Tentative Title: Modeling and Measurement of Intermolecular Interaction Forces between Cartilage ECM Macromolecules

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Brief Description of the Research:
The mechanical properties of cartilage tissue depend largely on the macromolecules that make up its extra-cellular matrix (ECM). I propose to study the interactions between the different ECM macromolecules using a combination of high resolution force spectroscopy experiments and modeling. These studies should contribute to a better understanding of the way cartilage works and of the factors that contribute to its degradation with age and disease.
1 Introduction

The motivation of this research is to determine the underlying molecular mechanisms responsible for the macroscopic compressive stiffness of cartilage tissue. It is believed that this compressive stiffness is largely due to large molecules called aggrecan found throughout cartilage tissue. Aggrecan is composed of a core protein to which are attached many highly charged glycosaminoglycan (GAG) chains (Fig. 1). The nanomechanical properties of aggrecan, primarily electrostatic repulsion between its GAG chains, are thought to be one of the major determinants of cartilage’s high compressive stiffness. For my thesis, I propose to undertake a thorough study of the interactions between aggrecan molecules. Such research should contribute to a better understanding of the way cartilage works and of the factors that contribute to its degradation with age and disease. From a broad perspective, my research will consist of a set of experiments to measure the molecular-level interactions between aggrecan, the results of which will be compared to and used to develop better theoretical models of these interactions.

High resolution force spectroscopy (HRFS) instruments, like the atomic force microscope (AFM) and the molecular force probe (MFP), make it possible to not only image macromolecules such as aggrecan but also measure the small nano-Newton scale forces associated with their interactions in a variety of environmental conditions. However, in order to better understand the origin of these forces one must establish a connection between the experimental data and mathematical models based on polymer theory. I propose to measure aggrecan interactions directly using HRFS and then compare the measured forces to continuum models. This will require the development of models designed for the specific molecular-level geometry of our system. These models should be able to account for specific features of the molecular structure important for their function, and may give good insight into the configuration of cartilage macromolecules under physiological conditions. To model electrostatic interactions, I plan to use a continuum Poisson-Boltzmann (PB) model in which the space that each individual molecule occupies is modeled as a cylindrical volume with fixed charge. This approach could have advantages since it incorporates some of the molecular structure of the experimental set-up and is applicable over a wide range of experimental conditions while still remaining computationally tractable.

2 Background

2.1 Cartilage Biology

Articular cartilage is the load bearing connective tissue found on the surface of movable joints. It normally sustains high compressive loads, 10-20MPa, without damage. This tissue is composed of 70-80% water by weight and contains only 20-40 thousand cells, called chondrocytes, per cubic millimeter [14]. This low density of cells is able to maintain the extracellular matrix (ECM) under normal conditions (Fig. 1). The biomechanical properties of cartilage, such as its high compressive resistance, are directly related to the molecular structure of extracellular macromolecules. The collagen molecules contribute to the tissues’ shear and tensile strength, while the highly charged proteoglycan molecules provide most of the compressive strength.

Proteoglycans make up 5-10% of the cartilage wet weight (35% by dry weight) [14]. They are com-
posed of a long core protein to which one or more glycosaminoglycan (GAG) chains are covalently bound. Aggrecan is the most abundant proteoglycan in cartilage (Fig. 2). The core protein of aggrecan contains three globular domains (Fig. 2b). The first, G1, is found near the amino-terminal and uses a link protein to attach to the binding region of hyaluronan, a glycosaminoglycan consisting of several thousand repeating disaccharide units. The second globular domain, G2, is found further down on the core protein and the third globular domain, G3, is found near the carboxyl-terminal of the core protein. Between G2 and G3 is a highly charged glycosaminoglycan rich region. The core protein is several hundred nanometers long and ~300kDa (Fig. 2a) [16]. Proteoglycans, such as aggrecans, form large aggregates with hyaluronan (Fig. 1).

Aggrecan contains three major types of glycosaminoglycans (GAG): chondroitin-6-sulfate, chondroitin-4-sulfate, and keratan-sulfate. The keratan-sulfate chains are the shortest (~5kDa) consisting of about 10 repeating disaccharides. These chains are located mainly near the G2 region of the core protein (Fig. 2b). The chondroitin-sulfate (CS) polyelectrolyte chains (Fig. 3a) occupy most of remaining GAG-rich region of aggrecan and comprises 95% of the molecular weight of the entire proteoglycan molecule (Fig. 2). The CS chains are 30-40nm long and are spaced approximately 2-4nm apart on the core protein. One end of the CS is covalently linked to the core protein while the reducing end points into the intra-tissue space such that aggrecan is presumed to have a bottle brush-like shape. The CS-GAG chain is composed of alternating glucuronic acid and N-acetyl-6(or 4)-sulfate galactosamine (Fig. 3b). Under normal physiological conditions, the carboxylic acid and the sulfate groups are negatively charged. CS-GAGs have been studied extensively, as their intermolecular electrostatic repulsive forces are responsible for more than 50% of the equilibrium compressive modulus of the articular cartilage [3]. Also, it has been found that the sulfation of GAG chains, and therefore its charge, is decreased in cartilage disease, such as osteoarthritis [19].

