Measuring the Electrostatic Repulsion Forces Between Glycosaminoglycans Using the Atomic Force Microscope

by

Jiang-Ti Kong

Massachusetts Institute of Technology, S.B, 1997

Submitted to the Department of Materials Science and Engineering in partial fulfillment of the requirements for the degree of Master of Science in Materials Science and Engineering at the MASSACHUSETTS INSTITUTE OF TECHNOLOGY

September 1999

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Abstract

Electrostatic repulsive interactions between glycosaminoglycan (GAG) molecules are the principle factor that contributes to the compressive strength of articular cartilage tissue. This thesis describes the theoretical and practical background to directly measure these repulsive forces between individual glycosaminoglycan molecules under aqueous conditions. Specifically, these interactions could be measured by attaching a glycosaminoglycan-coated silica microsphere to the cantilever of an atomic force microscope (AFM), which would then approach a flat silica substrate, also coated by the same glycosaminoglycans. The force will be deduced from the bending of the Hookian cantilever.

In this thesis, methodologies have been developed to coat the microsphere and the flat silica surface with chondroitin-sulfate GAG's, to remove all non-specifically absorbed molecules from the surface, and to characterize the density of coating. Specifically, it was found that sonicating the surfaces in 2% SDS solution for twenty minutes was enough to remove all non-specifically absorbed GAG's. Using radioactive chondroitin-6-sulfate from rat chondrosarcoma cells, we were able to coat it to carboxylic acid modified silica beads at a density of 27 ng/cm², which corresponds to 1 CS molecule per 10nm × 10nm. The activation chemistry involves the use of dicyclohexyl carbodiimide (DCC) and N-hydroxysuccinimide (NHS) in tetrahydrofuran (THF). The methodology to coat the plain silica surface involves one extra step - functionalization of the plain silica surface with carboxylic acids using a carboxy silane. Following the same coating protocol after the silane treatment, we were able to coat the flat substrate with CS at a density of 0.4 ng/cm², which corresponds to 1 molecule per 82nm × 82nm. The actual spacing between CS molecules in tissue is 2 to 4 nm.

Furthermore, we discovered that the CS concentration is not critical (between 0.1 to 5 mg/ml) when the coating is carried out over 16 hours. Under aqueous conditions, the best coating pH is 8.4. Because the very low solubility of GAG's in organic solvents (≈ 60 µg/ml), coating carried out in organic solutions (N-methyl-formimide, and dimethyl-sulfoxide) did not succeed.

Thesis Supervisor: Alan J. Grodzinsky
Title: Professor of Electrical, Mechanical and Bio-engineering
Acknowledgments

Although he didn’t manage to convert me into an avid fellow coffee drinker, Alan did succeed in teaching me the wonders of beers. If it were not for him, I still wouldn’t have recognized the difference between Boston Ale and Corona. Just like this induction to beers, gently, with patience, Alan has always pushed me to venture into realms that I first considered beyond me, be it foreign beers, strong coffee, bad jokes about Med school and cadavers, or doing research that no one has tried before. I would like to thank him sincerely for providing me the opportunity to develop an exciting project, teaching me the essential elements of a good scientist, and for guiding me along my path.

In addition to Alan, the development of the AFM project wouldn’t have been possible without the generous help from the following experts: Professor Anna Plaas, whose knowledge on chondroitin sulfate was crucial to our research; Professor Laibinis, who has been indispensable in making coating the flat substrates possible; Professor Ortiz, who not only did exciting works in AFM but also generously agreed to be my co-supervisor in DMSE; Professor Seeberger and Professor Evans, both of whom offered great opinions from their unique perspectives (chemistry and polymer physics). Last but not least, I would also like to express my sincere appreciation to Dr. Eliot Frank, who has always been patient with my never-ending questions and who has always offered the most efficient help when it comes to computers.

Ivan Lee has been absolutely indispensable to this project, too. His experience and expertise helped us especially in developing reliable controls. Delphine joined me three months ago, and has done a great deal of good work and learning in this three months. She is an excellent scientist and I am very lucky to have her help. I am sure that I’ll miss her sharp insights, her fine humor, and her impeccable french taste in lab fashion :) Libby Shaw from CMSE was the first person who taught me how to use the AFM, then I was also fortunate to get help from Gillian Brown of CBE.

My experience in this lab wouldn’t be complete without Linda, whose jokes always crack me up and, she is one of the few people that get my silly jokes. Her support
and wisdom has been a great source of help and comfort. I would also like to thank Han-Hwa for not only her help in lab, but also her encouragement and her care.

I was very fortunate to have received help from many other people: Parth, Cindi, Moonsoo, Nora, Alex, and Bodo. Their generosity and help are sincerely appreciated. Jiehyun, Namyoung, Murry, and Dan have also helped me while I worked in another lab.

I hope I didn’t forget anyone in this long list. Indeed, I would like to thank everyone for just being everyone, making life here as diverse, as fun an experience as it can be.

Finally, I would like to thank my mother, whose love and strength have sustained me and who taught me the principle of never giving up. I would like to thank my father and my sister for always being there for me. I love you all very much.

Just in case you are not tired of my long list of thank you’s, I’d also like to mention that this research is funded in part by NIH Grant AR33236 and AR45779, as well as by the National Science Foundation Fellowship.
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Chapter 1

Introduction

1.1 Articular Cartilage and Overall Objective

Articular cartilage is the dense, resilient tissue that lines the articulating surfaces of weight-bearing joints. Its equilibrium modulus is directly related to its molecular composition. [1] As shown in Figure 1.1, articular cartilage is composed of water, which comprises 75 to 80% of the total wet weight, and a dense extracellular matrix of collagens and proteoglycans. The strong collagen fibrils contribute to the tensile and shear strength of the tissue, while the highly charged glycosaminoglycan (GAG) chains attached to the proteoglycans are responsible for the compressive strength of cartilage due to electrical repulsive forces. In short, the GAG chains are highly negatively charged and densely packed. [2] Under compressive loading, the molecules are forced from their normal equilibrium spacing into positions much closer to each other. The resultant strong electrostatic repulsion force produces a swelling pressure that resists compressive loading; hence the contribution to compressive strength.

The long term goal of this research project is to directly measure the electrostatic repulsive forces between individual glycosaminoglycan chains as a function of intermolecular distance and chain density using the atomic force microscope.

The specific objectives of this thesis are to determine the methods and chemical procedures for attachment of chondroitin sulfate GAG chains to the cantilever microsphere and flat substrates used within the AFM, and to determine methods for
Figure 1-1: The Extracellular Matrix of Articular Cartilage
quantitative assessment of the density and configuration of GAG chains after attachment.

1.2 Structure and Chemistry of GAG’s

Glycosaminoglycans exist in cartilage as components of proteoglycan molecules. The large proteoglycan aggregates (Figure 1.2) consist of a hyaluronic acid backbone (whose radius of gyration is approximately 40 nm) to which many aggregan molecules are attached non-covalently, stabilized by by a link protein. [1] The aggregan molecule ($2 \times 10^6$ Da) is composed, in turn, of a core protein, to which the GAG chains are covalently attached like a bottle brush. The core protein ($\sim 300$ KDa) is is about several hundred nanometers long. The chondroitin sulfate GAG chains (30 to 40 nm long) are spaced approximately 2 to 4 nm away from each other, equivalent to several electrical Debye length. Shown in Figure 1.3 is a picture of a typical GAG molecule, chondroitin-6-sulfate, abundant in articular cartilage. [3] One end of the 40 nm long polymer is covalently linked to the core protein through a galactose-galactose-xylose-serine sequence, while the other end, the reducing end, points into the intra-tissue space. The CS-GAG chain consists of alternating glucuronic acid and N-acetyl-6-sulfate galactosamine. Both the carboxylic acid moiety (on GluA) and the sulfate
moiety (on GlcNAc) are negatively charged under normal physiological conditions. At 0.15N, the normal physiological ionic strength, the Debye length is approximately 1nm.

The high charge density, as well as the short spacing between the GAG chains, compels the polysaccharide chain to assume a rod-like configuration rather than a random coil. It is the strong repulsive forces between these rod-like molecules that contribute to more than half of the equilibrium modulus of cartilage. The correlation between the charge/architecture of the GAG’s and the compressive strength of articular cartilage have been extensively studied and modeled. [2, 4, 5] One crucial assumption in these models is the actual force between individual glycosaminoglycans. Up to now, no one has been able to measure it directly and quantitatively. Encouraged by the rapid advances in atomic force microscopy, we decided to tackle this problem with AFM.

If such intermolecular electrostatic forces can, indeed, be measured directly (e.g. via AFM), it would then be possible to relate such forces to the specific structures of the CS-GAG chains, which are known to vary with age and diseases.

1.3 The Atomic Force Microscope

1.3.1 Working Principle

The atomic force microscope works by the controlled approach of a tip (which is attached underneath a cantilever spring) to a sample on top of a piezoelectric mount (Figure 1.4). The deflection of the cantilever is recorded by a position sensitive laser diode (PSD), which registers the exact location of the laser beam that is reflected from the gold-coated tip of a cantilever. The force is monitored by recording the cantilever deflection. Under normal conditions, the force between the tip and the substrate is directly proportional to the bending of the cantilever in a manner described by Hooke’s law. To obtain a topographical image of the sample surface, one can either fix the position of the piezoelectric and monitor the cantilever deflection or vice versa.
Asn  Asparagine
Gal  Galactose
GalNAc  N-Acetyl-galactosamine
GlcNAc  N-Acetyl-glucosamine
GluA  Glucuronate
Man  Mannose
N  Nitrogen atom
NeuNAc  Sialic acid
O  Oxygen atom
Ser  Serine
Xyl  Xylose
\( \gamma \)  Carboxyl group
Sulfate group

Figure 1-3: Molecular Structure of Chondroitin-6-Sulfate
Figure 1-4: Structure of An Atomic Force Microscope
The AFM had been used primarily for imaging purposes until recently, when methods were developed to measure directly the force interactions at the molecular level. [6, 7, 8, 9, 10] Techniques such as osmotic stress, optical tweezers, and force balance may yield important results, but they can only be applied to a limited number of cases because the restrictions on the samples and often very complicated operating systems that defeat the purpose of a simple, direct force measurement. Because of simplicity in its structure, ease of operation, and high sensitivity (Ås in dimension and tenths of nanonewtons in force), more investigators are beginning to use the AFM as a versatile force measuring instrument.

1.3.2 Elements In Force Measurement

Cantilever Deflection

There are three important elements in measuring surface/molecular forces using the AFM. [11] First and foremost, we need to translate the voltage readings in the PSD to actual deflection of the cantilever. The voltage can be first converted to \( m \), the distance moved by the laser spot on the PSD using a standard calibration factor. Next, the deflection of the cantilever can be calculated by the following formula: [12]

\[
z = m \cdot l/3d
\]

(1.1)

where \( l \) is the length of the cantilever and \( d \) the distance between the cantilever tip and the PSD.

Spring Constant

A picture of a typical silicon nitride cantilever used for force imaging is shown in figure 1.5. The second element in force measuring is determine the spring constant of the lever to translate deflection to force. The simplest approach is by the following formula: [13]

\[
K = F/Z = 3EI/l^3
\]

(1.2)
Figure 1-5: A Standard Silicon Nitride AFM Cantilever
where $E$ is the Young's modulus of the silicon nitride cantilever, $I$ the moment of inertia, which is related to the width, $w$, and thickness, $h$, by $I = wh^3/12$. The drawback of this technique is that due to variations in manufacturing both the modulus and the thickness may deviate significantly (over 40% sometimes) from the supplier's estimate. [13]

Another method, trusted by many users, involves attaching a heavy tungsten bead to the end of the lever. [14] The spring constant can be measured either from the change in the resonant frequency before and after the attachment or from the actual deflection of the lever due to gravitational force from the bead. To carry out this method, one needs access to tungsten beads of known mass, attach it cleanly and reliably, and accurately measure the distance between the bead and the end of the cantilever. None of the above tasks are trivial.

The third method, which we will most likely use, works by using thermal cantilever noises. [15] In short, it models the long, thin, AFM cantilever as a simple harmonic oscillator with one degree of freedom along the Z direction. (Figure 1.6) The spring constant of the cantilever, $k$, is related to $T$, the temperature, and $< q^2 >$, the root mean square of the oscillatory displacement, in the following manner:

$$k = k_B T/ < q^2 > \tag{1.3}$$

In order to filter out noises from other sources which have distinctly different resonant frequencies, $< q^2 >$ is replaced by $p$ in the actual measurement of the spring constant, where $p$ is the area of the power spectrum of the thermal fluctuation along.

