Hierarchical Organization of Cartilage Proteoglycans

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Summary: The hierarchical organization of cartilage proteoglycans is investigated on different length and time scales using osmotic pressure measurements, small angle neutron scattering (SANS), small angle X-ray scattering (SAXS), static and dynamic light scattering and neutron spin echo techniques. Osmotic pressure measurements reveal association of aggrecan bottlebrushes into microgel-like assemblies. SAXS, SANS and light scattering results indicate weak interpenetration between neighboring aggrecan molecules. As opposed to DNA and many synthetic polyelectrolytes, which display great sensitivity to ion valence, aggrecan exhibits exceptional insensitivity to calcium ions in the physiological ion concentration range and beyond. This property allows aggrecan to play a role of ion reservoir that can mediate calcium metabolism in cartilage and bone.

Keywords: aggrecan; dynamic light scattering; neutron scattering; osmotic pressure; self-assembly

Introduction

Aggrecan is a high molecular weight bottlebrush-shaped polyelectrolyte ($1 \times 10^6 < M < 3 \times 10^6$) that governs the biomechanical properties of cartilage.[1] In the physiological concentration range (4–7%w/w) the high osmotic swelling pressure of aggrecan-hyaluronic acid assemblies, which are enmeshed in the collagen matrix, provides resilience to compressive load, controls lubrication of the joint and protects bone surfaces from wear during articular movement.[2–5] Aggrecan also participates in cartilage/skeletal metabolism, playing an active role in bone mineralization by accumulating calcium ions. The bottlebrush molecule consists of an extended protein core of approximate length 1500 Å, to which about a hundred rigid chondroitin sulfate and keratan sulfate side-chains (approximately 200 Å in length) are attached (Figure 1). The structure of aggrecan (e.g., length of bristles, charge density and distribution) varies with age and state of health. The multiple biological roles of aggrecan assemblies underline the need to better understand how the osmotic properties depend on the hierarchical organization at different length and time scales as a function of the ionic environment.

Experimental Part

Sample Preparation

Near-physiological solutions of aggrecan from bovine articular cartilage (Sigma) were prepared with 100 mM NaCl and different amounts of $CaCl_2$ (0–100 mM). The concentration of the aggrecan was varied in the range 0.01 wt% – 4 wt%. In all samples the pH was 7, at which the charged groups on the side-chains of the aggrecan molecule are dissociated.

Osmotic Pressure Measurements

The osmotic pressure of the aggrecan solutions was determined by equilibrating them with polyvinyl alcohol gel filaments of

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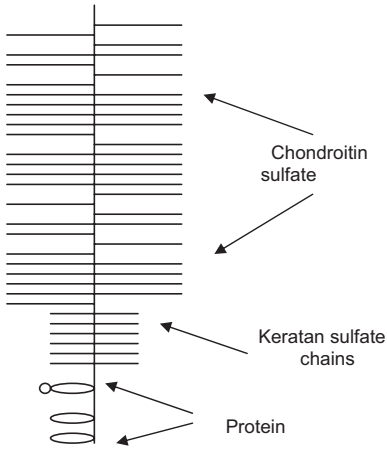

Figure 1.
Schematic representation of the aggrecan bottlebrush.

known swelling pressure.[6,7] The size of the PVA gels was measured by optical microscopy. The large size of the aggrecan molecule prevented penetration into the swollen gel. The osmotic pressure measurements were made with the following salt concentrations: 100 mM NaCl, 100 mM NaCl + 50 mM $CaCl_2$, and 100 mM NaCl + 100 mM $CaCl_2$.

Scattering Measurements

The structure of aggrecan in near physiological salt solutions was investigated by small angle neutron scattering (SANS) on the NG3 instrument at the National Institute of Standards and Technology (NIST) using three configurations: low-q, intermediate-q, and high-q, where the momentum transfer is $q = \frac{4\pi}{\lambda}\sin\left(\frac{\theta}{2}\right)$ and θ is the scattering angle. These experimental configurations covered the q-range $0.0035\ \text{Å}^{-1} < q < 0.4\ \text{Å}^{-1}$ corresponding to size scales from 0.18 μm (low-q) to 15 Å (high-q). For the SANS measurements solutions were prepared in D_2O. Standard corrections were applied for detector response and for cell window and incoherent background scattering.[8]

Small angle X-ray scattering (SAXS) measurements were made at the insertion device of Sector 5 at the Advanced Photon Source (APS) with an incident wavelength $\lambda = 1.55$ Å. The 2-dimensional scattering patterns were azimuthally averaged to yield the intensity curves $I(q)$. The q range explored was $0.015\ \text{Å}^{-1} \leq q \leq 0.7\ \text{Å}^{-1}$. Results were corrected for grid distortion, dark current, sample transmission and background scattering from the solvent. The intensity was put in absolute units by comparison with secondary standards obtained from ORNL and UNICAT at APS.

