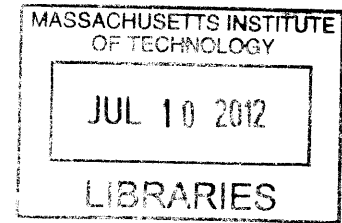**Developing Osteoarthritis Treatments through
Cartilage Tissue Engineering and Molecular Imaging**

by

Nicole Casasnovas Ortega

B. S. Chemical Engineering
University of Puerto Rico at Mayagüez

Submitted to the Department of Biological Engineering
in Partial Fulfillment of the Requirements for the Degree of

Master of Science in Biological Engineering
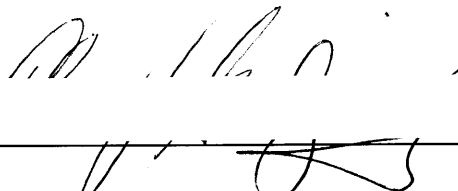
at the

Massachusetts Institute of Technology

June 2012

© 2012 Massachusetts Institute of Technology. All rights reserved.

Signature of Author _____

Department of Biological Engineering
[February 15, 2012]

Certified by _____

Alan J. Grodzinsky
Professor of Biological, Electrical, and Mechanical Engineering
Thesis Supervisor

Accepted by _____

Forest M. White
Associate Professor of Biological Engineering
Chair, Biological Engineering Graduate Program Committee

Developing Osteoarthritis Treatments through Cartilage Tissue Engineering and Molecular Imaging

by

Nicole Casasnovas Ortega

Submitted to the Department of Biological Engineering
on February 15, 2012 in Partial Fulfillment of the
Requirements for the Degree of Master of Science in
Biological Engineering

Abstract

Tissue engineering can be applied to develop therapeutic techniques for osteoarthritis, a degenerative disease caused by the progressive deterioration of cartilage in joints. An inherent goal in developing cartilage-replacement treatments is ensuring that tissue-engineered constructs possess the same properties as native cartilage tissue. Biochemical assays and imaging techniques can be used to study some of the main components of cartilage and assess the value of potential therapies.

Agarose and self-assembling peptides have been used to make hydrogels for *in vitro* culture of bovine bone marrow stromal cells (BMSCs) which can differentiate into chondrocytes, undergo chondrogenesis, and produce cartilage tissue. So far, differences in cell morphology that characterize chondrogenesis had been observed in peptide hydrogels like KLD and RAD but not in the 2.0% agarose hydrogels typically used for culture. A tissue engineering study was conducted to determine if a suitable environment for cell proliferation and differentiation could be obtained using different agarose compositions. BMSCs were cultured in 0.5%, 1.0%, and 2.0% agarose hydrogels for 21 days following TGF-β1 supplementation. Results indicate that the 0.5% agarose hydrogels are clearly inferior scaffolds when compared to the 1.0% and 2.0% agarose hydrogels, which are generally comparable. Since agarose gels appear to be suboptimal in promoting chondrogenesis, self-assembling peptides should be used in future studies.

In addition to the biochemical assays traditionally used in cartilage tissue engineering studies, atomic force microscopy (AFM) can be used to image aggrecan, one of the main components of cartilage. Imaging studies were carried out using fetal bovine epiphyseal aggrecan to optimize previous extraction and sample preparation procedures, as well as an AFM imaging protocol, for samples containing aggrecan. Experiments were conducted with 10, 25, and 50 µg/mL aggrecan solutions to find the minimum concentration needed to create aggrecan monolayers on APTES-mica that would yield acceptable AFM images (25

μg/mL). AFM instrument and software parameters were optimized to find the working range of the integral and proportional gains (0.2 - 0.4 and 0.6 - 0.8, respectively) and to increase the resolution, showing fields at the 800 nm level. Finally, an image processing protocol relevant to these molecules was established.

Thesis Supervisor: Alan J. Grodzinsky
Title: Professor of Biological, Electrical, and Mechanical Engineering

Acknowledgements

My time at MIT has been filled with a variety of experiences which have led to both professional development and personal growth. Taking part in my graduate program and contributing to an exciting area of biomedical research has helped me become a more skilled scientist and engineer. I have also had the opportunity to develop my capacity for analytical, strategic, and organizational thinking, particularly through leadership and volunteer positions within the MIT community. Furthermore, my graduate school experience has been an enriching and fulfilling one where I have become more aware of my interests, values, and ideals.

That being said, I would like to thank the people who have supported me over the past couple of years and who have helped make my experience at MIT a positive one.

First of all, I would like to thank Alan Grodzinsky for being a wonderful professor and my advisor. I have learned a lot through his example as a scientist and his enthusiasm towards both his work and his personal life. Al has been very encouraging and understanding as I have been conducting my thesis work. He has also supported me when I've taken time to pursue opportunities related to my professional interests. I will always be thankful for the role he has played in shaping my career.

I would also like to thank my fellow Grodzinsky lab members for maintaining a friendly work environment and for helping me out when I had to learn new lab techniques. A special thanks to Paul Kopesky, Rachel Miller, and Emily Florine for their help with cell culture techniques and biochemical assays. Hsu-Yi Lee and Lin Han were also helpful when working on aggrecan extraction and AFM imaging methods. Last, but not least, I would like to extend a very special thanks to Han-Hwa Hung for her help in performing a variety of lab protocols, as well as her welcoming, jovial, and supportive attitude.

Other key players in my graduate education were the MIT Department of Biological Engineering (BE) and the MIT-NIGMS Biotechnology Training Program (BTP). In addition to providing high-quality education and research funding, the BE department and BTP coordinators strive to maintain a community where students, staff, and faculty can exchange ideas and collaborate with one another. I have many good friends within these circles and always look forward to seeing them in special retreats, seminars, and discussion sessions. Specifically, I would like to thank Doug Lauffenburger, Forest White, K. Dane Wittrup, Dalia Fares, Darlene Ray, Daniel Darling, and Alan Parillo for their part in providing a positive graduate experience for me. My deepest appreciation goes out to Blanche Staton, the Senior Associate Dean for Graduate Students, and other members of the MIT administration who dedicate themselves to maintaining and improving the quality of life of students at MIT.

Another important part of my life at MIT has been my experience living at Ashdown House, a graduate residence with an active and diverse community of MIT graduate students. Many of my friends at MIT are fellow residents and we have shared fun activities in social settings both within MIT and the greater Boston area. I would like to thank Ann and Terry Orlando, the housemasters at Ashdown House, as well as fellow volunteer officers with whom I have worked with on different community projects for being a part of my life at MIT. Similarly, I am grateful of my time working with other multidisciplinary student groups like the MIT Science Policy Initiative and the MIT Graduate Student Council (GSC).

Finally, I would like to thank my family for their unconditional love and support. My mom, dad, and brother have always inspired and encouraged me to reach for the stars and trust my heart while making decisions. It is truly a privilege to have such a wonderful, kind, and loving family. I hope they are as proud of me as I am of them.

Table of Contents

List of Figures

Chapter 1: Introduction

Chapter 2: Culture of Bone Marrow Stromal Cells in Agarose Hydrogels

Chapter 3: Atomic Force Microscopy Imaging Assay for Cartilage Tissue Engineering

Chapter 1: Introduction

1.1 Osteoarthritis and Current Treatments

Osteoarthritis (OA), sometimes called degenerative joint disease, is the most common form of arthritis. It affects over 27 million people in the United States, particularly women over the age of 40 and the elderly [1.1]. People who are overweight, have suffered from joint injury, exhibit muscle weakness, or have bone deformities present an increased risk of developing this disease [1.2].

The main cause of osteoarthritis is the progressive deterioration of the cartilage that cushions bones in joints, specifically joints in the hands, hips, knees, and spine. As seen in Figure 1.1, the smooth surface of the cartilage becomes rough and, eventually, the cartilage may wear down completely, causing the bones at the affected joint to rub against each other [1.3]. As one would expect, this results in pain, stiffness, or loss of flexibility in the joints. Other characteristic symptoms of OA include swelling of the joints, a grating sensation when using the affected joint, and the presence of bone spurs or hard lumps that involve physical deformation [1.2, 1.3].

Osteoarthritis symptoms often develop slowly and worsen over time. Joint pain and stiffness may become severe enough to make getting through the day difficult, if not impossible. A French study found that more than 80% of clinical OA patients reported limitations in their daily lives, including basic tasks, work, and leisure activities [1.4]. As a result, people with OA can often feel desperate or frustrated when coping with their new limitations, which may lead to depression or other mental disorders [1.5].

Treatments depend on the severity of the disease. For mild to moderate OA, treatments include rest, exercise, physical therapy, losing weight, pain relievers, and drugs like acetaminophen or ibuprofen. Patients with severe OA may need to resort to stronger painkillers, cortisone shots, or even surgery such as joint replacement, realigning bones, and cleaning up the area around the joint. Unfortunately, there is no cure for osteoarthritis and the treatments available only alleviate symptoms temporarily [1.2, 1.6].

1.2 Cartilage and Aggrecan

Cartilage is an elastic and flexible connective tissue found in many areas of the body including intervertebral discs, the ear, the nose, and joints between bones. As such, some of the main functions of cartilage are providing structural support for body tissues, improving movement by reducing friction between bones, and preventing joint damage by acting as a shock absorber [1.7, 1.8].

11

Some of the main types of this tissue are fibrous, elastic, and articular cartilage. Fibrous cartilage may be found in areas like the intervertebral discs of the spinal cord, while elastic cartilage is located in places such as the outer ear, nose, and larynx. Articular cartilage, or hyaline cartilage, can be found in the joints between bones, notably in areas like the knees and the hips [1.7]. As observed in patients with osteoarthritis, articular cartilage does not regenerate in adults. Although superficial damage can heal in the very young, for all practical purposes injuries to hyaline cartilage heal with fibrous cartilage which has inferior weight-bearing properties [1.8, 1.9].

Hyaline cartilage functions as a low-friction, wear-resistant tissue designed to bear and distribute loads. This tissue is composed of specialized cells called chondrocytes that produce an extracellular matrix (ECM) rich in collagen fibers, proteoglycans, and other proteins and biomolecules, giving articular cartilage its characteristic mechanical properties [1.10]. Although cells make up only about 5% of the wet weight, chondrocyte metabolism is responsible for the maintenance of a stable and abundant ECM. The balance between anabolism and catabolism of the matrix is crucial for articular cartilage homeostasis and providing hyaline cartilage with viscoelastic and mechanical properties for efficient load distribution. [1.8]

Collagens are involved in defining structural integrity and some physiological functions of neighboring cells within cartilage tissue. Additionally, collagen degradation and disturbed metabolism are important in the course of OA [1.10]. There are many types of collagen, each with different properties. Type II collagen is the characteristic and predominant component of cartilage. It has the ability to assemble into highly oriented fibril aggregates that help mediate the interaction with proteoglycans. Fibrous cartilage contains type III collagen and is less durable than articular cartilage [1.7, 1.11].

Aggregating proteoglycans and other hyaluronan-binding proteins enmeshed in the collagen matrix provide the complex mechanism that allows cartilage tissue to resist deformation. These molecules contribute to the material and biological properties of the matrix and regulate cell and tissue function [1.11, 1.12]. The proteoglycan superfamily contains more than 30 full-time molecules that fulfill a variety of biological functions. For instance, proteoglycans act as tissue organizers, influence cell growth and the maturation of specialized tissues, play a role as biological filters, regulate collagen fibrillogenesis and skin tensile strength, and influence corneal transparency. The presence of aggregating proteoglycans in the ECM confers a high fixed negative charge density due to the numerous chondroitin sulfate chains, and can have important effects on the material properties and permeability of the matrix [1.12].

One of the prevalent molecules in the cartilage ECM is aggrecan, depicted in Figure 1.2 [1.13]. This aggregating proteoglycan has a high concentration of charged chondroitin

sulfates and has covalently attached glycosaminoglycan (GAG) side chains, as well as a small number of keratan sulfate chains. There may be about 100 chondroitin sulfates per aggrecan molecule. Aggrecan has a tridomain structure: an N-terminal domain that binds hyaluronan, a somewhat central domain that carries the glycosaminoglycan side chains, and a C-terminal region that binds lectins [1.12].

OA is characterized by the damage or destruction of the aforementioned components of cartilage. Molecules originating from articular cartilage, including aggrecan and type II collagen fragments, are usually released as degradation products as a result of catabolic processes. Therefore, these molecules serve as biomarkers that reflect quantitative and dynamic changes in the degradation products of cartilage, providing a means of identifying patients at risk for rapid joint damage and also for early monitoring of the efficacy of disease-modifying therapies [1.8, 1.10].

Aggrecan can also be used as a biomarker to test the potential of tissue-engineered constructs as cartilage substitutes. Thus, the ability to image aggrecan and assess its structural properties on a molecular scale is an asset to the field of cartilage tissue engineering. Atomic force microscopy (AFM) can be used to obtain such images and assist in our understanding of OA and, ultimately, in developing tissue-engineered cartilage replacement therapy [1.13].

1.3 Cartilage Tissue Engineering

Tissue engineering principles can be applied to develop therapeutic techniques to combat the progression of OA and even to produce cartilage substitutes to replace damaged tissue [1.14, 1.15]. Even though tissue engineering techniques have been used to produce cartilage-like tissues, it is important to ensure that these constructs maintain the same functional properties as native cartilage tissue.

A common cell source used in cartilage tissue engineering is bone marrow stromal cells (BMSCs), which can easily be cultured and expanded *in vitro*. These cells include mesenchymal progenitors capable of differentiating into cartilage tissue [1.16]. Cultured cells must be able to produce cartilage through a process called chondrogenesis. In culture, desired cellular differentiation and stimulation of chondrogenesis is achieved by using transforming growth factor β (TGFβ) [1.17].

One approach to tissue engineering involves inducing previously isolated cells to produce a tissue analog with composition, structure, and function approaching that of native cartilage tissue. Cells are usually encapsulated in three-dimensional matrices such hydrogels that serve as support structures and have been shown to help the cells maintain a chondrocytic phenotype during long-term culture [1.18].

13

Self-assembling peptide hydrogels, a new class of biomaterials, have shown to have many applications in biotechnology. These materials allow for study and manipulation of cells and may be used as novel scaffolds for tissue engineering and potential drug delivery vehicles [1.19]. Self-assembling peptide hydrogels have been applied in cartilage repair strategies and have been shown to stimulate the production of a mechanically functional ECM and cell division in constructs using chondrocytes [1.20]. Figure 1.3 shows a cartilage tissue engineering system where chondrocytes have been cultured in self-assembling peptide hydrogel disks. These successful constructs suggest the potential of peptide hydrogels in culturing BMSCs.

1.4 Atomic Force Microscopy Imaging

As previously mentioned, aggrecan is an important biomarker in cartilage tissue engineering, especially when assessing the viability of constructs as functionally equivalent substitutes for native cartilage tissue [1.22]. Atomic force microscopy (AFM) can be used to obtain nm-scale resolution images of numerous biological macromolecules, including notable ECM constituents, in fluid and ambient conditions [1.23, 1.24].

Direct visualization of aggrecan molecules through AFM imaging has been achieved while noting detailed intramolecular structures and probing mechanical properties [1.13]. Figure 1.2B shows the AFM height image of an individual aggrecan molecule isolated from fetal bovine epiphyseal cartilage where individual GAG chains can be observed and distinct resolution of the N-termianl globular domains from the CS/KS-substituted brush region has been achieved [1.13].

AFM imaging makes determination of the number, spacing, dimensions, and conformation of GAG chains and full-length molecules possible [1.25]. Consequently, the use of AFM imaging techniques to study aggrecan is advantageous both for improving our understanding of OA and for furthering the field of cartilage tissue engineering.

1.5 Thesis Overview

The objective of this thesis is to further the areas of cartilage tissue engineering and the molecular imaging of aggrecan through discovery and optimization studies.

This chapter has provided ample background on osteoarthritis and developing treatments for this degenerative joint disease using tissue engineering. Cartilage, the key molecules in this tissue, and the study of said molecules via AFM imaging have also been discussed.

Chapter 2 describes a cartilage tissue engineering experiment using bone marrow stromal cells aimed at finding the optimal agarose hydrogel composition for culturing these cells.

Chapter 3 expands on an atomic force microscopy assay for the study of native cartilage and tissue engineered constructs, including different measures that were taken to find the optimal sample preparation conditions and imaging settings.

Finally, Chapter 4 includes a summary of the main findings and suggestions for further work motivated by this thesis.

Note that all figures and references will be included at the end of each chapter.

1.6 Figures

Early Stages of Disease

Degeneration of cartilage

Reactive new bone

Late Stages of Disease

Cartilage particles

Loss of cartilage

Bone hypertrophy
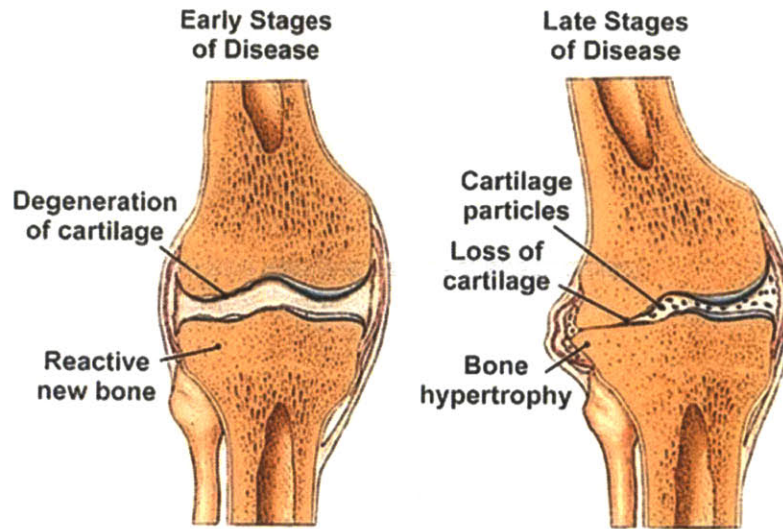
Figure 1.1 Progression of Osteoarthritis in the Knee. The early and late stages of osteoarthritis are depicted in a knee joint where we can see how articular cartilage is degenerated over time, affecting the adjoining bones in this joint. With generally asymmetric cartilage degradation, the joint becomes malaligned, which in turn causes abnormal joint wear and even reactive bone formations or osteophytes [1.21].

A.
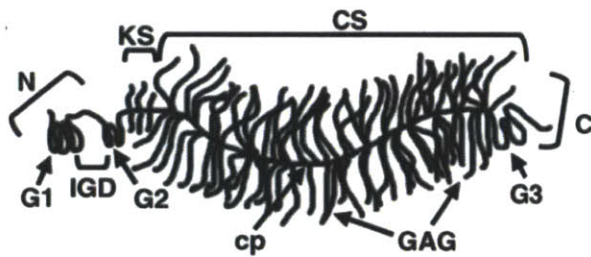
B.
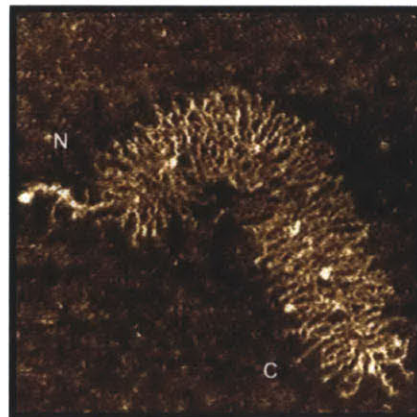
Figure 1.2 Aggrecan Molecule. (A) Diagram of a single aggrecan molecule indicating the G1, G2, and G3 domains, keratan sulfate chains (KS), chondroitin sulfate chains (CS), the interglobular domain (IGD), the core protein (cp), glycosaminoglycan chains (GAG), and the N- and C-terminus. **(B)** AFM height image of an individual aggrecan molecule isolated from fetal bovine epiphyseal cartilage [1.13].
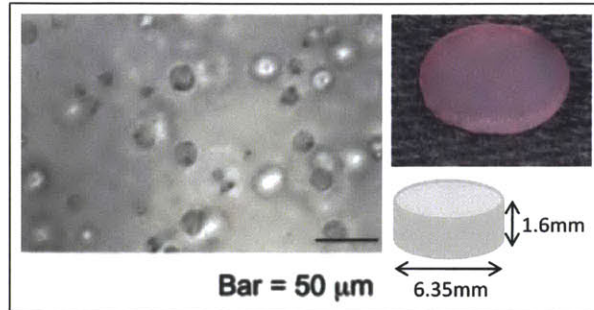
Figure 1.3 Cartilage Tissue Engineering System. Peptide hydrogels were used as scaffolds to culture chondrocytes. The left panel shows a light microscope image of chondrocytes encapsulated in peptide hydrogel. A chondrocyte-seeded, peptide hydrogel disk, along with its dimensions, can be seen in the panel on the right [1.20].

