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# Interaction of Cartilage Biopolymers

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**Summary:** Aggrecan is a charged biopolymer whose complexes with hyaluronic acid provide the compressive resistance of cartilage. In cartilage extracellular matrix aggrecan-hyaluronic acid complexes are enmeshed within a network of collagen fibrils. Osmotic pressure measurements and small angle neutron scattering (SANS) measurements are reported on solutions of aggrecan and aggrecan-hyaluronic acid complexes. These techniques probe the length scales relevant to the biological functions of cartilage. Osmotic pressure measurements indicate that dissolved aggrecan monomers form microgel-like assemblies. The osmotic pressure of aggrecan-hyaluronic acid complexes decreases with decreasing the ratio of aggrecan to hyaluronic acid. SANS reveals that there is no significant interpenetration between the neighboring aggrecan molecules. Both osmotic pressure measurements and SANS indicate weak interactions between the aggrecan bottlebrushes and collagen fibers.

**Keywords:** aggrecan; collagen; hyaluronic acid; osmotic pressure; small angle neutron scattering

## Introduction

Articular cartilage is a composite material consisting of a relatively small number of cells surrounded by a complex matrix.[1,2] Approximately 70 to 80% of the total weight of cartilage is water. The remaining part is composed primarily of proteoglycans (PGs) and collagen. Aggrecan, the major cartilage PG is consisting of an extended protein core to which glycosaminoglycans GAGs (mainly chondroitin sulfate and keratan sulfate chains) are attached in a bottlebrush arrangement (Figure 1).[3–6] In physiological conditions aggrecan is negatively charged due to the sulfate and carboxylate groups on the GAG chains. In cartilage, aggrecan molecules are condensed on a filament of hyaluronic acid (HA).

Collagen makes up 60 to 70% of the dry weight of the tissue. Type II collagen is the dominant collagen in cartilage, although other types are also present in smaller amounts. The swelling of the aggrecan-hyaluronic acid complexes enmeshed in the collagen matrix plays a critical role in cartilage function. Cartilage swelling is governed by the hydration of the PG molecules, the electrostatic repulsion between the charged carboxylate and sulfate groups, and entropic (mixing) contributions. Under compressive load, the negatively charged aggrecan molecules approach each other, and the repulsive forces increase the stiffness of the system. Changes in the structure and composition of the aggrecan-hyaluronic acid complex (e.g., number of aggrecan molecules condensed on the hyaluronic acid chain), as well as damage of the collagen matrix strongly affect the swelling and compressive properties of the tissue.

The biomechanical properties of cartilage are defined by its composition, the hierarchical organization of and the interactions among the polymeric components of the matrix. Osmotic swelling pressure measurements yield direct information on

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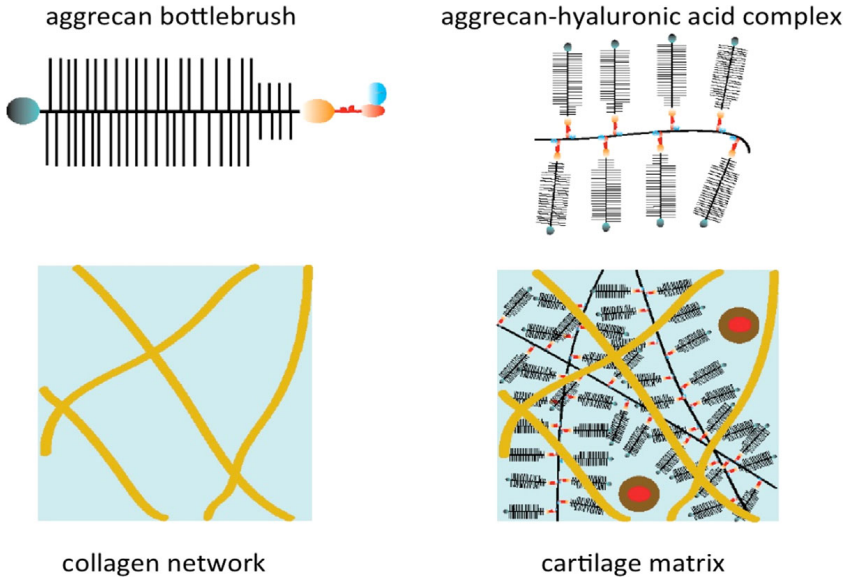


