BIOLOGICAL SELF-ASSEMBLY AND NANOSCALE INTERACTIONS IN AGGRECAN SOLUTIONS

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Introduction

Aggrecan is a bottlebrush shaped high molecular weight ($1x10^6 < M < 3x10^6$) proteoglycar. In the presence of hyaluronic acid and link protein, aggrecan molecules form a secondary bottlebrush structure.[1] This hydrated gel-like material enmeshed in a network of collagen fibres provides the osmotic properties necessary for the cartilage to resist deswelling under compressive load with minimum deformation.[2]

The biochemistry of cartilage formation has been extensively studied in the last couple of decades.[1,2] Aggrecan contains three globular domains. The N-terminal domain interacts specifically with hyaluronic acid to form large aggregates present in the extracellular matrix. Imaging techniques (electron microscopy, atomic force microscopy, etc) have revealed the size and structural pattern of large distinct aggrecan-hyaluronic acid complexes.[3,4]. However, the interactions that govern the equilibrium morphology and dynamics of these assemblies in solution, remain poorly understood. Owing to the complexity of the aggrecan-hyaluronic acid system the determination of the relationship between its structure and function requires a variety of complementary experimental techniques combining both biochemical and physical methods.

In solutions, knowledge of the statistical properties of the system is necessary to describe the conformation and motion of the components. Scattering techniques provide such information, since they explore a large volume of the investigated specimen. By varying the wavelength of the incident radiation and the scattering angle, i.e., the resolution, static and dynamic responses can be detected over a wide range of distance scales. At the spatial resolution of the light and neutron scattering measurements the scattering arises from density differences between the solute and the uniform background.

The aim of this work is to investigate the hierarchical organization of aggrecan and aggrecan/hyaluronic acid solutions in the length scale range between 10 Å and 5000 Å. The static properties of these solutions are studied by small angle neutron scattering (SANS) and static light scattering (SLS), while the dynamics are probed by dynamic light scattering (DLS). These noninvasive techniques provide information on the structure and motion of the structural elements. This knowledge is important to understand how the organization of aggrecan assemblies affect the biomechanical properties of cartilage. The changes at the molecular level (chemical composition, charge density, etc.) are detected by virtue of the average effect that they exert on the larger scale structures.

We address the following specific questions: What is the effect of the aggrecan concentration on the organization of the bottlebrush molecules in solution? Do the aggregates interpenetrate with increasing concentration? Do they diffuse freely or is their motion restricted?

Experimental

Sample Preparation. Aggrecan (Sigma-Aldrich) solutions were prepared in water containing 100 mM NaCl. The concentration of the aggrecan was varied in the range 0.02 - 0.5% by weight. An aggrecan/hyaluronic acid solution was also prepared in which the ratio of hyaluronic acid (Sigma-Aldrich) to aggrecan was set equal to 0.01. The ionic strength and pH (= 7) were the same in all samples.

Light Scattering. Static and dynamic light scattering measurements were made with an ALV DLS/SLS 5022F goniometer equipped with a fiber optic coupling and an avalanche diode, working with a 22 mW He-Ne laser and an ALV 5000E multi-tau correlator. The temperature of the refractive index matching toluene bath was maintained at 25 ± 0.1 °C. Measurements were made between 20° and 150° with accumulation times of 200 s.

Small Angle Neutron Scattering. SANS measurements were performed at NIST, Gaithersburg MD, on the NG3 instrument at an incident wavelength 8 Å. Solutions prepared in D_2O were placed in 2 mm thick sample cells. The temperature during the experiments was maintained at 25 ± 0.1 °C. After radial averaging, corrections for incoherent background, detector response and cell window scattering were applied.

Results and Discussion

Scattering methods provide information on the structure of a system in a range of length scales defined by the wavelength λ of the incident radiation and the angle of observation θ, and expressed through the scattering vector $q = 4\pi n/\lambda \sin(\theta/2)$, where n is the refractive index of the scattering medium. This technique explores characteristic structural features of size $1/q$ in the sample. Thus, in solutions containing extended objects such as large assemblies, information is obtained not only on the overall size of the assembly but also on the local internal arrangement. Combination of scattering techniques using different wavelengths, e.g., neutrons ($\lambda \approx 8$ Å) and light ($\lambda \approx 6328$ Å) enables a wide range of characteristic distances to be explored.