1.7 References

[1.1]   Lawrence RC, Felson DT, Helmick CG, Arnold LM, Choi H, Deyo RA, Gabriel S, Hirsch R, Hochberg MC, Hunder GC, Jordan JM, Katz JN, Kremers HM, Wolfe F, National Arthritis Data Wrokgroup. (2008) Estimates of the prevalence of arthritis and other rheumatic conditions in the United States. Part II. *Arthritis & Rheumatism* 58(1): 26-35.

[1.2]   Felson DT, Lawrence RC, Dieppe PA, Hirsch R, Helmick CG, Jordan JM, Kington RS, Lane NE, Nevitt MC, Zhang Y, Sowers M, McAlindon T, Spector TD, Poole AR, Yanovski SZ, Ateshian G, Sharma L, Buckwalter JA, Brandt KD, Fries JF. (2000) Osteoarthritis: New Insights; Part 1: The Disease and Its Risk Factors. *Annals of Internal Medicine* 133: 635-646.

[1.3]   Hunter DJ. (2007) In the clinic. Osteoarthritis. *Annals of Internal Medicine* 147(3): ITC8-1-ITC8-16.

[1.4]   Fautrel B, Hilliquin P, Rozenberg S, Allaert FA, Coste P, Leclerc A, Rossignol M (2005) Impact of osteoarthritis: results of a nationwide survey of 10,000 patients consulting for OA. *Joint Bone Spine* 72: 235-240.

[1.5]   Berenbaum F. (2008) New horizons and perspectives in the treatment of osteoarthritis. *Arthritis Research & Therapy* 10(S2): S1.

[1.6]   Madry H, Grün UW, Knutsen G. (2011) Cartilage Repair and Joint Preservation: Medical and Surgical Treatment Options. *Deutsches Ärzteblatt International* 108(40): 669-677.

[1.7]   Dandy DJ and Edwards DJ. (2009) *Esssential Orthopaedics and Trauma.* Churchill Livingstone Elsevier. 5: 33-37.

[1.8]   Pearle AD, Warren RF, Rodeo SA. (2005) Basic Science of Articular Cartilage and Osteoarthritis. *Clin Sports Med* 24: 1–12.

[1.9]   Strehl R, Tallheden T, Sjögren-Jansson E, Minuth WW, Lindahl A. (2005) Long-term maintenance of human articular cartilage in culture for biomaterial testing. *Biomaterials* 26: 4540–4549.

[1.10] Goldring MB and Marcu KB. (2009) Cartilage homeostasis in health and rheumatic diseases. *Arthritis Research & Therapy* 11(3): 224.

[1.11] Gelse K, Pöschl E, Aigner T. (2003) Collagens - structure, function, and biosynthesis. *Advanced Drug Delivery Reviews* 55: 1531-1546.

[1.12] Iozzo, RV. (1998) MATRIX PROTEOGLYCANS: From Molecular Design to Cellular Function. *Annu Rev Biochem* 67: 609–652.

[1.13] Ng L, Grodzinsky AJ, Patwari P, Sandy J, Plaas A, Ortiz C. (2003) Individual cartilage aggrecan macromolecules and their constituent glycosaminoglycans visualized via atomic force microscopy. *Journal of Structural Biology* 143: 242-257.

[1.14] Danisovic L, Varga I, Zamborsky R, Bohmer D. (2011) The tissue engineering of articular cartilage: cells, scaffolds and stimulating factors. *Experimental Biology and Medicine* 1–8.

[1.15] Hardingham T, Tew S, Murdoch A. (2002) Tissue engineering: chondrocytes and cartilage. *Arthritis Res* 4(3): S63-S68.

[1.16] Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* 284(5411): 143-147.

[1.17] Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. (1998) In Vitro Chondrogenesis of Bone Marrow-Derived Mesenchymal Progenitor Cells. *Experimental Cell Research* 238: 265–272.

[1.18] DiMicco MA, Kisiday JD, Gong H, Grodzinsky AJ. (2007) Structure of pericellular matrix around agarose-embedded chondrocytes. *Osteoarthritis and Cartilage* 15: 1207-1216.

[1.19] Zhang S, Marini DM, Hwang W, Santoso S. (2002) Design of nanostructured biological materials through self-assembly of peptides and proteins. *Curr Opin Chem Biol* 6(6): 865-871.

[1.20] Kisiday J, Jin M, Kurz B, Hung H, Semino C, Zhang S, Grodzinsky AJ. (2002) Self-assembling peptide hydrogel fosters chondrocyte extracellular matrix production and cell division: Implications for cartilage tissue repair. *PNAS* 99(15): 9996-10001.

[1.21] American College of Rehumatology. (2009) Osteoarthritis: Progression, Knee. *Rheumatology Image Bank.* <http://images.rheumatology.org>

[1.22] Chung C and Burdick JA. (2008) Engineering cartilage tissue. *Advanced Drug Delivery Reviews* 60: 243–262.

[1.23] Raspanti M, Congiu T, Guizzardi S. (2001) Tapping-mode atomic force microscopy in fluid of hydrated extracellular matrix. *Matrix Biology* 20: 601–604.

[1.24] Sheiko SS. (2000) Imaging of Polymers Using Scanning Force Microscopy: From Superstructures to Individual Molecules. *Advances in Polymer Science* 151: 61–174.

[1.25] Shao Z, Mou J, Czajkowsky DM, Yang J, Yuan JY. (1996) Biological atomic force microscopy: what is achieved and what is needed. *Advances in Physics* 45(1): 1-86.

Chapter 2: Culture of Bone Marrow Stromal Cells in Agarose Hydrogels

2.1 Introduction

Tissue engineering principles can be used to develop treatments for osteoarthritis and even to produce replacements for damaged cartilage tissue. The ultimate goal of tissue engineering approaches is to induce previously isolated cells to produce a tissue analog with composition, structure, and function approaching that of native cartilage tissue, which can later be implanted into a joint [2.1, 2.2].

Some of the main factors that must be established when designing a tissue-engineered construct are the type of cells that will be cultured, the type of scaffolds that will serve as their support structures, and the growth factors that will be used to obtain the desired tissue. A common cell source used in cartilage tissue engineering is bone marrow stromal cells (BMSCs), which can easily be cultured and expanded *in vitro*. These cells include mesenchymal progenitors capable of differentiating into cartilage tissue [2.3, 2.4].

BMSCs can be induced to undergo the desired cellular differentiation using transforming growth factor β (TGFβ), which serves to stimulate cartilage production through chondrogenesis [2.5]. Members of the TGFβ superfamily have been found to play roles in chondrocyte growth, differentiation, proliferation, migration, and extracellular matrix synthesis in different cell types, including chondrocytes and chondrogenic progenitor cells [2.6, 2.7].

Hydrogels composed of biomaterials like alginate, agarose, and self-assembling peptides are commonly used as scaffolds for tissue engineering [2.8, 2.9]. Investigators have found that agarose gels used to culture chondrocytes isolated from bovine, rabbit, and human sources help maintain cell phenotype and promote synthesis of a mechanically functional ECM with nominal levels of type II collagen and cartilage-specific aggrecan [2.10]. Similarly, self-assembling peptide hydrogels such as KLD and RAD have been applied in cartilage repair strategies and have been shown to stimulate ECM production and cell division in constructs using chondrocytes. This suggests their potential as peptide hydrogels for culturing BMSCs [2.11].

It is known that changes in cell morphology indicate proliferation and the start of chondrogenesis. As seen in Figure 2.1, morphology images of bovine BMSCs encapsulated in hydrogel scaffolds indicate that changes in cell morphology occurred in self-assembling peptide hydrogels (i.e., KLD and RAD) during the first four days of TGFβ supplementation but not in 2% agarose hydrogels [2.12].

Given that so far the differences in cell morphology that characterize chondrogenesis have been observed only in peptide hydrogels, it is important to verify if this favorable trend can be achieved using other materials. Agarose is a well studied gel that is more convenient to work with when compared to peptide gels. Studies typically include 2.0% agarose gels but this concentration may not provide the adequate mechanical properties for the optimal culture environment [2.10, 2.12]. What's more, studies using agarose gels in the past have indicated that 1% gels can cause chondrocytes to dedifferentiate while 2% gels are optimal for culture [2.13].

The main objective for this experiment is to verify if the differences in cell morphology that are typical of chondrogenesis and have been observed in peptide hydrogels can be achieved using agarose hydrogels. We also seek to gain a better understanding of the process of chondrogenesis, particularly in its initial stages. As a result, BMSCs will be cultured in agarose hydrogels of different concentrations to identify which scaffold best promotes chondrogenesis.

2.2 Materials and Methods

For this study, BMSCs were isolated from bovine calves and then encapsulated in 0.5%, 1.0%, and 2.0% agarose hydrogels assembled as disks such as those in Figure 1.3. The BMSC-seeded hydrogels were cultured for 21 days following TGFβ supplementation and several assays were performed to measure key properties and biochemical markers relevant in cartilage tissue engineering.

Isolation and Expansion of BMSCs

BMSCs were isolated from the femoral and tibial diaphyses of a bovine calf. After removing all connective tissue and muscle around each bone, sterilized hand-saw blades were used to bisect the bones at the mid-diaphysis. The exposed bone marrow was extracted from the medullary cavity with forceps and stored in PBS containing 1% PSA (100 U/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin). The bone marrow was mechanically disrupted and the fat content was removed via centrifugation at 1000 g for 15 minutes. The pellet was resuspended in PBS and further homogenized by subsequently passing the material through 16- and 18-gauge needles and a 70 μm cell strainer.

The nucleated cell count was then obtained by diluting a portion of the cell suspension in Ammonium Chloride-Tris Base using the Trypan Blue. After centrifugation at 200 g for 15 minutes, the cell pellet was resuspended in a culture medium that consists BMSC Expansion Medium (low glucose DMEM with 10% ES-FBS, 1% HEPES, and 1% PSA) supplemented with 1ng/mL bFGF. Approximately 75 x 10^6 cells were pre-plated and incubated for 30 minutes to allow rapidly-adhering cells to attach. The media and non-

adherent cells were transferred to another T-flask and additional BMSC Expansion Medium was added for approximately 1 x 10^6 cells/cm^2 in 15 mL of media.

After two days in culture, the media was exchanged for fresh BMSC Expansion Medium after and flasks were cultured until cells were nearly 75-80% confluent (Passage 0, P0). The cells that adhered to the T-flask wall were removed with 0.05% trypsin-EDTA, centrifuged at 200 g for 15 minutes, resuspended in PBS, and the nucleated cell count was obtained again via the Trypan Blue exclusion method. Finally, the cells were cryopreserved and frozen in aliquots of 5 million cells/vial using liquid nitrogen.

See Appendix A.1 for more details on this protocol.

Thaw and Expansion of Cryopreserved BMSCs

Cryopreserved cells were rapidly thawed at 37°C and resuspended in high glucose DMEM. Following centrifugation at 200 g for 8 minutes, the cell pellet was resuspended in BMSC Expansion Medium (low glucose DMEM with 10% ES-FBS, 1% HEPES, and 1% PSA) and the cells were counted via the Trypan Blue exclusion method in order to calculate the parameters for seeding the BMSCs into flasks for culture. Cells were cultured in T-flasks at 6000 cells/cm^2 in BMSC Expansion Medium with 5 ng/mL FGF at 37°C for three days.

BMSCs were detached from the culture-treated walls of the T-flasks by incubating the flasks with trypsin at 37°C for 4 minutes after having removed the media and washed with PBS. FBS Medium was added to each T-flask to inactivate the trypsin and all flasks were combined after subsequent washing with the new media. The cells were counted via the Trypan Blue exclusion method in order to calculate the parameters for the next passage. BMSCs were cultured in T-flasks at 6000 cells/cm^2 in FBS Medium with 5 ng/mL FGF at 37°C for three days in preparation for encapsulation in hydrogels.

See Appendix A.2 for more details on this protocol.

Encapsulation of BMSCs in Agarose Hydrogels for Culture

Custom three-piece casting molds were used to make 2% agarose rings whose hollow centers can be filled with hydrogels as seen in Figure 2.2. Once the acellular agarose rings solidified, the molds were removed and the rings were equilibrated in Casting Medium (high glucose DMEM with 25mM HEPES, 1% ITS+1, 1% NEAA, 1% Na Pyr, 1% PSA, and 0.4% Proline) at 37°C. The 0.5%, 1.0%, and 2.0% agarose solutions for cell encapsulation were prepared by dissolving the stock agarose solution to the desired concentration in sterile 10% sucrose.

Cells were treated with trypsin and inactivated with FBS Medium (low glucose DMEM with 10mM HEPES, 10% ES-FBS, and 1% PSA) for subsequent centrifugation and resuspension

in FBS Medium. Aliquots with 15 x 10^6 cells in 2 mL sterile tubes were centrifuged at 100 g for 5 minutes. Meanwhile, the agarose rings were prepared for casting by aspirating the media, leaving the wells completely dry.

The casting portion of this procedure is a two-person job given that it requires optimal timing and speedy work. The team must aspirate the media from the 2 mL tubes and resuspend the cell pellets in 300 µL of sterile 10% sucrose with 2.5mM HEPES. They must then inject the cell suspension into the desired hydrogel solutions and use a repeater pipette to dispense 50 µL of the cell-seeded hydrogel suspension into the center of each agarose ring. Finally, each well is covered with Casting Medium, which is then substituted by Culture Medium supplemented with 10ng/mL of TGFβ. Cells were incubated at 37°C with media changes every 2-3 days [2.14].

See Appendix A.3 for more details on this protocol. Appendix A.4 contains details for subsequent cell culture media changes and sampling.

Cell Viability and Morphology Assay

The Live/Dead assay was used to obtain a qualitative picture of cell viability and evaluate cell morphology. Sterilized tools were used to remove the exterior agarose rings from desired BSMC-seeded hydrogels and place them in microscope slide wells with PBS 1X. Samples were then dyed with 12.5 µg/mL of fluorescein diacetate for viewing live cells and 350 ng/mL of ethidium bromide for viewing dead cells. A Nikon Eclipse Fluorescent Microscope was used to image samples and identify live cells, which appear green in color, and dead cells, which appear red in color.

See Appendix A.5 for more details on this protocol.

Lyophilization and Proteinase K Digestion

Samples were freeze-dried and their final lyophilized weights were obtained. Hydrogel samples were then digested using 0.25 mg/mL Proteinase K so that specific biomolecules can be detected through the GAG Assay, DNA Assay, and Radiolabel Incorporation Assay.

See Appendix A.6 for more details on this protocol.

GAG Assay

This assay was used to determine the sulfated glycosaminoglycan (GAG) content in the tissue-engineered constructs. Samples were stained with dymethylmethylene blue (DMMB) dye, which indicates the presence of chondroitin sulfate and keratan sulfate found within GAG chains. A standard concentration curve was prepared using chondroitin-6-sulfate solutions in Tris Buffer. Samples and standards (20 µL) were distributed along a clear 96-well plate, stained with 200 µL DMMB dye, and readings were obtained at a 520

nm wavelength in the Maxy machine for data processing in terms of GAG concentration (μg/mL).

See Appendix A.7 for more details on this protocol.

DNA Assay

The DNA Assay uses bisbenzimidazole fluorescent dye Hoechst 33258 to stain DNA present in experimental samples. The interaction of Hoechst 33258 with DNA requires at least four consecutive A-T base pairs, as well as dissociation of DNA from proteins of the nucleoprotein complex. The former is satisfied by random chance, while the latter was accomplished by Proteinase K digestion.

A standard concentration curve was prepared using a 10 μg/mL DNA stock in Tris Buffer. Samples and standards (20 μL) were distributed along a black 96-well plate, stained with 200 μL 0.1 μg/mL Hoechst 33258 DNA Dye Solution, and readings were obtained in the Victor machine for data processing in terms of DNA concentration (μg/mL).

See Appendix A.8 for more details on this protocol.

Radiolabel Incorporation Assay

The objective of this assay is to examine cell proliferation and proteoglycan synthesis by radiolabeling samples with ^{3}H-Thymidine and ^{35}S-Sulfate, respectively. In order to label the samples, the ITS Medium was supplemented with calculated amounts of the desired isotopes (i.e., 5 μCi/mL ^{35}S and 10 μCi/mL ^{3}H) while preparing the ^{35}S and ^{35}S^{3}H standards needed for scintillation counts later on. Each cell-seeded hydrogel sample was cultured with 750 μL of the radiolabeled media at 37°C for 24 hours to ensure isotope incorporation.

Once at the 24-hour point, the radiolabeled media was removed from each well and samples were submitted to four consecutive washes to remove unincorporated isotopes. For each wash, 1mL of Rx Wash was added to all radiolabeled hydrogels within the 24-well plate, which was incubated for 30 minutes in a refrigerator to slow cell metabolism and maintain viability. Washed samples were then transferred to pre-weighed vials after removing the 2% agarose rings surrounding the plugs and the wet weight of each hydrogel was obtained.

A scintillation counter was then used to measure the levels of radioactivity given off by samples which have been tagged with ^{3}H and ^{35}S. Finally, the levels of protein synthesis (^{3}H) and proteoglycan synthesis (^{35}S) were calculated from the readings for the corresponding isotopes, converted to concentrations (nmol), and normalized by DNA content and gel weight (nmol/μg/mL/mg).

See Appendix A.9 for more details on this protocol.

2.3 Results and Discussion

Various assays were performed on the tissue-engineered constructs throughout the 21-day culture to measure several properties of interest such as cell viability and morphology, GAG content, DNA levels, gel contraction, cell proliferation, and proteoglycan synthesis.

Cell Viability and Morphology Assay

The Live/Dead Assay was used to assess the viability and get an idea on the morphology of cultured cells. A visual comparison of the live and dead cells, illustrated in Figure 2.3, indicates that viability was excellent and therefore not a cause for concern in proceeding with experiments and analyzing results. More importantly, it is evident that the BMSCs retained a spherical morphology in all agarose hydrogels both at Day 4 and Day 7 of culture. The fact that the cells did not exhibit changes in morphology like those observed in Figure 2.1 suggests that though agarose hydrogels with concentrations below 2.0% might provide a microenvironment more mechanically similar to *in vivo* surroundings, they are not suitable for promoting chondrogenesis. This conclusion points to the use of self-assembling peptide hydrogels as a superior scaffold for cartilage tissue engineering.