**Force Modeling**

After overcoming the first two elements, one is able to tell how much force there is between two surfaces as a function of distance. However, more work is needed to determine which forces are present and how they vary with parameters such as surface charge density and ionic strength of the liquid environment.

The interaction between the AFM tip and a flat substrate involves many types of
Model of an AFM tip-sample system. In this model, a spherical tip of radius $R$ is held at a distance $D$ from a planar sample by a spring of force constant $k$. The tip-sample interaction causes a tip deflection of $\delta$ from a position a distance $Z$ above the sample that the tip would occupy in the absence of any interactions.

Figure 1-6: Using Thermal Fluctuations To Measure the Cantilever Spring Constant
forces. However, not all the forces are high enough to be quantitatively considered. At the heart of the majority of the AFM force measurements is the DLVO theory. [16, 17] Several decades ago, Derjaguin, Landau, Verwey, and Overbeek independently proposed that much of the interactions between small charged particles in aqueous solutions can be explained by the interplay between the van der Waals force (vdW) and the electrostatic double layer repulsion force.

The vdW force of attraction arises from correlations between spontaneous, time-dependent dipoles in atoms and molecules, and is important only at very small separation distances (angstroms up to about 1nm). The following equation gives a rough estimate of vdW force between small particles in water: [18]

\[ F = \frac{-HR}{6D^2} \] (1.4)

Where \( H \) is the Hamaker's constant, \( R \) the radius of the particle and \( D \) the separation.

The electrical double layer repulsion force arises due to ionized charges on a solid surface in aqueous medium. Water has a high dielectric constant; thus, dissociation or adsorption of charged species at water-oxide interfaces is common. The charges at the solid surface are balanced by oppositely charged counterions in the solution phase. These counterions are attracted to the vicinity of the charged surface to minimize the electrical field energy. Entropic diffusion forces, however, result in motion of these ions to away from the surface. The charged surface and the counterions are referred to together as the electrical double layer. [19] Figure 1.7 shows a typical distribution of electrical potential, as well as the concentration of the counterion of a typical double layer system.

When two double layers approach each other, their electrical potentials overlap and repulsive (or attractive) forces result. The interaction surface force density (or surface stress) can be estimated by the following linearized equation (corresponding to surface potentials below 50mV): [19]
Figure 1-7: Distribution of the Electrical Potential Around A Double-Layer System
\[ F_{d-1} = \frac{2\sigma^2}{\epsilon} \times e^{-\kappa D} \]  

where \( \sigma \) is the double layer surface charge density, \( \epsilon \) is the permittivity of water, \( \kappa \) is the reciprocal of the electrical Debye length,

\[ \kappa^{-1} = \sqrt{\sum_{i=1}^{n} \frac{\epsilon RT}{2z_i^2F^2c_{io}}} \]

where \( c_{io} \) is the bulk concentration of the \( i \)th ion, \( z_i \) its valence, \( F \) the Faraday constant, \( R \) the universal gas constant and \( T \) the temperature. The force in general decays exponentially with distance in this linearized approximation.

The DLVO theory has been proven accurate in a variety of systems including silica-silica, glass-silica, gold-silica, alumina-alumina etc. Equation (1.4) corresponds to the repulsive electrical stress between two planar surfaces each having surface charge density \( \sigma \). In our experiment, the DLVO theory must be applied to surface charge densities on cylindrical GAG molecules, [2] since these molecules attached to the AFM substrates are the nearest neighbors. We may also need to consider the effect of unmasked charges on the relatively flat silica substrate and bead surfaces; however, this remains to be determined by direct measurement.

### 1.3.3 Recent Advances On Force Measurement

Ten years ago, scientists began to measure microscopic force interactions between the AFM imaging tip and a flat surface. [20, 21, 22] Although this endeavor yielded many insights on the nature of small scale force interactions, the results nevertheless were not ideal, because the shape of the individual tips were never the same, and force modeling involving irregular surfaces was cumbersome. In 1994, Ducker overcame this difficulty by replacing the sensing tip with a well-defined colloidal silica sphere. [23] He then measured the repulsion forces between this silica sphere and a flat substrate as functions of distance, \( \text{pH} \), and ionic strength. The results followed well the DLVO theory (Figure 1.8) and served as the foundation for many other more advanced
research probing the interactions between biological molecules.

AFM research involving intermolecular forces falls into two main categories. The first category, [9] directly adsorbs polymers such as extracellular polysaccharides (EPS) from marine algae to both the cantilever bead and a flat silica surface. They then let the cantilever approach and retract from the substrate. The difference of the force vs distance curve before and after the adsorption was attributed to the adhesion forces generated by the giant coils of EPS (extracellular polysaccharides from marine algae). In the second category, [7] scientists covalently attach molecules such as dextran to a surface (often gold to ensure high density coating), then probe it with an AFM tip also coated with dextran. Both approaches yield insights on the entropic forces of a single polymer and on the strength of the molecule. However, neither approach directly addressed the physical and chemical properties of the interacting surfaces (i.e. the density and orientation of the molecules fixed onto surfaces was not characterized). Moreover, most of these studies [6, 7, 9, 10] involved either the stretching of a polymer or the formation and breaking of a covalent bond. None of them addressed the interaction between surfaces coated with compact, rigid, highly charged molecules. This is the subject of this research.

1.3.4 Our Approach

We will also follow Ducker's example in using a silica microsphere instead of the actual tip for probing. Furthermore, we chose the flat substrate to be also silica, for simplicity and the fact that many investigators [23, 24] have dealt with this same system allowing comparison with their results.

In our study, we aim to covalently attach glycosaminoglycan molecules to both the bead and the flat substrate, and to rigorously characterize the density of the coating and the orientation of the molecules using techniques such as scintillation counting of radiolabeled GAG chains and ellipsometry. In addition, at high coating density, the glycosaminoglycans should become rigid rods rather than random coils under appropriate aqueous conditions. We wish to determine the transition point between these two configurations. Most importantly, we would like to characterize
FIG. 2. The force, \( F \), as a function of distance, \( D \), for a silica probe of radius \( R = 3.5 \mu m \). The force has been normalized by the sphere radius because \( F/2\pi R \) is equal to the energy per unit area between two equivalent flat surfaces (according to the Derjaguin approximation\(^2\)). These forces were measured using a cantilever of stiffness 0.58 N m\(^{-1}\) and an approach velocity of less than 200 nm s\(^{-1}\). Doubling the approach rate produced no change in the measured forces, indicating that hydrodynamic forces were insignificant.

Figure 1-8: DLVO Forces Between A Silica Colloid and A Flat Silica Substrate, As Measured by William Ducker
the repulsive interactions between two surfaces coated with dense, charged rod-like molecules, and relate these interactions to the actual behavior of glycosaminoglycans in physiological tissue.

1.4 Chemistry

The primary objective of this thesis was to first develop ways to reliably bind CS-GAGs to silica substrates at high density. In addition, we needed to find appropriate methods to characterize the coating density and molecular orientation.

1.4.1 Properties and Constraints of the Starting Materials

Flat Substrates

The materials we use place restrictions on the choice of coating chemistry. Silicon wafers were chosen for the flat substrates because under normal laboratory conditions, their surface is well oxidized to serve as a silica substrate. Moreover, reproducible imaging and force measurement both require a flat substrate. Polished silicon wafers offer unparalleled advantage in flatness and smoothness. The average roughness of our wafers are between 1 to 5 nm as estimated by the AFM.

A typical silica surface looks like that in Figure 1.9. [25] It consists of unreactive siloxane bonds (Si-O-Si), and reactive silanol groups (Si-OH or SiO−), which is the focus of our chemistry. The surface density of the reactive silanol groups depends on many factors including how the material is cleaned. Using conventional cleaning method involving the caustic mixture Piranha, the surface density of silanol groups should be on the order of 0.5 nmol/cm². [26]

The silanol groups limit the type of coupling chemistry that can be used with silica surfaces. The most common one involves reaction with silanes. As shown in Figure 1.10, a typical silane molecule has three parts. The first part consists of triols or tri-alkyl-oxy groups that attach to the central silicon atom. All three groups react with silanols. The second part is the fourth group on the central silicon, often a
The three main chemical groups of the silica surface which can be reversibly interchanged by addition or removal of water and protons: siloxane groups (Si–O–Si), silanol groups (Si–OH), silicic acid groups (Si–O\(^{-}\)). Silanol and silicic acid groups are hydrophilic, uncharged and charged, respectively; siloxane groups are hydrophobic. The isoelectric point (IEP) of silica surfaces in water is between pH 2 and 4.

Figure 1-9: Chemical Groups On A Typical Silica Surface
Figure 1-10: Structure of A Typical Silane Molecule
long aliphatic chain (n=3-12) that gives the silane length, which prevents it from extensive cross-linking. The third part is the moiety at the end of the aliphatic chain, often a reactive functionality such as an amine or carboxylic acid to facilitate further conjugation. A typical reaction between a silane molecule and a silica surface is shown in Figure 1.11.

**Chondroitin-6-sulfate**

We choose chondroitin-6-sulfate as our target glycosaminoglycan, because it is the major biomechanically functional molecule present in articular cartilage and its structure and chemistry have been well studied. The structure of CS was elucidated in detail in section 1.2. Here we will look at its reactivity. As shown in Figure 1.3, the polysaccharide contains many reactive moieties that can be directly coupled to a silica or silanized surface, such as carboxylic acids, sulfates, and hydroxyls. Unfortunately, none of these group will fit our purpose. All the functionalities mentioned above are present throughout the entire molecule. The resultant coupling reaction will therefore make the entire molecule lie flat on the surface. We want, however, to anchor only one end of the molecule (as occurs in vivo). This requirement forces us to look at the chemical groups that are present at one end only.

Only one chemical group fit the above description: the primary amine at the end of the amino acids which are attached to the terminal sugar through a serine residue. There is only one such group per CS molecule, and it is invariably located at the same one end of the molecule. This amine moiety offers simultaneously high specificity and versatile reactivity. It enabled us to couple the CS molecule to silica with known protein conjugation chemistry.

The premise to carry out the conjugation using the primary amine is that this group actually exists on the molecule. Specifically, we need to ensure that there is at least one amino acid remaining at the end of the chondroitin-6-sulfate. Unfortunately, the bond between the serine amino acid and the end of the polysaccharide can easily be broken by a beta-elimination reaction in base (0.05M NaOH). [27] We
Figure 1-11: Reaction Between A Silane Molecule and A Silica Surface
investigated on many commercially available CS products. Unfortunately, none of them had amino acids at the end of the molecule. Finally, we decided to produce our own naturally occurring chondroitin sulfate having 1-3 amino acid residues at the end. Such GAG chains were prepared by protease digestion and alcohol precipitation from rat chondrosarcoma cell cultures. No strong acid or base was used to cleave the serine-xylose linkage. (For details please refer to the Method section).

**Beads**

We wanted silica beads to be as large as possible so the average force is more meaningful. At the same time, we didn’t want the beads to be so large that they bend the cantilever. A reasonable diameter, which has been tried by many other AFM scientists is $\sim 3\mu m$. [9, 23, 24] The actual diameter of our beads is $2.9\mu m$. Dealing with beads requires more care than with flat substrates, in that the beads are much smaller, and consequently more difficult to wash and transfer. Therefore, the more steps we can skip in the coating chemistry the better. With this in mind, we chose carboxylic acid-modified solid silica beads instead of plain beads. With such premodified beads, we need only a one-step reaction that resembles protein-protein conjugation to couple the amine-terminated chondroitin sulfate to the carboxylic functions on the silica surface.

1.4.2 Coating Chemistry

**Coating Density Considerations**

Before going into the detailed chemistry, we will first look at the theoretical coating density maximum on the slabs and the beads, and compare them with physiological reality.

For the slabs, the limiting factor to maximum coating density is the surface density of the reactive silanol groups, which is approximately 0.5 $nmol/cm^2$. Knowing one silane molecule reacts with 3 silanol groups, and assuming each silane attaches to one CS molecule, the maximum coating density is therefore one third of 0.5, or
0.167 \text{nmol/cm}^2.\) Multiplying this number by Avogadro’s number and taking its reciprocal, we get the ‘parking area’, or surface area per molecule, which is approximately 1\text{nm}^2.

