

STRUCTURE, CONFORMATION, AND SELF-ASSEMBLY OF CARTILAGE POLYELECTROLYTE MACROMOLECULES STUDIED VIA ATOMIC FORCE MICROSCOPY

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Introduction

Cartilage is a highly specialized, dense connective tissue found between the surfaces of movable articular joints whose main function is to bear stresses during joint motion. This complex biocomposite possesses high stiffness, toughness, strength, resiliency, and shock absorption. The extracellular matrix of cartilage is composed of many different molecules and structures. The negatively charged, disaccharide *chondroitin sulfate glycosaminoglycan* (CS-GAG) macromolecules (**Figure 1a**) are a major determinant of the tissue's ability to resist compressive and shear loading in vivo (e.g., responsible for >50% of the equilibrium compressive elastic modulus under normal physiological conditions (0.15 M salt concentration)).¹ Approximately 100 CS-GAGs are covalently bound at extremely high densities (~2-4 nm separation distance), to a 250 kDa core protein forming the *aggrecan* molecule (**Figure 1b**). The high charge density of the CS-GAGs of conjunction with their close packing cause these polysaccharide chains to take on a rod-like conformation rather than a random coil. Multiple aggrecan molecules self-assemble further to form supramolecular *proteoglycan* aggregates by non-covalently attaching to a *hyaluronic acid* or *hyaluronan* (HA) central filament, an interaction that is stabilized by the adjacent binding of a small glycoprotein called a *link protein* (**Figure 1c**). These aggregates form the gel-like component of cartilage that is enmeshed within a network of reinforcing collagen fibrils.

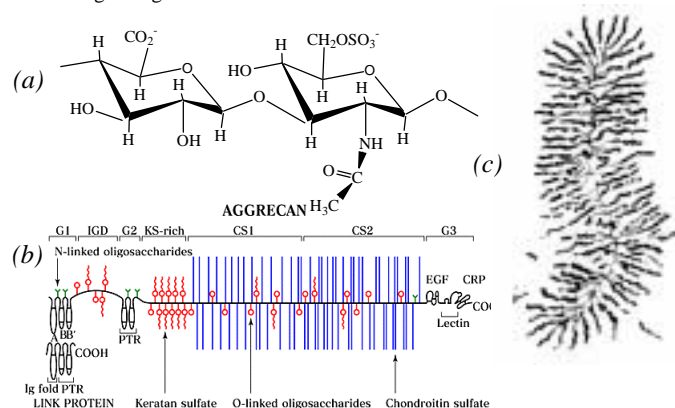


Figure 1. (a) Chemical structure of C6S-GAG, (b) schematic of aggrecan², and (c) rotary shadow electron micrograph of proteoglycan aggregate³

In this study, we have used *Atomic Force Microscopy* (AFM) to image the conformation of the molecular constituents of cartilage aggregating proteoglycans from the length scale of the aggregate (~ μm) down to the level of the individual glycosaminoglycan chains (~nm) in an ambient environment, as well as aqueous solutions of varying ionic strength.

Experimental

Materials. Aggrecan and other ECM molecules were extracted from fetal bovine articular cartilage under dissociative conditions using a 4 M-guanidinium HCl solution with protease inhibitors for 24-36 hours. A series of CsCl-density-gradient centrifugations were used to separate the aggrecan from collagen, hyaluronan (HA), and other extracted ECM components. The pure aggrecan obtained (denoted A1A1D1D1) was dialyzed against DI water to remove excess salts and diluted to concentrations of 5-25 $\mu\text{g}/\text{ml}$. Shark chondroitin sulfate glycosaminoglycan (Sigma) was diluted in MilliQ water 5-25 $\mu\text{g}/\text{ml}$ and filtered with a Centricon Y-100 to remove larger contaminants. Hyaluronan or HA-GAG (Healon sodium hyaluronate, Pharmacia & Upjohn) isolated from rooster combs was obtained as a 1% (w/v) solution in

physiological buffered saline (170 mM NaCl, 33 mM NaH_2PO_4 , 19.7 mM Na_2HPO_4).

AFM Sample Preparation. HA was diluted in MilliQ water 5-25 $\mu\text{g}/\text{ml}$. Aggregates of aggrecan and HA were formed *ex vivo* by combining 250 $\mu\text{g}/\text{ml}$ aggrecan with 2.5 $\mu\text{g}/\text{ml}$ HA and incubating at room temperature for 24 hours, and then diluted to appropriate concentrations. 2 μl of the desired solution was dropped onto freshly cleaved mica. The sample was air dried for at least 1 hour before imaging. For aggrecan imaging under fluid conditions, the mica was modified to form a positively charged surface by treatment with 3-aminopropyltriethoxysilane (APS). A dilution of 0.01% APS v/v MilliQ water was incubated on a cleaved mica surface for 30-45 minutes. The mica was then rinsed in a stream of MilliQ water, dried with N_2 , and used immediately. 60 μl of the aggrecan solution was allowed to incubate on the surface, then rinsed and dried.