GAG Assay

Figure 2.4 shows how this assay was used in determining the GAG content in the cultured chondrocytes at Days 0, 4, 7, and 21. For the most part, GAG concentrations gradually increased over time. However, the 0.5% agarose hydrogels are significantly lower. The overall levels of GAG concentration are low compared to the usual 50-100 µg/mL for agarose observed in similar samples at our lab. They are not very high when compared to Day 0 levels either. Importantly, the BMSCs cultured in 0.5% agarose hydrogels don't seem to produce GAG. This suggests that scaffolds made with 0.5% or less agarose are not fit for the development of chondrocytes or differentiated BMSCs. A lingering question is whether GAG is being lost to the media and if this effect could be due in part to the observed gel contraction. Though the media was saved during the course of this experiment, analyses could not be performed due to logistical difficulties in sample storage. The saved media samples are no longer available.

DNA Assay and Gel Contraction

Figure 2.5 shows the DNA concentration for the cell-seeded hydrogel samples at key time points throughout the experiment. It is apparent that DNA levels decreased by Day 21, which is expected as the culture period progresses. Levels are comparable to those obtained in previous experiments at our lab where samples contained 2-4 µg/mL DNA. As seen in Figure 2.5B, normalizing by DNA content shows higher amounts in the 0.5% and 1.0%, which is likely due to gel contraction.

Figure 2.6 depicts the weights of the gels and how they change over time. Gel weights and experimental observations show that 0.5% agarose gels contracted significantly more than 1.0% and 2.0% gels.

Radiolabel Incorporation Assay

In this assay, ^{3}H-Thymidine was used as an indicator for cell proliferation while ^{35}S-Sulfate was used to evaluate proteoglycan synthesis. As seen in Figure 2.7A and Figure 2.7C, cell proliferation seems to remain stable with the exception of 1.0% agarose gels at Day 7. This is still within the margin of error indicated by the error bars in the figure. It is clear that cells exhibit lower proliferation in 0.5% agarose gels.

Figure 2.7B and Figure 2.7D show proteoglycan synthesis through ^{35}S incorporation. It is apparent that proteoglycan synthesis is much lower for 0.5% agarose gels and slightly lower for 1.0% gels. Overall synthesis remains fairly stable. However, the 1.0% agarose gel at Day 7 seems to exhibit low or no proteoglycan synthesis. Since there is no reason to expect these results, it may be possible that this is due to an unforeseen experimental error.

2.4 Conclusions

The goal of this study was to determine if agarose hydrogel scaffolds could be used to promote chondrogenesis in TGFβ-supplemented BMSCs to the same extent as peptide hydrogel scaffolds have done so in the past. The latter have been used in experiments where the differences in cell morphology that are typical in chondrogenesis were observed and cells were able to maintain nominal levels of biochemical markers relevant in cartilage tissue engineering.

For our experiment, BMSCs were isolated from bovine calves, encapsulated in 0.5%, 1.0%, and 2.0% agarose hydrogel disks, and cultured for 21 days after TGFβ supplementation. It was evident that cells in all agarose hydrogel scaffolds did not undergo changes in morphology like those observed in peptide hydrogel scaffolds. What's more, all the agarose gels contracted over time, particularly the low-concentration 0.5% gels.

As for assays examining biochemical markers, overall results indicate that the 0.5% agarose hydrogels are clearly inferior when compared to the 1.0% and 2.0% agarose hydrogels, which are generally comparable. It was clear that though GAG concentrations gradually increased over time, cells in 0.5% agarose hydrogels had significantly lower GAG levels and did not even seem to produce GAG. DNA levels decreased as time progressed during the 21-day culture period. For the most part, cell proliferation and proteoglycan synthesis remained stable while corresponding levels for 0.5% agarose hydrogels were

notably lower. As a result, this experiment proves that scaffolds made with 0.5% or less agarose are not fit for the development of chondrocytes or differentiated BMSCs.

In order to gain a better understanding of the initial stages of chondrogenesis, future assays should include morphology imaging targeting actin networks and cell nuclei. This would be helpful in seeing the changes that BMSCs undergo on a deeper level. Western Blots and RT-PCR could also be used to verify the expression of genes characteristic of chondrocytes (e.g., collagen II). Performing the GAG Assay for media samples to examine GAG lost to the media would provide further insight into the BMSC culture process.

The overarching conclusion from this study is that agarose hydrogels, even those with concentrations below the customary 2.0% and thus more mechanically similar to *in vivo* cell surroundings, are not suitable for promoting chondrogenesis and cell proliferation. This suggests that self-assembling peptide hydrogels are indeed a superior scaffold for cartilage tissue engineering and should be used in future studies.

2.5 Figures

Scale Bar = 50µm, **Red = F-actin**, **Blue = DAPI (nuclei)**
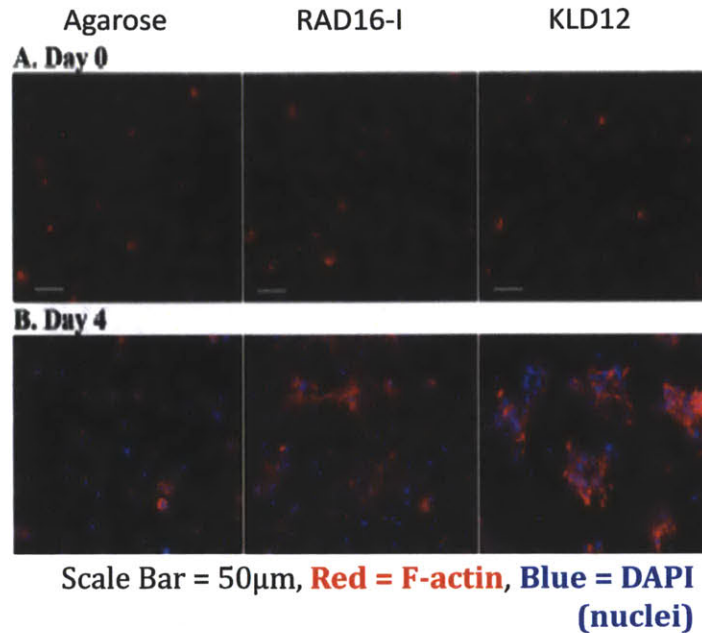
Figure 2.1 Morphology Images for Bovine BMSCs Encapsulated in Self-Assembling Peptide Hydrogel Scaffolds. Cells in different hydrogel scaffolds (i.e., agarose, RAD16-I, KLD12) were stained such that F-actin can be seen in red and the nuclei can be observed in blue. **(A)** Cells retain spherical morphology in all hydrogels at Day 0 after TGFβ supplementation. **(B)** Changes in cell morphology, which indicate proliferation and the start of chondrogenesis, occurred in self-assembling peptide hydrogels during the first four days of culture after TGFβ supplementation but not in 2% agarose [2.12].
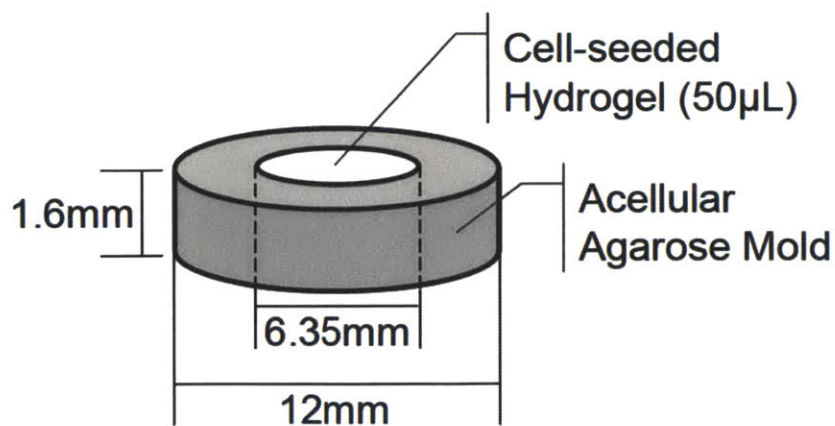
Figure 2.2 Cell Encapsulation in Hydrogels within Agarose Rings. Schematic of the agarose ring molds surrounding cell-seeded hydrogel disks, including the dimensions of each component. [2.12]

A. Day 4   B. Day 7
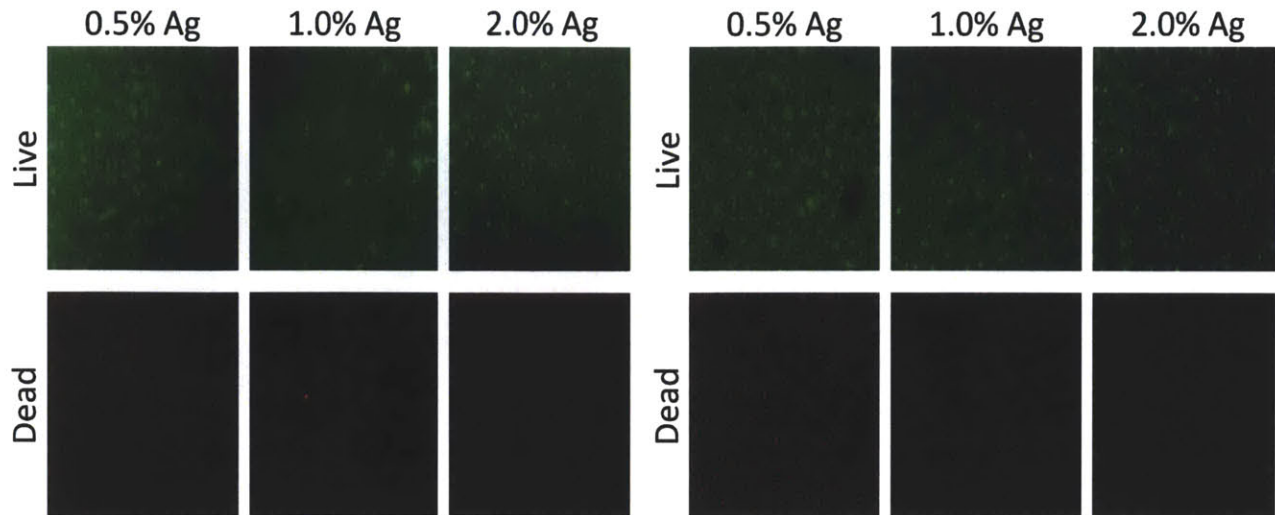
Figure 2.3 Cell Viability and Morphology for BMSC's Seeded in Agarose Hydrogels. Cells in 0.5%, 1.0%, and 2.0% agarose hydrogels were stained such that live cells appear green and dead cells appear red. Cells are viable both at Day 4 (A) and Day 7 (B) of culture. Their morphology remains spherical and changes such as those observed in self-asembling peptide hydrogels are not apparent.
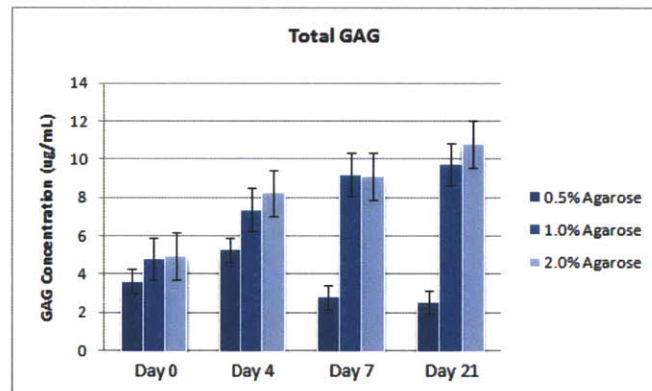
Figure 2.4 GAG Assay. The total GAG concentration (μg/mL) in the BMSC-seeded agarose hydrogels of different concentrations can be seen over the 21-day culture period. Though GAG concentrations generally increase over time, it is evident that the 0.5% agarose gels have significantly lower GAG.

A.

Total DNA

B.

Total DNA per Gel Weight
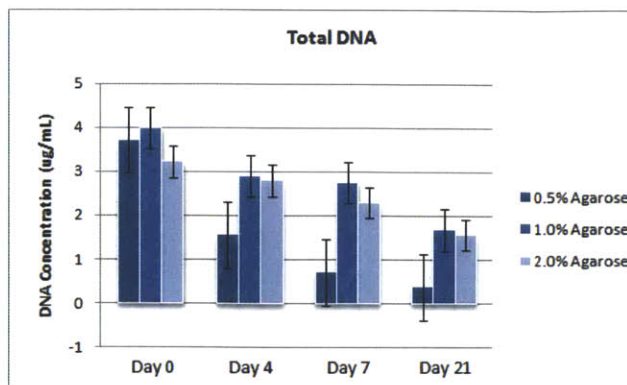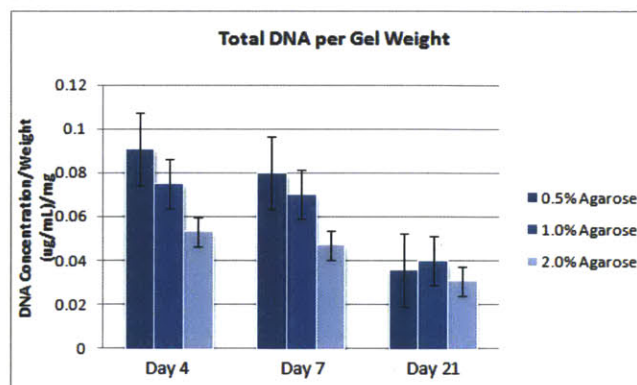
Figure 2.5 DNA Assay. (A) The total DNA concentration (µg/mL) in the BMSC-seeded agarose hydrogels of different concentrations can be seen over the 21-day culture period. **(B)** The DNA concentrations have been normalized by gel weight.

BMSC-Seeded Gel Weights
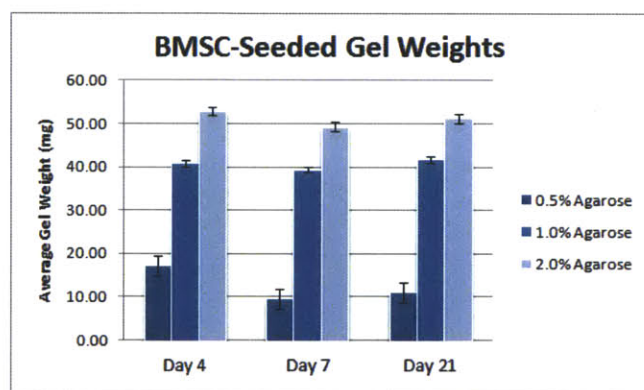
Figure 2.6 BMSC-Seeded Gel Weights. The average weight of the BMSC-seeded agarose gels can be seen over the 21-day culture period. Though 1.0% and 2.0% agarose gels remained fairly constant in weight, it is evident that 0.5% agarose gels contracted over time.
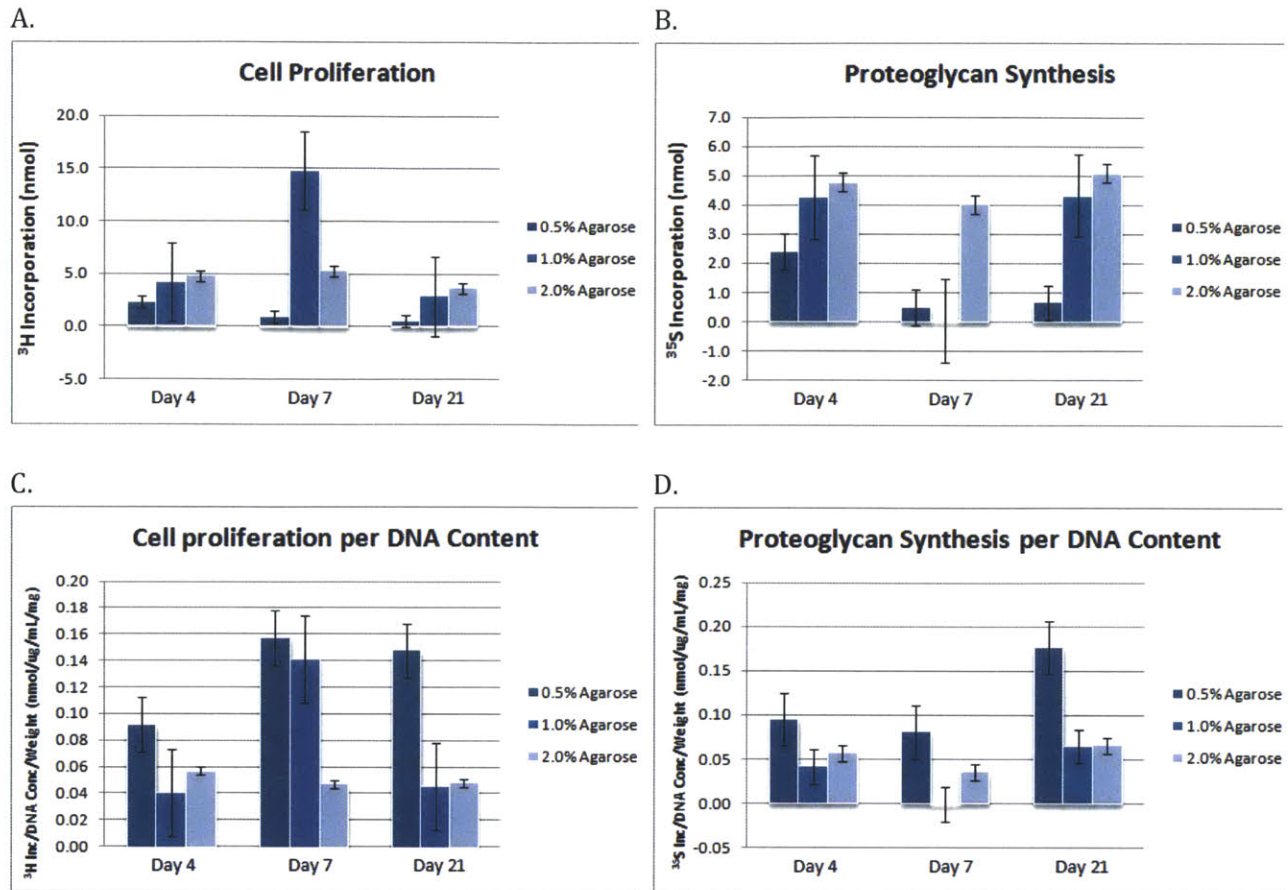
Figure 2.7 Radiolabel Incorporation Assay. **(A)** Cell proliferation is indicated by the level of ^{3}H incorporation. **(B)** Proteoglycan synthesis is indicated by the level of ^{35}S incorporation. **(C)** Cell proliferation normalized by DNA content and gel weight. **(D)** Proteoglycan synthesis normalized by DNA content and gel weight.

2.7 References

[2.1]   DiMicco MA, Kisiday JD, Gong H, Grodzinsky AJ. (2007) Structure of pericellular matrix around agarose-embedded chondrocytes. *Osteoarthritis and Cartilage* 15: 1207-1216.

[2.2]   Hardingham T, Tew S, Murdoch A. (2002) Tissue engineering: chondrocytes and cartilage. *Arthritis Res* 4(3): S63-S68.

[2.3]   Knippenberg M, Helder MN, Zandieh Doulabi B, Wuisman PI, Klein-Nulend J. (2006) Osteogenesis versus chondrogenesis by BMP-2 and BMP-7 in adipose stem cells. *Biochem Biophys Res Commun* 342(3): 902-908.

[2.4]   Mackay AM, Beck SC, Murphy JM, Barry FP, Chichester CO, Pittenger MF. (1998) Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. *Tissue Engineering* 4(4): 415– 428.

[2.5]   Roelen BA, Dijke P. (2003) Controlling mesenchymal stem cell differentiation by TGFBeta family members. *J Orthop Sci* 8: 740 –748.

[2.6]   Frenkel SR, Saadeh PB, Mehrara BJ, Chin GS, Steinbrech DS, Brent B, Gittes GK, Longaker MT. (2000) Transforming growth factor beta superfamily members: Role in cartilage modeling. *Plast Reconstr Surg* 105: 980 –990.