For the beads, the limiting factor is the surface density of the the carboxylic acid groups. Assuming each carboxylic acid couples to one chondroitin sulfate molecule, the maximum CS density is the surface density of the carboxylic acids. The manufacturer gave us the parking area, which is 90Å$^2$, which is close to 1\text{nm}^2. The fact that the beads had a similar parking area as the slabs made us suspect that the manufacturer of the beads used tri-oxy or triol silanes to modify their silica beads. The coating density of the beads therefore is the same as for the slabs, and not three times higher (due to trifunctional silanes).

In articular cartilage, chondroitin sulfates are densely packed along the core protein. Under normal physiological conditions, the spacing between two adjacent CS chains is 2 to 4 nm, [1] which implies a parking area of 4 to 16 \text{nm}^2. This is a more realistic estimate of the highest coating density, because strong repulsion forces will result at higher densities. Moreover, it is more difficult for the 40nm long CS molecule to maneuver around to find the correct docking position onto the surface if the density is too high.

**Beads Chemistry**

1. **Basic Chemistry:** The conjugation of an amine terminated chondroitin sulfate molecule to a carboxylic acid terminated surface is an already solved problem by protein biochemists, because it resembles a protein conjugation problem. With simplicity and efficiency in mind, we chose amongst a myriad of approaches [28, 29, 30] one that employs an activation of the carboxylic acid group by an ester, N-hydroxy-succinimide (NHS) via reaction with dicyclohexyl-carbodiimide (DCC). [32] As shown in the schematic in Figure 1.12, the hydroxyl group from the carboxylic acid forms an active complex with DCC, which is easily kicked off by NHS. The resultant NHS ester is an excellent leaving group which can be replaced by strong nucleophiles such as a primary amine.
Figure 1-12: Conjugation Between CS and -COOH Modified Sphere Using DCC/NHS
2. Major Considerations: Although the DCC/NHS chemistry has been proven to work, it is not a perfect system. Its major drawback is a side reaction with water: the NHS ester readily reacts with available nucleophiles. [31] Although the primary amine is a stronger nucleophile than water, water nevertheless replaces the NHS ester some 20 to 60% of the time. It is possible to reduce or eliminate this side reaction by either using a completely aprotic solvent (such as N-methal formamide or dimethyl sulfoxide), adjusting the pH, or use an extremely high concentration of CS. Due to limitations in the quantity of CS, we were not able try out this last approach. The second drawback in the DCC/NHS chemistry, which is common to many organic reactions, is the possibility of polymerization and multilayer formation during the activation step before adding CS. The DCC or NHS activated esters can react with their adjacent neighbors and form multilayers, which leads to a significant reduction in the active NHS esters at the outer surface, thus a reduced coating density. It is difficult to avoid polymerization. The best one can do is react the surface with DCC/NHS in a short time (15-20 min) at low concentrations (less than 0.5%). Last but not least is the problem of non-specific adsorption. Our coating procedure takes 12-16 hours, during which period CS molecules could attach to the bead surface without being covalently linked. To remove the non-specifically absorbed CS molecules, we sonicated the beads in a 2 wt% sodium dodecyl-sulfate (SDS) solution.

3. List of Experiments: The coating efficiency is affected by many factors, including temperature and pH of the coating bath, choice of solvent, concentration and total amount of CS, time of coating etc. With a total of 9 mg of CS from two batches, we carried out the following experiments to optimize the coating conditions: a) The concentration and pH matrix - we tried to coat at pH 4, 7, 10 and at concentrations 0.1, 1, and 5 mg/ml; b) The pH matrix - at 1 mg/ml CS, we tried to coat at a new set of pH values: 4, 7, and 8.4; c) Organic coating - to test the use of organic solvents, we coated the beads in ~ 60 μg/ml solutions
of CS in N-methyl-formimide (NMF) and in dimethyl-sulfoxide (DMSO). Additionally, we did a control experiment to determine how much sonication was needed to remove all non-specifically absorbed CS molecules.

Slab Chemistry

1. **Basic Chemistry:** The chemistry to coat the flat substrate is more complicated than for the beads, because it involves silanization of the silica surface which, sometimes, is still an unsolved problem in the silicon industry. As shown in Figures 12, 13, and 14, we tried several chemistries with the slabs. The first chemistry involved modification of the silica surface with an amino-silane \((N-(2\text{-AMINOETHYL})-3\text{-AMINOPROPYL-TRIMETHOXYSILANE, over chemical formula } C_8H_{22}N_2O_3Si)\), then linking the free amines from the silane and from the CS with glutaral-di-aldehyde (Figure 1.13). The second chemistry (Figure 1.14) used a carboxy-silane (CARBOXYETHYL SILANETRIOL SODIUM SALT, overall molecular formula \(C_5H_6Na_2O_5Si\)). After the surface was modified by the COOH groups, we used the same DCC/NHS chemistry to activate the surface. The third chemistry also aimed at modifying the silica surface with a carboxylic acid moiety (Figure 1.15). A vinyl silane (N-OCTENYL-TRICHLORO SILANE, overall molecular formula \(C_9H_{15}Cl_3Si\)) is first used to functionalize the surface with R-CH=CH2, which is then oxidized to \(R-CH_2-COOH\) by potassium permanganate \((KMnO_4)\). This chemistry has been previously shown to work well under stringent laboratory conditions, [26] while the other two chemistries were only introduced to us by their manufacturers.

2. **Major Considerations:** Unlike the beads for which we were able to optimize the coating conditions, the major consideration for the slabs was to find a practical coating protocol that would actually work. The silanization chemistry itself is highly variable and often yield multilayers which are susceptible to deactivation and contamination. Therefore the focus of our approach was to find a reliable way to silanize the surface of a silicon wafer, so that it would not
Figure 1-13: Aminosilane-Gluteraldehyde Chemistry
Figure 1-14: Carboxysilane-DCC/NHS Chemistry
Figure 1-15: Vinysilane-Permanganate Chemistry
only yield a dense monolayer but also a reasonable high density of reactive functionalities, such as carboxylic acids or primary amines.

3. **List of Experiments:** The following experiments were performed to discover a working coating protocol: 1. Just as the beads, glutaraldehyde activated slabs were coated in aqueous solutions of CS at pH 4, 7, 9 and concentrations of 0.01, 0.1, and 1mg/ml. This matrix is done to slabs activated by the glutaraldehyde chemistry; 2. Carboxy-silane chemistry - slabs activated with carboxy silane underwent DCC/NHS treatment and coated in 0.1 mg/ml CS at pH 7; 3. vinyl silane chemistry - the efficiency of two vinyl silanes were studied by ellipsometry. No CS was used in the last experiment.

### 1.4.3 Characterization

In short, the amount of chondroitin sulfate coated on both the beads and the slabs was measured by scintillation counting of the radiolabeled $^{35}S - CS$. The concentration of CS in the coating solution was determined by dimethylene blue dye (DMMB) colorimetric assay and by scintillation counting if the solution contained primarily labeled CS-GAG. The efficiency of silanization of the slabs are indirectly measured by ellipsometry, which gives an estimate of the thickness of a film on a silicon substrate by refractive index measurement. Please refer to the method section for details of all three characterization techniques.
Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chondroitin Sulfate

Amino acid terminated chondroitin-6-sulfate (CS) used to coat the surfaces was metabolically radiolabeled, harvested, and purified in the following manner:

Rat chondrosarcoma cells (which produce the desired CS attached to aggregan molecules) are grown in monolayer culture for 15 days. During the last two days, cells were incubated with $^{35}$S-sulfate, which was extensively incorporated into the CS molecules. Cells and their matrix-rich cell layer were then lysed, centrifuged, digested with proteinase K and the chondroitin sulfate was precipitated by ethanol. The CS was then purified by running through a Suprose 6 column and the molecular weight was estimated 20KDa. A typical Suprose 6 profile is shown in Figure 2.1. The purified sample was then lyophilized and stored at -20C until use.

2.1.2 Flat Silicon Slabs

The silicon wafers used in this experiment were of reject grade materials manufactured by Recticon Enterprises Inc. They were of $\overline{1}00\bar{1}$ orientation and was N doped with phosphorus. The quality control number (QC) was 25905. The maximum coating density of the surface was 0.167 nmolCS/cm$^2$, which, multiplied by 20KDa, the
Superose 6 profile of 35SO-labeled Rat Chondrosarcoma (CL11s) cell layer and medium

Figure 2-1: A Typical Suprose-6 Profile of Stock Chondroitin-6-Sulfate
average MW of CS, yield a maximum surface density of 3.33 \( \mu g/cm^2 \).

### 2.1.3 COOH-Modified Silica Beads

Solid, non-porous silica microspheres were obtained from Bangs Laboratories Inc, Fishers, IN. The diameter of these spheres was 2.9 \( \mu m \) and they were suspended 1:10 by weight in 0.1% SDS water. The density of the solid was 1.96 \( g/cm^3 \) and the surface area is \( 1.06 \times 10^{12} \ \text{um}^2/g \). The suspension contained approximately \( 4.2 \times 10^9 \) spheres per ml. These beads, when coated with CS, were to be attached to the cantilever of an AFM tip.

#### Chemical Modifications

All spheres were modified with -COOH moieties with a packing area of approximately 90 \( \text{Å}^2 \), which was equivalent to 0.184 \( \text{nmol/cm}^2 \). With the conversion shown in equation 2.1, the coating density can be translated to \( 4 \mu g/ul \) of the original bead suspension. It is easier in practice to deal with volume of the bead suspension than to deal directly with total surface area of the beads.

\[
\frac{0.184}{1\text{cm}^2} \times \frac{9 \text{mol CS}}{1\text{cm}^2} \times \frac{3.785 	imes 10^6 \text{spheres}}{1\text{cm}^2} \times \frac{4.2 	imes 10^6 \text{spheres}}{1\text{cm}^2} \times \frac{20,000 \text{g}}{1\text{cm}^2} \times \frac{\text{mol CS}}{4 \text{ul suspension}} \times \frac{\text{ul suspension}}{1\text{cm}^2} = 4 \\frac{\text{ug CS}}{1\text{cm}^2}
\]

### 2.2 Coating Protocols

The coating process for both the slabs and the beads includes the following major steps: cleaning the surface, activation of the surface, incubation with CS solution, and washing off non-specifically adsorbed CS molecules. The following are detailed protocols for coating each surface.
2.2.1 Coating Slabs

1. From a 3 in x 3 in silicon wafer, cleave out the desired number of 1 cm x 4 cm rectangular slabs. Blow away dust using a nitrogen gun for 5-10 seconds.

2. Place each slab in a clean 20 ml glass scintillation vial. Then pour into the vial just enough piranha solution to cover the slide. Let it sit bathed in the piranha for 30 min under a chemical hood. Piranha solution is prepared by mixing 1 part hydrogen peroxide to 3 parts pure sulfuric acid. (Handle with extreme care and ample protection.)

3. At the end of cleaning, decant the piranha solution down the drain. Rinse the slabs with five 20 ml portions of MilliQ (or other distilled) water. Dry them completely with a stream of nitrogen.

4. Obtain the baseline optical properties of the slabs using an ellipsometer.

5. Rinse a scintillation vial with MilliQ water followed by acetone, then dry the container thoroughly. To this container, add 20 ml of 0.1-0.5% octenyl trichlorosilane in anhydrous hexane.

6. Place two slabs back to back to the coating solution. Place in a deccicator or under dry nitrogen atmosphere for one hour. Make sure there is no moisture during the coating.

7. First rinse the slides in 20 ml portions of CH₂Cl₂ in a deciccatcr for 2-3 minutes. Then under normal laboratory air, rinse with another 20 ml portions of chloroform (CHCl₃) and ethanol in scintillation vials. Finally rinse the slabs with ethanol from a wash bottle. Monitor the change in thickness using ellipsometry.

8. Oxidize the vinyl bonds at the surface of the silane layer by submerging the slabs under 20 ml of water with 0.5 mM KMnO₄, 19.5 mM NaIO₄, and 1.8 mM K₂CO₃ at pH 7.5 for 24 hours.