***Figure 1.***
Organization of the main macromolecular constituents in cartilage extracellular matrrix.

the effect of hydration on the macroscopic properties.[7,8] In this experiment, the polymer concentration is varied by adjusting the water activity in the equilibrium bath. There are several other methods for studying the hydration behavior of cartilage constituents. For example, infrared (IR) spectroscopy yields important information on the binding and mobility of water molecules in the vicinity of the polymer chains.[9,10]

Although, previous studies have revealed important physical properties of cartilage extracellular matrix, less attention has been paid to understand the relationship between structural integrity and thermodynamic behavior.[11] For example, it is known that complex formation between aggrecan and hyaluronic acid is critically important to the biomechanical function of cartilage. However, it is not clear how the ratio of aggrecan to hyaluronic acid in the complex affects the compressive properties of the tissue. It is known that aggrecan-hyaluronic acid complexes are enmeshed in the collagen matrix.[12,13] However, it is unclear, how these aggregates interact with each other and with the collagen network. It is also unknown whether PG assemblies are mixed with the collagen fibers on the molecular level or do they coexist as separate entities.

The objective of this work is to answer the questions listed above. We systematically vary the ratio of aggrecan to hyaluronic acid in aggrecan-hyaluronic acid complexes and quantify the effect of these compositional changes on the osmotic pressure. We compare the osmotic response of aggrecan/collagen mixture with that of aggrecan-hyaluronic acid complex/collagen mixture. Small angle neutron scattering (SANS) is used to study the supramolecular structure of aggrecan-hyaluronic acid/collagen system.

## Materials and Methods

### Sample Preparation

Solutions of collagen (type II) (Sigma Aldrich) were prepared in $H_2O$ containing 100 mM NaCl. For SANS measurements the solutions were made in $D_2O$. The concentration of the collagen was varied in the range 0.003–0.04 g/cm$^3$. Samples were made at pH = 7. Aggrecan (bovine articular cartilage, Sigma) solutions were prepared in 100 mM NaCl. The concentration of the aggrecan was varied in the range 0.003–0.03 g/cm$^3$. The pH

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(= 7) was identical in all samples. Aggrecan-hyaluronic acid complexes were made at three ratios of aggrecan to hyaluronic acid (100:1, 50:1 and 25:1).

Mixtures of collagen and proteoglycan solutions (ratio 1:1) were prepared by mixing the appropriate volumes of the above solutions.

All measurements were performed at 25 °C.

### Osmotic Pressure Measurements

The osmotic pressure of the polymer solutions was measured as a function of concentration by bringing them to equilibrium with polyvinyl alcohol gels (PVA) of known swelling pressure.[7] The large size of the dissolved polymers (aggrecan, aggrecan-hyaluronic acid complex, and collagen) prevented penetration into the gel. The size of the PVA filaments was determined by optical microscopy after equilibration in the polymer solution (ca. 24 h). Osmotic pressure measurements were made in aqueous solutions containing 100 mM NaCl.

### Small Angle Neutron Scattering

SANS measurements were performed on the NG3 instrument at NIST, Gaithersburg, MD. Solutions were placed in sample cells with 2 mm quartz windows. Measurements were made at wavelength $\lambda = 8$ Å in the transfer momentum range $2.8 \times 10^{-3}$ Å$^{-1} < q < 0.02$ Å$^{-1}$, where $q = (4\pi/\lambda)\sin(\theta/2)$ and $\theta$ is the scattering angle. After azimuthal averaging, corrections for detector response and cell window scattering were applied. The incoherent background was subtracted following the procedure described in Reference 7. Normalization was carried out using standard NIST samples.[14]

## Results and Discussion

### Osmotic Observations

To gain a better understanding of how extracellular matrix components contribute to the biomechanical properties of cartilage first we investigate the osmotic behavior of PG assemblies in near physiological salt solutions. Our objective is to understand the implications of the hierarchical bottlebrush architecture of cartilage PGs, which ultimately defines the biological function of cartilage.