[2.7]   Grimaud E, Heymann D, Redini F. (2002) Recent advances in TGF-beta effects on chondrocyte metabolism: Potential therapeutic roles of TGF-beta in cartilage disorders. *Cytokine Growth Factor Rev* 13: 241–257.

[2.8]   Häuselmann HJ, Aydelotte MB, Schumacher BL, Kuettner KE, Gitelis SH, Thonar EJ. (1992) Synthesis and turnover of proteoglycans by human and bovine adult articular chondrocytes cultured in alginate beads. *Matrix* 12(2): 116-129.

[2.9]   Zhang S, Marini DM, Hwang W, Santoso S. (2002) Design of nanostructured biological materials through self-assembly of peptides and proteins. *Curr Opin Chem Biol* 6(6): 865-871.

[2.10] Buschmann MD, Gluzband YA, Grodzinsky AJ, Kimura JH, Hunziker EB. (1992) Chondrocytes in Agarose Culture Synthesize a Mechanically Functional Extracellular Matrix. *J Orthop Res* 10: 745-758.

[2.11] Kisiday JD, Jin M, Kurz B, Hung H, Semino C, Zhang S, Grodzinsky AJ. (2002) Self-assembling peptide hydrogel fosters chondrocyte extracellular matrix production and cell division: Implications for cartilage tissue repair. *PNAS* 99(15): 9996-10001.

[2.12] Kopesky PW, Vanderploeg EJ, Sandy JS, Kurz B, Grodzinsky AJ. (2009) Self-Assembling Peptide Hydrogels Modulate In Vitro Chondrogenesis of Bovine Bone Marrow Stromal Cells. *Tissue Engineering Part A* 16(2): 465-477.

[2.13] Benya PD, Shaffer JD. (1982) Dedifferentiated Chondrocytes Reexpress the Differentiated Collagen Phenotype when Cultured in Agarose Gels. *Cell* 30: 215-224.

[2.14] Mouw JK, Connelly JT, Wilson CG, Michael KE, Levenston ME. (2007) Dynamic Compression Regulates the Expression and Synthesis of Chondrocyte-Specific Matrix Molecules in Bone Marrow Stromal Cells. *Stem Cells* 25: 655–663.

Chapter 3: Atomic Force Microscopy Imaging Assay for Cartilage Tissue Engineering

3.1 Introduction

Tissue engineering principles can be applied to develop treatments for OA and even to produce replacements for damaged cartilage tissue. Even though tissue engineering techniques have been used to produce cartilage-like tissues, the challenge of creating constructs with biochemical, structural, and biomechanical properties that maintain the same function as *in vivo* cartilage still remains [3.1, 3.2].

As one of the prevalent macromolecules in the cartilage ECM [3.3], aggrecan is an important indicator when testing the potential of tissue-engineered constructs as cartilage substitutes [3.4]. Its expression, synthesis, organization, and turnover are often used as biomarkers of the chondrogenic potential of BMSCs [3.5, 3.6].

The overall composition of aggrecan can be assessed through biochemical [3.7], chromatographic [3.8], and electrophoretic techniques [3.9], while biophysical methods such as electron microscopy (EM) imaging can be used to observe details such as the thick CS-brush region in aggrecan molecules. Still, images obtained through these methods do not allow for a look at the fine details of molecular heterogeneity, conformation, and structure at the level of individual aggrecan molecules [3.10, 3.11, 3.12].

The recent development of high-resolution atomic force microscopy (AFM) imaging techniques allows for a direct visualization of detailed intramolecular structures and probing of nanoscale mechanical properties of various ECM constituents, particularly aggrecan [3.13, 3.14]. AFM imaging provides a better understanding of the molecule-to-molecule variability of aggrecan, making determination of the number, spacing, dimensions, and conformation of GAG chains and full-length molecules possible [3.15]. Thus, the ability to image aggrecan and assess its structural properties on a molecular scale is an asset to the field of cartilage tissue engineering.

Atomic force microscopy (AFM) can be used to gain further insights into key components of the ECM and assist in our understanding of OA and, ultimately, in developing tissue-engineered cartilage replacement therapy [3.13, 3.15]. As seen in Figure 3.1A, aggrecan molecules are immobilized on the APTES-mica substrate and imaged by a sharp silicon probe tip. Figure 3.1B shows an example of resulting images, in this case of newborn human aggrecan, where AFM allows for the distinct resolution of the G1 domain and the individual GAG chains.

The main objective for this experiment is to learn about the theoretical and practical aspects of atomic force microscopy while imaging aggrecan molecules. The sample

preparation conditions needed to immobilize aggrecan on the mica surface for subsequent imaging will be optimized. In addition, the range of instrument and software parameters relevant to tapping mode AFM for imaging aggrecan molecules will be explored. This will help in establishing a protocol for AFM imaging and image processing relevant to the selected molecules.

3.2 Materials and Methods

For this study, fetal bovine epiphyseal aggrecan samples were used to optimize sample preparation and imaging parameters with the goal of establishing a more thorough AFM imaging protocol.

Sample Acquisition

Purified samples of aggrecan molecules isolated from the epiphyseal region of fetal bovine femora, tibiae, and fibulae were acquired for this study. Fetal bovine cartilage was obtained from the epiphyseal growth plate region, processed, and stored as described previously [3.11, 3.16]. Purified aggrecan fractions (A1A1D1D1) were dialyzed consecutively against 500 volumes of 1M NaCl and deionized water to remove excess salts [3.13]. The GAG Assay was used to determine the concentration of aggrecan in purified samples as described in the preceding chapter and detailed in Appendix A.7.

Sample Preparation

Aggrecan molecules must be immobilized on the mica surface for subsequent AFM imaging. In order to achieve this, the surface was coated 0.01% v/v 3-aminopropyltriethoxysilane (APTES) for an incubation time of 30 minutes at room temperature. APTES is an immobilizing agent with a net positive charge to bind the aggrecan molecules [3.13].

The prepared APTES-mica surface was then incubated with the desired aggrecan solution at concentrations ranging from 50-to-500 µg/mL for an incubation time of 20 to 60 minutes. The excess aggrecan solution was rinsed off with 200 µL of purified water and the APTES-mica surface was dried overnight, resulting in an aggrecan monolayer.

See Appendix A.11 for more details on this protocol [3.13, 3.15].

AFM Imaging

The APTES-mica surfaces with aggrecan monolayer samples were then taken for AFM imaging using a NanoScope IIIa Multimode AFM. Sample surfaces were glued to the magnet with custom adhesive tabs while using tweezers to handle the samples and avoid contamination. Each containing a specific APTES-mica surface with an aggrecan monolayer sample, the magnets were placed on top of the microscope's piezo tube for imaging. The

key components of said microscope, as well as the manufacturer's schematic of the inner workings of the microscope head and cantilever holder, can be observed in Figure 3.2.

A Super Sharp Silicon AFM tip with 4 µm thickness was used to probe the sample surface through tapping mode AFM. Great care was taken to align the laser for optimum signal and select the parameters for the software's feedback loop. These include the integral and proportional gains, which are values ranging from 0 to 1 used to maintain constant amplitude for tapping mode. Once the laser is manually aligned and initial parameters are set up for imaging, the NanoScope IIIa Multimode AFM is placed in a platform and suspended in midair using a tripod and elastic cables in order to reduce ambient vibrations and therefore minimize noise during image capture.

See Appendix A.11 for more details on this protocol [3.13, 3.15].

Image Processing

The established heuristic for image processing requires the NanoScope 5.31r1 and the WSxM 4.0 Develop 10.1 software packages. The former was used to select the scan area, resolution, and flatten the image for a more accurate depiction of the aggrecan molecule height. The latter was used to adjust the color palette and equalize the image, allowing for better contour definition of the image molecules. Figure 3.3 shows three consecutive stages of image processing for a sample of fetal bovine epiphyseal aggrecan at a concentration of 50 µg/mL can be seen in three consecutive stages of image processing.

See Appendix A.11 for more details on this protocol.

3.3 Results and Discussion

A number of tests were conducted using fetal bovine epiphyseal aggrecan samples to optimize the sample preparation process for use with minimal sample volumes. The AFM imaging technique was optimized in terms of the integral and proportional gains, as well as the image resolution.

Sample Preparation: Test of Established Conditions

The recommended sample preparation conditions for aggrecan AFM imaging are 250 µg/mL of aggrecan incubated for 40 minutes. Given the need to minimize the aggrecan concentration needed to obtain quality images, the goal of this test was to test the range of the established sample preparation conditions using fetal bovine epiphyseal aggrecan. Samples were prepared with 200 and 250 µg/mL aggrecan and incubated for both 20 and 40 min. As seen in Figure 3.4, aggrecan molecules can be successfully visualized at both 200 and 250 µg/mL concentrations. Incubating the sample solutions for longer periods of time allows for more aggrecan molecules to attach to the APTES-mica surface, which is

evident when comparing the 20 and 40 min images. Therefore, when using lower aggrecan concentrations, longer incubation times will aid in promoting the attachment of molecules to the APTES-mica surface.

Sample Preparation: Minimization of Aggrecan Concentration

Decent images of aggrecan molecules were obtained at high aggrecan concentrations (200 - 250 µg/mL). Unfortunately, these conditions may still be too high for samples from tissue-engineered constructs which usually yield lower amounts of aggrecan. The goal of this imaging study was to find the minimum working conditions during aggrecan sample preparation that would yield good AFM images.

As seen in Figure 3.5, fetal bovine epiphyseal aggrecan samples were imaged at 10, 25, and 50 µg/mL concentrations where all samples were incubated for 60 min. Ideally, one would want to see distinct G1 domains and individual GAG chains along the length of the aggrecan molecule, which would allow for the qualitative and quantitative assessment of certain properties. It is evident that samples should be prepared at no less than 25 µg/mL of aggrecan to ensure representative images of the molecules. If the concentration is too low, the aggrecan molecules tend to curl. This phenomenon has been observed in previous studies and may merit further analysis [3.13].

AFM Imaging: Integral and Proportional Gains

The integral and proportional gains are fundamental components of the feedback control loop used to maintain constant amplitude for imaging via tapping mode AFM. The higher the gains are set, the better the tip will track the sample topography. However, higher gains tend to cause noise. The goal of this imaging experiment imaging was to find an optimal combination of parameters by imaging the same sample section while varying the integral and proportional gains.

Figure 3.6 shows the different parameter combinations alternated while imaging a fetal bovine epiphyseal aggrecan sample (25 µg/mL, 60 min). Results indicate that the optimal range for the integral gain should be 0.2-0.4, while the proportional gain works best at 0.6-0.8. Figure 3.7 shows further detailing of this data. It is important to note that the proportional gain is dependent upon the integral gain so these parameters should be adjusted simultaneously. For instance, if the integral gain is decreased, the proportional gain should be decreased as well.

AFM Imaging: Image Resolution

Previous images show aggrecan molecules within fields at the 2 µm level. Some of these images, unfortunately, do not show enough detail on the molecules. Thus, the goal of this test is to obtain higher-resolution images, preferably at the single-molecule level.

Figure 3.8 shows a fetal bovine epiphyseal aggrecan sample (25 μg/mL, 60 min) at 2 μm, 1 μm, 800 nm, and 500 nm levels. Results indicate that the resolution can be increased to show imaging fields at the 800 nm level with minimal noise.

3.4 Conclusions

The goal of this study was to learn about the theoretical and practical aspects of atomic force microscopy while imaging aggrecan molecules. Given the need to image aggrecan from tissue-engineered construct samples, which may contain low amounts of aggrecan, an important objective in this study was to find the minimum working conditions during aggrecan sample preparation that would yield good AFM images. Improving the imaging protocol in terms of some key instrument parameters and detailing the subsequent image processing methods were also significant objectives in this study.

The sample preparation conditions needed to immobilize aggrecan on the APTES-mica surface for subsequent imaging were optimized. After slightly testing the range of the established sample preparation conditions using fetal bovine epiphyseal aggrecan, a more driven test was conducted to find the minimum concentration at which aggrecan solutions could be used for sample preparation and subsequent AFM imaging. These experiments indicate that a minimum of 25 μg/mL of aggrecan should be used to ensure representative images of the aggrecan molecules. Since a very low concentration of aggrecan is being used, the incubation time must be on the longer side (e.g., one hour) to ensure attachment of aggrecan molecules to the APTES-mica surface.

In addition, the range of instrument and software parameters relevant to tapping mode AFM for imaging aggrecan molecules was explored. These include the integral and proportional gains, which are values ranging from 0 to 1 used to maintain constant amplitude for tapping mode, and the resolution, which is proportional to the size of the image field. Imaging studies showed that the optimal working range of the integral and proportional gains was 0.2 - 0.4 and 0.6 - 0.8, respectively. One must always strike a balance when controlling these and other parameters to keep the force with which the tip traces the surface of the sample high enough to provide accurate surface contour but low enough to prevent noise, as well as damage to the sample surface and the tip itself.

The resolution was increased to show imaging fields at the 800 nm level, as opposed to the default 2 μm level, while maintaining visibility of the desired structural details of the aggrecan molecules. It may be possible to enhance the resolution to get a more detailed view at the single-molecule level by finding ways to minimize noise, such as by suspending the NanoScope IIIa Multimode AFM mid-air via a tripod and improving the stability of the cantilever and tip holder. Special care must be taken when setting up the microscope for

imaging to make sure that the microscope head, the cantilever holder, the tip or probe, and other relevant equipment is stabilized and set up properly.

This study was very useful in establishing a thorough protocol for sample preparation relevant to aggrecan molecules, as well as subsequent AFM imaging and, particularly, image processing. The established procedures can be used to look at aggrecan from native cartilage and tissue-engineered constructs so that we can learn more about this molecule and how it is affected by different experimental conditions.

Finally, the approach taken to develop this AFM imaging assay can be refined for other relevant molecules in the cartilage ECM with the ultimate goal of broadening our understanding of osteoarthritis and developing treatment strategies. It may be interesting to observe the key players in the ECM both individually and as they interact with each other to perform their structural and functional roles.

3.5 Figures

A.
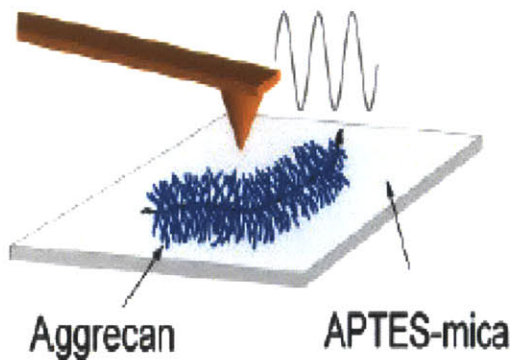
B.
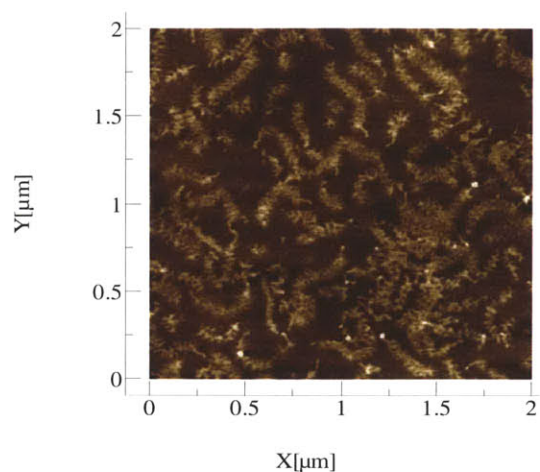
Figure 3.1 Aggrecan Imaging Using AFM Tapping Mode. **(A)** Aggrecan molecules can be immobilized on the APTES-mica substrate in a flattened conformation and imaged by a sharp silicon probe tip [3.15]. **(B)** Image of newborn human aggrecan (250 µg/mL incubated for 30 min).

A.

B.

C.
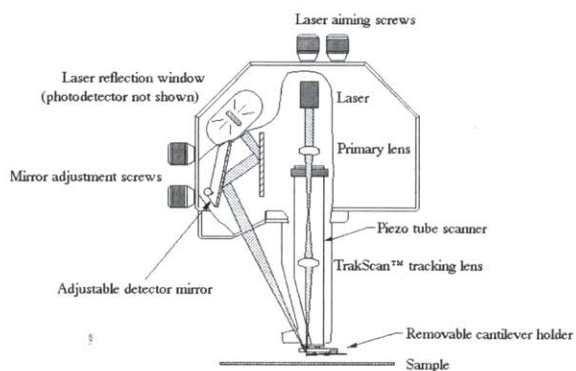
Figure 3.2 NanoScope IIIa Multimode AFM. **(A)** Piezo connected to the equipment controller. **(B)** Microscope head and cantilever holders. **(C)** Manufacturer schematic of the inner workings of the microscope head with a cantilever holder.
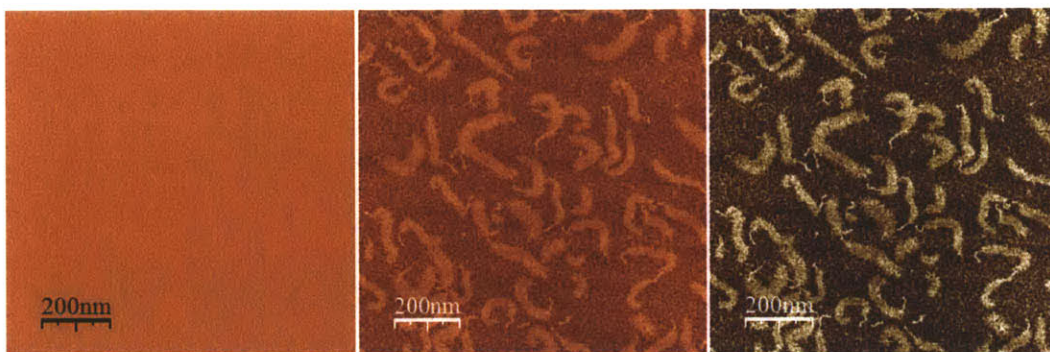
Figure 3.3 Image Processing Heuristic. A sample of fetal bovine epiphyseal aggrecan at a concentration of 50 μg/mL can be seen in three consecutive stages of image processing.

250 μg/mL		200 μg/mL	
20 min	40 min	20 min	40 min
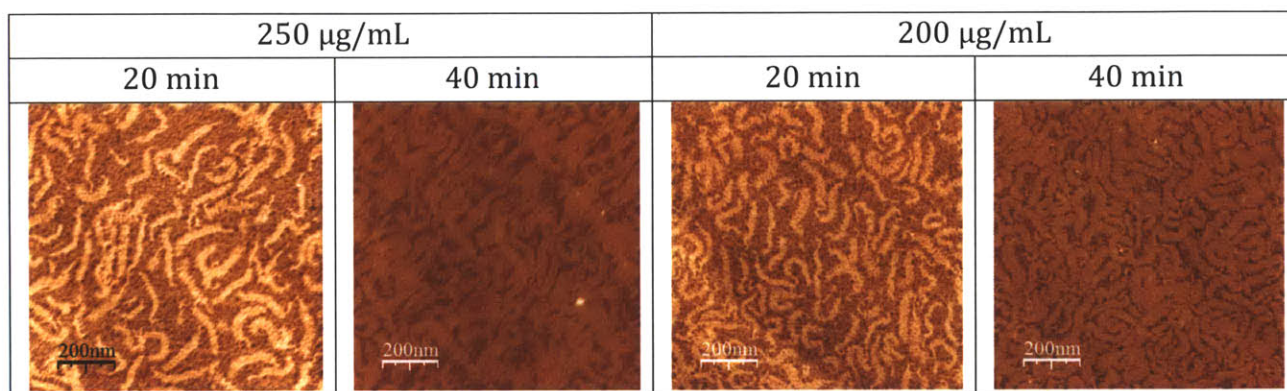
Figure 3.4 Test of Established Conditions for Sample Preparation. An initial test of the range of the established sample preparation conditions (i.e., 250 μg/mL of aggrecan incubated for 40 minutes) was conducted by preparing samples with variations in concentration and incubation time.

