IONIC EFFECTS AND SELF-ASSEMBLY IN THE SOLUTION OF THE BIOPOLYMER AGGRECAN

Ferenc Horkay

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

Introduction

Aggrecan is a biologically important high molecular weight ($1x10^6 < M < 3x10^6$) proteoglycan. It possesses a bottlebrush structure, consisting of an extended protein core, to which many chondroitin sulfate and keratan sulfate (sulfated polysaccharide) chains are attached.[1-3] In the presence of hyaluronic acid and a link protein, aggrecan molecules self-assemble into a supermolecular structure with as many as 100 (or more) aggrecan monomers condensed on a filament of hyaluronan.[6] This complex, when interspersed in a collagen matrix, forms an interpenetrating network that gives cartilage its resilience and its ability to withstand compressive load with minimal deformation.[4] It is noteworthy that degradative changes in the size and structure of aggrecan become more progressive with age. This process can be potentially deleterious to articular cartilage function as it reduces the charges on the aggrecan molecules and affects the mechanical properties of their assemblies.[5]

While recent research has focused mainly on the genetic and biochemical alterations associated with cartilage degeneration, relatively little is known about the physical properties that govern the interactions among the macromolecules that constitute the cartilage matrix itself. These interactions determine its osmotic resistance to compressive load. In the natural environment of aggrecan, in addition to other biological molecules, mono- and divalent ions are present. Since the negatively charged aggrecan molecules bind cations, it is essential to know how cations affect the structural properties of this system. Calcium ions are particularly important as they play a critical role in skeletal metabolism.

We investigated the effect of sodium and calcium ions on the organization of the aggrecan molecules in near physiological salt solutions by small angle neutron scattering (SANS) and small angle X-ray scattering (SAXS). We also measured the osmotic pressure of the aggrecan solutions in the presence of mono- and divalent ions. Scattering and osmotic pressure measurements were made on solutions of aggrecan from bovine cartilage (Sigma) prepared in water containing 100 mM NaCl and various concentrations of $CaCl_2$.

In aqueous solutions of synthetic and natural polyelectrolytes small amounts of multivalent ions generally induce major changes in the molecular conformation, followed by phase separation.[6-8] Here we report an unexpected finding for the biological polyelectrolyte aggrecan: addition of calcium chloride, even at concentrations as high as 200 mM, fails to precipitate this molecule, and does not even cause detectable changes in its conformation.

Experimental

Osmotic Pressure Measurements. The osmotic pressure of the aggrecan solutions was determined as a function of concentration by bringing them to equilibrium with polyvinyl alcohol (PVA) gels of known swelling pressure. The size of the PVA gel filaments was measured by optical microscopy after equilibration in the solution (ca. 24 h). The large size of the aggrecan molecule prevented penetration into the swollen gel. The osmotic measurements were made with the following constant salt concentrations: 100 mM NaCl, 100 mM NaCl + 50 mM $CaCl_2$ and 100 mM NaCl + 100 mM $CaCl_2$.

Small Angle Neutron Scattering. SANS measurements were made at NIST, Gaithersburg MD, on the NG3 instrument. The temperature during the experiments was maintained at 25° ± 0.1°C. Solutions prepared in heavy water (D_2O) were placed in sample cells with a 2 mm optical path. Measurements were made with an incident wavelength $\lambda = 8$ Å. After radial averaging, corrections for incoherent background, detector response and cell window scattering were applied.

Small Angle X-ray Scattering. SAXS measurements were carried out at the insertion device of Sector 5 at the Advanced Photon Source (DND-CAT), with an incident energy of 8 KeV. The 2-dimensional scattering patterns were azimuthally averaged to yield the intensity curves $I(q)$, where the wave vector is $q = (4\pi/\lambda)\sin(\theta/2)$ and θ the scattering angle. The q range explored was $0.0016 \leq q \leq 0.35$ 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 Oak Ridge National Laboratory and UNICAT at APS.

Results and Discussion

At the macroscopic level we use osmotic pressure measurements to quantify the thermodynamic interactions between the polymer and the solvent. **Figure 1** shows the osmotic pressure Π as a function of aggrecan concentration in solutions of 100 mM NaCl with different $CaCl_2$ concentrations, 0 mM, 50 mM and 100 mM. All the curves display the same characteristic features, but the osmotic pressure is progressively reduced with increasing calcium concentration.

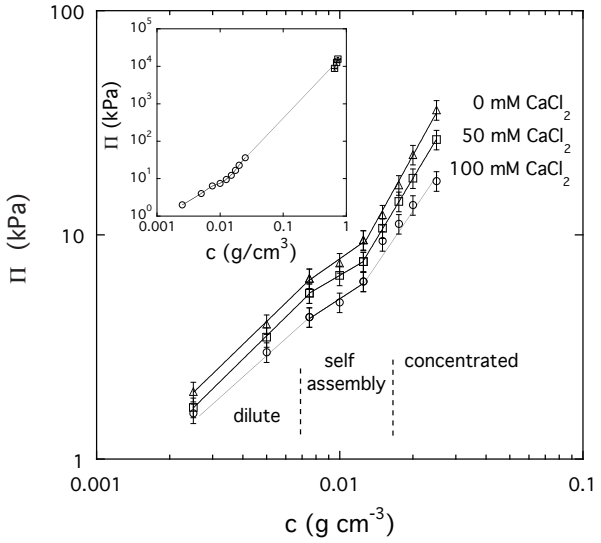

Figure 1. Variation of the osmotic pressure Π as a function of aggrecan concentration in 100 mM NaCl solution, with different amounts of $CaCl_2$. Inset shows Π(c) for aggrecan solution in 100 mM NaCl over an extended concentration range.