9. Rinse the oxidized slabs with 20 ml portions of 0.3 M NaHSO₃, water, 0.1 N HCl, water, and finally ethanol.
10. Prepare 10 mls of 0.2wt% N-hydroxy succinimide (NHS) and 0.4wt% dicyclohexyl carbodiimide (DCC) in anhydrous tetrahydrofuran (THF). Mix the solutions quickly. Immediately place in the two slabs and let the reaction go 15-20 minutes.

11. At the end of activation, take out slabs, thoroughly rinse with plenty of THF. Sonicate for 2 minutes if necessary. Dry the slabs quickly under a stream of nitrogen and immediately place in the chondroitin sulfate coating solution. Save one slab for ellipsometry measurement.

12. Let the coating run overnight. Take out the slabs, rinse with water, sonicate in 3-4 ml 2% SDS for 30 minutes to remove randomly adsorbed chondroitin sulfate molecules. Rinse again with water.

2.2.2 Coating Beads

1. Gently stir the suspension of stock beads for 5min to evenly disperse beads in the 1 wt% SDS solution. Under sterile conditions, take out the desired volume of beads suspension and pipette into an Eppendorf tube.

2. Separate the beads from the SDS solution by centrifuging the suspension at 30-40 % full speed for 3 mins. Remove the supernatant.

3. Add another 0.5 to 0.8 ml sterile de-ionized water to beads. Mix well with pipette. Centrifuge to separate beads from water. Remove supernatant.

4. Repeat step 3 four more times to completely clean the beads and to get rid of any residual SDS molecules.

5. Remove as much water as possible from the beads without disturbing the pellet. Lyophilize the remaining beads for at least five hours to drive off water completely. (Do NOT freeze beads at any time). During lyophilization, prepare 0.4% DCC and 0.2% NHS in separate bottles of anhydrous THF. Also prepare another sealed vial of anhydrous THF for washing purpose.
6. Quickly take out the lyophilized beads (to prevent re-absorbed moist) and place under the sterile hood. Quickly draw out equal volumes of DCC and NHS solution, mix well. Pipette into the beads an appropriate amount of the reaction mixture so that the total number of the both the NHS and DCC molecules is at least a hundred times that of the COOH groups on the beads. Mix well.

7. Place the tube of beads and NHS/DCC solution on a roller. Let the reaction run for 15 to 20 minutes.

8. Spin down the beads and remove the supernant. Wash any residual NHS/DCC off the beads by adding 500-800 ul portions of anhydrous THF, mix, spin down beads, and removing the supernant five times. Now the bead surface is activated and ready to react with the chondroitin sulfate solution.

9. Add a small amount of THF to the beads, mix well. Pipette out desired portions of beads into separate eppendorf vials. Spin down all beads, and remove as much liquid as possible without disturbing the bead pellet.

10. Add to the beads an appropriate amount of 1 mg/ml chondroitin sulfate solution buffered at pH 8.4. The total amount of CS should be 10 times the theoretical maximum on the surface. Mix the beads well with the coating buffer. Immediately place the eppendorf vials on a roller and let the reaction run overnight (approx. 16 hours) at 4°C.

11. At the end of coating reaction, spin down the beads. Remove and save coating solution. Take out 5 μl for scintillation analysis. Wash beads twice with 500-800 μl portions of sterile water. Save all washes for scintillation analysis.

12. Remove any non-specifically absorbed CS by sonicate the beads in 500 μl of 2 wt% aqueous SDS solution for 40 minutes.

13. Spin down mixture. Remove the SDS solution. Wash beads 3-4 more times with 500-800 μl portions of water. (After the SDS wash, the beads tend to
persistantly stick to the wall of the eppendorf. One may want to increase both
the speed and time of centrifuging).

14. Now the beads are clean and coated, and hopefully intact. They are ready to
be attached to the AFM cantilever.

2.3 Characterization of Coatings

The purity of the chondroitin sulfate, the density of the coating, as well as the evenness
of the layers were determined by the following assays.

2.3.1 DMMB – Assay to Quantify the Concentration of Chondroitin Sulfate

The Dimethyl Methylene Blue (DMMB) dye forms a complex with chondroitin sulfate
molecules and absorbs visible light at a wavelength of 520nm. [35] The absorbance
at 520nm is directly proportional to the level of chondroitin sulfate in solution. A
standard calibration curve of absorbance vs CS concentration is shown in Figure 2.2.

Because the DMMB assay only works in liquid, it is best to characterize the origi-
nal reconstituted chondroitin sulfate stock. This assay can not be used to measure
the CS concentration at the surface of the slab. The presence of CS at the surface
of the silica beads does cause an increase in the absorbance. However, this increase
is sensitive to the number of beads present in solution, which is difficult to normal-
ize. Due to these limitations, we resort to scintillation counting of the $^{35}$S-sulfate
radiolabel to ultimately characterize the coating density.

2.3.2 Scintillation – Assay To Characterize Level of Radioac-
tivity

Scintillation counting is the most sensitive technique to quantify radiolabeled chon-
droitin sulfate. It can detect as little as nanograms of CS. When mixed with scin-
Figure 2-2: Standard Calibration Curve For the DMMB Colorimetric Assay
tillant, the solvated radioative substance causes release of fluorescent light from the scintillant, which is then detected and recorded.

The lyophilized chondroitin sulfate is first reconstituted with 0.5 to 1 ml sterile water. Next both DMMB assay and scintillation are performed on the reconstituted stock diluted 50 times. Finally a conversion factor is established to translate scintillation counts to micrograms of chondroitin sulfate in the particular stock.

The radioactivity on the coated silica beads is measured by removing as much water as possible from the beads and then mixing the beads with 3 ml of scintillation fluid. Measuring the radioactivity on the slabs takes more effort because the slab will block the reading mechanism of the counter. Therefore, after thorough washing the slabs, bound CS molecules are removed from the surface by beta-elimination at the serine-sugar linkage. Specifically, the slabs are placed in a small volume of 0.5mM NaOH overnight. The digest is then neutralized by a few drops of 2M HCl, and then lyophilized overnight. The lyophilized digest is finally reconstituted using a minimum amount of scintillation fluid and the radioactivity counted.

2.3.3 Ellipsometry – Indirect Measurement of Layer Thickness

For the slabs, in addition to defining the final coating density by scintillation counting, the quality of each chemical treatment at the surface can also be characterized indirectly by ellipsometry. As shown in Figure 2.3, an ellipsometer estimates the thickness of a film on a substrate by applying Snell’s law. On average, we take 6-10 readings per slab to determine both the thickness of its film and its eveness from the standard deviation.

To operate the ellipsometer, one must first clean the surface of the silicon wafer. Immediately after cleaning, the baseline optical parameters (i.e. the real and the imaginary refractive index) are measured. Next, as the slab reacts with silanes and goes through subsequent chemical treatments, the thickness of the film is measured each time.
Figure 2-3: Working Principle of the Ellipsometer

\[ \pi \Delta \chi \cdot \sin \theta \]
Although not 100% accurate, ellipsometry gives us a good sense of how well each layer is coated onto the silicon substrate.

2.4 Mounting of Beads

The beads are mounted onto the AFM cantilever (spring constant is approximately 0.06N/m) by a thermoset epoxy. The entire procedure is carried out by Bioforce Laboratory, Inc, Ames IA.

2.5 Operating the Atomic Force Microscope

Need the model number of our AFM. Please refer to Appendix F for a protocol for running the Multimode AFM.
Chapter 3

Results and Discussions

3.1 Characterization of Chondroitin Sulfate GAG Chains

3.1.1 Amino Acid Analysis of Commercial Glycosaminoglycans

Shown in Fig 3.1 is the amino acid analysis of chondroitin-6-sulfate from Calbiochem, Inc. The amino acid analysis was performed on an Edman degradation machine from Perkin Elmer. As chondroitin sulfate molecules are attached to aggrecan protein core only by a serine residue, the presence of CS can be inferred from the serine concentration. Unfortunately, this source of chondroitin sulfate had negligible serine compared to proline and hydroxy-proline, the latter being a characteristic trademark of collagen. This discovery points to two facts: a. the Calbiochem chondroitin sulfate has a significant amount of collagen in addition to glycosaminoglycans; b. the chondroitin-sulfate present (which was verified by a decent result from DMB assay) was NOT attached to any amino acids. Based on these results, we decided to obtain our own stock of amino-acid terminated chondroitin-6-sulfate.
Protein Microsequence Report

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Figure 3-1: Amino Acid Analysis of CS from Calbiochem, Inc.
3.1.2 Characterization of Metabolically labeled Chondroitin-6-Sulfate Synthesized by Rat Chondrosarcoma Cells

The cellular production, protease digestion, and column purification of chondroitin-6-sulfate from rat chondrosarcoma cell line were described in detail in section 2.1. After purification by Suprose 6 column, the CS was lyophilized and sent to us overnight.

The preparation and characterization of the CS involved the following four steps:

1. reconstitute the lyophilized sample by 500 to 1000 ul of sterile di-ionized water, mix well, remove supernatant from the particulate debris. 2. Take out a small portion (2-5 ul) then dilute to 50x. 3. Assay the dilutions for both glycosaminoglycan content using the DMBB assay and radioactivity by scintillation counting. 4. Using data from DMBB and scintillation counting, determine the CS concentration of the reconstituted stock and the conversion factor between the cpm (scintillation counts per minute) obtained from radioactive counting and $\mu$g of CS for further dilution and preparation of coating solutions.

We received two batches of chondroitin-6-sulfate from our collaborators. The raw data describing both batches are included in Appendix A and their quantities summarized below. The first batch included two samples, 1.25 mg radioactive (hot) CS in 0.3 ml $H_2O$, and 4.43 mg non-radioactive (cold) CS in 0.3 ml $H_2O$. The conversion factor for the hot CS is 74,000 $cpm/(\mu g \text{CS})$. The second batch consisted of hot CS only, 2.30 mg CS harvested from cell layer in 0.7 ml water, and 1.02 mg CS harvested from the feeding medium in 0.7 ml water. The conversion factor for the cell layer was 53,000 $cpm/(\mu g \text{CS})$ and that for the medium CS was 44,000 $cpm/(\mu g \text{CS})$.

3.2 Coating of the Beads

3.2.1 The Time Curve of SDS Wash

One important aspect of our experiment is to establish a reliable control for all samples to compare. Specifically, we needed a control for non-specifically absorbed chondroitin sulfate. By submerging beads in the coating solution for more than 12 hours, there
are covalently linked CS as well as non-specifically absorbed CS at the surface. We needed to develop a method that effectively removed all the non-specifically absorbed CS without disrupting the covalently linked CS. To accomplish this, we used sodium dodecyl sulfate (SDS), a well known detergent specifically targeted to break down any non-covalent interactions.

In the actual control experiment, we placed COOH modified beads (not treated by DCC/NHS) into 1 mg/ml CS coating solutions for 16 hours. Beads were taken out of the solution, washed twice in water, sonicated in 2wt% SDS for various time periods, and then washed another three times in water. The beads and all washes including the SDS were saved. Shown in Figure 3.2 are the counts of all bead samples sonicated for different times in SDS after all six washes. The data indicate clearly that 20 minutes of sonication in SDS together will five other washes in water were sufficient to remove all randomly absorbed CS. Figure 3.3 shows the counts of all washes of the beads sonicated for 40 minutes. The concentration of chondroitin sulfate steadily decreased after each wash, except the SDS wash, which apparently removed a great deal of CS from the beads, all non-specifically absorbed. It seemed that by the fifth wash, most of the chondroitin sulfate was removed.

Based on results from the control experiment, we were able to devise a standard coating protocol for all future experiments, which included washing the freshly coated beads twice with 500ul portions of sterile water, followed by sonication in 500 µl 2% SDS for 35 minutes, and then two more washed in 500 µl portions of water.

For the flat substrates, we adopted a similar washing protocol: rinse thoroughly with water, SDS wash for 35 minutes, and rinse again with water. The reason for using the same protocol was that the slabs were much less likely to adsorb CS because the slab surface is more negatively charged than the beads surface. As chondroitin sulfate molecules are highly negatively charged, it is difficult for them to stick (non-specifically) to another negatively charged surface. We tried once by submerging plain slides in non-radioactive, commercial Sigma CS solution. At the end of the incubation, the results from ellipsometry showed that there was almost no increase in surface thickness (0-0.2 nm). This slight thickness increase went back to zero.
Figure 3-2: Control: Absorbed CS vs Sonication Time In SDS
Figure 3-3: Control: CS Removed From Beads IN Successive Washes
by merely submerging the slides in 2% SDS for several hours. Our metabolically labeled CS might be different than the Sigma CS, but could not withstand half-hour sonication. To make sure there will be no randomly adsorbed CS at the surface, we decided to sonicate the slabs as well.