10 μg/mL	25 μg/mL	50 μg/mL

Figure 3.5 Minimization of Aggrecan Concentration Needed During Sample Preparation. Given that samples from tissue-engineered constructs usually yield low amounts of aggrecan, there is an interest in finding the minimum concentration of aggrecan that will yield good AFM images. Results indicate that samples should be prepared at no less than 25 μg/mL of aggrecan to ensure representative images of the molecules. Aggrecan molecules tend to curl at low concentrations, as seen in the left panel.

51

Field 1	Integral Gain		
	0.2	0.3	0.4
Proportional Gain 0.5			
0.6			
0.7			
0.8			

Figure 3.6 Integral and Proportional Gains During AFM Imaging. Images of the same sample section were captured while varying the integral and proportional gains to find the optimal combination of these parameters when dealing with aggrecan molecules. Results indicate that the best working ranges for the integral gain (IG) and proportional gain (PG) are 0.2-0.4 and 0.6-0.8, respectively. Higher-quality images in this figure are (1) IG 0.4 PG 0.5, (2) IG 0.3 PG 0.6, (3) IG 0.3 PG 0.7, (4) IG 0.4 PG 0.7, (5) IG 0.3 PG 0.8, and (6) IG 0.4 PG 0.8, which are outlined in black.
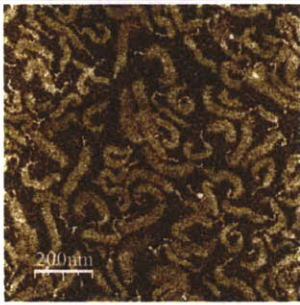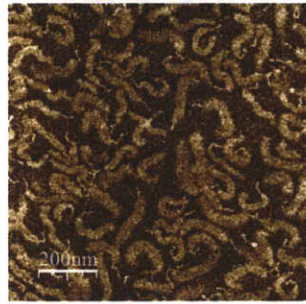
Field 2	Integral Gain		
	0.2	0.3	0.4

Figure 3.7 Integral and Proportional Gains During AFM Imaging. Images of the same sample section were captured while varying the integral and proportional gains to find the optimal combination of these parameters when dealing with aggrecan molecules. Results indicate that the best working ranges for the integral gain (IG) and proportional gain (PG) are 0.2-0.4 and 0.6-0.8, respectively. Higher-quality images in this figure are (1) IG 0.3 PG 0.6, (2) IG 0.3 PG 0.7, (3) IG 0.4 PG 0.7, and (4) IG 0.2 PG 0.8, which are outlined in black.

	2 μm	1 μm	800 nm	500 nm
Field 1				
Field 2				
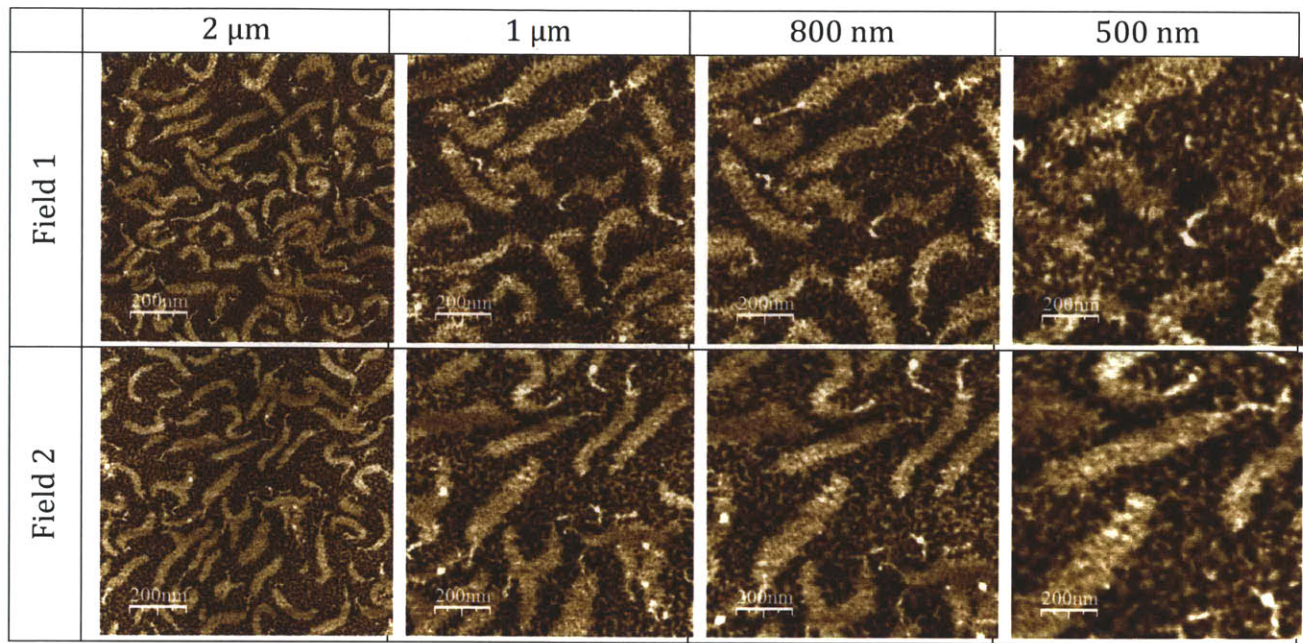
Figure 3.8 Image Resolution During AFM Imaging. Images of the same sample section were captured while at different resolutions to find the best image while preventing distortion and noise. Results indicate that the resolution can be increased to show imaging fields at the 800 nm level with minimal noise. For Field 1, the integral gain was 0.3 and the proportional gain was 0.6. For Field 2, the integral gain was 0.2 and the proportional gain was 0.8.

3.6 References

[3.1]   Hunziker EB. (2002) Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects. *Osteoarthritis and Cartilage* 10(6): 432-463.

[3.2]   Langer RS and Vacanti JP. (1999) Tissue engineering: the challenges ahead. *Scientific American* 280(4): 86-89.

[3.3]   Dudhia J. (2005) Aggrecan, aging and assembly in articular cartilage. *Cellular and Molecular Life Sciences* 62: 2241-2256.

[3.4]   Mauck RL, Yuan X, Tuan RS. (2006) Chondrogenic differentiation and functional maturation of bovine mesenchymal stem cells in long-term agarose culture. *Osteoarthritis and Cartilage* 14(2): 179-189.

[3.5]   Im GI, Jung NH, Tae SK. (2006) Chondrogenic Differentiation of Mesenchymal Stem Cells Isolated from Patients in Late Adulthood: The Optimal Conditions of Growth Factors. *Tissue Engineering* 12(3): 527-536.

[3.6]   Connelly JT, Wilson CG, Levenston ME. (2008) Characterization of Proteoglycan Production and Processing by Chondrocytes and BMSCs in Tissue Engineered Constructs. *Osteoarthritis and Cartilage* 16(9): 1092-1100.

[3.7]   Farndale RW, Buttle DJ, Barrett AJ. (1986) Improved quantitation and discrimination of sulfated glycosaminoglycans by use of dimethylmethylene blue. *Biochimica et Biophysica Acta* 883: 173-177.

[3.8]   Brown MP, Trumble TN, Sandy JD, Merritt KA. (2007) A simplified method of determining synovial fluid chondroitin sulfate chain length. *Osteoarthritis and Cartilage* 15(12): 1443-1445.

[3.9]   Calabro A, Midura R, Wang A, West L, Plaas A, Hascall VC. (2001) Fluorophore-assisted carbohydrate electrophoresis (FACE) of glycosaminoglycans. *Osteoarthritis and Cartilage* 9(S1): S16-S22.

[3.10] Rosenberg L, Hellmann W, Kleinschmidt AK. (1970) Macromolecular Models of Proteinpolysaccharides from Bovine Nasal Cartilage Based on Electron Microscopic Studies. *Journal of Biological Chemistry* 245(16): 4123-4130.

[3.11] Buckwalter JA and Rosenberg LC. (1982) Electron Microscopic Studies of Cartilage Proteoglycans: Direct evidence for the variable length of the chondroitin sulfate-rich region of proteoglycan subunit core protein. *Journal of Biological Chemistry* 257(16): 9830-9839.

[3.12] Morgelin M, Paulsson M, Hardingham TE, Heinegard D, Engel J. (1988) Cartilage proteoglycans. Assembly with hyaluronate and link protein studies by electron microscopy. *Biochemical Journal* 253(1): 175-185.

[3.13] Ng L, Grodzinsky AJ, Patwari P, Sandy J, Plaas A, Ortiz C. (2003) Individual cartilage aggrecan macromolecules and their constituent glycosaminoglycans visualized via atomic force microscopy. *Journal of Structural Biology* 143(3): 242-257.

[3.14] Dean D, Han L, Grodzinsky AJ, Ortiz C. (2006) Compressive nanomechanics of opposing aggrecan macromolecules. *Journal of Biomechanics* 39(14): 2555-2565.

[3.15] Lee H-Y, Kopesky PW, Plaas A, Sandy J, Kisiday J, Frisbie D, Grodzinsky AJ, Ortiz C. (2010) Adult bone marrow stromal cell-based tissue-engineered aggrecan exhibits ultrastructure and nanomechanical properties superior to native cartilage. *Osteoarthritis and Cartilage* 18: 1477-1486.

[3.16] Hascall VC and Sajdera SW. (1969) Proteinpolysaccharide Complex from Bovine Nasal Cartilage: The function of glycoprotein in the formation of aggregates. *Journal of Biological Chemistry* 224(9): 2384-2396.

Chapter 4: Summary and Conclusions

Affecting over 27 million people in the United States alone, osteoarthritis (OA) is a degenerative joint disease caused by the progressive deterioration of cartilage tissue in joints. Unfortunately, there is no cure for OA and current treatments are not enough to fully alleviate disease symptoms which can have a profound impact on the health and everyday activities of affected patients. Thus, there is a drive among the research community to develop alternative therapies for this disease.

Tissue engineering principles can be applied to develop therapeutic techniques for OA. Materials such as agarose and self-assembling peptides have been used to make hydrogels for *in vitro* culture of bovine bone marrow stromal cells (BMSCs) which can differentiate into chondrocytes and undergo chondrogenesis to produce cartilage tissue. Prior to the experiment documented in Chapter 2, the differences in cell morphology that characterize chondrogenesis had been observed in peptide hydrogels such as KLD and RAD but not in the 2.0% agarose hydrogels typically used for culture.

A tissue engineering study was conducted to determine if a suitable environment for cell proliferation and differentiation could be obtained using different agarose compositions, or if self-assembling peptides are more favorable scaffolds. BMSCs were cultured in 0.5%, 1.0%, and 2.0% agarose hydrogels for a period of 21 days following TGF-β1 supplementation. Results from a variety of experiments such as the Live-Dead Imaging Assay, the GAG Assay, the DNA Assay, and the Radiolabel Incorporation Assay indicate that the 0.5% agarose hydrogels are clearly inferior scaffolds when compared to the 1.0% and 2.0% agarose hydrogels, which are generally comparable. Since agarose gels appear to be suboptimal in promoting chondrogenesis, self-assembling peptides should be used in future studies.

A main concern when developing cartilage-replacement treatments is ensuring that tissue-engineered constructs possess the same properties as native cartilage tissue. As described in Chapter 3, atomic force microscopy (AFM) can be used to image key molecules in this tissue to help in developing treatments based on tissue engineering techniques. Aggrecan, a molecule intrinsic to cartilage, was used as a model to develop an AFM imaging assay useful for cartilage tissue engineering. The assay includes a sample preparation stage, manipulation of instrument and software parameters, and an image processing protocol.

Fetal bovine epiphyseal aggrecan was used to optimize the sample preparation conditions such as the concentration and incubation time needed to create the aggrecan monolayer on a mica surface for imaging, especially since the 250 µg/mL concentration previously used would require excessive amounts of sample materials. AFM imaging experiments were conducted with 10, 25, and 50 µg/mL of aggrecan while using a 60 min incubation period.

Results indicate that aggrecan samples should be prepared at no less than 25 µg/mL to ensure representative images of the molecules. Next, the instrument and software parameters were optimized by focusing on finding the working range of the integral and proportional gains (0.2 - 0.4 and 0.6 - 0.8, respectively) and by increasing the resolution to show imaging fields at the 800 nm level as opposed to the 2 µm level previously used in similar experiments in our lab. Finally, an image processing protocol relevant to these molecules was established. The resulting protocols for sample preparation, AFM imaging, and image processing have been included with thorough details in Appendix A.11. It is important to note that the approach taken to develop the aforementioned AFM imaging assay could be refined for other molecules relevant to cartilage with the ultimate goal of broadening our understanding of osteoarthritis and developing treatment strategies.

Though it was not detailed in this thesis, another project of mine was to use the AFM Imaging Assay for Aggrecan to explore samples of tissue-engineered constructs. These consisted of BMSCs enclosed in RAD peptide, with and without dexamethasone. Unfortunately, only very low volumes (i.e., ~50 µL) of these samples were available at nominal aggrecan concentrations and initial images proved that more aggressive sample purification measures were needed to obtain the purity required for effective aggrecan imaging. An aggrecan extraction protocol using cesium chloride density centrifugation had been used in the past with about ten times the starting sample volume, with nominal aggrecan concentrations. Pilot tests using readily-available native cartilage were conducted to see if the extraction protocol would work for our low-volume samples. Results indicated that the protocol could not be satisfactorily altered to accommodate for low-volume samples, so images from the available samples of BMSCs enclosed in RAD peptide could not be obtained. Still, details on how to effectively carry out the aggrecan extraction protocol were established and updated procedures can be found in Appendix A.10. This protocol should be used to effectively extract and purify aggrecan from tissue-engineered constructs in preparation for AFM imaging.

The *in vitro* work discussed in this thesis is only a part of the greater efforts in the field of cartilage tissue engineering. This exciting area continues to thrive with creative studies pursuing goals like improving cell culture techniques, testing the effect of multiple cytokines on constructs, binding particular molecules for enhanced functionality, comparing different matrices or scaffolds, among others. Given that numerous challenges are associated with the application of engineered tissues as reliable and effective treatments *in vivo*, collaborative animal studies that test the potential of these therapeutic systems in veterinary and, eventually, human therapy should be conducted. Using *in vitro* studies helps to further our knowledge of OA and provides a foundation for designing *in vivo* studies to find promising technology for cartilage repair and regeneration.

Appendix

A.1 Isolation and Expansion of BMSC's

Objective:

Isolate bone marrow stromal cells (BMSCs) from the femoral and tibial diaphyses of bovine calf joints. Expand the isolated cells and culture or cryporeserve for future experiments.

Procedure:

Bone Marrow Harvest and Processing

Materials:
- Bovine calf joints (Research 87, Marlborough, MA)
- 50 mL Falcon conical tubes, two for each bone to be harvested
- PBS 1X
- PSA (100 U/mL penicillin, 100 µg/mL streptomycin, and 250 ng/mL amphotericin)
- 70% Ethanol
- Sterilized carving knife and hand-saw blade
- Sterilized forceps and scoop spatulas
- 25 mL and 5 mL pipettes
- 16-gauge and 18-gauge needles
- 70 µm cell strainers

1) Prepare a 50mL Falcon conical tube with 25 mL PBS and 1% PSA for each bone to be harvested.
2) Remove all the connective tissue and muscle around the mid-diaphysis of each bone using a sterilized carving knife.
3) Clean the bone with 70% ethanol.
4) Cut the bone at the mid-diaphysis with a sterilized hand-saw blade and transfer to the tissue culture hood.
5) Carefully remove the bone marrow from the medullary cavity with a forceps and a scoop spatula and transfer it to the prepared 50mL conical tubes with PBS and 1% PSA. (Note: Avoid scraping the interior surface of the bone to preserve the purity of the extracted bone marrow.)
6) Break up the bone marrow through vigorous pipetting using a 25 mL followed by a 5mL pipette. Mechanical disruption through subsequently smaller pipettes helps unravel the large pieces. (Note: You may also need to use a syringe without a needle attached to apply enough pressure.)

7) Continue to homogenize the bone marrow by passing the material through a 16-gauge needle.
8) Centrifuge the homogenized bone marrow at 1000 g for 15 minutes.
9) Carefully aspirate the supernatant and the fatty layer. Discard these materials.
10) Resuspend the cell pellet in fresh PBS. Mix well.
11) Pass the cell suspension through an 18-gauge needle and then through a 70 µm cell strainer onto a new 50 mL Falcon conical tube.

Nucleated Cell Count and Cell Plating

Materials:
- Cell suspension in 50 mL Falcon conical tubes
- Ammonium Chloride-Tris Base (7.6 mg/mL Ammonium Chloride, 2.4 mg/mL Tris Base)
- Eppendorf 0.5 mL microcentrifuge tubes for cell counting dilutions
- Pipettes and micropipettes with sterile tips
- Hemocytometer for cell counts under light microscope
- BMSC Expansion Medium (LG-DMEM with 10% ES-FBS, 1% HEPES, and 1% PSA)
- bFGF stock solution (25 ng/µL) from freezer (R&D Systems, Minneapolis, MN)
- 0.05% Trypsin-EDTA
- T-flasks for culture as needed

1) Prepare a 1:20 dilution of the cell suspension in Ammonium Chloride-Tris Base for nucleated cell count in an Eppendorf 0.5 mL microcentrifuge tube.
   Note: Dilutions may need to be adjusted for each harvest depending on the cell density of the suspension.
2) Obtain the approximate nucleated cell count via the Trypan Blue exclusion method using a hemocytometer.
3) Centrifuge the cell suspension at 200 g for 15 minutes.
4) Aspirate the supernatant and resuspend the cell pellet in BMSC Expansion Medium with 1 ng/mL FGF.
5) Pre-plate approximately 75×10^6 cells in 5mL on 100 mm tissue culture treated petri dishes.
6) Incubate for 30 minutes to allow rapidly-adhering cells to attach.
7) Transfer the media and non-adherent cells to a T-flask and add another 10mL of BMSC Expansion Medium (approximately 1×10^6 cells/cm^2 in 15 mL media; $\sim 75 \times 10^6$/T-75).
8) After 2 days in culture, aspirate out the media and add fresh BMSC Expansion Medium.
9) Continue to culture flasks until cells are nearly (75-80%) confluent [P0].
10) Remove the cells that have adhered to the T-flask wall with 0.05% trypsin-EDTA.
11) Centrifuge at 200 g for 15 minutes and resuspend in PBS.

12) Obtain the approximate nucleated cell count via the Trypan Blue exclusion method using a hemocytometer.

13) If you want to cryopreserve the cells for future experiments, freeze them in aliquots of 5 million cells/vial using liquid nitrogen. Make sure to use appropriate freeze tubes and don't tighten the caps too much.

14) If you want to culture the cells immediately, re-plate at approximately 6,000 cells/cm^2.

15) Culture until 60-70% confluent (2-3 days), passage [P1], and reseed again at 6,000 cells/cm^2.

16) Culture again until 60-70% confluent (2-3 days), passage [P2], and seed into 3-D constructs for chondrogenesis.

A.2 Thaw and Expansion of Cryopreserved BMSC's

Objective:

Thaw and expand cryopreserved bovine bone marrow stromal cells (BMSCs) for experimental use.