In the osmotic response of the aggrecan solution three regions are distinguishable.[9] At low aggrecan content (below 0.05 g/cm^3), Π increases linearly with concentration, as expected for dilute systems where the osmotic pressure is dominated by the independent particles. The decrease in slope in the range 0.005 to 0.015 g/cm^3 is a sign of clustering. Above 0.015 g/cm^3 the slope increases to 2, corresponding to increasing interpenetration of the aggrecan assemblies. In this region the osmotic pressure is no longer due to the number of individual molecules but to the repulsion between each 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 very high concentrations, up to $c \approx 0.75$ g/cm^3 (see inset). The effect of calcium is to decrease Π in all three regions, at concentration thresholds that are independent of the calcium content. In the dilute region the decrease in Π is a sign of a reduction in the number of independent bottlebrushes coexisting with the clusters and demonstrates that calcium promotes self-assembly.[10]

In **Figure 2** are plotted the combined SANS and SAXS spectra from 0.01 g/cm^3 aggrecan solutions containing 100 mM NaCl, in the absence of and with calcium chloride at concentrations of 100 and 200 mM. At low and intermediate values of q the three sets of data points coincide with each other. At low values of q the scattering intensity exhibits a power law behavior with an exponent of -1.9. Over a limited q-range in the intermediate region of

Figure 2, another power law behavior is observed, with a slope of -2.7, characteristic of a slightly interpenetrating branched system. At high q (> 0.08 Å^{-1}) the scattering intensity varies as q^{-1} as expected from the rigid side-chains. In this region $I(q)$ increases slightly with increasing calcium content indicating that the electronic density of the chain increases as calcium ions replace the sodium ions.[10] In all other regions, however, the scattering response of the aggrecan solutions is insensitive to the addition of calcium ions, conferring on aggrecan the role of an ion reservoir mediating calcium metabolism in cartilage and bone.

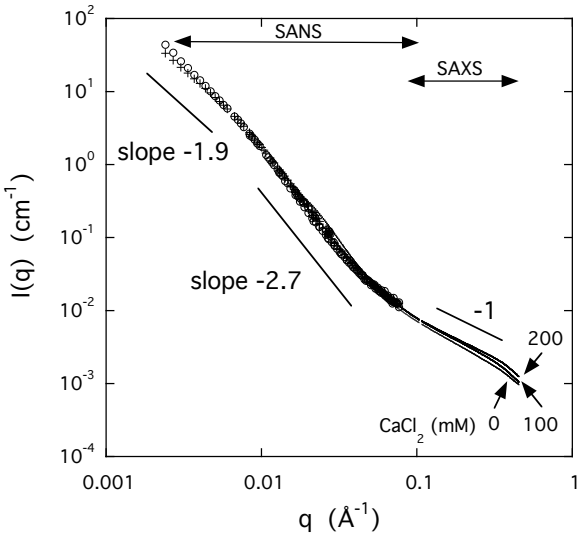

Figure 2. Combined SANS and SAXS spectra of aggrecan solutions (c = 0.01 g/cm^3) in 100 mM NaCl with 3 different $CaCl_2$ concentrations, 0, 100 and 200 mM.

Conclusions

The absence of detectable effect of calcium ions on the structure of aggrecan solution distinguishes this polymer from solutions of linear polyelectrolytes such as charged vinyl polymers. In the latter, multivalent cations adsorb along the polymer chain and generate weak attractive multipolar interactions between adjacent chains. Increasing the calcium ion concentration increases the number of bridging contacts and hence the attractive interaction among the mutually interpenetrating flexible chains. In this process, temporary molecular associations such as bundles are formed. Although the calcium ions are not localized, the cumulative effect of their large number is sufficient to create a strong attraction between chains that ultimately leads to phase separation. By contrast, the rigid bottlebrush structure of the aggrecan molecule and of its aggregates favors the formation of microgel particles, as detected in the SANS observations. Addition of calcium ions, i.e., replacing sodium counterions on the polyanion and changing the electrostatic interactions in the system, does not alter the rigid aggrecan structure. These microgel particles are unable to interpenetrate significantly, i.e., the interior of the aggregates remains inaccessible. As only the molecules at the interface can interact, the number of possible contacts between the aggregates is limited and is insufficient to cause phase separation.

In conclusion, the exceptional insensitivity of the supermolecular structure of aggrecan to the presence of calcium ions can be attributed to the formation of rigid impenetrable gel-like particles. When calcium is introduced, the sodium ions are replaced inside these particles, but the intrinsic rigidity of the aggrecan molecules prevents their collapse. This behavior, which is unique in polyelectrolyte systems, enables aggrecan to act as a reservoir of calcium for its metabolism in bone.

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