### 3.2.2 The Concentration, PH Matrix

As mentioned in section 1.4.1, many factors influence the coating efficiency, such as time, temperature, pH, concentration, total chondroitin sulfate, solvent etc. In the first experiment, we fixed the coating time to be 16 hours (overnight) hoping that this would be long enough to ensure complete reaction. In standard protein conjugation literature, coating times vary between 2 to 24 hours. We also fixed the temperature to be 4°C to prevent bacterial infection. Similar or higher coating temperatures were quoted in the literature. Thirdly, we fixed the total CS to be 5 or 10 fold excess of the maximum total amount needed. Results showed that our highest coating density was about 1% of the expected maximum, so the CS in solution was by far in excess of what was needed at all concentrations used. Fourth, we fixed the solvent to be water exclusively.

The parameters we varied were pH (4, 7, 10) and concentration (5, 1, 0.1mg/ml). Please refer to Appendix B for details. In addition to these nine coating solutions, we also had a control of just plain COOH-modified beads in a coating solution at 1 mg/ml, pH 7. Both labeled and unlabeled chondroitin sulfate from the first batch were used. Because the total amount of hot CS was much less than that of unlabeled cold CS, one part of hot CS was mixed with 3 parts cold CS. Due to limited supply, we were not able to have more than one sample per condition. After the coating experiment, the beads were collected, washed, their radioactivity counted and translated to μg CS coated. The washes were also counted to ensure complete non-bounded CS.

As this was our first try on coating beads, the results were in general sporadic (Figure 3.4). No trend was observed with either pH or concentration. However, all samples showed definite above-background radioactivity, indicating substantial coating. Except for 5 mg/ml, pH 4, all readings varied varied between 2000 and 200
Figure 3-4: CS Concentration and pH Experiment: Beads
which translates to 0.1 to 0.01 $\mu g$ CS. Compared to the maximum coating estimated by available surface COOH groups (about 15$\mu g$ CS), we obtain a coating efficiency between 0.72% and 0.072% of the theoretical maximum. A coating density of 0.72% correspond to a parking area of $\frac{90}{0.0072} = 12500(\AA)^2$, equivalent to a $11nm \times 11nm$ dimension. Our goal is to reach a $3nm \times 3nm$ dimension.

Although no trends were observed with either concentration or pH, we could still make the following comments. First, the fact that all concentrations were able to produce at least one high coating density ($\geq 0.05\mu g$) implied that over long enough coating time (i.e. $\geq 16hr$), the exact concentration didn’t matter in the range used. The 5 mg/ml samples produced some low results, which we attribute to the small volume of coating solution, which was hard to handle. The best results were found in 1 mg/ml samples, which we believe is due to the fact the volume of coating solution used was appropriate for these samples. (Because we held the total CS to be constant in all samples while varying the concentration, the volume of the coating solutions unfortunately had to change.)

Second, the fact that we didn’t observe any trend in pH did not mean that there wasn’t any pH dependence, because there was much to improve in our method. For example, we used different chemicals for the different buffers (Appendix B), which may have affected the results. Also, we first added the concentrated CS stocks to the activated beads, then after two or three minutes, we added the buffer. Because the ester replacement reaction is fast, much of the reaction may have taken place already before the buffer was added. Last but not least, we may have lost many beads during washing (about 2 out of 5 $\mu l$). We only had one sample for each condition; therefore, it was hard to check the consistancy of our method. Due to all of the above reasons, we decided to run another experiment, focusing on the effect of pH, and fixing the CS concentration to be 1 mg/ml, the optimal concentration discovered from this first experiment. The other conditions including the time and temperature of coating remained the same.
3.2.3 The pH Matrix

With experience from our set of experiments, we then tried 1 mg/ml using pH values 8.4, 7, and 4. (Please refer to Appendix C for detailed recipes of the coating solutions). Since at pH 10, many water molecules will be deprotonated, becoming $-OH^-$, which compete with the CS to replace the NHS ester. At pH 8.4, on the other hand, $-NH_2$ will still be a strong nucleophile, while water will be unchanged. Our prediction was that samples treated at pH 8.4 should have the highest coating density, because at lower pH's, the $-NH_2$ would become $-NH_3^+$, an unreactive species.

In an effort to correct other problems, we used the same components (different salts of sodium phosphate and phosphoric acid) for all pH buffers. Furthermore, we mixed the CS stock with the buffers before adding them together to the activated beads. Two bead samples were used per condition, so that we could see some reproducibility of our results. We also tried to recover all the beads after washing by warming the eppendorf vials in a 60°C bath and then rinsing with scintillation fluid. Indeed we observed that much of the beads that had previously stuck to the wall of the vials were now successfully washed off. All CS used in this test came from the second (all-radioactive) stock.

The results from the pH experiment are shown in Figure 3.5. As we expected, the coating density at pH 8.4 was significantly higher than the rest (almost ten times). The reproducibility between duplicates were good. The best coated sample from pH 8.4 showed 6500 cpm which, divided by the conversion factor $[53,000 \text{ cpm}/(\mu g \text{ CS})]$, yeild 0.13 $\mu g$. Divide this again by the expected maximum, 15 $\mu g$, we again obtained the same coating density as the previous experiment, approximately 0.86% of the theoretical maximum.

This pH experiment had the following significance. First, it showed that whatever highest coating density we were able to achieve in the first experiment, was reproducible with a different batch of CS and buffers. Second, it showed convincingly that pH did matter and that the best pH for coating so far is 8.4, as suggested by Laibinis. Third, although our methodology was still not perfect, it improved much from
Figure 3-5: Beads: CS Coating Density Vs. Bath pH
the previous run. The point that needs much improvement this time is the washing step: we observed higher than background cpm in control samples (plain beads in CS overnight). This was mostly likely due to cross contamination during washing, and/or that the CS time was all labeled whereas last time we had one part hot to 3 parts cold. We plan to improve the control sample by not using the sample pipette tip for different samples, prolonged SDS wash (45 min), and larger wash volumes (750 \( \mu l \)). Despite the high background readings, all test samples nevertheless had cpm significantly above background.

### 3.2.4 Results From Coating With Organic Solutions

**Solvation Experiments and Preparation of Organic Coating Solutions**

As mentioned in section 1.4.2, the coupling reaction by DCC/NHS is sidetracked by the presence of water molecules. Therefore, one way to improve the coating efficiency would be to dissolve CS in organic solvents that exclude water. Thus we tried to dissolve Sigma brand CS (cheap, pure, widely available, but does not contain amino acids at the end) in NMF and DMSO. The concentrations of CS in the resultant solution were quantified by the DMB assay. As shown in Figure 3.6, NMF can dissolve a maximum of approximately 57ug/ml CS and DMSO 63ug/ml. These concentrations are low compared to aqueous solutions of CS, which can reach as high as 10mg/ml.

**The Coating Experiment**

Simultaneous with the pH experiment, we also tried to coat some beads in NMF and DMSO. Specifically, two samples of activated beads, and two samples of plain beads were each placed in 1 ml of CS dissolved in NMF. The same was done for the DMSO solution. The coating was carried out under room temperature for 48 hours. The washing protocol was almost the same as for the aqueous coated beads, except organic solvents (NMF and DMSO) were used for the first two washes before SDS.

As shown in Figure 3.7, the results of organic coating was not great.

First, the background of the control beads was high, indicating inadequate wash-
Figure 3-6: Solubility of CS In NMF and DMSO
Figure 3-7: Beads: Coating Experiment With CS In Organic Solvents
ing. Second, the NMF samples were barely above background, implying little or no coating. The results from the DMSO was slightly better, but not by much. The highest coating density achieved was 0.007 ug, much less than the aqueous coating.

We attribute the low coating efficiency in organic solvent to several possible factors. First, the solubility of CS in either solvent was low, so there was probably not enough CS in solution for easy coating. Second, there might be residual water at the surface of beads, thus preventing the organic solvent to reach the surface. Additionally, we first carried out the coating at 4°C, at which temperature the DMSO froze. We will increase the temperature to 36°C in our next experiment. Other solvents such THF will also be tested.

3.3 Results From Coating the Flat Substrates

3.3.1 Glutaraldehyde: pH and Concentration Matrix

At the same time we conducted our first set of experiment with the beads, we performed a similar study on the flat substrates, following the same nine pH and concentration conditions. Additionally, we used two slabs per condition.

The total expected chondroitin sulfate on the slabs (about 1µg/slab) was much less than that on the beads, since the beads have a much greater collective surface area. As a result, to get a strong signal, one needs more radioactive material. We therefore decided to use only the radioactive CS from the first stock in this experiment. We allocated 10µg total CS in each coating solution. (Please refer to Appendix B for details).

The slabs were approximately 3mm x 7mm in dimension. They were cut from bigger slabs (1.5cm x 4cm) which were cleaned, treated with amino-silane and then activated by glutaraldehyde. After cutting, the tiny slabs were then placed immediately in their respective coating solutions over night. The washing protocols followed that described in section 3.2.1. For a protocol describing the entire procedure from cleaning the slides with strong base to final washing, please refer to Appendix D.
After washing, the slides were placed in a scintillation tube and their radioactivity counted.

The results of the scintillation readings are shown in Figure 3.8. They were in general extremely low, only barely above the background. There was one higher reading, 74 cpm. Assuming a surface area of 20 mm², the maximum possible amount of CS is 0.66ug, which translates to about 49,000 cpm. Dividing 74 cpm by 49,000 cpm, we obtain about a 0.1% of the theoretical maximum, which is ten times less than the beads. This gives us a parking area of approximately 30nm x 30nm, a coating density of $2.5 \times 10^{-15} \text{mol/mm}^2$. Unfortunately, these results did not appear reproducible, possibly due to contamination rather than real coating.

We attribute the poor outcome of this experiment to two factors: the difficulty in reading the radioactivity from a solid slab, and the basic activation chemistry. The orientation and the shape of the slabs seemed to affect the readings a great deal, even at a level barely above the background. At first, the slabs were all sitting at the bottom of the scintillation tube and there was no signal at all. Suspecting the bottom of the tube was out of the detection range, we elevated the slabs with a stub of paper towels. That increased the reading significantly, but still we could not obtain reproducible results, most likely due the slight differences in the inclination angles of the slabs to the horizontal plane. Second, although the glutaraldehyde activation chemistry has been used by other researchers, the results seemed to vary a great deal, [31, 33, 34] because glutaraldehyde is extremely reactive, and any lapse in between the activation step and the coating step could easily deactivate the surface (i.e. block the aldehyde bond). In order to get significant improvement from the first try, we need to focus on developing a sensitive characterization of the coated flat surface, as well as a reliable method to activate the silica surface for conjugation.

3.3.2 Carboxy-silane treatment

With the above two considerations in mind, we carried out another set of experiments to coat the slabs. To improve the method of detecting radioactivity from the slabs, we used bigger slabs (1cm x 4cm), and we removed CS from the slabs after coating by
Figure 3-8: CS Concentration and pH Matrix: Flat Substrates
submerging them in 1M NaOH overnite, in order to more accurately determine the coating density of the CS. The base digest was then neutralized with equal amount of acid, and lyophilized. The resultant powder was then reconstituted with scintillation fluid for counting. Additionally, we also tried to read the slides directly in the scintillation counter, after digesting with proteinase K overnight. This gave a total of six samples, two digested with NaOH, two with proteinase K, and two untreated and read directly.

In addition to the changes in the characterization protocols, we also completely revamped the coating chemistry. Since the COOH functionalized beads were coated reasonably well with CS, we decided to modify the flat silica surface also with COOH. This was achieved by using a carboxy-silane (carboxy-ethyl silanetriol) purchased from Gelest Inc. After the COOH silane modification, we carried out the DCC/NHS modification the same way as the beads. For a complete description of the protocol, please refer to Appendix D. After staying in 0.1 mg/ml pH7.2 coating solution overnight, the slabs were washed and digested according to plan.

The radioactivity of the two untreated slabs, as well as the proteinase K and base digests are shown in Figure 3.9.