Neutron spin echo (NSE) measurements were performed on the IN15 instrument at the Institute Laue Langevin. Time delays were extended from 0 to 170 ns in the range $0.03\ \text{Å}^{-1} \leq q \leq 0.05\ \text{Å}^{-1}$.

Static and dynamic light scattering measurements (SLS and DLS) were made using an ALV 5022F goniometer with a 22 mW HeNe laser between 20° and 150° with 200 s accumulation times.[9] To avoid shear degradation of the large aggrecan molecules the solutions were not filtered prior to the light scattering measurements. The combined SLS, SANS and SAXS observations spanned the wave vector range $4 \times 10^{-4}\ \text{Å}^{-1} \leq q \leq 0.7\ \text{Å}^{-1}$.

All measurements were performed at 25 °C.

Results and Discussion

Osmotic Pressure Measurements

Figure 2 shows the variation of the osmotic pressure Π as a function of aggrecan concentration in 100 mM NaCl solutions with different $CaCl_2$ concentrations, 0 mM, 50 mM and 100 mM. In the figure is also displayed the concentration dependence of Π for a solution of chondroitin sulfate (CS) in 150 mM NaCl (dashed line).[10] The CS solution models the free side chains of the bottlebrush. The osmotic pressure is smaller by a factor of roughly two in the aggrecan solution than in the CS solution. Such a reduction in the osmotic pressure can be attributed to the loss of entropy due to the bottlebrush arrangement of the polymer chains.

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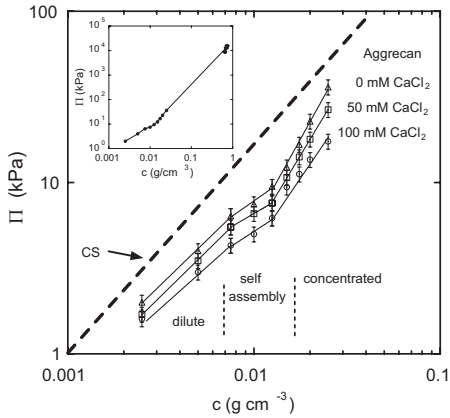

Figure 2.
Variation of the osmotic pressure Π as a function of aggrecan concentration in 100 mM NaCl solution, with different amounts of $CaCl_2$. Dashed line is the osmotic pressure of chondroitin sulfate solution in 150 mM NaCl from reference [10]. Inset shows Π(*c*) for the calcium-free aggrecan solution over an extended concentration range.

In the osmotic response of the aggrecan solution three concentration regions are distinguishable. At low aggrecan content (below 0.05 g/cm^3) Π increases linearly with concentration, as expected for dilute systems in which the osmotic pressure is governed by independent particles. In the double logarithmic plot the decrease in slope to about 0.6 in the concentration range 0.005 to 0.015 g/cm^3 is a sign of clustering. Above 0.015 g/cm^3 the slope increases to approximately 2, which corresponds to increasing packing density of the aggrecan assemblies. This exponent indicates that the osmotic pressure is governed by the repulsion between each aggrecan molecule and its nearest neighbor, and is thus proportional to the square of the concentration, i.e., $\Pi \propto c^2$. It is notable that this power law behavior for Π extends to rather high concentrations, up to $c \approx 0.75$ g/cm^3 (see inset). The observed reduction in Π relative to the CS solution suggests that the aggrecan system can be viewed as a suspension of microgel particles.

The effect of calcium ions is to decrease Π in all three regions. It can be seen that the concentration thresholds are practically independent of the calcium content. Also the shape of the three curves remains similar over the entire concentration range investigated. Addition of 100 mM $CaCl_2$ reduces the value of Π by a factor of approximately 2. The change in the solvent quality and the modification of the electrostatic interactions associated with the calcium-sodium exchange process promote association among the aggrecan molecules.