Atomic Force Microscopy (AFM). The Nanoscope IIIa Multimode AFM was used to image all samples with the EV or JV scanners (Digital Instruments, Santa Barbara CA). Both contact and tapping modes were employed in ambient temperature and humidity and various aqueous solutions including MilliQ HOH (pH 4.9, IS=0.00006M) and NaCl solutions of ionic strength 0.5M and 0.05M (both at pH=5.9). Nanosensors Pointprobe® silicon tips, driven slightly below the resonant frequency of ~260 kHz, were used for tapping in air. DI silicon nitride tips with a nominal spring constant of 0.32 N/m and length of 100 μm were used for fluid imaging. To minimize sample damage, a slow scan rate of 1-3 Hz was used for a scan size of 5 μm . To obtain high resolution, the set point was minimized and a sample size of 512 was used. Images were processed using WXM software version 1.1 (Nanotec). Height data was flattened to remove background curvature.

Results and Discussion

Individual Aggrecan Molecules. **Figure 2** shows typical AFM contact mode deflection images of low density surfaces with isolated aggrecan molecules in aqueous solutions on APS mica.

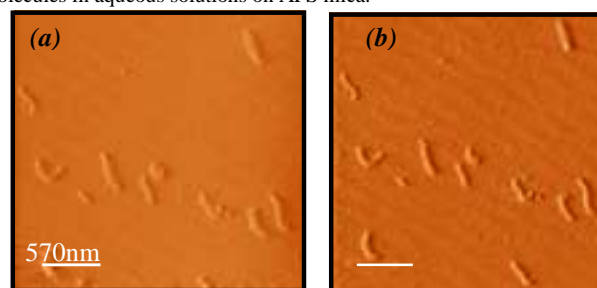


Figure 2. AFM contact mode deflection images taken of aggrecan molecules on APS mica : (a) MilliQ water (IS=0.00006M) and (b) 0.5 M NaCl aqueous solution

It is suspected that the aggrecan molecules remain fixed to the surface via the negatively charged sulfate ($\text{pK}_{\text{aSULFATE}}=2-2.5^4$) and carboxylic acid groups ($\text{pK}_{\text{aCOOH}}=3.5-4^5$) on the CS-GAG chains which bind ionically with the surface amine groups of the APS mica which are positively charged below neutral pH⁶. **Figure 3** shows the mean and standard deviation values for the aggrecan dimensions measured from these images as a function of the ionic strength solution conditions, as well as for air imaging.

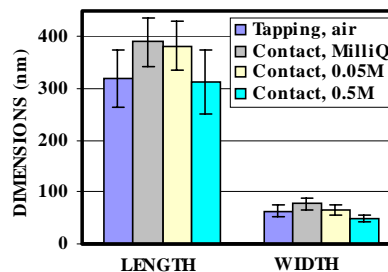


Figure 3. Plots of the means and standard deviation values for the lengths and widths of individual aggrecan molecules as a function of solution conditions and in air as measured by AFM

For the lowest ionic strength solution (MilliQ HOH, IS=0.00006M) the average dimensions were largest; length= 389 ± 47 nm, width= 77 ± 12 nm, and height= 1.7 ± 0.4 nm. This swollen, expanded structure is due to intra- and intermolecular electrostatic repulsion of the negatively charged GAG chains that are fully extended and create a rigid-rod, "bottle brush"-like structure. The average dimensions reduced significantly in the highest ionic strength solution (0.5M) to a length= 313 ± 63 nm, width= 48 ± 0.7 nm, and height= 0.63 ± 0.2 nm, which were similar to the dimensions in air. As the ionic strength is increased, the GAG electrostatic repulsion is screened by counterions that reduce the Debye length causing the GAGs to collapse to a more random-coil like conformation. For air imaging, there is presumably a thin layer of water (few nm) on the hydrophilic mica substrate which keeps the structures partially hydrated. The measured heights are much smaller than expected for native aggrecan (~50 nm) which we attribute to compression by the probe tip, attractive interactions with the surface, and tip-shape effects. **Figure 4** shows a higher magnification AFM tapping mode amplitude image taken in air on mica of an individual aggrecan molecule with identifiable domain regions. A thinner section of the molecule is clearly observed at the top of the structure that is 50 nm in length and 20 nm in width which presumably includes the G1 binding domain, the interglobular (IGD) domain, and the G2 domain. Below this region is the thicker CS-GAG domain with apparent dimensions 250 nm in length and 55 nm in width.

Figure 4. AFM tapping mode amplitude image in air of an individual aggrecan molecule on APS-mica with proposed domain regions labeled

Figure 5 shows noticeably swollen and smoothed individual aggrecan molecules in MilliQ water with the G1, IGD, G2 domains indistinguishable and globular, 60 nm in diameter while the CS-GAG region is 110 nm in width and 350 nm in length.