Procedure:

<u>Thawing Cells and Seeding Flasks</u>

Materials:
- Vial with cryopreserved cells from liquid nitrogen tank
- HG-DMEM (4.5 g/L glucose) from refrigerator
- BMSC Expansion Medium from refrigerator
- FGF stock solution (25 ng/μL) from freezer
- Falcon conical tube (15 mL)
- Pipettes and micropipettes with sterile tips
- Hemocytometer for cell counts under light microscope
- Bottle for Seed Medium
- T-185 culture flasks

1) Make BMSC Expansion Medium (LG-DMEM with 10% ES-FBS, 1% HEPES, and 1% PSA) according to "MSC Media Calcs" spreadsheet.
2) Remove desired vial of BMSCs from the cryopreservation chamber. Quickly loosen the cap to keep the vial from exploding due to rapid changes in temperature.
3) Thaw the vial of BMSCs as quickly as possible by dipping it in the 37°C water bath and shaking vigorously.
4) Resuspend the cells in 10 mL of HG-DMEM in a 15 mL Falcon conical tube.
5) Spin down cells at 200 g (200 rcf) for 8 min using the centrifuge in the Zhang Lab. Make sure to take a Falcon tube with 10 mL of water to use as a counterbalance.
6) Carefully aspirate the supernatant through the vacuum to leave the cell pellet at the bottom of the tube.
7) Resuspend the cells in 5 mL of BMSC Expansion Medium. Mix well by using the 1000 μL micropipette.
8) Count cells using the hemocytometer and determine the following using the "BMSC Thaw" spreadsheet:
   Note: Each T-185 will have 25 mL of Seed Medium (BMSC Expansion Medium with cells and FGF)
   - Amount of T-185 flasks to seed (usually 4 flasks)

- Input: Cell count (viable cells using Trypan Blue), total volume ($\sim$ 5 mL), dilution factor (2)
- Output: # T-185s
- Amount of FGF solution needed in Seed Medium for 5 ng/mL FGF

$$\_\_\_\_ \ flasks \times \frac{25 \ mL \ Seed \ Media}{flask} \times \frac{5 \ ng \ FGF}{mL \ Seed \ Media} \times \frac{\mu L \ FGF \ soln}{25 \ ng \ FGF} = \_\_\_\_ \ \mu L \ FGF \ soln$$

- Amount of cell suspension needed in Seed Medium for 6000 cells/cm² in the T-185s

$$\_\_\_\_ \ flasks \times \frac{mL \ cell \ suspension}{flask} = \_\_\_\_ \ mL \ cell \ suspension$$

- Amount of BMSC Expansion Medium needed in Seed Medium

$$\_\_\_\_ \ flasks \times \frac{25 mL \ Seed \ Media}{flask} - \_\_\_\_ \ mL \ cell \ suspension = \_\_\_\_ \ mL \ BMSC \ Exp \ Med$$

9) Make Seed Medium using the calculated amounts of BMSC Expansion Medium, FGF solution, and cell suspension. Mix well by pipetting up and down.

10) Add 25 mL of Seed Medium to the culture-treated side of each T-flask. Be careful not to get any media on the mouth of the flasks to prevent growth in that area.

11) Place the T-flasks in the incubator at 37°C. Make sure the culture-treated sides are facing down and that the liquid is level throughout the flasks. Don't stack more than 2-3 flasks on top of each other.

12) Leave the flasks in the incubator for 3 days before passaging cells for further expansion.

Passage Cells (3 Days After Seeding)

Materials:
- T-185 culture flasks from incubator
- PBS 1X from refrigerator
- Trypsin from freezer
- FBS Medium from refrigerator
- FGF stock solution (25 ng/µL) from freezer
- Pipettes and micropipettes with sterile tips
- Hemocytometer for cell counts under microscope
- Bottle for Passage Medium
- T-185 culture flasks

1) Remove the Seed Medium from the T-flasks by aspirating through the vacuum.
2) Add 10 mL of PBS to the culture-treated side of each T-flask.
3) Wash by aspirating with a pipette and rinsing the culture-treated side of each T-flask 2-3 times.
4) Remove the PBS from the T-flasks by aspirating through the vacuum.

5) Add 2 mL of trypsin to each T-flask. Lay down the flasks and tilt them to make sure that the entire surface is covered in trypsin. Place the flasks in the incubator at 37°C for 4 min.

6) Add 8 mL of FBS Medium to each T-flask to inactivate the trypsin. Wash by aspirating with a pipette and rinsing the culture-treated side of each T-flask 2-3 times.

7) Combine the media from all flasks into one T-flask.

8) Count cells using the hemocytometer and determine the following using the "BMSC Thaw" spreadsheet:

   Note: Each T-185 will have 25 mL of Passage Medium (FBS Medium with cells and FGF)

   - Amount of T-185 flasks to seed (usually 15 flasks)
     - o Input: Cell count (viable cells using Trypan Blue), total volume ($\sim$ 3-4 mL), dilution factor (20)
     - o Output: # T-185s
   - Amount of FGF solution needed in Passage Medium for 5 ng/mL FGF

     $$\text{\_\_\_\_ } flasks \times \frac{25 \ mL \ Passage \ Media}{flask} \times \frac{5 \ ng \ FGF}{mL \ Passage \ Media} \times \frac{\mu L \ FGF \ soln}{25 \ ng \ FGF} = \text{\_\_\_\_ } \mu L \ FGF \ soln$$

   - Amount of cell suspension needed in Passage Medium for 6000 cells/cm^2 in the T-185s

     $$\text{\_\_\_\_ } flasks \times \frac{mL \ cell \ suspension}{flask} = \text{\_\_\_\_ } mL \ cell \ suspension$$

   - Amount of FBS Medium needed in Passage Medium

     $$\text{\_\_\_\_ } flasks \times \frac{25 mL \ Passage \ Media}{flask} - \text{\_\_\_\_ } mL \ cell \ suspension = \text{\_\_\_\_ } mL \ FBS \ Medium$$

9) Make Passage Medium using the calculated amounts of FBS Medium, FGF solution, and cell suspension. Mix well by pipetting up and down.

10) Add 25 mL of Passage Medium to the culture-treated side of each T-flask. Be careful not to get any media on the mouth of the flasks to prevent growth in that area.

11) Place the T-flasks in the incubator at 37°C. Make sure the culture-treated sides are facing down and that the liquid is level throughout the flasks. Don't stack more than 2-3 flasks on top of each other.

12) Leave the flasks in the incubator for 3 days and prepare for casting.

A.3 Encapsulation of BMSC's in Hydrogels

Objective:

Prepare agarose rings using custom molds for BMSC encapsulation. Cast BMSC-seeded hydrogels for cell culture. Hydrogels can be agarose or self-assembling peptides such as RAD and KLD.

Procedure:

Casting Acellular Agarose Rings

Materials:
  * Custom 3-piece casting mold (Ring, Cap, Post)
  * 2% LMP Agarose (or stock 3% LMP Agarose, Invitrogen) from refrigerator
  * 24-well non-tissue culture treated plates
  * Repeater pipette (beige Eppendorf)
  * 1.25 mL sterile, individually-wrapped repeater pipette tips
  * 1X PBS without Ca^{2+} or Mg^{2+}, sterile, pH=7.2 from refrigerator

1) Autoclave the following materials on the dry cycle for 20 min:
    - Agarose ring molds (metal rings, metal caps, and white center posts in separate beakers)
    - Spatula with thin tip
    - Tweezers with long tips
    - Tweezers with grip
2) Sterilize the 3% agarose stock by autoclaving on the wet cycle for 20 min.
3) Prepare 2% agarose solution based on the calculations below. Mix well by pipetting up and down and leave the solution in the water bath at 66°C so that it stays melted.
    Notes: Melt agarose by placing it in a water bath at 66°C. Refrigerate agarose to solidify for storage. Each 24-well plate requires ~3mL of 2% agarose for making all the rings.
    - Amount of 2% agarose needed for the desired number of rings
      $$\_\_\_\_ \; rings \times \frac{0.125 \; mL \; 2\% \; agarose}{ring} = \_\_\_\_ \; mL \; 2\% \; agarose$$
    - Amount of 3% agarose needed to make the desired 2% agarose
      $$\_\_\_\_ \; mL \; 2\% \; agarose \times \frac{2\% \; Ag}{3\% Ag} = \_\_\_\_ \; mL \; 3\% \; agarose$$
    - Amount of PBS to add to the 3% agarose to make the desired 2% agarose
      $$\_\_\_\_ \; mL \; 2\% \; agarose - \_\_\_\_ \; mL \; 3\% \; agarose - \; = \_\_\_\_ \; mL \; PBS$$
4) Assemble casting molds in 24-well plates. Use the tweezers to align the pieces correctly.
    - Place metal rings with the smooth side facing up using tweezers with long tips.

- Place metal caps with the smooth side facing down using tweezers with long tips.
- Align the holes of the rings and caps such that the 2% agarose poured through the holes leaks into the space in between the metal parts to form the rings.
- Place the white posts in the center of the rings and check their stability using the tweezers with grip.

5) Add 125 µL of 2% agarose to each ring through the holes of the metal caps using a repeater pipette.

   Notes: Repeater pipette (beige Eppendorf) should be set at 5 for 125 µL. The pipette allows casting of 8 rings per full tip. Before discharging the first 125 µL dose, discharge a 25 µL dose (set at 1).

6) Refrigerate the plate for at least 5 min so that the agarose rings can solidify.

7) Remove the molds carefully and aspirate excess agarose from the center or surroundings of the rings.

8) Add PBS to wells (enough to cover the rings) and store in the refrigerator at 4°C for up to 4 days.

Note: Make sure to complete the agarose rings 1-2 days prior to BMSC trypsinization during casting.

Casting Cells in Agarose Rings (2 Person Procedure)

Materials:
- 24-well non-tissue culture treated plates with 2% agarose rings
- Casting Medium: HG-DMEM with 25mM HEPES, 1% ITS+1, 1% NEAA, 1% Na Pyr, 1% PSA, and 0.4% Proline
- Culture Medium: HG-DMEM with 10mM HEPES, 1% ITS+1, 1% NEAA, 1% Na Pyr, 1% PSA, and 0.4% Proline
- FBS Medium: LG-DMEM with 10mM HEPES, 10% ES-FBS, and 1% PSA
- 1X PBS, sterile, pH=7.2
- 0.05% Trypsin/EDTA
- 10% Sucrose, Sterile
- 10% Sucrose, Sterile with 2.5mM HEPES
- Desired hydrogel solution for cell encapsulation
  a) 2% LMP Agarose (or stock 3% LMP Agarose, Invitrogen) from refrigerator
  b) Peptide powder, sterile
     Example: For 0.35% KLD12, you need 1.5mL x 3.5mg/mL x 1.2 = 6.3 mg/cast.
- 2mL screw top tubes, sterile
- 5mL polypropylene snap top tubes, sterile (BD Falcon)
- Repeater pipette
- 1.25 mL sterile individually wrapped repeater pipette tips

1) Aspirate PBS from each agarose ring and clean up loose agarose debris.
2) Add ~0.5mL of Casting Medium (above) to each well.
3) Place plate(s) in 37°C incubator.
4) Prepare hydrogel solutions for cell encapsulation:
    a) For agarose hydrogels, dissolve the stock agarose solution to the desired concentration in sterile 10% sucrose by vortexing and sonicating. Leave the solution in the water bath at 42°C to prevent solidification of the agarose.
    b) For peptide hydrogels, dissolve the peptide at 4.375 mg/mL (for 3.5 mg/mL final concentration) in sterile 10% sucrose by vortexing & sonicating. Leave the peptide solution in the sonicator.
5) Trypsinize cells and inactivate with FBS Medium.
6) Centrifuge cells and resuspend in FBS Medium at $> 10 \times 10^6$ cells/mL. Estimate this count based on the previous passage yield.
7) Count cells via the Trypan Blue exclusion method using a hemocytometer.
8) Aliquot 15×10^6 cells into 2 mL sterile screw top tubes. Store tubes at 4°C.
9) Prepare 5 mL tubes with 1.2 mL of the prepared hydrogel solutions. Return the tubes to the water bath or sonicator as appropriate.
10) Centrifuge desired 2 mL tubes with 15×10^6 cells at 100 g for 5 minutes.
11) While spinning, aspirate the media out of 23 acellular agarose rings in the 24-well plate, leaving the center of each ring dry. (Only 23 rings will be cast with cell-seeded hydrogels due to repeater pipette limitations.)
12) Assemble a repeater pipette with 1.0 mL or 1.25 mL tip and set it to 50 μL.
13) Person #1: Aspirate media from the 2 mL tubes and resuspend the cell pellet in 300 μL of sterile 10% sucrose with 2.5mM HEPES.
14) Person #2: Hold the 5 mL tubes with 1.2 mL of prepared hydrogel solutions.
15) Person #1: Inject the cell suspension into the 5 mL tube with the desired hydrogel.
16) Complete the following in 60 seconds, starting from the time the cells are added to the hydrogel solution: (Speed is of the essence to prevent solidification prior to casting.)
    a) Gently vortex the cell-seeded hydrogel suspension to ensure homogeneous cell distribution.
    b) Gently draw the cell-seeded hydrogel suspension into the repeater pipette. Make sure to avoid bubbles.
    c) Rapidly, but gently, dispense 50 μL of the cell-seeded hydrogel suspension into the center of up to 23 agarose rings.
17) Wait at least 5 minutes and add 300 μL of Casting Medium to each well, making sure the tops of the hydrogel disks are covered.
18) Aspirate the Casting Medium from each agarose ring while taking care of not damaging the ring or hydrogel disks.
19) Add 750 μL of Culture Medium to each well.
20) Incubate at 37°C.

A.4 Cell Culture Media Changes and Sampling

Cell Culture Media Changes

Objective:

Change the cell culture media every 2-3 days to promote growth during extended periods.

Procedure:

Materials:
- Cell culture media stock from refrigerator shelf
- Media supplements from freezer (e.g. A2P + Dex)
- Falcon tube for required media
- Pipettes and Micropipettes
- Labeled mini tubes for saving media from gels
- Waste container
- Culture plates from incubator

Preparation of Culture Media

1) Calculate the amount of media required for the gels that need a media change.
$$\_\_\_\_ \ gels \times \frac{0.750mL\ media}{gel} = \_\_\_\_ \ mL\ media$$
2) Use the "MSC Media Calcs" spreadsheet to determine the amount of media supplements that must be added to the required media.
$$\_\_\_\_ \ mL\ A2P + Dex$$
3) Sterilize hood in Tissue Engineering Lab by wiping with 70% ethanol.
4) Transfer the amount of required media to a Falcon tube using an appropriate pipette.
5) Add the calculated amounts of media supplements to the Falcon tube using appropriate micropipettes.

Media Change

1) Sterilize hood in Tissue Engineering Lab by wiping with 70% ethanol.
2) Use a 1000 µL micropipette to aspirate old cell culture media out of each well in the culture plates and into the corresponding labeled mini tube for saved media. Dispose of undesired media in the waste container.
3) Add 750 µL of new cell culture media to the desired wells in the culture plates.
4) Place the culture plates back in the incubator at 37°C and freeze the saved media from the gels at -20°C.
5) Empty the waste container in the hood's vacuum waste system.

Cell Culture Sampling

Objective:

Obtain sample plugs from tissue culture plates for different assays.

Procedure:

Materials:
- Freezer vials (2mL) and tube rack for samples
- Bent spatula and pointed spatula
- Burner and igniter for sterilization
- Culture plates from incubator

1) Sterilize hood in Tissue Engineering Lab by wiping with 70% ethanol.
2) Sterilize bent and pointed spatulas by spraying them with 70% ethanol and then flaming over the burner.
3) Remove agarose rings from desired plugs and place them in their corresponding freezer vials.
   Method 1: Use the pointed spatula to gently tap the edges of the ring and detach it from the well. Use the bent spatula to lift the edge of the ring slightly and release it. The fluid motion should allow the plug to float out of the ring. Remove the ring from the well using the pointed spatula and carefully place the plug in the desired vial using the bent spatula.
   Method 2: Use the bent spatula to remove both the ring and the plug from the well and place them on a petri dish. Use the pointed spatula to carefully cut the ring and detach it from the plug. Place the plug in the desired vial using the bent spatula.
4) Place the culture plates back in the incubator.
5) Store the freezer vials in the Wet Lab freezer for future assays.
6) Discard the detached agarose rings in the hood's vacuum waste system.

A.5 Live-Dead Imaging Assay

Objective:

Image sample plugs during cell culture to obtain a qualitative picture of cell viability. Using the dyes listed below, live cells will appear green and dead cells will appear red. Cell morphology may also be explored.

Procedure:

<u>Microscope Setup</u>

1) Go to Microscope Room and check the logbook. If the mercury lamp is off, make sure it has been off for at least 30 min before turning it on. If it is on, fill in the logbook and proceed with your work.
2) Turn on the mercury lamp (3rd switch box) and wait 30 min before imaging any samples.
   Note: Make sure that the other three switches are off, since turning them on before the mercury lamp could cause voltage issues and damage the equipment.

<u>Preparation of Samples</u>

Materials:
- PBS 1X from refrigerator
- Microscope slide with 4 wells
- Bent spatula and pointed spatula
- Micropipette (1000 µL)
- Burner and igniter for sterilization
- Culture plates from incubator

1) Sterilize hood in Tissue Engineering Lab by wiping with 70% ethanol
2) Sterilize bent and pointed spatulas by spraying them with 70% ethanol and then flaming over the burner.
3) Remove agarose rings from desired plugs and place them in the microscope slide with 4 wells.
   Method 1: Use the pointed spatula to gently tap the edges of the ring and detach it from the well. Use the bent spatula to lift the edge of the ring slightly and release it. The fluid motion should allow the plug to float out of the ring. Remove the ring from the well using the pointed spatula and carefully place the plug in the microscope slide using the bent spatula.

Method 2: Use the bent spatula to remove both the ring and the plug from the well and place them on a petri dish. Use the pointed spatula to carefully cut the ring and detach it from the plug. Place the plug in the microscope slide using the bent spatula.

4) Add 1mL of PBS to plugs in the microscope slide.
5) Place the culture plates back in the incubator.
6) Discard the detached agarose rings in the hood's vacuum waste system.

<u>Live/Dead Imaging</u>

Materials:
- Microscope slide with 4 wells
- Fluorescent dyes (Red and Green) from refrigerator
  - Ethidium bromide (Red), 350 ng/mL, for dead cells
  - Fluorescein diacetate (Green), 12.5 µg/mL, for live cells
- White box next to the sink in the Tissue Engineering Lab with:
  - Tips for micropipettes
  - Radioactive tip disposal box
  - Liquid waste container
  - Micropipettes from Wet Lab
- Nikon Eclipse Fluorescent Microscope

1) After the 30 min wait for the mercury lamp to warm up, go to the Microscope Room and turn on the microscope using the other three switches.
2) Setup the software (Openlab 4.0.4) for imaging.
3) Add dyes to the first sample and mix with 1000 µL pipette by gently aspirating up and down.
   a. 0.35 µL of red dye to stain nuclei (dead)
   b. 0.10 µL of green dye to stain cytoplasm (live)
4) Remove the dye solution with the pipette while making sure that the plug remains around the center of the well. This will allow better visibility under the microscope.
5) Place under the objective (set to 10X, Phase 1) and turn on UV lamp (upper left button on the control panel). Move the filter switch LEFT to view green dye and RIGHT to view red dye. Keep the white light in the microscope and the room light off, since the dyes can photobleach.
6) Capture images using the Auto Exposure button (AE) and then the Camera button. Repeat for a total of 3 fields per plug, making sure that the fields are representative of what is observed in the plugs.
7) Color the images by going to "Enhance Image" and selecting green or red, depending on the filter being used for the desired field. Also, go to "Enhance Contrast" (Image>Contrast>Best Guess>OK).