From the Π(c) curves the osmotic compression modulus $K = c\partial\Pi/\partial c$ can be estimated. Extrapolation of the power law dependence to the physiological aggrecan concentration ($c = 0.07$ g/cm^{-3}) yields $K = 520$ kPa, which is consistent with values reported by Treppo et al.[11] The increased slopes in the aggrecan systems in Figure 2 illustrate the fact that in the physiological concentration range the bottlebrush architecture significantly enhances the osmotic modulus relative to the linear chondroitin sulfate solution.

Scattering Measurements

Osmotic pressure measurements reveal that aggrecan bottlebrushes start to assemble in the dilute concentration range and can be approximated by three different power law regimes. Scattering observations provide spatial information about the coexisting structures and the effect of calcium ions on them over a range from the size of the disaccharide units to that of the assemblies.

Figure 3 shows the SANS spectra of the aggrecan solutions at three different concentrations. The measured intensities *I*(c) are normalized by the aggrecan concentration c. At low *q*, in the light scattering region (see inset), the intensity decreases according to a power law the exponent of which is about −2. This implies that the packing density of aggrecan assemblies is close to that of Gaussian clusters for which the intensity varies as q^{-2}. Absence of a plateau regime at low *q* shows that the size of these objects exceeds the resolution of the light scattering measurements, i.e., several hundred nanometers.

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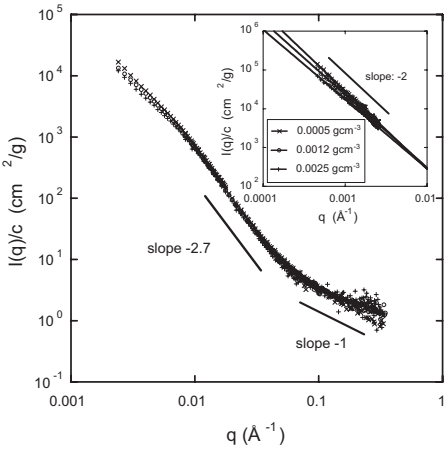

Figure 3.
Variation of the reduced SANS intensity $I(q)/c$ of aggrecan solutions at three different concentrations (x: 0.0005 gcm^{-3}, o: 0.0012 gcm^{-3}, +0.0025 gcm^{-3}). Inset: reduced light scattering intensity $I(q)/c$ for the same samples with the corresponding power law fits.

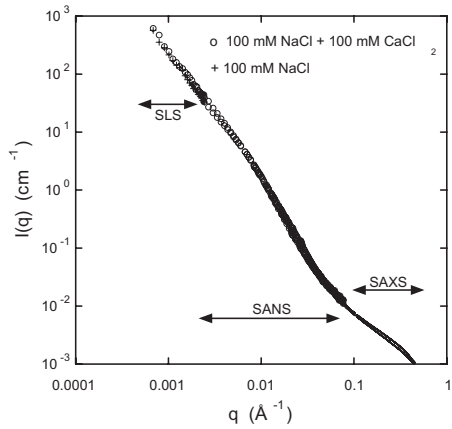

Figure 4.
Combined SLS, SANS and SAXS spectrum of aggrecan solution ($c = 0.0025\ gcm^{-3}$) in 100 mM NaCl (+) and in 100 mM NaCl + 100 mM $CaCl_2$ (o).

The normalized intensity decreases with increasing aggrecan concentration indicating that the packing density of the large assemblies increases. The power law behavior extends into the SANS domain up to the knee at $q \approx 0.008$ Å^{-1}, where a change in slope is observed. Beyond the knee in the range 0.012 Å$^{-1} < q < 0.08$ Å^{-1}, the scattering response exhibits a power law behavior, with slope –2.7, characteristic of percolating branched polymer clusters.[12] This length scale range from about 500 Å to 100 Å is of the order of the length of the side-chains (bristles). In this region the normalized intensity is practically independent of the concentration, i.e., the degree of interpenetration between neighboring bottlebrushes does not vary. In the high-q range (corresponding to 5–10 Å), the intensity decreases as q^{-1} reflecting the linear (rod-like) structure of the side chains.[13]

In Figure 4 are shown the results of combined SLS, SANS and SAXS measurements in the presence of 100 mM calcium chloride. Within experimental error, the data sets with and without calcium chloride coincide. The lack of any change in scattering intensity over the entire q range signifies that calcium ions induce neither phase separation nor densification of the clusters. Thus, replacing sodium counterions on the polyanion with calcium ions, i.e., changing the electrostatic interactions, does not alter the structure of the aggrecan assemblies.[14] Even under conditions where the electrostatic interactions are screened, the random orientation of each bottlebrush axis and its rigid bristles limits interpenetration and prevents the development of compact structures. The most striking result from these combined scattering observations is that the ion-induced structural changes do not propagate to higher length scales.

