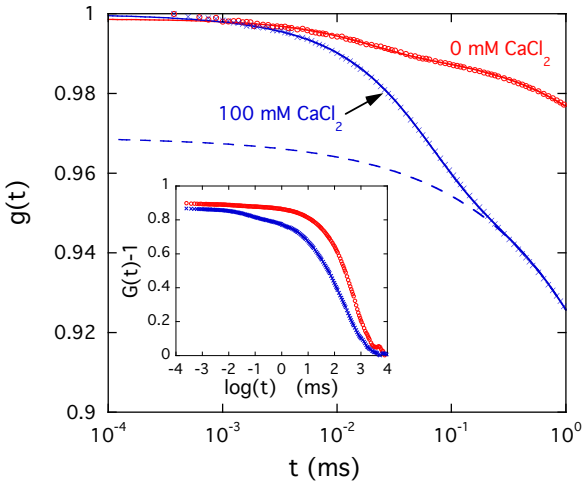

Figure 2. Field correlation functions $g(t)$ of 1% w/w HA solutions measured at 150° scattering angle. O: 100 mM NaCl; ×: 100 mM NaCl and 100 mM $CaCl_2$. Continuous curves are fits to eq. 4; dashed line: slow mode term in the calcium-containing solution. Inset: intensity correlation functions $G(\tau) - 1$ for the same solutions.

As is seen from eq 3, DLS measurements yield information on the diffusion properties of the HA solution. Figure 2 shows the intensity correlation functions[9] $G(t)$-1 (inset) and an expanded view of the corresponding field correlation functions $g(t)$, measured by DLS for two HA solutions of concentration 1% w/w, one containing 100 mM NaCl, the other containing in addition 100 mM $CaCl_2$. Both solutions exhibit two distinct relaxation processes, separated by about 3 orders of magnitude, that are visible in the zoom figure of $g(t)$. The first process, which appears around 0.1 ms, is due to the relaxation of the HA molecules and is approximately a simple exponential decay. The slower process, which is more complex as it contains a broad range of decay times, corresponds to fluctuations inside clusters and can be described by a stretched exponential decay. The normalized intensity correlation function is thus[5]

$$g(t) = a \exp(-\Gamma t) + (1-a) \exp[-(t/\tau)^{\mu}] \qquad (4)$$

where a and Γ 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. The least squares fits of eq. 4 to both curves are shown in Figure 2. The relative amplitude of the fast component in the calcium-containing sample becomes apparent on comparing with the stretched exponential term (dashed line), which constitutes about 97% of the scattered intensity. The value found for the exponent μ is close to 0.6, similar to what is reported for microgels.[10]

The above results show that the presence of calcium ions significantly influences the relaxation rate of the matrix. With regard to the biological importance of relaxation processes, it must be noted that while κ_{os} defines the osmotic resistance of the tissue, D_c governs the rate of volume change under compressive load.

CONCLUSIONS

Combination of SANS and SAXS measurements enables to determine the effect of ions on the organization of the HA molecules in solution. Divalent ions provide increased screening of the repulsive interaction between the fixed charges located on the polymer backbone. Reduced repulsion favors cluster formation, which is consistent with the increased scattering intensity observed in the calcium ion containing solution. DLS is used to obtain information on the diffusion processes that govern the transport of solvent molecules within the HA matrix. The results show that calcium ions strongly affect the relaxation rate of the matrix.

ACKNOWLEDGEMENT

This work was supported by the Intramural Research Program of the NICHD/NIH. We acknowledge the support of the National Institute of Standards and Technology, U.S. Department of Commerce, in providing the neutron research facilities used in this work. This work utilized facilities supported in part by the National Science Foundation under Agreement No. DMR-0944772.

REFERENCES

1. Alberts, B.; Johnson, A.; J. Lewis, J.; Raff, M.; Roberts, K.; Walter, P. Molecular Biology of the Cell, 4th edition, Garland, New York, Chapter 19. 2002.
2. Radin, E.L.; Swann, D.A.; Weisser, P.A. *Nature* **1970**, *228*, 377-378.
3. Iozzo, R. *Proteoglycans: Structure, Biology, and Molecular Interactions*. Marcel Dekker, New York 2000.
4. NIST Cold Neutron Research Facility. NG3 and NG7 30-meter SANS Instruments Data Acquisition Manual, January 1999.
5. Horkay, F.; Basser, P.J.; Londono, D. J.; Hecht, A.M.; Geissler, E. *J. Chem. Phys.* **2009**, *131*, 184902.
6. Horkay, F.; Burchard, W.; Geissler, E.; Hecht A.M. *Macromolecules* **1993,** *26*, 1296-1303.
7. Horkay, F.; Basser, P.J.; Hecht A.M.; Geissler, E.; *Macromolecules* **2012,** *45*, 2882-2890.
8. de Gennes, P.G. *Scaling Concepts in Polymer Physics*, Cornell, Ithaca NY 1979
9. Berne, R.; Pecora, R. Dynamic Light Scattering, Academic, London 1976.
10. Ngai, T.; Wu, C.; Chen, Y. J. Phys. Chem. B. **2004,** *108*, 5532-5540.