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Feasibility of a Genipin-Crosslinked Fibrin Gel as a Patch for Annulus Fibrosus Repair in the Intervertebral Disc

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Feasibility of a Genipin-Crosslinked Fibrin Gel as a Patch for Annulus Fibrosus Repair in the Intervertebral Disc

by

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Abstract

Tears in the annulus fibrosus (AF) of the intervertebral disc are a significant clinical problem resulting in pain and disability for patients. This thesis investigated a tissue engineering solution using fibrin gels crosslinked with genipin and seeded with mesenchymal stem cells to repair defects in the annulus and promote generation of new tissue. The addition of an antifibrinolytic agent was required to maintain gel stability over 30 days, and gel stiffness was significantly increased by the addition of 0.100mg/mL genipin without affecting cell viability. Gene expression indicated that genipin crosslinking upregulated expression of chondrogenic-specific markers. Cells responded to being placed in a defect in tissue and loading may have promoted a more fibrochondrogenic type of lineage differentiation. Histology showed genipin gels promoted a more fibroblastic type of morphology than fibrin gels. Genipin-crosslinked fibrin gels were shown to have sufficient properties for annulus fibrosus repair and should be investigated further.

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List of Symbols, Abbreviations and Nomenclature

Symbol/Abbreviation	Definition
ACL	Anterior Cruciate Ligament
AF	Annulus Fibrosus
ASTM	American Society for Testing and Materials
BMP-7	Bone Morphogenic Protein 7
$CaCl_2$	Calcium Chloride
cDNA	Complementary DNA
CO_2	Carbon Dioxide
CS	Chondroitin Sulfate
CT	Computed Tomography
DDD	Degenerative Disc Disease
dH_2O	Distilled Water
DMEM	Dulbecco's Modified Eagle Medium (Low Glucose)
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
GAG	Glycosaminoglycan
IDD	Internal Disc Disruption
IVD	Intervertebral Disc

MAPs	Marine Adhesive Proteins
MRI	Magnetic Resonance Imaging
MSCs	Mesenchymal Stem Cells
mTG	Microbial Transglutaminase
n	Sample Size
Nectrostatin-1	Necrosis Inhibitor
NP	Nucleus Pulposus
O_2	Oxygen
p	Probability Value
PBS	Phosphate Buffered Saline (1X)
PCL	Polycaprolactone
PEGDA	Polyethylene Glycol Diacrylatel
PLGA	Poly-(Lactic-Co-Glycolic Acid)
PRG4	Lubricin
RGD	Arginine-Glycine-Aspartic Acid
RNA	Ribonucleic Acid
RNase	Ribonuclease
rt-PCR	Real Time Polymerase Chain Reaction
SF-HBC	Silk Fibroin-Hydroxybutyl Chitosan
TGF-β	Transforming Growth Factor-β
tTG	Tissue Transglutaminase
Z-VAD-FMK	Apoptosis Inhibitor

Chapter One: Introduction

1.1 Introduction

One in fifty Canadians will become disabled by back pain in their lifetimes, with this pain accounting for 40% of all workplace absences (Kandel et al., 2008). Back pain can be caused by a variety of disorders, but many of them can be associated with degeneration of the intervertebral disc (IVD) (Kalichman et al., 2010; Luoma et al., 2000; Millecamps et al., 2012; Urban and Roberts, 2003), with the primary cause of disc failure being rupture of the outer region of the disc, or disc herniation. The IVD is aneural, avascular, and contains a low density of cells. These characteristics result in poor regeneration and healing abilities in the disc. Most current treatment options aim to reduce pain, but do not treat the underlying cause of back pain, creating a need for innovative solutions to repair or replace the degenerated disc.

Tissue engineering may be an effective method of combining scaffolds, cells, and various biomaterials to develop a regenerative solution for biomedical problems and may provide a means of developing solutions for annulus fibrosus repair. It has been acknowledged that other tissue engineering solutions for various components of the IVD will not be successful unless the structural integrity of the annulus is retained. The various tissues within the IVD together can withstand a greater load than any of the tissues individually (Urban and Roberts, 2003), and therefore, maintaining the structural integrity of all the tissues is required for proper physiological function. It is critical to the success of any

tissue engineering constructs intended for IVD repair that a successful method for annulus closure is developed.

1.2 The Intervertebral Disc

1.2.1 Anatomy

The human spinal column consists of twenty six bones: twenty four vertebrae, the sacrum and the coccyx. Together these structures bear and transfer the weight of the head, neck and trunk, protect the spinal cord, and help to maintain the body in an upright position (Martini, 2001). The intervertebral discs lie between the vertebral bodies, linking them together. These discs, ranging from about 7-10mm in height, account for approximately one third of the total spinal column length (Urban and Roberts, 2003). The IVD's main role is to provide height in order to separate the vertebral bodies and must be pliable enough to allow for movement, while being stiff enough to absorb large compressive loads (Adams et al., 2006). The IVD are also required to transmit loads created by the body's weight and muscle activity throughout the spinal column and provide flexibility which allows for bending, flexion and torsion (Nerurkar et al., 2008).