There were little or no counts shown from either the untreated or the proteinase K treated slabs. However, the radioactivity of the NaOH digest was significantly above background (80 cpm). This count was approximately equivalent to 1.5 ng. Dividing 1 ng by the total area (4cm$^2$), we obtain a coating density of 0.4 ng/cm$^2$. The theoretical maximum coating density is 3 $\mu$g/cm$^2$, giving a 0.013% coating efficiency (which translates to $\frac{90\AA^2}{0.0013} = 675,000\AA^2$, approximately 82nm x 82nm). This is an improvement over the first experiment.

After re-examining the experiment, we decided that the procedure to clean the silica surface, silanization chemistry, and reliable characterization will be the focus of our next experiment. Results from ellipsometry showed that the refractive indices of the silicon wafers varied significantly after base wash, indicating an unclean surface. Further, the standard deviation in the thickness of the film produced by the carboxy-silane (15 Å) greater than the actual size of the silane (10 Å, pointing strongly to
Figure 3-9: The Efficiency of Different Digestion Protocols
polymerization and multi-layer formation, both of which will reduce the amount of free COOH groups at the surface. According to experts in the field (P. Laibinis, I Lee), the carboxy silane is highly prone to form multilayers, and it is best to try another chemistry. Our characterization chemistry must also be improved. Specifically, the use of strong base and acid left behind a large piece of strongly ionic solid, which was hard to dissolve in the scintillant. As a result, the scintillation mixture often showed phase separation during counting, which lead to results very possibly much lower than the actual value.

3.3.3 Vinyl-silane Treatment

With all of the above considerations in mind, we tried one more coating experiment with the following modifications from the previous experiment. First, we aggressively cleaned the silica surface with piranha, a mixture of two parts fuming sulfuric acid to 1 part hydrogen peroxide at 90°C. Second, we adopted a silanization protocol developed by Wasserman et al. [26, 33] Instead of directly applying a carboxy silane, this protocol first coat the silica surface with a monolayer of vinyl silane, then converting the vinyl group to carboxylic acid using permanganate and periodate salts. For the detailed protocol, please refer to section 2.2.1. Third, we plan to treat the coated surface with minimal amount of 50mM NaOH instead of 6ml of 1M NaOH. With fewer salt crystals, the scintillation count should be hopefully more accurate.

Unfortunately, due to timing constraints, we were not able to carry out our plan beyond the silanization step. Shown in Figure 3.10 are the standard deviation of the real and imaginary refractive indices (Ns, Ks) of the silica surfaces after different cleaning procedures. It showed that cleaning the slabs with Piranha resulted in more uniform surface properties. This difference was also visible: one could observe an even water layer at the surface immediately after the piranha treatment, whereas after the base treatment, there were still many patches of hydrophobic areas at the surface. The silanization itself, however, did not look as good as described in Wasserman et al’s paper. The average thickness of the layers produced by the vinyl silane, as well as the amino and carboxy silane, are shown in Figure 3.11.
Figure 3-10: The Effect of Different Cleaning Procedures On Ns, Ks
Figure 3-11: Average Thickness of Layers Produced By Different Silanes
The thickness of the vinyl silane layer (3.3 nm) was almost three times the thickness of the monolayer predicted (1.2 nm) by Wasserman et al. More debugging is needed to improve the original monolayer formation.
Chapter 4

Conclusions and Future Work

4.1 Summary of Results

4.1.1 Selection of Source Materials

Chondroitin Sulfate Stocks

Chondroitin-6-sulfate (CS) with 1-3 amino acids at one end was obtained by digestion of rat chondrosarcoma cell layers and their glycosaminoglycan rich feeding medium. The average molecular weight of the suprose-6 column purified CS was 20 KDa and its length $\sim 40$ nm. Additionally, to facilitate characterization of the density of CS covalently attached to surfaces, the CS was radiolabeled with $^{35}$S-sulfate. A total of 2 batches of CS were used. In the first batch, 1.25 mg CS was labeled with a radioactivity of 74,000 $cpm/(\mu g$ CS). There was also 4.43 mg cold, non-labeled CS. In the second batch, both the CS harvest from the cell layer (2.30 mg) and the CS from the medium (1.02 mg) were labeled. The conversion factor for the cell-layer CS was 53,000 $cpm/(\mu g$ CS) and that for the medium CS was 44,000 $cpm/(\mu g$ CS).

Microspheres For the AFM Cantilevers

Solid, non-porous silica spheres of 2.9 $\mu m$ diameter were chosen. All spheres were functionalized with -COOH, with a parking area of 90 Å$^2$ per -COOH group.
Flat Substrate For AFM Measurement

Standard three inch silicon wafers were chosen as the flat substrate. Their roughness varied between 1 to 5 nm. All of them are rejection grade, manufactured by Recticon Enterprises, Inc.

4.1.2 Coating Chemistry

Spheres

We were able to coat CS to COOH-modified microspheres at a density of 27 ng/cm², equivalent to a parking area of approximately 10nm x 10nm. The coating chemistry involved activating the surface by di-cyclo hexyl carbodiimide (DCC) and N-hydroxy succinimide (NHS) in tetrahydrofuran (THF), and then placing the beads in aqueous solutions of CS overnight. Our results indicated that at long enough coating times (over 16 hr), the exact concentration of CS (between 0.1 to 5 mg/ml) was not critical. The pH of the coating solution, however, did make a difference. pH’s at or near 8.4 gave the highest coating density.

Sonication of the beads in 2% SDS for twenty minutes were shown to be sufficient to remove all non-specifically absorbed CS. Methodologies need to be developed to successfully coat the spheres in organic solutions of CS.

Flat Substrate

First, the silica surface was functionalized with carboxylic acids by a carboxy silane. Next, just as for the beads, the -COOH modified surface was treated with DCC and NHS in THF, then aqueous solutions of CS. By digesting the final coated surface with 1M NaOH followed by lyophilization, we were able to obtain scintillation counts equivalent to a coating density of 0.4 ng/cm², which can also be expressed as a parking area of 82nm x 82nm.

Coating the silica surface with amine, then activating it with glutaral-di-aldehyde did not seem to work. Currently, to improve the quality of the -COOH layer at the surface, we are trying to first produce a dense mono-layer of vinyl groups at the
surface with a vinyl silane, then oxidize the terminal vinyl group to $-CH_2-COOH$ by the use of potassium permanganate.

4.2 Future Plans

4.2.1 Improvement On the Flat Substrates

Almost all wafers used were reject grade (based on criteria for microfabrication). We would like try the same vinyl silane chemistry on wafers of better quality. Furthermore, if functionalizing bare silica surface is indeed variable and extremely difficult, we will try to plasma coat the silica with gold, then functionalize the gold surface. Protein conjugation to gold surface is done routinely and is known to have a high yield. [7, 8, 33]

4.2.2 Alternative Chemistry

So far, for both the beads and the flat substrates, we used a DCC/NHS activation chemistry, which is susceptible to competition from water. We would like to try another approach, which, instead of using DCC/NHS, converts the adjacent COOH groups to anhydrides, [36] then conjugates the CS directly to the anhydrides. This approach requires NO water to be present. We will try to dissolve CS in NMH, DMSO, or even THF, then coat at higher temperatures (36 – 45°C).

4.2.3 Better Detection Technique

We will continue to work on improving our techniques to detect $^{35}S$ – sulfate on the CS covalently linked to the flat substrates. We will try to reduce both the concentration and the volume of the base digest.
Appendix A

Characterization of Stock

Chondroitin-6-Sulfate: Raw Data
**First Batch of CS, Received 6/1/99**

<table>
<thead>
<tr>
<th>Hot ul 1:50</th>
<th>AVG Abs</th>
<th>raw gag</th>
<th>conv. GAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.203</td>
<td>3.9264244</td>
<td>78.528488</td>
</tr>
<tr>
<td>2</td>
<td>0.222</td>
<td>8.5903419</td>
<td>85.903419</td>
</tr>
<tr>
<td>3</td>
<td>0.2375</td>
<td>12.395117</td>
<td>82.634111</td>
</tr>
<tr>
<td>4</td>
<td>0.2555</td>
<td>16.813565</td>
<td>84.067824</td>
</tr>
<tr>
<td>5</td>
<td>0.276</td>
<td>21.845686</td>
<td>87.382745</td>
</tr>
<tr>
<td>10</td>
<td>0.354</td>
<td>40.992295</td>
<td>81.98459</td>
</tr>
</tbody>
</table>

**AVG Hot:** 83.416863  
**Std Dev:** 2.8546818

**Dividing the radioactivity of the hot sample, 30,250cpm/ul, by the GAG concentration obtained from DMMB, 4.17ug/ul, we obtain the conversion factor, 73,000 cpm/(ug CS)**

<table>
<thead>
<tr>
<th>Cold ul 1:50</th>
<th>avg Abs</th>
<th>raw gag</th>
<th>conv. gag</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
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<td>59.647965</td>
<td>238.59186</td>
</tr>
<tr>
<td>2</td>
<td>0.3155</td>
<td>31.541725</td>
<td>315.41725</td>
</tr>
<tr>
<td>3</td>
<td>0.365</td>
<td>43.692458</td>
<td>291.28305</td>
</tr>
<tr>
<td>1.5</td>
<td>0.2725</td>
<td>20.986544</td>
<td>279.82058</td>
</tr>
<tr>
<td>1</td>
<td>0.25375</td>
<td>16.383994</td>
<td>327.67987</td>
</tr>
</tbody>
</table>

**AVG Cold:** 295.63972  
**Multiply by 50:**

<table>
<thead>
<tr>
<th>actual tot</th>
<th>1251.2529</th>
</tr>
</thead>
</table>

<p>| Cold 19.918928 | 14781.986 |
| actual total | 4434.5958 |</p>
<table>
<thead>
<tr>
<th>Medium</th>
<th>Tot. GAG (ug)</th>
<th>[CS], ug/ml</th>
<th>Cell Layer</th>
<th>Tot. GAG (ug)</th>
<th>[CS], ug/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>vol (ul)</td>
<td></td>
<td></td>
<td>vol (ul)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>8.01</td>
<td>2670</td>
<td>3</td>
<td>12.16</td>
<td>4053.3333</td>
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<tr>
<td>5</td>
<td>11.078242</td>
<td>2215.6484</td>
<td>5</td>
<td>17.363014</td>
<td>3472.6028</td>
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<td>13.108707</td>
<td>2184.7844</td>
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<td>21.810699</td>
<td>3635.1164</td>
</tr>
<tr>
<td>10</td>
<td>18.813346</td>
<td>1881.3346</td>
<td>10</td>
<td>30.416002</td>
<td>3041.6002</td>
</tr>
<tr>
<td>12</td>
<td>22.197454</td>
<td>1849.7878</td>
<td>12</td>
<td>35.637197</td>
<td>2969.7664</td>
</tr>
<tr>
<td>AVG. CONC (ug/ml)</td>
<td>2032.8888</td>
<td>AVG CONC (ug/ml)</td>
<td>3279.7714</td>
<td></td>
<td></td>
</tr>
<tr>
<td>std. dev.</td>
<td>168.05356</td>
<td>std. dev.</td>
<td>281.19506</td>
<td></td>
<td></td>
</tr>
<tr>
<td>count: 90166cpm/ul</td>
<td>90166</td>
<td>count: 174605.5</td>
<td>174605</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conversion factor</td>
<td>44353.631</td>
<td>Conversion factor</td>
<td>53236.941</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total ug CS</td>
<td>1016.4444</td>
<td>Total ug CS</td>
<td>2295.84</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Appendix B

Recipe For the pH/CS Concentration Matrix Experiment
### Recipe Used To Coat the Beads

<table>
<thead>
<tr>
<th>Conc (ug/ul)</th>
<th>5</th>
<th>1</th>
<th>0.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tot Vol (ul)</td>
<td>60</td>
<td>300</td>
<td>1500</td>
</tr>
<tr>
<td>Tot CS (ug)</td>
<td>300</td>
<td>300</td>
<td>150</td>
</tr>
<tr>
<td>Hot CS (ug)</td>
<td>75</td>
<td>75</td>
<td>37.5</td>
</tr>
<tr>
<td>Cold CS (ug)</td>
<td>225</td>
<td>225</td>
<td>112.5</td>
</tr>
<tr>
<td>vol hot CS (ul)</td>
<td>17.985612</td>
<td>17.985612</td>
<td>8.9928058</td>
</tr>
<tr>
<td>vol cold CS (ul)</td>
<td>15.222245</td>
<td>15.222245</td>
<td>7.6111224</td>
</tr>
<tr>
<td>vol buffer</td>
<td>26.8</td>
<td>266.8</td>
<td>1466.8</td>
</tr>
</tbody>
</table>