8) Merge green and red images by selecting both files (hold Shift key) and dragging them to "New."

9) To save all images as TIFS, select them in blue and then go to Image>Depth>Millions of Colors>Selected Layer>OK. Then go to File>Save as Multiple>Naming>Start with Document Name & Append a Numerical Subscript. Finally, save to my file and copy to my USB flash drive. To remove the USB flash drive, go to File>Eject "No Name." (If this is not done, the files will not copy.)

10) Turn off the last three switches of the microscope and then the Mercury lamp. (Turning off the Mercury lamp first could cause voltage issues and damage the equipment.) Record the time in the logbook.

11) Dispose of samples by aspirating them out of the wells with a pipette and placing them in the liquid waste container. Its contents can then be discarded in the vacuums under the hoods.

12) Return all pipettes, tips, and white box to their appropriate places.

A.6 Lyophilization and Proteinase K Digestion

Objective:

Lyophilize samples to dry and obtain final weights. Digest samples with Proteinase K so that specific biomolecules can be detected by appropriate assays (e.g., GAG, DNA, radiolabel incorporation).

Procedure:

Lyophilization

Materials:
- Pre-weighed red-cap freezer tubes containing hydrogel samples
- Box for placing sample vials in the lyophilizer, can be plastic or cardboard

1) Verify if the lyophilizer's lid is placed correctly over the refrigerator coil. Turn on the machine.
2) Set the temperature using the "Manual" button and wait until it reaches the lowest point in curve displayed on the lyophilizer.
3) Prepare the samples by loosening the caps of the tubes. Make sure they are still tight enough to stay on the vials when the vacuum is applied.
4) Set them on the lyophilizer's sample chamber inside a box. Keep the box open.
5) Press the "Vacuum" button to start the vacuum and wait 2 hours while the samples undergo the freeze-drying process.
6) After 2 hours, release the valve at the bottom of the machine to let the vacuum air out. Be careful not to do this too quickly, since samples could be damaged.
7) Remove samples and turn off the lyophilizer.
8) Weigh the vials again to obtain the lyophilized weight of each hydrogel sample by subtracting the weights of the empty tubes.

Note: Don't lyophilize the Day 0 samples or the radiolabel standards.

Proteinase K Digestion

Materials:
- Tris Buffer (50 mM Tris, 1 mM $CaCl_2$, pH = 8.0, Sterile), stored in refrigerator
- ProK (5 mg/mL Proteinase K, recombinant PCR Grade, Roche Applied Science), stored at 2-8°C freezer
- Box with red-cap freezer tubes containing lyophilized hydrogel samples
- Day 0 sample vials

1) Set the water bath in the Injury Room to 66°C and the oven in the back of the Wet Lab to 60°C. Check the temperatures periodically to ensure they reach appropriate levels.
2) After lyophilization, add 0.95 mL Tris Buffer to each hydrogel sample tube.
3) Melt the agarose-containing hydrogel samples, as well as the Day 0 samples, by placing the vials in the water bath at 66°C.

4) Add 50 μL of ProK to the samples and the Day 0 vials for a final concentration of 0.25 mg/mL Proteinase K.
5) Mix well by vortexing and shaking fervently.
6) Place the samples and Day 0 vials in the oven at 60°C overnight. Make sure the oven does not reach 65°C given that Proteinase K denatures at this temperature.
7) Samples can then be frozen or used to perform desired assays.

A.7 GAG Assay

Objective:

Determine the sulfated glycosaminoglycan (GAG) content in samples. The GAGs (CS6, CS4, HS, KS) are stained using dymethylmethylene blue (DMMB) dye and chondroitin-6-sulfate is used as a standard due to its prevalence in articular cartilage. Samples must be previously lyophilized and digested using Proteinase K.

Procedure:

Materials:
- Samples from freezer
- GAG Standard from freezer
- Tris Buffer from refrigerator
- DMMB dye solution from bench
- Mini test tubes
- Clear 96-well plate
- Wet lab micropipettes, repeater pipette, and tips
- Maxy machine and software in Wet Lab

1) Prepare a map for the clear 96-well plate used in this assay. Have duplicates of standards and samples.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 100	Std 100										
B	Std 75	Std 75										
C	Std 50	Std 50										
D	Std 25	Std 25										
E	Std 12.5	Std 12.5										
F	Std 100	Std 50										
G	Std 75	Std 25										
H	Std 0	Std 0										

2) Prepare GAG standard solutions in mini test tubes using the Tris buffer. Make sure to vortex the GAG standard stock and to mix the standards well by repeatedly pipetting up and down. These standards will be used to relate the GAG concentrations to the instrument readings.

GAG Standard Concentration (µg/mL)	Volume of GAG Standard Stock (µL)	Volume of Tris Buffer (µL)
100	80	720
75	60	740
50	40	760
25	20	780
12.5	10	790

Note: The buffer used to prepare the standards should be the same as the buffer used in the samples being tested. Tris buffer is the most common but other buffers or cell culture media could be used.

3) Turn on the Maxy machine so that it has time to warm up.
4) Add 20 µL of each standard and sample according to the plate map. Make sure to vortex all solutions right before adding them to ensure that they are well-mixed.
5) Add 200 µL of DMMB dye into each well using a repeater pipette. Remove any bubbles left over in wells and dry any minor spills if necessary.
6) Scan plate in the Maxy machine.
   - Click on "Read Plate" while at 520 nm wavelength.
   - Save results by copying to a text document.
7) Dispose of plate contents in appropriate waste containers and return materials.

A.8 DNA Assay

Objective:

The DNA Assay uses bisbenzimidazole fluorescent dye Hoechst 33258 to stain DNA present in experimental samples and obtain readings for data processing in terms of DNA concentration (μg/mL). The interaction of Hoechst 33258 with DNA requires at least four consecutive A-T base pairs, as well as dissociation of DNA from proteins of the nucleoprotein complex. The former is satisfied by random chance, while the latter can be accomplished by digestion with Proteinase K, papain, or by a high salt dye buffer. The stability of the dye and dye-DNA complex is great enough that the fluorescence will decrease by less than 5% over 2 hours after the initial mixing of the dye solution and the digested plug.

Procedure:

Preparation of DNA Dye Solution

Materials:
- 1x TEN Buffer (10 mM Tris, 1 mM Na$_2$EDTA, 0.1 M NaCl, pH = 7.4, Sterile)
  Note: The 1x TEN Buffer contains Tris, a buffer needed to maintain the DNA Dye Solution's pH given that the DNA Assay is sensitive to pH. EDTA serves as a chelating agent by binding up free ions such as Mg^{2+}, which usually serve as co-enzymes for DNAases, RNAases, and other degradation-type proteins.
- Hoechst 33258 Dye Solution (10,000x, Sterile)
  Note: The Hoechst 33258 Dye Solution (10,000x, Sterile) is a stock solution of 1 mg/mL Hoechst 33258 dye powder in sterile DI H$_2$O. Because it degrades from exposure to light, the solution is kept in brown bottle at 4°C. Stored like this, it should remain viable for at least 6 months. Note that the final DNA Dye Solution will have a concentration of 0.1 μg/mL Hoechst 33258 dye.
- DI H$_2$O, Sterile
- 50 mL Falcon conical tube

1) Prepare 1x TEN Buffer by adding 45 mL of DI H$_2$O and 5 mL of 10x TEN Buffer into a 50mL conical tube. Mix well.
2) Prepare the DNA Dye Solution by adding 5 μL of 1 mg/mL of Hoechst 33258 Dye Solution into the 1x TEN Buffer in the 50 mL conical tube. Mix well.
   Note: The DNA Dye Solution is simply a mixture of 1x TEN Buffer and Hoechst 33258 Dye Solution (10,000x). All buffers and solutions must be kept sterile, so use sterile technique when handling these reagents.
3) Cover the 50 mL conical tube containing the DNA Dye Solution with aluminum foil to prevent degradation from exposure to light.
   Note: The DNA Dye Solution is a possible carcinogen so it should be handled and disposed of with care (i.e., Organic Liquid Disposal Jar, or Rx DNA Dye Disposal Jar if mixed with something radioactive. Both containers are located in NE47-365.).

85

Preparation of DNA Standard Curve and Sample Readings

Materials:
- Samples from freezer
- DNA Stock (10 µg/mL)
- Tris Buffer from refrigerator
- DNA Dye Solution (0.1 µg/mL Hoechst 33258 Dye)
- Mini test tubes
- Black 96-well plate (Microfluor 2, Thermo #7805 from VWR)
- Wet lab micropipettes, repeater pipette, and tips
- Victor machine and software in Wet Lab

1) Prepare a map for the black 96-well plate used in this assay. Have duplicates of standards and samples.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

2) Prepare DNA standard solutions in mini test tubes using the amounts of DNA Stock and Tris Buffer listed in the table below. Make sure to vortex the DNA Stock and to mix the standards well by repeatedly pipetting up and down. These standards will be used to relate the DNA concentrations to the instrument readings.

Standard Solution	DNA Conc. (µg/mL)	DNA Stock (µL)	Tris Buffer (µL)
1	10	0	200
2	5	100	100
3	4	80	120
4	3	60	140
5	2	40	160
6	1	20	180
7	0	0	200

3) Prepare DNA standard solutions in mini test tubes using the amounts of DNA Stock and Tris Buffer listed in the table below. Make sure to vortex the DNA Stock and to mix the standards well by repeatedly pipetting up and down. These standards will be used to relate the DNA concentrations to the instrument readings.
4) Turn on the Victor machine so that it has time to warm up.

5) Add 20 µL of each standard and sample according to the plate map. Make sure to vortex all solutions right before adding them to ensure that they are well-mixed.
6) Add 200 µL of DNA Dye Solution (0.1 µg/mL Hoechst 33258 Dye) into each well using a repeater pipette as needed. Remove any bubbles left over in wells and dry any minor spills if necessary.
7) Put the plate in the Victor machine and read the plate according to the instructions posted on the machine.
8) Dispose of plate contents in appropriate waste containers and return materials.

For more detailed information, please refer to:

Kim YJ, Sah RL, Doong JY, Grodzinsky AJ. (1988) Fluorometric assay of DNA in cartilage explants using Hoechst 33258. *Analytical Biochemistry* 174(1): 168-176.

A.9 Radiolabel Incorporation Assay

Objective:

Depending on the radiolabel used, this assay can be used to quantify proteoglycan synthesis (^{35}S-Sulfate), protein synthesis (^{3}H-Proline), or cell proliferation/DNA synthesis (^{3}H-Thymidine).

Procedure:

Radiolabeling Samples

Materials:
- Bottle for ITS Medium Stock (HG-DMEM with 1% ITS+1, 1% HEPES, 1% NEAA, 1% Na Pyr, 1% PSA, 0.4% Proline, and 1% Ascorbate-2-Phosphate) for radiolabeling day
- Bottle for ITS Medium with radiolabels, mark with tape for radioactive materials (Rx tape)
- Bottle for ITS Medium without radiolabels
- Tube for stock radiolabeled media where isotopes will be added
- Tubes for different media groups that are radiolabeled
- Tubes for different media groups that are not radiolabeled
- Two red-cap freezer tubes for radiolabel standards (^{35}S and ^{35}S^{3}H), mark with Rx tape
- Micropipette (1000 µL) and sterile tips
- Box for discarding radiolabled pipette tips
- PBS 1X with 1% PSA for empty wells
- Stock isotope solutions (^{35}S and ^{3}H) from locked refrigerator
- 24-well plate(s) with cell-seeded hydrogel samples

1) Calculate the amount of ITS Medium Stock needed for samples with and without radiolabels. Include the volumes for the initial radiolabeling day and two media changes. Determine how much ITS Medium will be separated for each sample group.
2) Make ITS Medium Stock and split it into two groups: ITS Medium *with* radiolabels and ITS Medium *without* radiolabels.
   Note: Do not split up the radiolabeling media into subgroups because then you need standards for each subgroup and it gets hard to compare between them.
3) Make sure you have labeled the mini test tubes for saving media from gels and the two red-cap freezer tubes for radiolabel standards.
4) Mark tubes and plates that will contain radiolabeled samples with Rx tape where appropriate.
5) Use the Grodzinsky Lab "Calculation Sheet for Solutions" spreadsheet to determine the volume of each isotope (^{35}S and ^{3}H) needed and note their associated radiation (µCi) for lab records.
   a) Check the logbook to see what label number/letter we are on and how much is left.
   b) Make sure that the calibration date in the spreadsheet is correct.
   c) Enter 0.75 mL per hydrogel disk and number disks that will be radiolabeled.
   d) Enter 1 mL for the standards. (There are two standards: ^{35}S and ^{35}S^{3}H).

e) Keep the concentrations of ^{35}S and ^{3}H at 5 and 10 µCi/mL, respectively.

f) Print the sheet of what you are using to keep for your records.

6) Remove your watch and other metal items when working with isotopes.

7) Remove the appropriate isotope vials from the locked refrigerator in the Tissue Culture Room. Check the labels to make sure they match the logbook records. Note which label you are using on the printed sheet.

8) Prepare the tissue culture hood with all necessary media bottles, tubes, and pipettes.
Note: Only use micropipette tips to handle isotope solutions or radiolabeled media. Do not use 5 mL or 10 mL pipette tips because they are difficult to dispose of as radioactive materials (e.g., large tips must be broken in half so they fit in our containers for radioactive materials). Also, do not aspirate radiolabeled media into the regular media waste container. This would make the entire waste solution a radioactive material with specialized disposal needs.

9) The ^{35}S isotope must be handled first. Add the calculated amount of ^{35}S isotope to the tube for stock radiolabeled media and mix well.

10) Transfer 1 mL of the media at this time for the ^{35}S standard to the appropriate red-cap freezer tube. If you forget to do this, you must start over.
Note: Be extraordinarily careful not to spill anything that is radiolabeled to avoid strict cleanup procedures. Try not to set bottle or tube caps down on the surface of the hood to avoid getting the hood contaminated with radioactive materials. Also, protect micropipettes by not touching the sides of tubes containing isotopes with these instruments.

11) Add the calculated amount of ^{3}H isotope to the tube for stock radiolabeled media and mix well.

12) Transfer 1 mL of the media at this time for the ^{35}S^{3}H standard to the appropriate red-cap freezer tube.

13) Freeze both standards along with the day 0 samples from this experiment.

14) As soon as you are done with the stock isotope solutions, place them back in the locked refrigerator. Record the amount of each isotope used/remaining in the logbook.

15) Add 750 µL of the radiolabeled media to each designated hydrogel sample.

16) Write down the start time for the 24-hour radiolabeling period on the printed sheet.

17) Add 750 µL of PBS 1X with 1% PSA to empty wells to prevent contamination.

18) Make sure to mark all the plates that contain radiolabeled media with Rx tape. Specify which wells are radiolabeled or empty.

19) Place the plates back in the incubator at 37°C for 24 hours so that the isotopes can be incorporated into the cell-seeded hydrogel samples.

20) Clean up tubes with leftover media and verify micropipettes
a) Once you are done with the tubes that had radiolabeled media in them, clean them by pouring any leftover media into the radioactive waste bin in the wet lab. Rinse the tubes with a small amount of DI water which must be poured into the bin as well. The empty tubes must be disposed of in the radioactive waste trashcan for ^{3}H, not ^{35}S, since ^{3}H has the longer half-life of the two isotopes.
b) Fill out the tags on both the bin and the trashcan to specify the amount of radioactive waste. The required information can be calculated using the results of the "Calculation Sheet for Solutions" spreadsheet.

c) Verify if the micropipettes were contaminated with radioactive materials by using the Geiger counter in the wet lab. If they are contaminated, you should wipe the micropipettes with 70% ethanol and dispose of them in the radioactive waste trashcan for ^{3}H.

Radiolabel Takedown

Materials:
- Pre-weighed red-cap freezer tubes for storing hydrogel samples, mark Rx as needed
- Labeled mini test tubes for saving radiolabeled media from gels, mark with Rx tape
- Labeled mini test tubes for saving unlabeled media from gels
- Sterile spatulas (bent spatula and pointed spatula) to remove hydrogels from their wells
- 24-well plate(s) for transferring non-radiolabeled hydrogels
- 50 mL Falcon conical tube for Rx Wash
- 50 mL Falcon conical tube for radiolabeled wash waste, mark with Rx tape
- Micropipette (1000 µL) and sterile tips
- Box for discarding radiolabled pipette tips
- Rx Wash (DI Water with 10% PBS 10X, 0.4% Proline, and 0.8 mM Sodium Sulfate)
- Petri dish for discarded 2% agarose rings
- 24-well plate(s) with cell-seeded hydrogel samples

1) Make sure that the following materials are ready before the 24 hours for radiolabel incorporation and gel takedown are up. Prepare the tissue culture hood with these materials.
   a) Labeled red-cap freezer tubes for storing the actual hydrogels. The weights of these tubes when empty must be recorded to allow for wet weight measurements later on.
   b) Labeled mini test tubes for removing the radiolabeled media.
   c) Rx Wash (DI Water with 10% PBS 10X, 0.4% Proline, and 0.8 mM Sodium Sulfate)
      - Calculate amount of Rx Wash needed (4 washes with 1mL/wash for each sample)
      - To make Rx Wash, you may use one of the 1 mL aliquots of 250X Rx Wash Stock (DI Water with 11.5 mg/mL Proline and 35.5 mg/mL Sodium Sulfate) stored in the refrigerator in the Tissue Culture Room. The 1 mL Rx Wash Stock must be mixed with either 249 mL of PBS 1X, or 225 mL of DI Water and 25 mL of PBS 10X.
      - Transfer the calculated amount of Rx Wash needed to a 50 mL Falcon conical tube to avoid pipetting in and out of the large Rx Wash bottle.
   d) Sterilized spatulas to move the hydrogels from their plates to the red-cap freezer tubes.
2) Move non-radiolabeled hydrogel samples to a new 24-well plate using sterilized tools. Transfer the media accompanying each hydrogel to its corresponding new location using a 1000 µL micropipette with sterile tips. Place the 24-well plate with non-radiolabeled hydrogel samples in the incubator at 37°C. The tips used must be discarded in the radioactive waste bin.

Note: The reason for the transfer is to separate the non-radiolabeled samples from the radiolabeled ones that must be refrigerated during the washes.

3) Once at the 24 hour point, transfer the radiolabeled media from each well to its designated labeled mini test tubes using a 1000 µL micropipette.

4) Immediately add 1mL of Rx Wash to each well containing a radiolabeled cell-seeded hydrogel. Place the 24-well plate in the refrigerator for 30 minutes. This is Wash #1.
   Note: The plates are placed in the refrigerator to slow cell metabolism and maintain viability.

5) After the 30 minutes are up, remove the used Rx Wash solution and discard it in the 50 mL Falcon conical tube for radiolabeled wash waste. Add 1 mL of fresh Rx Wash to each desired well and place the plate back in the refrigerator for another 30 minutes. This is Wash #2.

6) Repeat the previous step for a total of 4 washes.

7) After the last wash, separate the hydrogel plugs from the 2% agarose rings and transfer them to their corresponding pre-weighed red-cap freezer tubes.
   Method 1: Use the pointed spatula to gently tap the edges of the ring and detach it from the well. Use the bent spatula to lift the edge of the ring slightly and release it. The fluid motion should allow the plug to float out of the ring. Remove the ring from the well using the pointed spatula and use a Kimwipe to remove excess water from the spatula without touching the hydrogel. Carefully place the plug in its designated red-cap freezer tube using the bent spatula.
   Method 2: Use the bent spatula to remove both the ring and the plug from the well and place them on a petri dish. Use the pointed spatula to carefully cut the ring and detach it from the plug. Lift the hydrogel with the bent spatula and use a Kimwipe to remove excess water from the spatula without touching the hydrogel. Carefully place the plug in its designated red-cap freezer tube using the bent spatula.
   Note: Method 1 is preferred when working with peptide hydrogels, since these are more fragile and could break apart easily. Both methods can be used for agarose hydrogels.