The intervertebral disc is composed of three distinct tissues: the annulus fibrosus (AF), the nucleus pulposus (NP), and the cartilage end plates (Figure 1.1). The annulus fibrosus is a fibrocartilaginous material composed of 10-20 tightly packed sheets of collagen called lamellae arranged circumferentially around the periphery of the disc (Adams et al., 2006). This fibre reinforced tissue is a key contributor to the discs mechanical function due to its complex hierarchical structure and the stiff lamellae are

able to sustain large tensile loads. The tissues high collagen content allows it to maintain its pliability, allowing enough deformation to enable bending and motion of the disc. The outer portion of the annulus is responsible for withstanding primarily tensile stresses (circumferential and longitudinal), but as it transitions towards the inner portion of the disc, it becomes increasingly more involved in absorption of compression (Kandel et al., 2008).

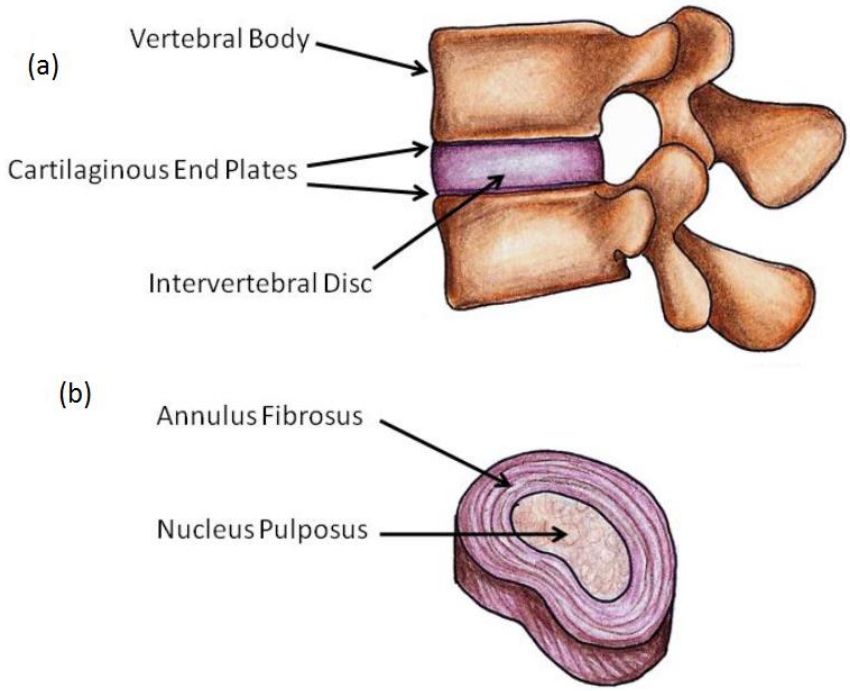


Figure 1.1: Anatomy of the intervertebral disc (a) within the spine and (b) as an individual structure (Image courtesy of Stephanie Fisher (Fisher, 2011)).

The nucleus pulposus is a hydrated gel located in the centre of the disc that absorbs compression and transmits load radially to the AF, preventing the AF from buckling during movement and allowing it to maintain its stiffness (Adams et al., 2006). These

structures are sandwiched by the superior and inferior cartilage end plates, structures composed of hyaline cartilage that covers almost the entire surface of the vertebral bodies adjacent to the IVD (Adams et al., 2006). The end plates are typically less than 1mm thick and act as an interface between the disc and the vertebral bodies.

1.2.2 Microstructure

The unique composition and microstructure of the AF is critical to its mechanical function within the disc. The fibres are oriented at a $\pm 30°$ angle from the transverse plane, passing obliquely from one vertebral body to the next and alternating to the left and right in each adjacent lamellae (Adams et al., 2006) (Figure 1.2). This unique configuration allows for the annulus to resist tension in multiple directions while at the same time, preventing a plane from occurring through which the NP could seep through.

The AF is composed primarily of types I and II collagen, approximately 70% by dry weight, with the concentration of type I collagen being predominant in the outer portion of the disc and diminishing towards the centre, and the opposite trend being observed for type II collagen. This distribution of collagen reflects the mechanical role played by the AF within the disc, with a greater tensile requirement in the outer region of the disc and a combination of tensile and compressive resistance being required increasingly towards the central region of the disc (Adams et al., 2006). Type I collagen fibres are typically found in tensile structures such as ligaments, while type II collagen is common in compressive structures such as articular cartilage.

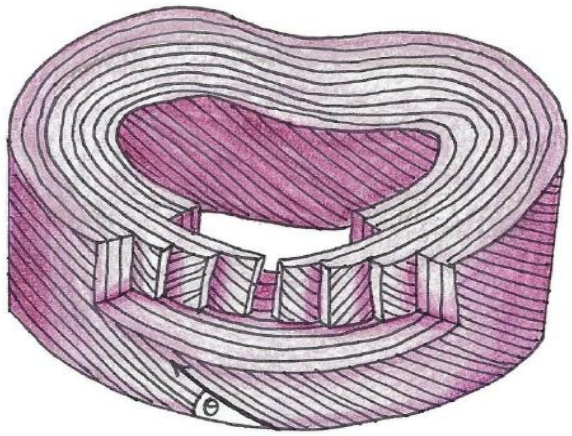


Figure 1.2: Microstructure of the annulus fibrosus with oriented fibres in alternating +/- 30 degrees from the traverse plane (Image courtesy of Stephanie Fisher (Fisher, 2011)**).**

The largest proteoglycan component in the annulus is aggrecan, which helps to maintain tissue hydration and osmotic pressure through its constituent chondroitin and keratin sulphate chains (Urban and Roberts, 2003). Elastin fibres are also present within the annulus between the lamellae, and may contribute to retention of the structure's shape after deformation (Roberts et al., 2006; Yu et al., 2005).