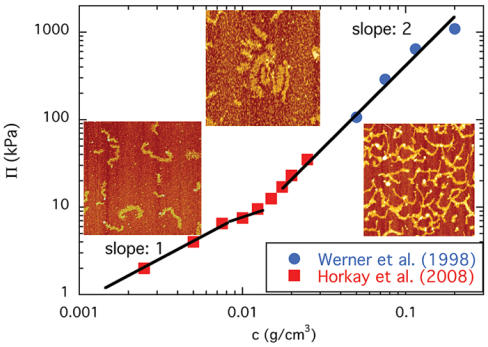


***Figure 2.***
Variation of the osmotic pressure Π with the aggrecan concentration in 100 mM NaCl solution.[7,11,15] The AFM images illustrate the changes in the organization of the aggrecan molecules with increasing concentration.

Figure 2 shows that at low concentration ($c < 0.08$ g/cm$^3$) Π increases linearly with increasing aggrecan concentration. The linear behavior is typical of dilute polymer solutions, and implies that Π is governed by the individual aggrecan molecules as illustrated by the Atomic Force Microscopy (AFM) image. At higher aggrecan concentration, self-assembly of the aggrecan molecules results in a shoulder ($0.008 < c < 0.015$). Self-assembly can be attributed to the difference between the water affinity of the hydrophobic groups at the end of the aggrecan core protein and the hydrophilic, negatively charged GAG bristles. The corresponding AFM image illustrates that individual bottlebrush subunits coexist with loosely packed, microgel-like assemblies. Above the shoulder ($c > 0.015$ g/cm$^3$), Π exhibits power law behavior with an exponent close to 2.

Figure 3 shows the concentration dependence of the osmotic pressure for solutions containing aggrecan-HA complexes. The ratio of aggrecan to HA was set at 100:1, 50:1 and 25:1. In the aggrecan-HA system, the osmotic pressure increases smoothly over

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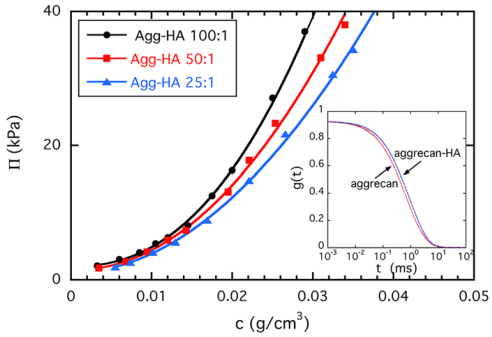


***Figure 3.***
Variation of the osmotic pressure Π of aggrecan-hyaluronic acid solutions with the polymer concentration in 100 mM NaCl solution. Inset: autocorrerlation function of aggrecan and aggrecan-HA solutions measured by dynamic light scattering at 90°.

the whole concentration range without any singular feature. With increasing HA content, the osmotic pressure is gradually reduced indicating that complexation removes free aggrecan bottlebrushes from the solution.

The stability of the aggrecan-HA assemblies is critically important in providing the unique mechanical properties of cartilage, including its high resistance to different loading conditions and shock absorbing properties. In the aggrecan-HA complex the aggrecan molecules are packed around the central HA filament. A high affinity between HA and aggrecan is required to ensure the integrity of such structures and overcome the strong repulsive electrostatic forces. In cartilage the aggrecan-HA complexes are reinforced by a link protein that stabilizes the complex within the extracellular matrix.[16,17] (In the present study we did not add link protein to the system because (i) we did not apply loading that may lead to disintegration of spontaneously formed complexes, and (ii) we tried to minimize the chemical complexity of our model system.)

The inset in Figure 3 shows the autocorrelation functions measured by dynamic light scattering for a 0.1% aggrecan solution with and without HA. The small difference between the two curves indicates that the dynamics of the system is only weakly affected by the connectivity.