**Summary:** A total of 2.25mg CS is required, out of which 0.5625mg is hot and 1.6875mg cold.

### Recipe Used To Coat the Slides

<table>
<thead>
<tr>
<th>Conc (ug/ul)</th>
<th>1</th>
<th>0.1</th>
<th>0.01</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tot Vol (ul)</td>
<td>30</td>
<td>300</td>
<td>1500</td>
</tr>
<tr>
<td>Tot CS (ug)</td>
<td>30</td>
<td>30</td>
<td>15</td>
</tr>
<tr>
<td>Hot CS vol (ul)</td>
<td>7.1942446</td>
<td>7.1942446</td>
<td>3.5971223</td>
</tr>
<tr>
<td>Cold CS Vol (ul)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Buffer Vol (ul)</td>
<td>22.8</td>
<td>292.8</td>
<td>1496</td>
</tr>
</tbody>
</table>

**Summary:** A total of 0.225mg hot CS is used. No cold CS was added because the slides have a smaller surface area, and a minimum of 100cpm is preferred for detection by the scintillation counter.
Appendix C

Recipes For the pH Matrix

Experiment: Beads Only
Recipe For the pH Experiment On Beads

**CS:** radiolabeled cell layer CS, conversion factor 53,000 cpm/ugCS

**Buffer:** All made from mixing any of the following three:

- H$_3$PO$_4$, pH 1.
- NaH$_2$PO$_4$, pH 7
- Na$_2$HPO$_4$, pH 9

After mixing, the pH’s were verified with Litmus paper

<table>
<thead>
<tr>
<th>[CS]</th>
<th>1 mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>V(CS)</td>
<td>55ul = 300ug</td>
</tr>
<tr>
<td>V(buffer)</td>
<td>245ul</td>
</tr>
<tr>
<td>Tot. vol</td>
<td>300ul</td>
</tr>
</tbody>
</table>
Appendix D

Protocols For Activating Flat Substrate Surfaces With Glutaraldehyde
Revised Protocol For Coating Plain Silica Surface
With Chondroitin Sulfate GAG

1. Cut up wafer in 1cm x 3cm rectangles

2. Spray wash with ethanol.

3. Submerge in 12.5 wt% NaOH for 2hrs, NO LONGER than that.

4. Wash with dI water, then ethanol or acetone, blow dry.

5. Measure baseline optical properties of the subtrate for future thickness determination using the ellipsometer. (n=6)

6. Coat with 0.5 wt% A8TES (N-2-aminoethyl-aminopropyl-triethoxy silane), which is approximately 0.1ml /20ml of ethanol. Duration = 15-24 hours. Make sure all slabs stand straight up in the same vial.

7. Wash with ethanol thoroughly, blow dry.

8. Measure the thickness of the A8PES layer using ellipsometry (n=12), making sure the absorption maximum setting is constant with each other and with previous previous tests.

9. Prepare 1 wt% glutaraldehyde in milli-Q water (pH=? buffer needed?). Stir the solution for 10 min to homogenize. Chill in 4C fridge for 10 minutes.

10. Submerge slabs in the glut. vial, for 2hrs at 4C.

11. Wash thoroughly with water, dry thoroughly. (Can acetone be used?)

12. Take thickness measurement again with ellipsometry. Make sure the max. absorption is constant.

13. Dissolve lyophilized C-S GAG samples in distilled (or doidized?) water. Should it be buffered at pH9, using carbonate buffer? Mix thoroughly. Take the scintillation reading of total initial radioactivity.

14. Cut up the wafer most evenly coated with glutaraldehyde into two to three 0.3 x 0.5cm^2 rectangles. Place each facing up in an eppendorf. Inject buffered C-S solution. Let it sit for ? hours (2, 6, 24 hrs are quoted in literature).

15. Take thickness measurement using ellipsometry.
16. Wash off all non-specifically absorbed chondroitin sulfate, by sonication for two hours in 2 wt% SDS solution. Use ellipsometry again to quantify the amount of C-S actually covalently bound to surface.
Revised Protocol For Coating COOH-Modified Silica Beads

1. Take initial count of the radioactive C-S. Determine how many spheres are needed to give a significant reading in the scintillation counter.

2. Take out appropriate amount of spheres from SDS suspension. Rinse thoroughly with dd H2O to get rid of any SDS. Then rinse with 190 proof ethanol to facilitate drying. Lyophilize 1hr to get rid of any water or alcohol.

3. Prepare an anhydrous, aprotic solution of DCC/NHS (di-cyclohexyl-carbodiimide/N-hydroxysuccinimide) in tetrahydrofuran. The concentration for DCC is 0.4wt%, and 0.2wt% for NHS. Mix thoroughly with a stirrer for 10-15min.

4. After the beads are dried, inject 100x molar excess of the DCC/NHS solution to the beads. Mix completely by tapping. Let the reaction go on for 20min on a shaker, so that so sedimentation will occur.

5. Get rif of the reaction solution. Wash beads thoroughly with THF solution. Dry in lyophilizer for 30min.

6. Dissolve stock C-S GAG in 0.1 - 1mg/ml solution in carbonate-bicarbonate buffer (pH=9 - 9.5). Inject 10x molar excess of the C-S to the dry beads. Let the reaction take place for ?? hours (perhaps 6?) at room temperature.

7. Remove the C-S solution. Count the radioactivity for total surface C-S. Then wash with 2 wt% SDS under sonication for 1.5hrs. Count radioactivity again for covalently linked C-S.
List of Questions On Coating Chondroitin Sulfate On to Modified Surfaces:

1. To achieve a certain surface coating concentration (say one molecule per 90 anstrom sq), what bulk concentration should we use? Or, alternatively, to coat x ug of molecules onto some surface, how many ug (y) is needed in the bulk coating solution?

2. How much coating time is needed. I supposed this is dependent on the solution concentration as well. The more concentrated the solution, the less time. Is this true?

3. How reusable is the solution? Say if we need a high concentration and large volume for coating, would it be okay if we use the solution several times?

4. Once solubilized in carbonate buffered solution, might the GAG’s precipitate out of the solution during coating?
Appendix E

Protocols For Modifying Flat Substrate Surface With Carboxy Silane, and then With DCC/NHS
Most Current Coating Protocols

Coating -COOH functionalized Silica Beads

1. Stir the stock silica sphere suspension for five minutes, making sure that the spheres are evenly dispersed in the SDS-water solution.
2. Sterily, transfer 40ul suspension (ideally, 0.1g solid/ml suspension) from the stock bottle to a 1.5ml Eppendorf tube. Centrifuge at 0.4x of max speed for 2min. Remove supernatant.
3. Wash with sterile, tissue-culture grade water, 10x the volume of the original suspension (400ul). Centrifuge. Remove liquid. Repeat to wash the beads a total of five times. Remove excess liquid.
4. Lyophilize the beads to complete dryness (4-5hr under vacuum).
5. Measure out 0.08g DCC (dicyclohexyl carbodiimide, 0.4wt%) and 0.04g NHS (N-hydroxysuccinimide, 0.2wt%). Place them in two separate bottles capped by septums. To each vial, inject 10ml anhydrous THF (tetra-hydro-furan) under nitrogen. Shake vials to mix, but do NOT invert, the THF will dissolve the rubber. To a separate, septum capped bottle, inject 20ml anhydrous THF.
4. Inject 50ul DCC and 50ul NHS solution to well dried beads (from an original 40ul suspension, scale down or up as necessary). Mix well the beads and liquid. Let the reaction run for 15 to 20min, no longer than 20min.
5. Centrifuge the rxn mixture. Remove supernatant. Wash with five 400ul portions of anhydrous THF. Remove excess liquid. Lyophilize beads again (10-30min). Now the beads are ready to be coated with aqueous solution of chondroitin sulfate.
6. After 12-16hr of CS coating, spin down beads, remove CS solution. Wash once with 400ul water. Then sonicate in 400ul, 2wt% SDS solution to get rid of non-specifically adsorbed CS. Wash two more times to remove any free CS floating in solution. Remove liquid.

Protocol For Functionalization of Flat Silica Substrate

1. Scribe an optically clean silica wafer into 1.4cm x 3cm rectangles.
2. Rinse the rectangles with Milli-Q water followed by ethanol. Dry the slides with nitrogen, then immerse in 12.5wt% NaOH solution for 2 hours.
3. During the base bath, dissolve N- aminoethyl-N- aminopropyl -triethoxy silane (C8) to 0.5% w/w in ethanol.
4. At the end of the base wash, carefully take out the slides, rinse immediately with water and ethanol thoroughly, use sonication if necessary. Dry the slides under nitrogen blow. Measure baseline Ns and Ks values using the ellipsometer.
5. Choose slides with even distributions of Ns and Ks values, submerge them into the CS/ethanol solution for 16+ hours at room temperature.
5. Wash silica substrate with absolute ethanol. Dry under nitrogen blow. Measure the thickness by ellipsometry, using Ns, Ks obtained earlier.
6. Prepare 1wt% glutaraldehyde in Milli-Q water. Submerge slides in this solution for two hours at 4 degree celcius.
8. Wash glutaraldehyde treated slides with Milli-Q water. Dry under nitrogen. Cut slides into 2mmx5mm tiny rectangles. Now they are ready to be coated with CS.
9. Submerge two rectangles into one Eppendorf tube of CS solution. Let the reaction take place overnite (12-16hrs).
10. Rinse the coated rectangles with water. Sonicate each slide in 300ul of 2wt% SDS solution for 0.5hr. Rinse again with water. Save the SDS soup for scintillation counting.

Revised Protocol For Coating Flat Silica Substrate
Rationale: Due to the unpredictable nature of the glutaraldehyde chemistry (i.e. many, many possible side reactions that deactivate the surface for CS coupling), we decided to change the coating strategy. Specifically, we tried to functionalize the silica surface with carboxylate moieties, then use the same DCC/NHS chemistry to conjugate CS to the carboxylate groups.
1. & 2. Cleave and clean the bare silica surface the same way as described above.
3. Dissolve carboxysilane (Gelest, Product #2263.0) in 0.5wt% in Milli-Q water. Stir for 2min to ensure even suspension. Place one slide at a time, inside the solution. Gently agitate the coating mix for 3 minutes (sonication is okay).
4. Take out the slide from coating solution. Rinse with Milli-Q water thoroughly. Place all slides inside an oven (80-100C) for 45 to 60min to cure the silane. Measure thickness using the ellipsometry.
5. Make NHS/DCC solution the same way as for the beads. Place slides in the solution for 15-20min. Take out slides, rinse quickly with THF, then sonicate in anhydrous THF for 3-5min to remove all unreacted DCC and NHS.
6. Dry slides thoroughly and quickly. Submerge in CS coating solution immediately after drying (prolonged exposure in air will introduce side reactions which inactivate the surface by replacing the activated esters with hydroxyls).
7. Rinse, sonicate, and count the slides the same way as in the Glutaraldehyde protocol above. We have not developed a way to reliably remove the radioactive CS from the surface yet. We will try both proteinase K and sodium borohydride.
Appendix F

A Brief Guide On How To Operate the Multimode AFM In Contact Mode
Operating the Multimode AFM In Tapping and Contact Mode

This is a protocol for operating the Multimode AFM (Digital Instrument, Nanoscope III) in 56-353. The objective is to crystallize the detailed instructions from the manual to a few pages, so that new users can find a relatively easy start.

Part I. Principle of the Multimode AFM

1. Basic Principles In Contact Mode

As shown in Figure (2.2), the atomic force microscope works by approaching a tiny tip (which is attached to a cantilever) to a surface. The force of the interaction between the needle and the surface can be measured by the deflection of the cantilever, and then converting it to force by Hooke's law. The force vs. deflection of the cantilever is linear over a reasonable range. The z-deflection of the cantilever is measured by a photo-diode array (Fig 2.2).

In addition to force measurement, the AFM can be used for imaging. As soon as the tip is in contact with the surface, it will "stick" due to Van der Waal's interactions (Figure A). By setting the cantilever at a fixed deflection (i.e. fixed interaction force, and hopefully a fixed distance between the probe and the surface) and then scanning the probe over an area (using a piezo-electric controller), a topographical image can be obtained.