The nucleus consists largely of proteoglycans, large molecules composed of complex sugars and proteins that have the ability to retain large amounts of water. It is this property of proteoglycans that allows for the hydrodynamic properties of the nucleus, with water constituting 70-85% of its composition. Proteoglycans and collagens account for approximately 50% and 20% of the NP dry weight respectively. If the NP loses proteoglycans, it significantly affects its water retention, preventing it from properly bracing the annulus during movement (Adams et al., 2006).

1.2.3 Cells of the IVD

The AF and NP contain a very low density of cells, approximately 9000 cells/mm^3 and 5000 cells/mm^3 respectively (Urban and Roberts, 2003), with the cells sometimes located in lacunae or capsules within the matrix that are distinct from the rest of the matrix. This pericellular matrix consists of components such as collagens I (mainly in the annulus), II (mainly nucleus), III, IX and XI as well as aggrecan, fibronectin, and laminin. It differs from the pericellular matrix found around chondrocytes in articular cartilage in that it also contains collagen VI (Cao et al., 2007). Similarly to collagen content, the cells within the AF vary by region, likely as a result of the varied mechanical environment. Towards the outer portion of the disc, the cells are more elongated and fibroblast-like, orienting themselves in the direction of the fibres. On the inner portion of the disc the cells are more similar to a chondrocyte phenotype (Adams et al., 2006). The NP contains rounded cells that are notochordal during childhood and resemble articular cartilage chondrocytes later in life.

Due to the avascularity of the disc, nutrient transport and metabolic waste disposal are limited. A large portion of disc nutrients are obtained by diffusion at the periphery from blood vessels at the margins, while the pathway for NP cells is primarily from capillaries that originate in the vertebral bodies (Adams et al., 2006). Nutrients to the NP must diffuse through the cartilage end plates and dense ECM to reach the NP cells that can be as far as 8mm from a capillary bed.

Both the cells of the AF and NP respond to load in a similar fashion as articular chondrocytes, with very high and very low hydrostatic pressure resulting in a decrease in proteoglycan synthesis. Moderate loads, on the other hand, increase proteoglycan synthesis (Adams et al., 2006).

1.2.4 Biomechanics

Loading in the spine is complex as it experiences 6 degrees-of-freedom motion (Kulkarni and Diwan, 2005). Key loads experienced throughout the disc at various locations and tissue levels are compression, tension, and torsion (Figure 1.3). The NP primarily experiences compressive forces. Due to the imbibing properties of the proteoglycans in the NP, it has the ability to absorb high compressive stresses. Upon compression, the disc height is narrowed and the NP bulges outwards, transmitting axial and radial compressive forces to the AF (Nerurkar et al., 2010).

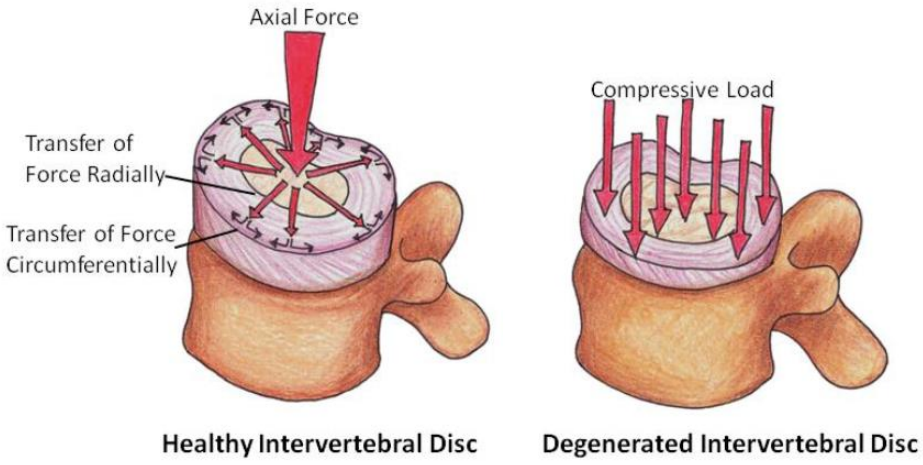


Figure 1.3: Distribution of compressive load in a healthy and degenerated intervertebral disc (Image courtesy of Stephanie Fisher (Fisher, 2011)**).**

The AF also experiences large tensile stresses in the circumferential direction. The fibre-reinforced structure of the annulus results in the biomechanical properties of the material: it's both anisotropic (directionally dependent) and has a non-linear stress strain response. This tensile non-linearity is common in fibre-reinforced soft tissues and results in a stress-strain curve that contains a toe region, a linear region, and a transition region between the two. The uniaxial tensile modulus of the AF is one or two orders of magnitude higher in the circumferential direction than either the radial or axial directions (Nerurkar et al., 2010), displaying the marked anisotropy of the tissue. The AF and NP both also experience shear forces during torsional movements and bending. During shear, the AF also responds anisotropically and viscoelastically, and due to the prominent shearing produced in the AF during torsion, the circumferential shear properties of materials developed for AF tissue engineering may be crucial for proper physiological function (Nerurkar et al., 2010). The mechanical properties of the intervertebral disc are summarized in Table 1.1.

Table 1.1: Mechanical properties of the annulus fibrosus and nucleus pulposus in the healthy intervertebral disc.