Figure 4a shows the concentration dependence of the osmotic pressure for solutions of aggrecan (circles), collagen (squares), and a 1:1 w/w aggrecan collagen mixture (triangles). As expected Π of the mixture is between those of the two components. With increasing polymer concentration, the aggrecan contribution

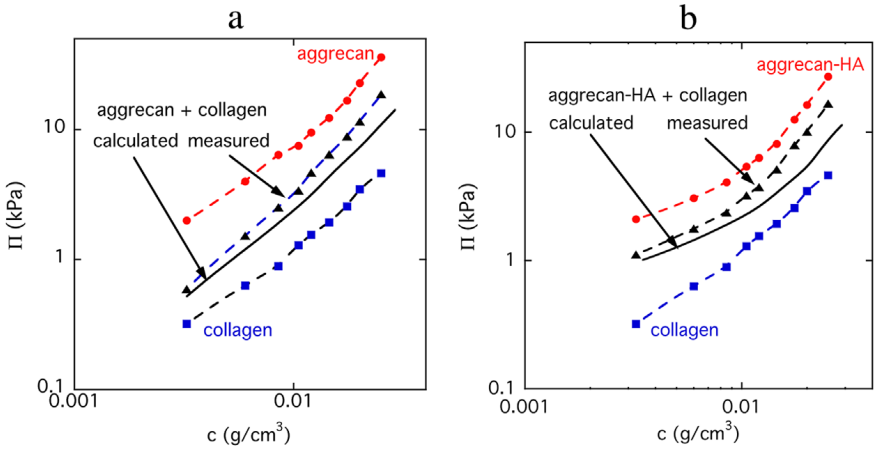


***Figure 4.***
(a) Variation of the osmotic pressure Π as a function of the concentration of aggrecan (circles), collagen (squares) and 1:1 w/w mixture of aggrecan and collagen (triangles). (b) Variation of the osmotic pressure Π as a function of the concentration of aggrecan-HA (circles), collagen (triangles) and 1:1 w/w mixture of aggrecan-HA and collagen (triangles). Continuous curves: calculated dependences of Π of the mixtures assuming additivity of the osmotic pressures of the components. Dashed lines through the data points are guides for the eyes.

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dominates implying that the contribution of collagen to the osmotic pressure is significantly smaller than that of the aggrecan. The continuous line shows the calculated dependence of Π for the 1:1 aggrecan-collagen mixture. In this calculation we assumed that there was no interaction between the two polymers, i.e., $\Pi(c) = \Pi_{aggrecan}(c/2) + \Pi_{collagen}(c/2)$. The calculated curve lies below the measured values indicating that the osmotic contributions of the two polymers are not additive. The deviation from additivity may be the consequence of electrostatic and/or steric interactions between the aggrecan buttlerbrushes and the collagen fibers.

Figure 4b shows the osmotic pressure concentration dependences for solutions of aggrecan-hyaluronic acid complex, collagen, and 1:1 w/w mixture of aggrecan-hyaluronic acid complex and collagen. The continuous curve was calculated by assuming additivity of the osmotic pressure of the aggrecan-hyaloronic acid solution (circles) and the collagen solution (squares). The experimental data lie above the calculated curve. This behavior is qualitatively similar to that observed in the aggrecan-collagen system (see Figure 4a) indicating that complex formation does not significantly influence the interaction between the aggrecan bottlebrushes and collagen fibers.

## Small Angle Neutron Scattering

In the previous section we have seen that osmotic pressure measurements provide important information on the thermodynamic properties of PG assemblies and their interaction with the collagen fibers. Although the thermodynamic response is governed by the organization of the macromolecular constituents and the interactions among them, osmotic pressure measurements do not yield direct information on the underlying molecular and supramolecular structure. To probe the systems at higher resolution we made small angle neutron scattering measurements.

In Figure 5 are shown the SANS profiles of aggrecan solutions measured at different aggrecan concentrations. At low values of $q$,

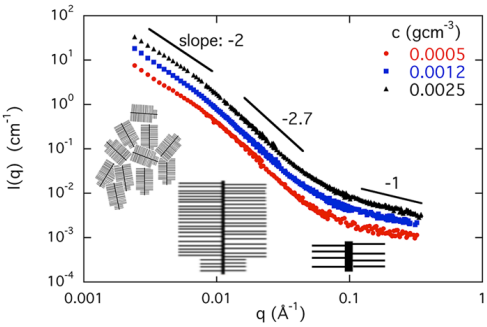


***Figure 5.***
SANS profiles from aggrecan solutions measured at three aggrecan concentrations in $D_2O$ containing 100 mM NaCl. The drawings illustrate typical structural features probed in different $q$ regions.