Figure 5 summarizes the characteristic structural features identified in the aggrecan solution as a function of q. In the present experiment the flat region at low q (region 1) lies below the accessible range owing to the large size of the aggrecan microgels. In the light scattering region (region 2), $I(q)/c \propto q^{-2}$, corresponding to randomly packed assemblies. Below $q \approx 0.01$ Å^{-1} the packing density is q-dependent and the slope of the power law decreases as the assemblies progressively interpenetrate. Above $q \approx 0.01$ Å^{-1} (region 3), where the scattering response is governed by the bottlebrush structure of the aggrecan subunit, the normalized

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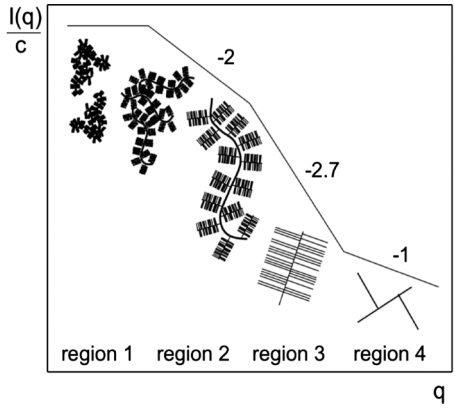

Figure 5.
Schematic diagram of $I(q)/c$ illustrating the main structural features probed at different resolutions.

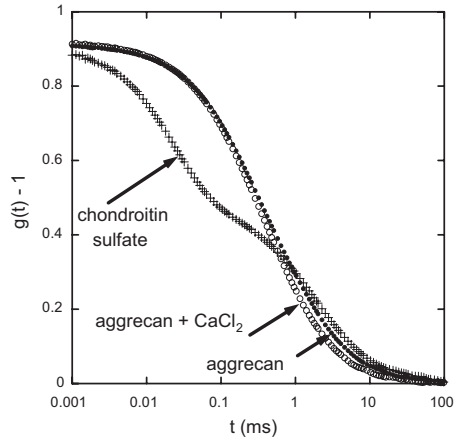

Figure 6.
Intensity correlation functions $g(t)$ - 1 for aggrecan solutions ($c = 0.6\%$w/w) in 100 mM NaCl without $CaCl_2$ (•) and with 25 mM $CaCl_2$ (○), and for a 2%w/w chondroitin sulfate solution (+) showing the fast and slow components at scattering angle 150°.

scattering curves superimpose, implying that these rigid structures resist deformation. Region 4 yields information on the linear structure of the bristles.

Dynamic Properties of Aggrecan Assemblies

We investigated the dynamic properties of aggrecan assemblies by dynamic light scattering (DLS) and neutron spin echo (NSE). In solutions of flexible polymers the relaxation rate Γ of the thermodynamic concentration fluctuations is governed by diffusion processes and the intensity correlation function $g(t)$ - 1 is given as[15,16]

$$g(t) - 1 = \beta \exp(-2\Gamma t) \quad (1)$$

where t is the time delay and the optical coherence factor $\beta \approx 1$ is defined by the light collection geometry.

In Figure 6 are displayed $g(t)$-1 for a 0.6% aggrecan solutions in 100 mM NaCl without $CaCl_2$ and with 25 mM $CaCl_2$. The difference between the two data sets is very small indicating that the calcium ions hardly affect the dynamic properties. In the figure is also shown $g(t)$-1 for a CS solution ($c = 2\%$w/w). The latter solution, like many other linear polyelectrolyte solutions, such as DNA and polyacrylic acid (PAA), exhibits two distinct relaxation processes separated by approximately two orders of magnitude in delay time. The fast process displays a simple exponential decay while the slow mode can be described by a stretched exponential. In general, in linear polyelectrolyte solutions, when the monovalent counter-ions are replaced by divalent counter-ions, the fast relaxation process becomes slower and the slow becomes faster.[17] The behavior of the aggrecan solution is entirely different: (i) this system displays a broad range of relaxation times (10^{-2} ms $< \tau <$ 10 ms), (ii) no fast component possessing a single exponential decay is distinguishable in the autocorrelation function, and (iii) the relaxation process is practically unaffected by the calcium ions.