Material Parameter	Value in the Annulus Fibrosus	Value in the Nucleus Pulposus	Reference
Porosity	0.70	0.75	(Antoniou et al., 1996)
Aggregate Modulus (Compression)	0.56 MPa [ref1]	0.3 MPa [ref2]	[ref1](Iatridis et al., 1998) [ref2](Iatridis et al., 1997)
Shear Modulus	0.1 MPa [ref3]	0.025 MPa [ref4]	[ref3](Iatridis et al., 1999) [ref4](Iatridis et al., 1997)
Tensile Modulus	20 MPa [ref5]	0.04 MPa [ref6]	[ref5](Acaroglu et al., 1995) [ref6](Panagiotacopulos et al., 1987)

1.3 Degenerative Disc Disease

1.3.1 What is Degenerative Disc Disease

With age, there is an increase in degenerative changes within the IVD including cell death, loss of cell proliferation, granular change and concentric tears (Urban and Roberts, 2003). More than 50% of cells in adult discs are necrotic and there is a limited ability of the tissue to regenerate and heal itself. Over 80% of the adult population is affected by low back pain (Shvartzman et al., 1992), with surgical procedures being performed on approximately 5% of those affected (Whatley et al., 2011). Degenerative disc disease has a large economic impact, with direct medical costs of $33 billion and indirect costs around $100 billion in the United States annually (Waddell, 1996).

Degenerative disc disease (DDD) is poorly defined, but can be described as the structural failure of the IVD as a result of degeneration to one or several of the structures of the IVD resulting in issues such as radial AF tears, inward buckling of the inner AF, reduced disc height, end plate defects, and vertical bulging of the endplates into adjacent vertebral bodies (Adams et al., 2006). Back pain is strongly associated with DDD (De Schepper et al., 2010) and is associated with disc herniation, prolapse, and sciatica (Luoma et al., 2000; Macnab, 1986), although it can be asymptomatic in many cases. Degeneration can alter the disc height and mechanics of the spinal column, adversely affecting other spinal structures such as muscles and ligaments. It can also result in spinal stenosis, a major cause of pain and disability in the elderly (Hadjipavlou et al., 2008; Kandel et al., 2008).

Degeneration of the disc can occur at any age, but there is a correlated increase in degenerative changes with ages. Disc degeneration occurs earlier than in other musculoskeletal tissues with the first findings of degeneration reported as early as 11-16 and as many as 20% of people in their teens have minor degeneration. At the age of 50, 10% of people have discs that are severely degenerated with this percentage increasing to 60% in those aged 70 years or older (Kandel et al., 2008).

1.3.2 Degradative Changes in the Intervertebral Disc

There are also many biochemical changes in the IVD, the most significant being a loss of proteoglycans. With increasing age and loss of proteoglycans, there is a decrease in water content of the disc, affecting its ability to maintain hydration under load and bear compressive forces. Major changes in disc behaviour may affect the function of other spinal structures or predispose them to injury (Koepsell et al., 2010). There is also reduced matrix turnover in aged discs, with collagen fibrils becoming more crosslinked and the types and distribution of collagen changing, leading to less flexibility and a decreased ability to withstand compressive and tensile forces (Masuda and Lotz, 2010).

During degeneration the NP becomes stiffer, and the structural organization and biochemical composition of the AF are compromised, which ultimately affects the biomechanical function of the tissues. The boundary between the AF and the NP becomes less defined, and the region of the inner AF, which normally withstands hydrostatic pressure, is reduced. This results in more of the compressive load being distributed to the outer annulus, particularly in the posterior of the disc (Adams et al.,

2006). The tensile properties of the annulus also decrease with age as it becomes increasingly disorganized and damage leads to cracks and fissures (Urban and Roberts, 2003).

Annular tears tend to occur in early stages of disc degeneration and are associated with more rapid nuclear degradation after they have occurred (Sharma et al., 2009). There are three types of annular tears that can occur: concentric tears, radial tears, and rim lesions with radial tears being the most common. Concentric tears are also referred to as "delaminations" and result in the splitting apart of the lamellae in a circumferential direction. Rim lesions, or peripheral rim tears, are the horizontal tearing of the very outer annular fibres near where they attach to the vertebral bodies. Radial tears are full thickness annulus tears that originate in the NP and propagate outwards, which can ultimately result in a defect from which the NP can herniate (Adams et al., 2006). The least invasive and most accurate way of identifying such tears is through the use of MRI (Benjamin and Evans, 1990). If NP material migrates down through a radial fissure, it is detected on the MRI as a "high intensity zone" or HIZ (Adams et al., 2006), however there is much controversy over how significant these HIZ are as a pain indicator and diagnosis tool (Lam et al., 2000).

1.3.3 Mechanisms of Degeneration

The mechanisms of degenerative disc disease are largely unknown; however there are many factors that are attributed to its development and propagation. Genetics accounts for 50-75% of IVD degeneration in addition to other individual factors such as height,

weight, flexibility, strength, fatigability, and fitness (Adams et al., 2006). Environmental factors, such as mechanical loading, may also contribute to degeneration. Abnormal mechanical loads, resulting in overloading or structural damage, may result in degenerative changes in the disc (Urban and Roberts, 2003).

The other major contributing factor thought to cause degeneration is the poor nutritional pathways to the disc. Cells within the IVD require nutrients such as glucose and oxygen and are highly sensitive to the extracellular oxygen and pH levels. In low oxygen and acidic pH conditions, matrix synthesis rates fall and cells cannot survive in these conditions, or with low glucose concentrations, for long. The disc is largely avascular and depends on blood vessels at the margins or originating in the vertebral bodies for nutrient supply and metabolic waste removal (Adams et al., 2006). This can become an even greater issue as degradative changes cause the ECM to become increasingly crosslinked, making diffusion of nutrients more difficult. Calcification of the endplates also limits diffusion of nutrients to the NP (Kandel et al., 2008).