Static Scattering Properties of Aggrecan Solutions. **Figure 1** shows the SLS response, R_θ/Kc, of aggrecan solutions in 100 mM NaCl at 4 different concentrations c in the range 0.02 - 0.27 % w/w. Here, R_θ is the Rayleigh ratio and K the optical contrast factor.

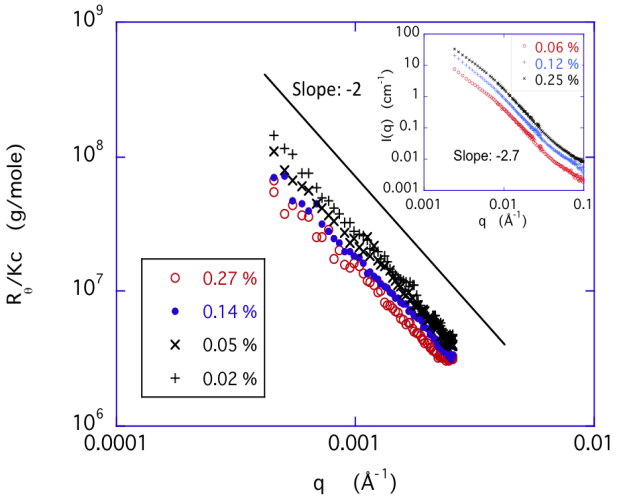

Figure 1. Variation of the reduced static light scattering intensity R_θ/Kc of aggrecan solutions at different concentrations as a function of the scattering vector q.

All the curves exhibit straight-line behavior with a slope of approximately -2. No plateau region that defines an upper size limit for the scattering centers is visible, even at the lowest q vector. It follows that the solution contains extended clusters the size of which exceeds several thousand ångströms and whose molecular weight is indeterminately large. The solutions thus consist of a suspension of microgel-like particles, which is in agreement with rheological observations.[5] If the clusters were rigid and impenetrable, the scattering intensity should be proportional to the concentration, i.e., the data points, normalized by the aggrecan concentration, should fall on a master curve. The figure shows, however, that $R_\theta//Kc$ decreases with increasing aggrecan concentration. This behavior corresponds to an increase of the concentration inside the clusters over the length scale range explored by SLS ($400 \leq 1/q < 2500$ Å).

To explore the structure at higher resolution, SANS measurements were performed at 3 different aggrecan concentrations, 0.06, 0.12 and 0.25 % w/w (see inset). The curves are roughly parallel to each other and the intensity increases with increasing concentration. In each case, power-law behavior is

distinguishable in the q range above 0.01 Å^{-1}, with a slope approximately -2.7. The value of a fractal exponent between 2.5 and 3 is typical of a weakly interpenetrating disordered array of branched sructures, such as the aggrecan molecules. The upper size of these primary aggregates, calculated from the position of the knee at $q \approx 0.01$ Å^{-1} in the scattering curve is approximately 500 Å, i.e., the size of a small number of aggregated aggrecan monomers.

In summary, the static light and neutron scattering results reveal the presence of very large clusters composed of loosely connected aggregates. At low q, variation of the scattered intensity with aggrecan concentration is weaker than linear, indicating that the clusters become more densely packed with increasing overall concentration. At higher q ($q \geq 0.01$ Å^{-1}), the power law behavior of slope -2.7 implies that the system contains aggregates, inside which the aggrecan bottlebrushes interpenetrate weakly.

Dynamic Light Scattering of Aggrecan Solutions. Since the static scattering observations show that the clusters are compressible and possess a loose structure, it is reasonable to expect that the connection between the primary aggregates is not rigid. Information on the mobility inside the clusters can be obtained from DLS. This technique probes the motion of diffusing particles and, through the Stokes-Einstein relation, yields their hydrodynamic radius R_H. The intensity correlation function G(τ)-1, expressed in terms of the delay time τ, is given by[6]

$$G(t)-1 = \beta \exp(-2\Gamma\tau) \qquad (1)$$

where Γ is the relaxation rate of concentration fluctuations and the optical coherence factor $\beta \approx 1$ is defined by the geometry of the experimental set-up.