Analysis of the correlation functions allows us to determine the effect of calcium ions on the characteristic size of the fluctuating units. In dilute polymer solutions the relaxation rate of the concentration fluctuations can be described in terms of the Stokes-Einstein relationship[16]

$$\Gamma\eta = \frac{kTq^2}{6\pi\xi} \quad (2)$$

where k is the Boltzmann constant, T the absolute temperature, η the viscosity of the solvent and ξ is a characteristic length. For a

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given temperature, the normalized relaxation rate $\Gamma\eta$ depends only on the geometrical properties of the polymer chain. When $q\xi > 1$, the relaxation detects modes inside the correlation volume (internal modes) that are limited only by the resolution of the observation, i.e., Γ is no longer determined by ξ but instead by $1/q$. It has been found for a variety of polymeric structures ranging from flexible polymer chains to percolating clusters in critical fluids that

$$\Gamma\eta \propto q^m \tag{3}$$

For ideal flexible chains with hydrodynamic interactions, $m = 3$ (Zimm model).[18] In the case of screened hydrodynamic interactions (Rouse model), [15] $m = 4$.

The upper data set of Figure 7 shows the relaxation results for a solution of a typical flexible polymer, poly(dimethyl siloxane) in toluene.[19] In this system the two regimes are clearly distinguishable. By contrast, the results from the aggrecan solutions, both without and with calcium chloride, lie on a single straight line with an exponent $m \approx 3$ over the entire q-range explored (lower data set of the same figure). The q^3 dependence of is in agreement with observations reported for dispersions of microgel particles.[20] Our finding implies that there is no intrinsic characteristic length scale governing the dynamics of aggrecan solutions over an exceptionally wide range of length scales, spanning from $2\pi/q_{max} = 13$ Å to $2\pi/q_{min} = 2.2\,\mu$m, i.e., from the size of the disaccharide unit to that of the bottlebrush clusters. The presence of microgel-like aggrecan assemblies is consistent with the results of the osmotic and static scattering observations discussed in the previous sections.

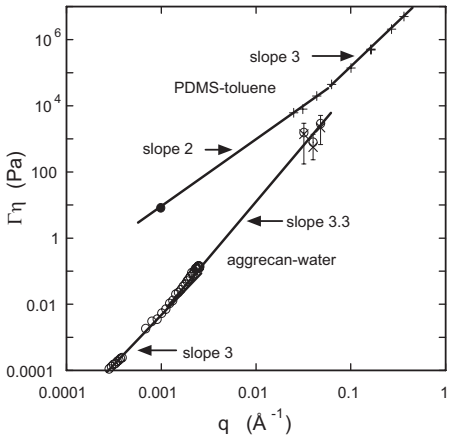

Figure 7.
Dependence of the characteristic relaxation rate $\Gamma\eta$ on the wave vector q, where η is the solvent viscosity. Measurements by dynamic light scattering and neutron spin echo for solutions of 0.6%w/w aggrecan solution in 100 mM NaCl (×) and in 100 mM NaCl with 40 mM $CaCl_2$ (○). At high q the behavior is intermediate between the Zimm model $\Gamma \propto q^3$ and the hydrodynamically screened model (Rouse), where $\Gamma \propto q^4$. Data are also shown for poly(dimethyl siloxane)/toluene system measured by NSE (+) and DLS (•), in which the region where collective diffusion dominates ($\Gamma \propto q^2$) is clearly distinguishable.

Conclusion

In conclusion, the present results show that aggrecan in near-physiological salt solutions self-assembles into microgel-like particles that display remarkable insensitivity, both in structural and in dynamic properties, to changes in the ionic environment, notably to calcium ions. This insensitivity of the structure of aggrecan assemblies to calcium ions contrasts with the behavior of linear polyelecrolytes such as DNA or PAA. For aggrecan, the persistence length of the side chains, 100 -200 Å, is comparable to their total length. Thus, owing to the bottlebrush architecture and the intrinsic rigidity of the bristles, the aggrecan molecule does not possess the highly entropic character of flexible polymer chains. Moreover, the bottlebrush topology prevents propagation of ion-induced structural changes among the crowded bristles, the spatial separation of which is 3–4 nm.

These results are consistent with the biological role of aggrecan as an essential structural component in the load bearing function of cartilage, and as an ion-exchange matrix in bone metabolism. Furthermore, since entanglement formation among the rigid highly charged chains

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is not favored, the microgel nature of the solutions can facilitate articular lubrication. The gel-like structure also constitutes a reservoir of low viscosity fluid that may be released under external pressure.

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