1.4 Current Treatments for Degenerative Disc Disease

One of the main challenges with degenerative disc disease is that currently, treatments are palliative and don't address the underlying issues of the disease. While these solutions may reduce pain, they won't result in long term stability and health of a degenerated disc.

1.4.1 Conservative Treatments

The first and most predominant method of treatment taken with patients presenting with back pain or herniation are conservative measures aimed at reducing pain. This includes the use of muscle relaxants or injection of corticosteroids as well as local anaesthetic and manipulation therapies (Urban and Roberts, 2003).

1.4.2 Surgical Interventions

If conservative treatments fail, surgical procedures such as discectomies and spinal fusions are used to maintain stability of the disc or spinal segment. Discectomies are a surgical practice directed at removing all or part of the damaged tissue. While these procedures often reduce pain, they do not restore the disc to its original height or load bearing capacity. Additionally, the loss of the NP tissue has been associated with further pathogenesis of DDD. In 2002, there were 200,000 spinal fusions performed in the United States (Kandel et al., 2008). However, this is not an optimal treatment as it limits flexibility of the spine and may induce degeneration in adjacent vertebrae.

Surgeries are performed on approximately 5% of the population presenting with back pain (Whatley et al., 2011), with similar success rates in both types of procedures. However, 70-80% of patients who present with obvious surgical indications eventually recover, regardless of whether a surgery is performed or not (Whatley et al., 2011).

In an attempt to preserve mobility of the spine, partial or total disc replacements may be completed instead of spinal fusions. The use of full disc replacements is very

controversial as the complications can be catastrophic leading to paralysis or death. Long term data is required before their effectiveness can be confirmed (Kandel et al., 2008). Full disc replacements have the advantage over spinal fusions in that they have been shown to reduce the occurrence of adjacent spinal segment degeneration in short term studies (Harrop et al., 2008), however they face issues of mechanical wear, displacement, failure, and debris. There are many commercially available disc replacements, with some of the most widely used over a significant time period including the CHARITE (Depuy) and ProDisc (Synthes Spine). Short term studies of these devices showed positive patient outcomes with reduced pain and mobility while preserving spinal segment motion. However, long term studies have shown aggravation and degenerative processes in many patients, indicating that facet arthrosis adjacent segment disease may still be a concern with long term implantation of these devices (Shim et al., 2007).

1.5 Current Research in Tissue Engineered Treatments

Due to the lack of treatments for underlying causes of degenerative disc disease, there is a growing interest in alternate therapies such as tissue engineering that could be used to regenerate the original structures of the AF or NP for treatment of degeneration. These types of solutions create a new need for a method of fixation of tissue engineered constructs to native cartilage. There have been a variety of materials investigated for implementation of tissue engineering solutions such as collagen (Alini et al., 2003; Attia et al., 2011; Barnes et al., 2007; Calderon et al., 2010; Madhavan et al., 2010), chitosan (Dang et al., 2006; Nandagiri et al., 2011; Tan et al., 2009; Yu et al., 2011; Zhang et al., 2010), alginate (Augst et al., 2006; Bhattarai et al., 2006; Bron et al., 2011; Li et al.,

2005), silk fibroin (Chang et al., 2007; Chang et al., 2010; Meinel et al., 2006; Roohani-Esfahani et al., 2012; See et al., 2012), hyaluronan (Ji et al., 2006; Park, 2005; Segura et al., 2005; Vanderhooft et al., 2009) and fibrin (Dare et al., 2009; Eyrich et al., 2007; Jung et al., 2010; Osathanon et al., 2008; Schek et al., 2011) in the form of gels, fibres, and composites with varying degrees of success.

Although significant research has gone into the development of replacement materials for the NP in order to maintain disc height, these solutions will be unable to be successfully implemented unless the AF can be repaired as well to maintain the natural biomechanics of the disc. The following section will summarize some of the current research in the area of tissue engineering to regenerate the structures of the IVD with a focus on the biomaterials studied for repair of the annulus fibrosus.

1.5.1 Natural & Synthetic Biomaterials

In tissue engineering, there are a large variety of both natural and synthetic biopolymer-based materials used that are typically polysaccharide-based (such as hyaluronic acid, chondroitin sulphate, chitosan, alginate, and agarose) or protein-based (collagen, gelatin, fibrin and silk) (Tibbitt and Anseth, 2009). There are many advantages and disadvantages of each type of material that need to be considered for each individual tissue engineering application (Figure 1.4).