2.2 High-Resolution Force Spectroscopy (HRFS)

Recent developments in high-resolution force spectroscopy (HRFS) have made it possible to measure the nanoscale interaction forces between molecules [8, 23, 24]. Atomic force microscopes (AFMs) have been popular tools for imaging surfaces and recent work has shown that they are reliable tools
1.1 nm

Figure 2: (a) Tapping-mode atomic force microscope image of an aggrecan monomer in air on a mica substrate (courtesy of Laurel Ng[16]) (b) Chemical structure of aggrecan[28]

Ser = Serine
Xyl = Xylose
Gal = Galactose
GlcNAc = N-Acetyl-galactosamine

GlcNAc = N-Acetyl-galactosamine-6-sulfate

Figure 3: (a) The structure of Glycosaminoglycan, (b) The molecular structure of Chondroitin-6-Sulfate.

for force measurements as well [1, 4]. The molecular force probe (MFP) (Fig. 4) is very similar to the AFM but has been optimized specifically for the measurement of force. It overcomes some of the optical interference problems inherent to standard AFMs and has slightly better resolution.

In HRFS, a planar substrate is slowly approached by a small cantilever or spring with a tip of known size held by a piezoelectric ceramic translator. As the tip feels repulsive or attractive forces, the cantilever bends and its deflection is measured by a laser (Fig. 5a). Thus, a cantilever deflection versus piezoelectric actuator distance curve (deflection curve) is obtained. The cantilever spring constant is measured by analyzing its resonant frequencies [4, 12]. The data is then converted into a force versus distance curve (force curve) using the measured cantilever spring constant (Fig. 5b). These measurement techniques are particularly useful for biological applications because they can be carried out in an aqueous environment without harming the sample. Therefore, using these techniques, it is now possible to make molecular level measurements of the repulsion forces between the cartilage macromolecules, leading to a better understanding of how cartilage behaves in a variety of conditions.
2.3 Modeling Polyelectrolyte Brushes

Since the GAG region of aggrecan is a highly charged brush, it may be useful to compare the forces measured to polyelectrolyte brush models. There have been several different approaches used in the literature for modeling polyelectrolyte brush interactions. Molecular dynamic simulations of individual polyelectrolyte macromolecules have provided information on chain conformation and supramolecular structure. However, since brush layers involve the interactions between many molecules (e.g. polymer chains, ions, and water molecules), this technique is computationally intensive and currently has limited application for predicting brush interaction forces at physiological conditions [6]. Scaling theory is another method used to characterize polyelectrolyte brush interactions [18, 20, 26], and provides straightforward analytical solutions [18]. However, each scaling law can only be applied to certain distinct sets of experimental conditions (e.g. solution ionic strength, pH, polymer density, chain length) [26]. Continuum theory [9, 17, 25] is applicable to a wide range of experimental conditions while still remaining computationally tractable and allows direct quantitative comparison with experimental data. However, the continuum approach does not account for structure and interactions at the atomic level.
Figure 5: (a) An example of a deflection curve, (b) An example of a converted force curve.

Figure 6: Schematic of experimental setup: probing aggrecan with a negatively charged tip

3 Proposed Methods

3.1 Proposed Experimental Methods

To measure the interaction forces between aggrecan monomers, aggrecan must be attached to a substrate and probe tip which will then be placed in an MFP for force measurement. In order to get accurate and meaningful measurements of aggrecan intermolecular forces, a method for the robust attachment of aggrecan to an atomically flat surface must be developed. The attachment method must make a dense monolayer of aggrecan with minimum debris which should at the same time be able to withstand changes in pH and ionic strength of the bath. After modification, the substrate will first be imaged to verify the presence of the monolayer and to approximate the density of the monomers on the surface. The substrate will initially be probed with a variety of functionalized AFM tips of known surface chemistry, such as carboxyl self-assembled monolayer (SAM) (Fig. 6) [22], sulfate monolayers [23], and GAG brushes [24]. Finally, if a suitable method of attachment is found, aggrecan will be attached to the tip and the forces between two approaching aggrecan monomers will be measured.
3.1.1 Attachment of Aggrecan to Mica Substrate