2. Tapping Mode

Based on the above force interaction and imaging principles, a variety of applications have been developed for AFM. The most popular one is tapping mode AFM. The original AFM is contact mode, meaning when the probe scans, it is constantly in contact with the surface. This is okay for rigid, well defined surfaces, but can be damaging to soft and/or biological surfaces. To overcome this obstacle, scientists developed the tapping mode, in which the cantilever is constantly vibrated. Without contact, the lever vibrates at its natural resonant frequency with a fixed amplitude. Upon contact with the surface, the vibration is dampened, and the amplitude reduced. Again, by setting the amount of amplitude reduction, one monitors the distance between the vibrating tip and the surface and an image can be obtained. Tapping mode is not designed for force measurement.

3. Lateral Force Mode

The AFM can also be used to measure friction forces (LFM, lateral force microscope). In addition to bending in the z-direction, the cantilever can also incline in the y (lateral, perpendicular to the tip axis) direction. The amount of y-inclination can be measured the same way the z-direction bending is measured, using an array of photo-detectors.

Part II. The Structure of the Multimode

1. Orientation of Key Scanning Elements.

The relative orientations of the scanning and photo-detecting elements are shown in Figure 2.2. When everything is intalled correctly, the tip should be right above the sample, which is mounted on the piezo-electric head. The essential feature that distinguishes the Multimode from the Dimension 3000 is that in the Multimode, the
scanning tip is held stationary and the sample is moved during imaging. In Dimension 3000, the sample is held stationary and the tip is moved by the piezo-electric.

2. Overall Structure of the Microscope
As shown in Figure 2.1, the Multimode microscope is consisted of three major components:

1) the base, which houses most of the electronics, connector to the computer, mode selecting switch (contact vs. tapping vs SPM), stepper motor switch for the height of the scanner, scanner support ring, and direct displays of signals from the photodiode. For details on what information each display window shows, please refer to figure 2.3.

2) the scanner, which consists the cylindrical, piezo-electric, scanning head, two manual screws to adjust the height of the optical head, and a small, cylindrical protrusion to connect to the stepper motor.

3) the optical head, this is where the tip, the laser, and the photo-diodes are housed.

Part III. Overall Protocols For Operating In Tapping and Contact Mode.
The overall approach to the set-up of both mode is similar. In the following paragraphs, the generic set-up procedure is outlined. An asterisk signifies that the details for this step differ between the contact and Tapping mode. (The major difference between the contact and the tapping is in the feedback signals they each take. For contact, the most important feedback signal is the top/bottom differential output from the photo-diode. For tapping, it is the RMS of the cantilever vibration amplitude. For LFM, it is the left/right differential output.)

Step 1: Mount scanner onto base (usually it is already done)
Step 2: Mount sample onto the piezo-electric head of scanner
Step 3: Mount Tip to tip holder*
Step 4: Mount tip holder into the optical head/scanner
Step 5: Mount the optical head/scanner on top of the support ring, connect laser.
Step 6: Locate tip with overhead microscope
Step 7: Dim light, locate laser spot, place the laser spot on the tip of cantilever*
Step 8: Maximize total signal by rotating the reflective mirror angle

Step 9: Adjust the photo-diode for maximum, optimal signal collection*

Step 10: If necessary, repeat step 7 to 9 till optimal alignment and signal is obtained (optional).
Step 11: Lower tip closer to surface for engagement
Step 12: Set up the software parameters for scanning*
Step 13: Place the microscope inside the vibration-proof chamber
Step 14: Engage tip, start scanning.

Part IV. Detailed Procedures For the Contact Mode
Step 1: Fit scanner into the triangular hole in base, with the piezo-electric facing up. Make sure the cylindrical protrusion from the bottom of the the scanner fit into the hollow tube from the stepper motor in the base. This stepper motor, together with the two manual knobs, control the height of the three metal balls on top of the scanner, which in turn determine support and balance the optical head.

Step 2: Attach sample surface to a standard 0.5" steel disc using double stick tape. Then place the metal disc on top of the magnetic piezo-electric head.

Step 3: Place new tip of interest in the vicinity. Gently press the tip holder onto a leveled surface, so the spring in the bottom is compressed and the wire clip securing the old tip is released. Using a specialized AFM tweezer, take out the old tip from the groove, while the spring is compressed. Replace with the new tip, making sure it is fitted snugly into the groove. Gently relieve pressure on the spring. If possible, look at the tip holder and the tip under microscope, making sure the set-up looks the same as that in Figure z in the manual.

Step 4: Slowly and gently, place the tip holder onto the bottom opening of the optical head. Make sure the two intrusions in the tip holder fits onto the tiny metal balls from the the bottom of the scanner. Secure the tip holder by tightening the screws at the center of the back of the optical head.

Step 5: Place the optical head on top of scanner, make sure that metal balls from the scanner is fitted to the appropriate holes. Also, very important, make sure the cantilever does not touch sample surface, or the tip will be destroyed. While holding the head with left hand, attach the two springs from the base to the head. Make sure the head does not topple over.

   It is absolutely crucial to make sure the head is levelled, otherwise the scanning results will be insignificant. The levelness is adjusted by the two manual screws from the scanner and the stepper moter at the base. The use of a precise level is highly recommended, if not required.

Step 6: Lower the microscope head to about 1cm away from the optical head. Ajust the lateral position of the microscope using the x-y translater located at the bottom of stage, so that the cantilever of interest is in the bright spot from the microscope lens. Slowly lower the microscope head to focus.

Step 7: Dim the light source to setting #1, move the x-y translator to look for the red spot from the laser. If necessary, twich the laser translater knobs (front right- for axial direction along the substrate, back-for perpendicular direction). Once located the laser spot, move it onto the tip of the cantilever of interest.

   For contact mode, we use triangular silicon nitride cantilever. The way to bring laser to its tip is
a: move laser spot to the edge of substrate, at the midpoint between the two legs.
b: move laser spot axially till the sum signal, as represented by the lower elliptical bar graph reaches a maximum (should be between 4 to 7V, approx). You should be able to see the laser spot defuse as it moves between the legs, and then focus again as it is moved onto tip. Toggle the crew back and forth till you are confident it is right at the center of tip.

For tapping mode, a long, thing, needle-like, silicon cantilever is used. Unlike the SiN cantilever which is coated with gold, the silicon cantilever is less reflective, therefore its total sum is about 3V. Again, move the laser to the end of substrate right next to the cantilever. Then move the spot horizontally using the axial knob toward the end of the cantilever. While the laser is on the cantilever, you should read an aprox. constant sum. As the end is reach, you will observe a maximum, which then drops as the laser is moved off from the tip. You want o position your laser just at point ned the end of the tip, right before the maximum is reached.

Step 8: Adjust the lever at the back of the optical head so as to reach the maximum total signal (shown by the elliptical bar graph).

Step 9: For contact mode AFM, adjust the top left screw (photo-diode positioner), so that the number at the top window is -2V. For contact mode, this number is the differential voltage between the upper and lower diodes, which is a direct measure of the deflection the cantilever is during contact. Usually, we set the before contact voltage to be -2. Upon contact, the cantilever will bend upward, so the lower output will read less and upper output more. It is a general rule define the point of engagement when the cantilever is bent for about 2V, therefore the setpoint is 0V.

For the tapping mode, we care about the RMS vibration amplitude of the cantilever, which is shown in the upper window, and which is adjusted during the cantilever tune routine. Before engaging, we need to make sure the top-bottom differential is zero. In tapping mode, the top-bottom differential is displayed in the lower winder, enclosed by the elliptical bar graph. This is again adjusted by the same photo-diode positioning knob as mentioned in the previous paragraph.

Step 10: Recheck everything. If necessary, repeat steps 7 through 9.

Step 11: This is a crucial step in that a: we want to make sure the tip is close enough to surface but not too close to be crushed; and b: while we approach tip to surface, we still want to make sure the optical head is leveled, so that during imaging, no parts from the cantilever, except the tip is in touch with the surface.

Knobs of interests: the two knobs from the scanner that control the height of the optical head, the stepper-moter, which also controls the height of the head, the two x-y knobs at the bottom of the optical head, which moves the head (therefore the tip) relative to the surface in minute distances; the focusing knob from the microscope.
**The microscope method** for approaching tip to surface: the idea is to use the microscope video to moditor the levelness of the optical head. The assumption is that at the starting point, the head is perfectly leveled.

*a.* Turn the focusing knob of the microscope to focus on first the tip and then the surface, so to get a rough idea of how far apar they are. Then focus on tip.

*b.* Using the tiny the x-y translator knob at the bottom of the optical head, move the tip to the farthest upper right corner of the microscope screen.

*c.* Turn the two scanner knobs CW to lower the front part of the optical head, till the image of the tip hit the lower corner of the screen.

*d.* Use the stepper motor control, press "down," till the image of the tip returns to its original position on screen.

*e.* Refocus tip and surface, and then back to tip. If they are still far apart, repeat step a thru c. If they are close enough (i.e. about 1/4 rotation of knob away), the procedure is successfully completed.

**Step 12.** Go to the computer. If in DOS mode, type 'cd: spm,' then type 'z,' to enable the microscope operation window. Now we can set up the scanning parameters.

**For Contact Mode:** go to Microscope from the pull-down menu, select 'small sample,' 'Multimode,' and 'E-scanner.' Set up the rest of the parameters using those quoted in manual Chapter 2.

**For Tapping Mode:** choose "tapping mode." Click on the little fork on top of screen to go to "cantilever tune" routine. Then click on "manual" button of the next window. Now the Manual tune window should pop up: set sweep width to 300KHz. The center of drive frequency should be around 260 KHz. Drive amplitude should be around 80mV. Input attenuation should be 8X.

*a.* set the "set-point" to be 0, to see the baseline. If baseline is horizontal and the peak is narrow, we have a good tip. If baseline is tilted, consider change tip.

*b.* Raise set-point to 3V.

*c.* While the sweep width is 300KHz, center the drive frequency to the resonant frequency by first finding the peak, then move the green line onto the peak. Click on Offset, which mobilizes the green line. Then move the green line to the center of peak, click the left button to immobilize the green line. Then click on "execute" or double click the right button to execute.

*d.* Narrow down sweep width to 2KHz, and reduce input attenuation to 1x to observe the details.

*e.* adjust the drive amplitude so that the max of the peak is right at 3V, the set-point.

*f.* Move the drive amplitude to 2 units to the left of peak. Click execute.
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the contact AFM and LFM, or the RMS value of the cantilever vibration amplitude for the Tapping Mode AFM. For the AFM techniques, an optical head is used to sense the cantilever deflection by sensing the change in position of a laser beam which is reflected off the back of the cantilever.

Figure 2.1 Multimode SPM with AFM Head (Front View)

The STM uses a Tip-view head which holds the atomically-sharp probe and the tunneling current preamplifier. The Multimode base contains switches
Quick Start Procedures

which change the function of the base by changing the way the output signal from the head is processed. Figure 2.1 shows a front view of the Multimode microscope and the major components.

Two different heads and four scanners are available for use with the Multimode base. The scanners which vary in length provide different maximum scan ranges. The two heads, the optical AFM head and the Tip-view STM head, provide several operating modes. Together, the different combinations of heads and scanners provide a wide range of scanning options.

Each scanner consists of a cylindrical piezoelectric tube which is attached to an outer shell at one end while the sample is mounted to the other end of the tube. Three fine-pitched screws which form the mount for the head run through the outer shell of the scanner. The head rests on the tips of the screws which are used to adjust the position of the head relative to the sample.

The Tip-view STM head supports the tip over the sample and contains the preamplifier circuit for the tunneling current. It senses the tunneling current between the tip and a conductive sample.

The optical head is used for TappingMode™ AFM, contact AFM, and LFM measurements. It relies on a very sharp probe on a flexible cantilever to sense the topography of the sample. Different styles and shapes of cantilevers are available but, regardless of the cantilever selection, the optical sensing system, shown schematically in Figure 2.2, monitors the deflection of the cantilever.

![Figure 2.2 Tapping Mode and LFM Optical Sensing System](image)

The beam from a laser diode is focused onto the back of the cantilever. The beam reflects off the back of the cantilever onto a segmented photodiode. The amplified differential signal between the upper and lower photodiodes provides a sensitive measure of the cantilever deflection. The differential
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