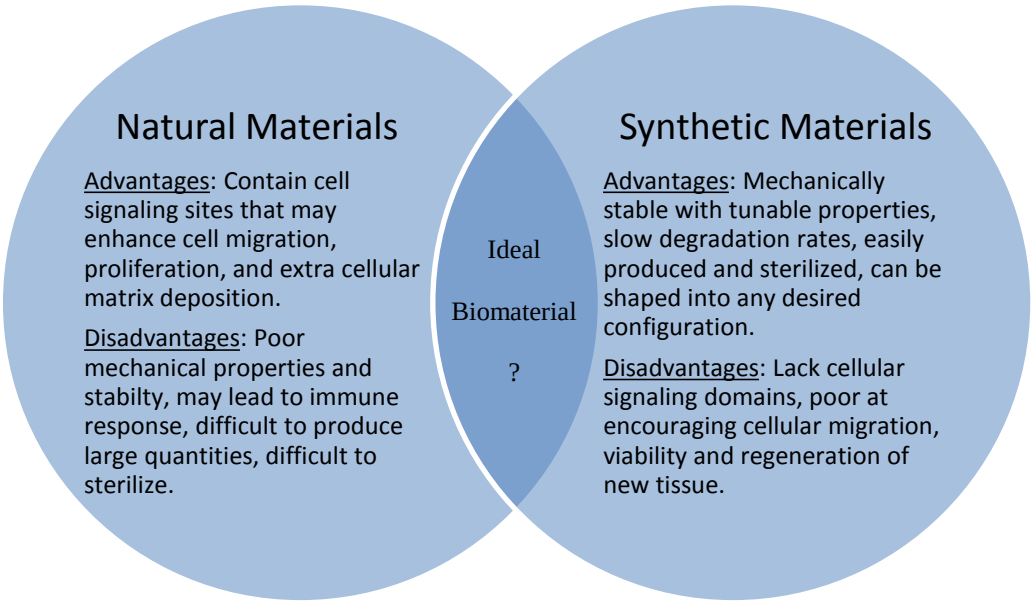


Figure 1.4: Advantages and disadvantages of natural and synthetic biomaterials for tissue engineering applications.

The advantages of these types of natural biopolymer based materials are that they are often components of the extracellular matrix or molecules that play a critical role in activities such as cell signaling and adhesion (Balakrishnan and Banerjee, 2011; Barnes et al., 2007; Segura et al., 2005). Unfortunately, many biopolymers have poor mechanical properties, degrade rapidly, and often cause an immunogenic response (especially in the case of protein-based materials) and difficulties can arise in producing large enough batches and maintaining protein stability during sterilization (Nam et al., 2011; Vanderhooft et al., 2009; Zhang et al., 2010).

Synthetic materials on the other hand are easily produced in a reproducible manner, are mechanically stable, have slower degradation rates and allow for easy manipulation of mechanical properties (Burke et al., 2007; Tibbitt and Anseth, 2009). What synthetic

materials lack is the potential for containing cell signaling domains often found in natural materials making them less effective at promoting cellular migration and ECM proliferation and deposition (Kim et al., 2011). Likely a combination of natural and synthetic materials will be required to obtain successful tissue engineering solutions.

1.5.2 Hydrogels & Fibre Biomaterials for Tissue Engineering

Hydrogels are crosslinked, water-swollen, hydrophilic polymers that are frequently studied for tissue engineering applications in the IVD. The properties of a hydrogel are highly dependent on their environment and their mechanical properties can be tuned by controlling the type of monomers used for synthesis, the polymerization conditions and crosslinking density, the degree of swelling and the medium in which the material is swollen (Anseth et al., 1996; Strehin et al., 2010; Zhu, 2010). While some hydrogels have been studied for tissue engineering of the AF, they are primarily investigated as materials for NP replacement.

Fibres have been extensively investigated for tissue engineering of meniscus, tendons, ligaments and the intervertebral disc, particularly in the annulus due to its highly organized lamellar structure and high collagen fibre content. Fibres have the ability to mimic the microstructure of the annulus, tend to have a higher tensile modulus than hydrogel materials, and can be manufactured into porous scaffolds or highly aligned fibre bundles. The following are common materials used for tissue engineering of the IVD and the repair strategies investigated to date.

1.5.2.1 Collagen

Collagen is one of the most studied materials for tissue engineering of cartilage, tendon and ligaments and has been shown to be compatible with a wide range of cells including AF cells (Alini et al., 2003), chondrocytes (Roche et al., 2001), and mesenchymal stem cells (MSCs) (Calderon et al., 2010). Collagens type I and II are major ECM components in the annulus which immobilize other proteoglycans in the ECM and have intrinsic cell-binding sites that are naturally degraded by enzymes (Calderon et al., 2010). Collagen is already present in the AF, thus it may be an ideal material to use for tissue engineering solutions as it degrades naturally and mimics the biochemical, mechanical, and microstructural properties of the ECM.

A key factor for successful integration of a tissue engineered construct in the IVD is the ability of cells from the surrounding tissue to migrate into the tissue engineered scaffold. A study by Bron et al. (Bron et al., 2012) demonstrated that scaffolds of type I collagen, fabricated by plastic compression, were able to support cellular invasion into the scaffold, showing promise for a tissue engineering material either in the AF or NP.

Type II collagen scaffolds were combined with hyaluronan to create a scaffold for tissue engineering in the IVD, specifically for regeneration of the NP. Calderon et al. (Calderon et al., 2010) found that the collagen-hyaluronan hydrogels, when crosslinked with a low concentration of EDC, were stable and had the ability to support proliferation of rat mesenchymal stem cells. The cells were also observed to differentiate into a

chondrogenic lineage, indicating that this may be an appropriate cell carrier for regeneration of cartilaginous materials.