One non-covalent attachment method that has been successfully applied \[16\] creates an aggrecan monolayer using the electrostatic interaction between the negatively charged monomers and the positively charged AP mica. Purified A1A1D1D1 aggrecan monomers from the fetal bovine epiphyseal growth plate are dialyzed first against 500 volumes of 1 M NaCl and then against HOH to remove excess salts to 5-25 mg/ml. The surface of freshly cleaved muscovite mica 1cm × 1cm squares (Pelco International, Redding, CA) is made to be positively-charged by amine-functionalization (Fig. 7). The substrates are treated with \( \sim 50\mu l \) 0.01 \% 3-amino-propyltriethoxysilane (APS) for 20-30 min (Sigma Aldrich Co., St. Louis, MO) v/v MilliQ water (Millipore Corp, Bedford, MA.). \( \sim 50\mu l \) of 500 5g/ml aggrecan solution is allowed to incubate on the APS-mica for 30-40 min, then rinsed, and dried in air for at least 10 hrs before imaging in tapping mode in ambient conditions with a Nanoscope IIIa Multimode atomic force microscope (TMAFM) (Digital Instruments (DI), Santa Barbara, CA) or a 3-D Molecular Force Probe (3-D MFP) (Asylum Research) using Olympus AC240TS-2 Si cantilevers (probe tip radius \(<10nm\), spring constant=2 N/m).

This non-covalent attachment method creates a dense layer on the substrate and also a relatively clean surface free of debris. However, this method may have some drawbacks since the strength of the electrostatic varies greatly with changes in the environment pH and ionic strength. Specifically, it appears that some of the aggrecan may detach from the surface at bath ionic strengths slightly higher than physiological conditions (IS > 0.1M). Also, this method is not appropriate for tip modification as any aggrecan on the tip would be attracted to substrate while the tip and substrate are in contact and therefore the aggrecan on the tip would mostly likely come off. Therefore, I am also planning to try to use a covalent attachment method if a suitable chemistry can be found. To date, several candidate covalent attachment methods have been evaluated with limited success. The major setback seems to be the impurities in the chemicals which adhere to the substrate before the aggrecan has a chance to react.

3.1.2 Probe Tip Modification

In order to understand measured aggrecan forces, a succession of measurements from differently modified tips on an aggrecan monolayer will be used to isolate different aspects of the interaction forces.
Figure 8: Diagram of how to apply an electric field between the tip and an electrode to increase the coating density of a negatively-charged polymer on the tip.

- **Carboxyl SAM Tip.** Probe tips of known negative surface charge density (-0.018C/m$^2$) can be made by modifying Au-coated Thermomicroscopes V-shaped Si$_3$N$_4$ cantilevers (probe tip radius, R$_{tip}$ ~ 50nm, spring constant ~ 0.01N/m) incubated in 2 mM solution of 11-mercaptoundecanoic acid (HS(CH$_2$)$_{10}$COOH (Aldrich) in ethanol for 48 hrs, followed immediately by rinsing with DI water before experimentation. This forms a well-ordered self assembled monolayer (SAM) on the surface of the tip and therefore the charge density is very consistent from tip to tip. The forces will be measured at different ionic strengths (0.0001M-0.1M NaCl) to see how the interactions change as the salt is increased to shield the charge interactions. This tip will be used first so that the experiments on aggrecan may be directly compared to the experiments on GAG brushes. However, carboxyl groups have a pKa ~ 4 and so both the carboxyl groups on the aggrecan and those on the tip will loose their charge at low pH. Therefore, it is necessary to make a tip with a negative that has pKa much lower than 4.

- **Sulfate Tip.** Probe tips with a sulfate monolayer can be made by immersing Au-coated Thermomicroscopes V-shaped Si$_3$N$_4$ in a 5mM ethanol solution of mecaptoethanesulfonic acid (Aldrich) for 24 hours then backfilling using ethanethiol (Aldrich). This will create a negatively charged tip whose charge density remains relatively constant at pHs above ~ 2. Therefore, using this tip, I can measure the difference in force as the aggrecan’s carboxyl groups lose their charge in a low pH environment. However, this is not a well ordered packing and so the charge density (~ -0.001C/m$^2$) is not as consistent with each tip. It is therefore a little more difficult to directly compare measurements on aggrecan to the results found with GAG brushes with these tips.