Figure 2 shows intensity correlation functions for three aggrecan solutions. The curves all decay to zero at a rate that decreases with increasing concentration, indicating that on the length scale of a few thousand ångströms the motion of the scattering centers becomes increasingly hindered.

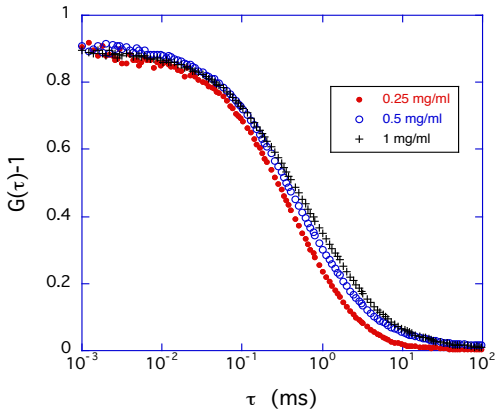

Figure 2. Correlation functions G(t)-1 for aggrecan solutions in 100 mM NaCl at three different aggrecan concentrations.

Aggrecan-Hyaluronic Acid Solutions. The addition of hyaluronic acid produces long-range structural changes in the organization of aggrecan assemblies. In the presence of this large negatively charged polyelectrolyte of molecular weight 1.2 10^6, aggrecan forms elongated supermolecular structures, several microns in length and about half a micron in diameter.

To determine the characteristic length scale of the aggrecan-hyaluronic acid complex, static light scattering measurement was performed on a solution containing 0.02 mg/ml aggrecan and 0.2 μg/ml hyaluronic acid. **Figure 3** shows the intensity of the aggrecan-hyaluronic acid complex in a Kratky representation[7] ($q^2R_\theta//Kc$ *vs* q). The continuous line through the data points is the least squares fit to the expression

$$I(q) = \frac{a}{q}\left[\frac{2J_1(qr)}{qr}\right]^2 + bq^{-2} \qquad (2)$$

where $J_1(x)$ is the Bessel function of order 1, and a and b are constants. In this fit, it is found that r = 3250 Å, in agreement with the result reported in the literature[8] obtained by electron microscopy (ca. 3200 Å) for a hyaluronic acid/aggrecan complex extracted from a bovine epiphyseal cartilage.

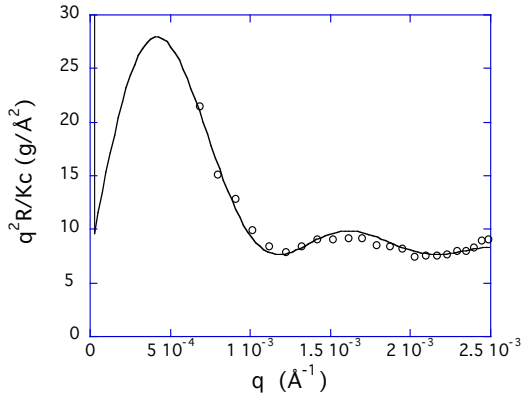

Figure 3. Kratky plot of static light scattering intensity for aggrecan/hyaluronic complex formed in a 100 mM NaCl solution containing 0.02 mg/ml aggrecan and 0.2 μg/ml hyaluronic acid after equilibration. The continuous curve shows the least squares fit to equation 2.

Conclusions

Small angle neutron scattering in dilute physiological salt solutions of aggrecan reveals fractal behavior of dimensionality D = 2.7 over a limited q-range above 0.01 Å^{-1}, indicating that the aggregates are composed of weakly interpenetrating branched structures. Light and neutron scattering measurements below 0.01 Å^{-1} display a q^{-2} power-law dependence of the intensity that can be attributed to a random association of the primary aggregates. These clusters have a size of several thousand ångströms. The packing density of the clusters increases with concentration, while that of the primary aggregates remains constant.

In aggrecan/hyaluronic acid solutions static light scattering measurements detect a cylindrical structure, which forms spontaneously in dilute solution. The size of this assembly, in which the aggrecan molecules are condensed along a central filament of hyaluronic acid, is in good agreement with that determined by electron microscopy.

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