1.5.2.2 Silk Fibroin

Silk fibroin is a naturally occurring polymer that is derived from the silkworm *Bombyx mori*, and has been studied for tissue engineering in both gel (Kim et al., 2004) and fibre (Bhattacharjee et al., 2012; Chang et al., 2010; Nazarov et al., 2004; Roohani-Esfahani et al., 2012; See et al., 2012; Yan et al., 2011; Zhang et al., 2010) forms. Silk is one of the strongest known natural fibres and displays resistance to failure in compression (Chang et al., 2007), making it an ideal candidate for tissue engineering of cartilage, ligament and tendon. The filament core protein of the fibre, fibroin, is surrounding by an immunogenic protein coating of sericin which must be removed prior to implementation in tissue engineering. Hydrogels can be formed by sol-gel transition in the presence of acids, ions, or other additives (Balakrishnan and Banerjee, 2011). Silk fibroin has many advantages including its high biocompatibility, slow degradation rate and strong mechanical properties. Additionally, it has been shown to support stem cell adhesion, proliferation and differentiation. The main disadvantage of silk fibroin is that it is brittle (Zhang et al., 2010).

Chang et al. (Chang et al., 2007) studied the effect of modifying porous silk scaffolds with RGD (arginine-glycine-aspartic acid) in order to enhance cell attachment for applications in tissue engineering the annulus fibrosus. The study found that while AF cells were able to adhere to the porous scaffolds and synthesize collagen and

proteoglycans after attachment, the presence of RGD didn't enhance cell attachment. Furthermore, collagen accumulation and tissue formation as determined by histology weren't affected by addition of RGD to the scaffolds as cells were able to produce collagen types I and II and aggrecan, regardless of the scaffold on which they were cultured. One of the main challenges for AF tissue engineering identified by the authors was the difficulty of forming properly aligned AF tissue within this scaffold.

Another group investigated the effect of varying the concentration of silk fibroin to improve the physiochemical and mechanical properties of the construct for tissue engineering of cartilage and meniscus. The compressive modulus and strength increased dramatically with increasing silk fibroin concentration between 8 and 15%. The compressive modulus increased from 0.81MPa to 15.14MPa and compressive strength from 0.05MPa to 0.79 MPa (Yan et al., 2011). Increasing concentrations also produced an increase in trabecular thickness in the constructs, decreased porosity, and decreased the swelling capabilities – factors that may decrease the ability of cells to infiltrate the scaffold and deposit new tissue (Koepsell et al., 2011). Therefore, a balance between mechanical properties and cultivation of new tissue development must be found for an optimal tissue engineering construct to generate new AF tissue.

An IVD-like assembly was developed by See et al. (See et al., 2012) and contained sheets of bone marrow derived MSCs adhered to silk scaffolds. The cells were cultured for five weeks prior to being incorporated into the scaffolds and produced tissue with approximately 92% type I collagen and 8% type II collagen. The cell sheets were then

attached to the silk scaffolds and the constructs were wrapped around a central silicone NP-like structure and loaded to simulate physiological conditions. After 4 weeks in culture, extensive modeling had taken place resulting in a tissue containing 30% type I collagen and 70% type II collagen, a tissue resembling the composition of the inner AF. However unlike the AF, both types of collagens were evenly distributed throughout the ECM.

Silk fibres are naturally brittle, with pure scaffolds showing an average tensile strength of approximately 2.72 MPa and an elongation fracture of 3.85%. To improve the stability of these fibres, Zhang et al. (Zhang et al., 2010) compared the effects of crosslinking silk fibroin-hydroxybutyl chitosan (SF-HBC) scaffolds with glutaraldehyde (GA), ethanol, and genipin. The best results were obtained from scaffolds containing a 20:80 ratio of SF:HBC and produced a tensile strength of 18.65MPa and an elongation at break of 8.88%. All the constructs showed improved tensile strength and lower elongation at break after crosslinking, with ethanol providing a greater tensile strength and elongation (21.37MPa, 5.45%) compared to genipin (18.65 MPa, 8.88%) and GA (16.71 MPa, 3.20%). Genipin-crosslinked scaffolds, however, showed the greatest cell compatibility.

1.5.2.3 Alginate & Chitosan

Alginate is a linear, negatively charged polysaccharide that forms a hydrogel upon ionic crosslinking with divalent cations such as calcium (Jeong et al., 2010) and has been used for tissue engineering materials including skin, cartilage, bone and nerves. Alginate is widely used for microencapsulation of a variety of cell types and is used as a wound

dressing (Matthew et al., 1995). It is biocompatible, relatively inexpensive and has good hydrophilicity. Chitosan is a positively charged, linear polysaccharide that is biodegradable, biocompatible, has low toxicity and has been shown to support cell adhesion (Shao and Hunter, 2007). Both alginate and chitosan have been approved by the FDA for clinical use, making them attractive candidates for tissue engineering.

Alginate and chitosan have been developed into a unidirectional fibrous scaffold for tissue engineering applications in the AF by Shao and Hunter (Shao and Hunter, 2007). The goal of the study was to develop fibres that more closely mimic the anatomic microstructure of the AF, and were synthesized using a wet spinning method to form fibres with diameters ranging from 40-100μm. AF cells were able to proliferate on the normal control and both alginate and alginate/chitosan scaffolds. PCR studies showed that the cells were capable of expressing functional genes including collagen types I and II and aggrecan on both scaffolds. Although this study didn't look at the mechanical properties of the constructs, another study has shown that a chitosan/alginate scaffold had a Young's modulus that increased significantly compared to a pure chitosan construct (8.15 MPa versus 2.56 MPa respectively) (Li et al., 2005), however this strength is still too low to use as a replacement material for annulus.