- **GAG Tip.** The CS-GAG, obtained from rat chondrosarcoma cell cultures, can be end-grafted onto Au-coated Thermomicroscopes V-shaped Si$_3$N$_4$. In this method, an electric field is applied between the probe tip and a Pt electrode immersed in a 1mg/ml CS-GAG solution using the closed liquid cell of an AFM (Fig. 8) for 9 hrs. The probe tip is grounded and a negative voltage (-0.15V) is applied to the Pt cathode via a cap on the piezoelectric scanner. The separation distance between the probe tip and the Pt electrode is about 100nm. The sharp probe tip geometry results in about a 10-fold higher E-field strength near the tip apex compared to the E-field at the Pt surface below. The voltage will kept low so that the resulting small, non-Faradaic current (< 200 nA) minimizes chemical reactions at the probe tip that
could lead to electrolysis. The probe tip is then immersed in 5mM 11-mercaptoundecanol ethanol solution to backfill the unreacted portions of the surface. This tip could be used to probe first a GAG brush then an aggrecan monolayer. It would be very interesting to compare the two measurements to see if there are added forces just due to arrangement of GAGs along the core protein of aggrecan versus the GAGs arranged in a uniform brush on the substrate.

- **Aggrecan tip.** Eventually, the goal of this research would be to measure the interaction forces between 2 approaching aggrecan monomers. First a suitable chemical scheme for attaching the aggrecan to Si$_3$N$_4$ or Au needs to be developed. Then, because the AFM probe tip is small, it still might be difficult to attach the aggrecan to the probe tip using a chemical reaction that is found to be effective on flat substrates. If that is the case then I will try to increase the local concentration of aggrecan monomers near the tip during attachment by using an electric field as described for the GAG tip (Fig. 8).

### 3.2 Electrostatic Model Methods

I will use a continuum approach to model the electrostatic component of interactions between polyelectrolyte molecules in a brush layer. The applicability and accuracy of three increasingly refined theoretical models (Fig. 9) based on the Poisson-Boltzmann (PB) equation were first examined via a rigorous quantitative comparison with high resolution force spectroscopy (HRFS) experimental data on a model CS-GAG brush system [23]. The PB approach predicts the electrostatic double layer force between charged surfaces due to electrical and osmotic interactions associated with polyelectrolyte fixed charge and the mobile ions in solution. The first two models have been reported previously in the literature [4, 17] and the third is a new model whose development represents a significant portion of the work to date on this research [7]. Each of the three models employs increasingly more geometrically-specific representations of the polyelectrolyte macromolecular fixed charge.

In the first model (Fig. 9a), a polyelectrolyte brush layer is represented as a uniform, flat constant surface charge density [4]. The second model (Fig. 9b) approximates the polyelectrolyte brush as a uniform volume charge density [17]. Even though this model takes into account the height of the brush, the molecular shape and charge distribution along the polyelectrolyte chain backbone are not included. The third model (Fig. 9c) represents the time average space occupied by the individual polyelectrolyte macromolecules in the brush as cylindrical rods of uniform volume charge density and finite height. This approach attempts to account for additional aspects of polymer molecular geometry and nonuniform molecular charge distribution inside the brush. It should be noted that this model is different from the “unit cell” model [13] where each polyelectrolyte macromolecule is represented as an infinitely long cylinder having a fixed surface charge.

First, the electrical potential and the spatial distribution of ions were computed in the region between a planar brush layer and a charged planar surface situated above the brush as a function of separation distance D, shown in Fig. 9a-c. Then, the electrostatic forces between the brush and the charged planar surface predicted by each of these 3 models were compared to each other using a range of model parameters and bath ionic strengths. The models were then adapted to the experimental configuration [23] of Fig. 6, incorporating the geometry of a hemispherical probe tip.
with known surface charge density situated above the brush (Fig. 9d-f) instead of the charged planar surface [2]. Model predictions were then compared to HRFS measurements of the total repulsive force reported in the literature by us [23]. Because the equations relating the electrostatic force to the known system parameters (e.g., GAG charge density and bath ionic strength) are nonlinear and are difficult to solve analytically, finite difference methods (FDM) were used to obtain numerical solutions of the models.

4 Collagen Proteoglycan Interactions

The interactions between proteoglycans and collagen has been shown to regulate collagen fibril fusion and creates strong interfibrillar binding in tendons [11, 21]. It has also been shown with AFM images that this interaction is responsible for the highly regular interfibrillar collagen spacing
distinctive to corneal stroma.[21] Light absorption studies on solutions of collagen from bovine nasal cartilage mixed with proteoglycans showed that different proteoglycans (such as aggrecan, byglycan, and decorin) changed how the collagen fibrils formed in solution.[27] However, these studies only indirectly measure collagen-proteoglycan interactions and so the molecular details of collagen-proteoglycan interaction are still not completely understood. Therefore, I propose to study this interaction through a combination of modeling and HRFS experiments. I plan to measure the forces between surfaces coated with different types of collagen and tips coated with either aggrecan or a GAG brush (Fig. 10). These would be direct measurements of the interactions between proteoglycans and collagen. Comparisons of these measurements to theoretical models of intermolecular forces may lead to insight on their mechanisms.

References