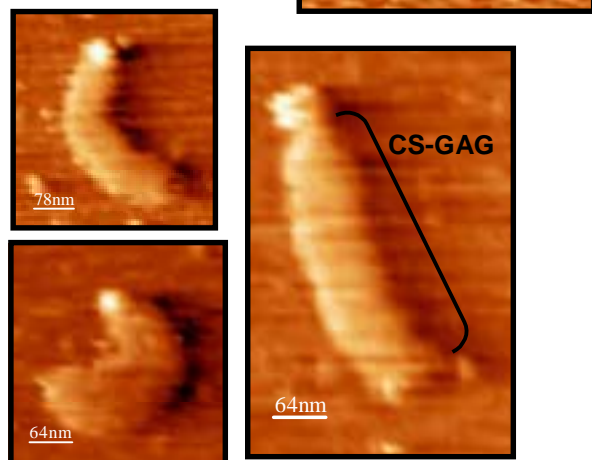


Figure 5. AFM contact mode deflection images taken in MilliQ HOH of individual aggrecan molecules on APS-mica

Individual Hyaluronan Macromolecules. Hyaluronan (HA) (**Figure 6a**) is a linear, negatively charged, self-associating and network forming disaccharide of D-glucuronic acid-beta-1, 3-N acetylglucosamine-beta-1,4- which can reach up to 10,000 repeat units or more. HA acts as a scaffold for aggrecan self-assembly in the extracellular matrix of the tissue. In a dilute physiological solution, HA is an expanded random coil that is stiffened by hydrogen bonds and solvent interactions. The axial hydrogen atoms form a non-polar, relatively

hydrophobic face while the equatorial side chains form a more polar, hydrophilic face, thereby creating a twisting ribbon structure⁷. **Figure 6b** shows an amplitude image taken in tapping mode in air of individual HA chains deposited on mica that have an average height of 0.3 nm.

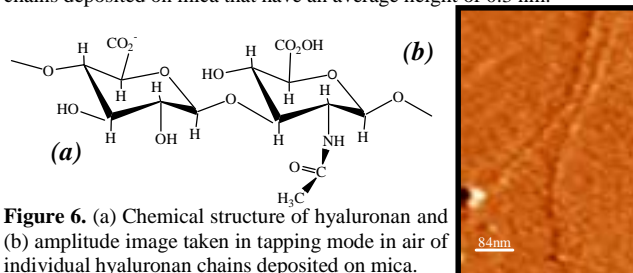


Figure 6. (a) Chemical structure of hyaluronan and (b) amplitude image taken in tapping mode in air of individual hyaluronan chains deposited on mica.

Proteoglycan Aggregate. The globular N-terminal G1 domain of aggrecan contains a lectin-like binding site with high affinity for hyaluronan that is thought to be responsible for the formation of proteoglycan aggregates. It is highly thermally stable but easily perturbed by alterations in the structure of several amino acids². Each hyaluronan chain can bind a large number of aggrecans to form aggregates up to several hundred million Da in molecular weight. **Figure 7** shows an AFM tapping mode amplitude image taken in air on mica of a proteoglycan aggregate (aggrecan + HA) deposited on mica. In many images, it was observed that the aggrecan molecules aligned in pairs on opposite sides of the HA molecule at relatively low densities, separated by an average distance of 250-350 nm. It was difficult to observe the individual HA chains of aggregates due to the smaller height of HA compared to aggrecan

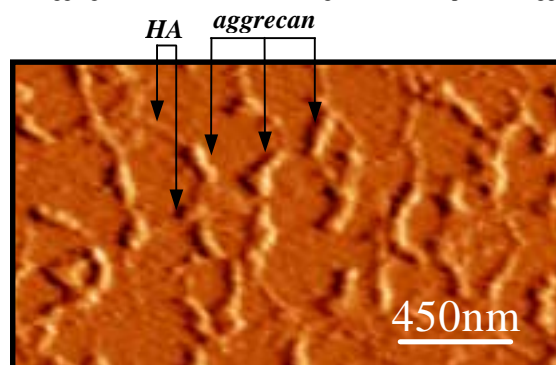


Figure 7. AFM tapping mode amplitude image taken in air on mica of a proteoglycan aggregate (aggrecan + HA) deposited on mica

Conclusions

AFM is a powerful tool for imaging the nanoscale dimensions and shapes of biological macromolecules in solution. Using AFM, we were able to directly visualize the domain structure of individual aggrecan molecules and the variations in aggrecan conformation as a function of ionic strength. As expected, a large increase in height was observed when the aggrecan monomers were fully hydrated at low ionic strength. As the ionic strength was increased, the counterions shielded the negative charges of the GAG chains, thereby reducing the Debye length and collapsing the molecule. The self-assembly process of aggrecan and hyaluronan to form aggregate was also directly observed. Ongoing experiments include studies of effects of ionic strength and pH on aggrecan conformation, characterization of the kinetics of the aggregate self-assembly process, and the use of specific G1- and G3-antibodies to further confirm aggrecan structures visualized by these methods.

Acknowledgements. Funding was provided in part by the Whitaker Foundation (L. Ng), the Dupont-MIT Alliance, and NIH AR45779.

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⁷ <http://www.glycoforum.gr.jp/science/hyaluronan/HA01/HA01E.html>