the curves exhibit power-law behavior, the exponent of which is close to −2. This slope corresponds to swollen branched polymers.[18,19] The upper size limit for these objects exceeds the resolution of the SANS experiment indicating that the aggrecan solutions consist of a suspension of large, microgel-like particles. At values of $q$ greater than 0.01 Å$^{-1}$, a stronger power law behavior is observed. The value of the slope is –2.7, characteristic for branched clusters with screened excluded volume interactions. In this region the scattering response is governed by the bottlebrush shape of the aggrecan molecule. In the highest $q$-range of the figure the SANS response decreases approximately as $q^{-1}$, as expected from the rod-like side chains. The figure also illustrates that the scattering intensity of the solutions $I(q)$ is proportional to the aggrecan concentration, i.e., there is no significant interpenetration between the neighboring bottlebrushes.

In cartilage the aggrecan-HA complexes are confined to the collagen matrix. The interaction between the PG assemblies and collagen fibers is expected to modify the local structure of both components. SANS allows us to identify the characteristic length scales affected by the interactions.

Figure 6 shows the SANS response for an aggrecan-HA solution, a collagen solution and a 1:1 mixture of these components. The signal from the mixture is compared

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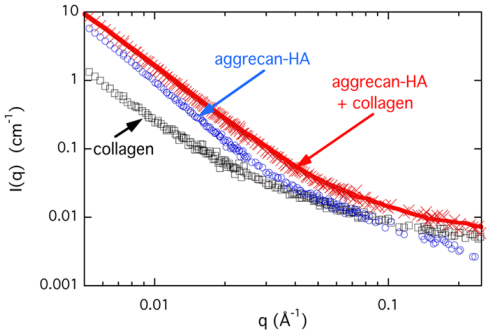


***Figure 6.***
SANS profiles of solutions of aggrecan-HA (circles), collagen (squares), and their 1:1 mixture (x). Continuous curve is calculated from the scattering curves of the components [$I(q) = I(q)_{\text{aggrecan-HA}} + I(q)_{\text{collagen}}$].

with the sum of the signals from the solutions (continuous red curve through the aggrecan-HA data points). The measured and calculated curves are practically identical. This finding implies that each component acts like an independent scatterer, i.e., the interaction between the aggrecan-HA complex and the collagen fibers is weak.

The microgel nature of the aggrecan-HA complex has important consequences for the biological function of cartilage. Microgels are swollen polymer structures that display finite elasticity and, consequently, they exhibit enhanced mechanical resistance to external stress. These features are essential in cartilage biomechanical properties such as its function as a shock absorber. Furthermore, microgels are in equilibrium with the surrounding solution, and they are capable of releasing fluid under hydrostatic pressure. When cartilage is deformed some of the hydration water is forced out and provide enhanced lubrication of the joint surfaces.

## Conclusion

Osmotic pressure measurements made on solutions of aggrecan and aggrecan-hyaluronic acid complexes reveal the presence of microgel-like assemblies. The size of the aggrecan assemblies is of the order of several thousand ångströms.[7] The osmotic pressure of aggrecan-hyaluronic acid complexes decreases with decreasing the ratio of aggrecan to hyaluronic acid.

At low values of $q$ ($<0.01$ Å$^{-1}$) SANS measurements made on aggrecan solutions display a $q^{-2}$ power-law dependence of the intensity that can be attributed to a random association of the primary aggregates. In the intermediate $q$ range ($0.01$ Å$^{-1} < q < 0.1$ Å$^{-1}$) SANS reveals fractal behavior of dimensionality $D = 2.7$, indicating that the aggregates are composed of weakly interpenetrating branched structures. At high $q$ ($>0.1$ Å$^{-1}$) the scattering signal decreases as $q^{-1}$ reflecting the rod-like character of the side chains in the aggrecan bottlebrushes.

Both osmotic pressure measurements and SANS indicate weak interactions between the aggrecan molecules and the collagen fibers. The present results show that combination of osmotic pressure measurements and SANS sheds light on the supramolecular organization of proteoglycan assemblies, and provides important insight into the biomechanical properties of cartilage.

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