8) Weigh the vials again to obtain wet weight of each hydrogel sample by subtracting the weights of the empty tubes.
   Note: The gel weights should be around 50 mg at most.

9) Once the samples have been weighed, place the tubes in the freezer. Make sure all tubes and boxes are appropriately labeled and stored, especially radioactive materials.

10) Clean up and discard all radioactive materials appropriately.
   a) Discard all the waste wash from the plate and the 50 mL conical tube in the liquid radioactive materials bin in wet lab.
   b) Discard the petri dish with 2% agarose rings and 24-well plate in the solid radioactive materials trashcans.
   c) Discard micropipette tips in the radioactive materials sharps containers in the Tissue Culture Room.
   d) Clean spatulas by rinsing them with DI water, spraying with 70% ethanol, and wiping them down with a Kimwipe.
   e) Fill out the tags on all radioactive materials containers as needed.

<u>Measuring Radioactivity</u>

Materials:
- Red-cap freezer tubes with Rx hydrogel samples
- Red-cap freezer tubes with radiolabel standards (^{35}S and $^{35}S^3H$)
- 96-well plates for samples and radiolabel standards
- Optiphase Supermix Scintillation Fluid
- Micropipette (20 µL) and sterile tips
- Repeater pipette with 5 mL sterile tips
- Sticky, hard plastic and roller for covering plates
- Scintillation counter

1) Thaw hydrogel samples and radiolabel standards in a water bath at 37°C
2) Prepare plate map(s) for samples. Do not read the Day 0 samples, since they were not radiolabeled. For the sample plate, you must leave one row between each type of sample. Also, the scintillation counter reads certain rows together (i.e., A&E, B&F, C&G, D&H) so repeats should be placed accordingly to minimize errors.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

3) Prepare plate map for radiolabel standards. For the standards plate, you must leave one row and once column between standards. They should be located at a diagonal from each other. This prevents the highly-concentrated radiolabeled materials from exchanging between wells and causing errors in readings. See sample map below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	^{35}S A				^{35}S B							
B												
C			$^{35}S^3H$ A				$^{35}S^3H$ B					
D												
E	^{35}S A				^{35}S B							
F												
G			$^{35}S^3H$ A				$^{35}S^3H$ B					
H												

4) Vortex each sample/standard and immediately add 20 µL of the sample/standard to its corresponding well using the 20 µL micropipette with sterile tips.
5) Add 300 µL of Optiphase Supermix Scintillation Fluid to each well using a repeater pipette set at 3 with a 5 mL tip. (Note: Use the beige repeater pipette in the Wet Lab.)

6) Cover the prepared 96-well plates with sticky, hard plastic using a small roller to prevent air bubbles.
7) Vortex the covered 96-well plates.
8) Set the 96-well plates in the scintillation counter chamber. The "stop plate" should be at the top and the plates that will be read should be right below it. Plates should have the same orientation and labels should always face out.
9) Software: Micro Beta Windows
   a) Set start shelf
   b) Set wells to be read
   c) Set protocol: Adjusted Dual Label 96 Well (found in General Group Protocols)
   d) Read samples by clicking "Start" when the program is "READY"
      Note: It takes 90 seconds to read one well. They are read two at a time every 4 rows (A&E, B&F, C&G, D&H).
10) Use "Rx Calcs" MS Excel sheet to convert CCPM1 and CCPM2 readings into ^3H and ^{35}S concentrations (nmol). The highlighted (yellow) areas must be input. The concentrations can then be used to see the amounts of protein and proteoglycans in the samples, which should be expressed in terms of the DNA content. These normalization values are obtained from the DNA Assay.

A.10 Aggrecan Extraction

Objective:

This protocol describes how to extract aggrecan from tissue-engineered constructs or native cartilage. The goal is to obtain samples that have undergone sufficient purification such that aggrecan molecules can be better observed and studied through atomic force microscopy (AFM).

Procedure:

<u>Extraction Solution Preparation</u>

Materials:
- Sodium Acetate, Anhydrous, ACS Grade (CAS # 127-09-3)
- Guanidine Hydrochloride (Gu HCl), Molecular Biology Grade, Ultra Pure (CAS# 50-01-1)
- Protease Inhibitor Cocktail Tablets (Roche, Catalog # 11697498001)
- Milli Q Water in 15 mL Falcon Tube
- Four 1.5 mL tubes for prepared solutions

1) Determine the volume of Extraction Solution needed for samples
   _____ µg sGAG in samples * (1mL Extraction Solution / 120 µg sGAG) = _____ mL Extraction Solution
   Note: For tissue-engineered samples, make 1 mL of Extraction Solution for every ~40mg of tissue wet weight with sGAG content of ~120 µg (i.e., plug sGAG content is ~3 µg/mg wet weight). Double the amount of Extraction Solution needed if using native tissue instead of tissue-engineered samples.
2) Make desired volume of Guanidine HCl Solution (4M Guanidine HCl in 100mM Sodium Acetate, pH ~7.2)
   a) Decide what volume of Guanidine HCl Solution should be prepared (_____ mL)
      Note: This volume should be at least as much as the volume of Extraction Solution needed, though it may be useful to have extra Guanidine HCl Solution (e.g., to make GAG Assay standards).
   b) Prepare 100 mM Sodium Acetate (FW 82.03)
      Weigh _____ g of Sodium Acetate and dilute in _____ mL Milli Q Water
   c) Prepare 4 M Guanidine HCl (FW 95.53)
      Weigh _____ g of Guanidine HCl and dilute in _____ mL of 100 mM Sodium Acetate
      Note: Guanidine HCl powder is an irritant and is toxic to the nervous system so it should be handled in the fume hood. When diluting Guanidine HCl in 100 mM Sodium Acetate, start with less than half of the desired total volume and add 100 mM Sodium Acetate as needed. This is due to the fact that Guanidine HCl expands when dissolved and rapidly increases the total volume.
   d) Adjust pH to ~7.2 using appropriate buffers
3) Make desired volume of Protease Inhibitor Stock Solution (12.5x)
   Dissolve ½ tablet in 1mL of Milli Q Water to obtain a 12.5x solution

Note: The above is based on the manufacturer's instructions for the Protease Inhibitor Cocktail Tablets. This stock solution can then be added to the Guanidine HCl Solution at 1x concentration to inhibit a broad range of proteases and protect the aggrecan molecules in the samples.

4) Prepare final Extraction Solution by mixing appropriate fraction of Protease Inhibitor Stock Solution with Guanidine HCl Solution
Volume of PI Stock Solution = (1x / 12.5x) * (Volume of Extraction Solution for Samples)
Volume of Gu HCl Solution = (Volume of Extraction Solution for Samples) – (Volume of PI Stock Solution)
Dilute: _____ μL of Protease Inhibitor Stock Solution + _____ uL of Guanidine HCl Solution

5) Use the Extraction Solution as soon as possible, since the Protease Inhibitor Stock Solution is only good for 1-2 weeks if stored at 2 to 8°C, or 12 weeks if stored at -15 to -25°C.

Guanidine Hydrochloride Aggrecan Extraction

Materials:
- Samples
- Scalpel
- Extraction Solution
- Shaker in Cold Room
- Microcentrifuge in Cold Room
- New tubes for each sample

1) Mechanically disrupt each sample. Using a scalpel to dice works well for tissue-engineered plugs. If using native cartilage tissue, it may be good to pulverize and homogenize.
2) Add appropriate volume of Extraction Solution to each sample (determined in previous section).
3) Mix on shaker at 4°C for 48 hours. (This is probably excessive but will maximize proteoglycan extraction.)
4) Spin the samples for 0.5 – 1 hr at ~16,000 x g in microcentrifuge at 4°C.
5) Transfer the clarified supernatant to a new tube and discard the pellet.

Cesium Chloride Density Gradient Centrifugation

Materials:
- Samples
- Cesium Chloride (CsCl), Molecular Biology Grade (CAS# 7647-17-8)
- Microbalance in Main Lab
- Centrifuge Tubes (Beckman, Polycarbonate Tubes, 7/16 x 1 3/8 in., Product #343778)
- Ultracentrifuge in Main Lab
- Fraction collection tubes and post-dialysis sample tubes
- Slide-A-Lyzer Dialysis Cassettes, 10,000 MWCO (Thermo Scientific, Product # 66383)
- Autoclaved beakers for dialysis

- Sodium Chloride (NaCl), Molecular Biology Grade (CAS# 7647-14-5)
- Milli Q Water in beaker for dialysis

1) Add CsCl powder to each sample until solution density is 1.58 g/mL.
   a) Approximate the amount of CsCl that must be added based on the sample volume.
   b) Check the density by weighing a known volume of solution in a microbalance.
2) Transfer each sample to the Centrifuge Tubes appropriate for spinning in the ultracentrifuge. Make sure to balance the ultracentrifuge rotor exactly!
3) Spin the samples for 72 hrs at ~500,000 x g in the ultracentrifuge at 4°C.
   Note: Spin all samples as long and hard as possible to produce a good density gradient.
4) Immediately at the end of the spin, carefully pipette fractions of the sample.
   Notes: Splitting 1 mL into ~10 x 100 μL fractions works well. Start by pipetting off the very top and working down the tube. Be aware that a hard pellet of CsCl may form at the bottom; the density gradient will likely still be sufficient for PG purification.
5) Measure the density of the fractions by weighing a known volume of solution in a microbalance.
6) Combine fractions based on density in the fraction collection tubes.
   D1 > 1.54 g/mL; 1.46 g/mL < D2 < 1.54 g/mL
7) Dialyze with 500 volumes of 1M NaCl for 12-24 hrs.
8) Dialyze with 500 volumes of MilliQ filtered, distilled H20.
9) Store purified samples in freezer at -20°C

A.11 AFM Imaging Assay for Aggrecan

Objective:

Atomic force microscopy (AFM) can be used to image key molecules in cartilage tissue-engineered constructs. This protocol describes how to prepare mica with an aggrecan monolayer and how to image samples with AFM.

Procedure:

Preparing APTES-Mica for Imaging

Materials:
- APTES (3-aminopropyltriethoxysilane)
- One 50mL Falcon tube filled with MilliQ water
- Mica film
- Scissors, tweezers, and tape
- Box for treated mica storage
- Filter paper (Whatman, 90 mm ø, Catalog # 1002090)

1) Mix 3 µL of APTES (3-aminopropyltriethoxysilane) with 30 mL MilliQ water (0.01% v/v). Shake gently by inverting the vial a few times.
2) Cut square pieces of mica (1 cm x 1 cm) inside a bio-hood. Cleave the mica by pressing the square piece against paper tape and peeling slowly. Make sure the cleaved plane comes off cleanly in one piece. Place the cleaved mica in a closed container with the cleaved plane side up. Do not touch the cleaved plane.
3) Immediately after cleavage, place 50 µL (or enough to cover the entire surface but not spill off) of the 0.01% APTES solution on the cleaved mica. Keep the mica in a closed container with water to minimize evaporation of the solution. For example, use a pipette tip box filled with water in the bottom. Mica can be place on the divider in the middle. Incubate for 30 minutes at room temperature.
4) Rinse gently in a stream of 200 µL MilliQ water for about 10 seconds. Dry the mica by holding it vertically with tweezers and blotting its edge on a piece of filter paper. (Nitrogen drying is another option.)
5) Store in a closed container to prevent dust from contaminating the surface. Use the treated mica to prepare the aggrecan monolayer on the same day.

Preparing Aggrecan Monolayer

Materials:
- Aggrecan sample solution
- Box for temporary sample storage
- One 50 mL Falcon Tube filled with MilliQ water
- Filter paper (Whatman, 90 mm ø, Catalog # 1002090)
- One petri dish for final sample storage

1) Thaw the frozen aggrecan solution at room temperature.

2) Dilute the aggrecan solution with MilliQ water to the desired concentration and volume (i.e., 10-100 μg/mL and 50-100 μL/mica). Mixed the diluted sample well by pipetting up and down a few times.
3) Incubate 50-100 μL of the diluted aggrecan solution on the treated mica in a closed container at room temperature. Again, prevent sample evaporation by having water in the container. The incubation time can range from 20-60 minutes, depending on the desired final monolayer density of aggrecan.
4) Rinse gently in a stream of 200 μL MilliQ water for about 10 seconds. Dry the mica by holding it vertically with tweezers and blotting its edge on a piece of filter paper.
5) Store the mica with aggrecan samples in a closed petri dish to prevent dust from contaminating the surface. Image when the sample is completely dry. This may take at least 4 hours. Overnight drying is preferred.

AFM Imaging

Materials:
- Petri dish with mica aggrecan samples
- Magnetic disks and custom adhesive tape
- AFM probe tip (NANOSENSORS, SuperSharpSilicon, 4 μm thick, Tip Type SSS-NCHR-10)
- Specialized tweezers with grove for AFM cantilever or tip handling
- NanoScope IIIa Multimode AFM

Notes:
- AFM facilities are located in 13-5037
- Computer: Username = administrator, Password = afm2007

Start the Instrument

1) Open the Nanoscope 5.31r1 program on the computer. ALWAYS open the program before anything else.
2) Click the microscope icon on the left to enter imaging mode.
3) Turn the NanoScope IIIa Controller on using the switch on the upper right corner of the back panel.

Setting up for Imaging

1) Select the appropriate piezo. Current images are obtained with the J scanner.
   a) E scanner: x-y direction: 10 μm; z-direction 2.5 μm
   b) J scanner: x-y direction: 100 μm; z-direction 5 μm
2) Glue the mica sample on the magnet with adhesive tabs. Use tweezers to handle the samples and avoid touching the sample surface.
3) Place the magnet on the top of the piezo tube.
4) Place the AFM head on the piezo and attach the springs to the head. Hold the head tightly while you do this. Make sure it is ALWAYS securely attached.
5) Move the sample down by pressing "Tip Up" (the base moves down). This prevents damage to the tip and sample when you slide in the tip holder.

6) Select a cantilever or tip. Place the tip holder facing up (handle should be on the left) and press down on the base to open the golden clasp that holds the chip. Carefully slide the chip into the holder until you hit the groove. Make sure the cantilever chip is right-side up (the step around the chip should be on the bottom).

7) Place the holder into the head at a 45° angle to the surface (or at an angle so the holder and cantilever don't hit the stage). To do this, turn the holder upside down (handle should be on the right). Fasten the holder on the AFM head by turning tightening the knob in the back to bring down the platform with the screws.

8) Move the sample up by pressing "Tip Down" (the base moves up). This brings the sample and the tip closer together for imaging and laser alignment.

9) Switch the mode to AFM & LFM. It is better to align the laser in this mode because it is more sensitive and aligned parameters will transfer over to TMAFM mode. If this proves difficult, switch the mode to TMAFM and then align.

10) Plug in the laser and align the laser spot onto the tip of the cantilever. Use the paper method to obtain a concentrated laser spot and use the light microscope to optically guide the laser spot to the tip of the cantilever by moving its position with the knobs in the head. Take special care to optimize the parameters as follows: maximize the sum (e.g., 6.8-7.2), set the vertical difference to 0 V, and set the horizontal difference to 0 V.

11) Switch the mode to TMAFM.

12) Placing the AFM on a tripod during imaging improves the image quality significantly by reducing vibrations. Use extreme caution when setting the AFM on the tripod.

Setting up the Nanoscope Program

1) Go to auto tuning mode by clicking the tuning icon to find the cantilever resonant frequency. Set the frequency to 50-500 kHz, the target amplitude to 3V, and the peak offset to 10%. Click Auto Tune button. You should see an amplitude profile with one clear peak.

2) Go back to image mode.

3) Check the selected scanner: Go to MICROSCOPES > SCANNER > E or J

4) Check the selected profile: Go to MICROSCOPES > SCANNER > Tapping Mode

5) Other parameters:
   Scan Controls
   a) Scan Size: 2 μm
   b) Aspect Ratio: 1:1
   c) X Offset: 0 nm
   d) Y Offset: 0 nm
   e) Scan Angle: 0
   f) Scan Rate: 0.5-1.0 Hz
   g) Samples/Line: 256 (pre-scan image); 512 (high-quality image)
   h) Slow Scan Axis: Enabled
   Feedback Controls
   a) SPM Feedback: Amplitude
   b) Integral Gain: 0.2-0.4 (Make sure it is low enough to prevent feedback oscillation.)
   c) Proportional Gain: 0.6-0.8

d) Amplitude Setpoint: 0.6-2.0, or higher (For tapping mode, the lower the value, the greater the contact force on the tip. Use a larger value to prevent tip damage, but if this value is too large then the tip may lose contact with the surface.)

e) Drive Frequency: Should be determined by Auto Tune

f) Drive Amplitude: Should be determined by Auto Tune

Other Controls

a) Microscope Mode: Tapping

b) Z Limit: According to the piezo

c) Units: Metric

d) Engage Setpoint: 0.8-1.0 (The lower the value, the greater the engage force. Use a larger value to prevent tip damage, but if this value is too large then the tip may lose contact with the surface later on.)

e) Parm Update Retract: Disabled

Channel 1

a) Data Type: Height

b) Data Scale: 5 μm

c) Line Direction: Trace

d) Scan Line

e) Realtime Planetfit: Line

f) Offline Planefit: None

Channel 2

a) Data Type: Amplitude

b) Data Scale: 1 V

c) Line Direction: Retrace

d) Scan Line

e) Realtime Planetfit: Line

f) Offline Planefit: None

6) Press the Engage icon to bring the tip to the sample surface.

7) Once the tip is engaged, you should hear a beep sound and see images appear.

8) During imaging, pay attention to the piezo Z center position. Make sure it doesn't exceed ± 150 V.

9) Avoid scanning any large dust by offsetting the imaging area with the software or disengaging and changing the scanning area with the adjustment knobs. DO NOT use the adjustment knobs if the tip has not been disengaged. This could damage both the sample surface and the tip.

10) Click the Capture button to capture an image. The software will capture the next complete image. If any parameters have been changed after the capture function is activated, the capture function will be held until the next frame.

11) When you are done or want to move another area on the sample, disengage the tip.

Clean up

1) Disengage the tip from the sample.

2) Unplug the laser.

3) Raise the tip from the sample so you do not damage the tip and the sample when you take out the holder.

4) Take out the cantilever chip from the hold and put it back in the tip box.
5) Detach the springs from the head.
6) Put the sample away.
7) Turn off the controller.
8) Close the software.

Image Processing

Materials:
- AFM Image Files
- NanoScope 5.31r1 Software
- WSxM 4.0 Develop 10.1 Software

1) Using the NanoScope 5.31r1 Software, select the scan area and resolution for the image file.
2) Flatten the image to allow for a more accurate height image. Note that the WSxM 4.0 Develop 10.1 Software also has a flatten feature but this seems to work better with the NanoScope 5.31r1 Software.
3) Save the modified image file for further processing.
4) Using the WSxM 4.0 Develop 10.1 Software, open the image file through the heuristic interface.
5) Adjust the color palette to the desired aggrecan molecule colors. Click on the palette settings button, which looks like a rectangle with lines on top. Choose the Nanolux color scheme.
6) Equalize the image for a more accurate depth representation. Click on the equalize button, which looks like a triangle with lines on top. Move the left and right boundaries by clicking the mouse buttons (left click on desired location of left boundary, right click on desired location of right boundary).
7) Remove any undesired noise lines on the image. Click on the remove lines button, which looks like three intersecting lines. Move the indicator in the scroll bar to the line you want to remove and click OK.
8) Save the final image in a high-quality file.