1.5.2.4 Polycaprolactone (PCL)

Polycaprolactone (PCL) is a synthetic, biodegradable, highly elastic polymer that can readily be made into nanofibres via electrospinning. Electrospinning has been shown to form aligned nanofibrous PCL fibres that mimic the mechanical anisotropy and

nonlinearity of fibre-reinforced soft tissues such as the AF (Nerurkar et al., 2009). PCL is nontoxic, inexpensive, has good mechanical properties and a degradation rate of one to two years, making it an ideal biomaterial for biomedical applications (Koepsell et al., 2011).

A study by Nerurkar et al (Nerurkar et al., 2009) aimed to achieve functional parity with the natural AF by developing an electrospun nanofibrous PCL scaffold that replicated the anatomical features of the AF. The PCL fibres were fabricated into fibrous sheets and then formed into a laminate with opposing bilayers and seeded with MSCs. The fibres were cultured *in vitro* for 2 weeks then coupled into bilayers with the long axis rotated 30° to the prevailing fibre direction, mimicking the collagen fibres in the native AF. The study then compared bilayers that had fibres in parallel with those that had bilayers with opposing fibre directions and found no difference in cell, glycosaminoglycan or collagen quantity and localization between the two layers. There was, however, significantly higher tensile moduli found in opposing bilayer laminates. The opposing bilayer samples had a uniaxial tensile modulus of 14.2MPa, approximating the tensile modulus of the AF within 15% (17.3MPa). The bilayers with fibres in parallel to one another had a tensile modulus of only 10.5MPa. The major limitation of this study is that the bilayer laminates require 10 weeks of culture *in vitro* prior to achieving these mechanical strengths, posing a significant clinical challenge.

Koepsell et al. (Koepsell et al., 2011) examined whether the fibre alignment in electrospun PCL fibrous scaffolds would affect the orientation, proliferation and ECM

production of the cells. They found higher rotational speeds (1500 and 2000 rpm) created fibres that were smaller in diameter, resulting in a smaller pore size in scaffolds, and had a tensile modulus along the fibre direction of 7.25 MPa. *In vivo*, the fibres of the inner annulus and outer annulus have tensile moduli of 28 MPa and 78 MPa, respectively. At slower rotational speeds, larger pore sizes were observed with tensile moduli that were slightly lower than the small diameter fibres (2.5-6.26 MPa). This study again highlights the trade-off between mechanical integrity of fibrous scaffolds and promotion of ideal cell conditions. The study also supported previous findings that scaffolds with oriented fibres strongly affected the orientation of cells (Koepsell et al., 2010), and ECM production on scaffolds with aligned fibres was generally higher than those with random fibre arrangements.

As with many synthetic polymers, inclusion of natural material components is often investigated with PCL fibres to enhance their biocompatibility and cell signaling potential. Gee et al. (Gee et al., 2012) hypothesized that the inclusion of biomimetic elements, such as collagen I fibres, in a synthetic PCL scaffold would improve initial cell adhesion and result in greater cell infiltration. This study found that that crosslinking the collagen fibres with genipin and incorporating them into PCL scaffolds produced a mechanically robust nanofibrous network that stabilized the fibres. The inclusion of collagen, however, had the opposite effect as hypothesized with higher percentages of collagen inclusion resulting in the poorest infiltration and matrix deposition. There was also little additional collagen and almost no proteoglycans deposited in the constructs containing collagen while the pure PCL constructs showed increases in both ECM

components with time in culture. The authors concluded that the high levels of collagen in the cellular microenvironment may have resulted in down-regulation of new collagen production via a negative feedback mechanism. Another study incorporated gelatin into genipin-crosslinked PCL fibres and found that the proliferation of myoblasts was higher on the PCL-gelatin scaffolds than the PCL fibres alone, indicating that addition of gelatin improved the PCL fibres performance for potential applications in muscle tissue engineering (Kim et al., 2010).

1.5.2.5 Fibrin & Fibrin Glues

Fibrin is a non-globular protein involved in blood clotting and is commonly used for tissue engineering scaffolds. It is also frequently used as a soft tissue adhesive as an adjunct to sutures and is commercially available. Fibrin glue was first used as a haemostatic agent over a century ago and has successfully been used as an adhesive since 1940 (Patel et al., 2010). Fibrin glues most commonly work by converting fibrinogen to fibrin in a reaction catalyzed by thrombin, mimicking the end stages of the coagulation cascade (MacGillivray, 2003). Fibrin has the advantage of being highly biocompatible and has been shown to support MSCs as well as chondrocytes (Acosta et al., 2011) and allows for ECM generation and deposition within the scaffold.

One study by Buser et al. (Buser et al., 2011) tested the *in vivo* effects of intradiscal injection of a commercially available fibrin adhesive, BIOSTAT BIOLOGX Fibrin sealant. In the study, porcine lumbar IVDs were studied by comparing untreated controls, degenerative injuries (nucleotomy), and a nucleotomy with a fibrin sealant

injection. The study showed that in the nucleotomy group, there was a progressive invasion of the annular tissue into the nucleus and a reduction in proteoglycan content. However in the group where the nucleotomy was supplemented with the fibrin injection, it inhibited nuclear fibrosis and facilitated an increase in proteoglycan content over time, indicating that a fibrin sealant injection into denucleated disc helped to facilitate the structural and mechanical repair of surgically damaged discs.

In a similar study, Acosta et al. (Acosta et al., 2011) injected denucleated porcine intervertebral discs with fibrin glues containing either chondrocytes or allogenic MSCs. Discs were harvested and studied at 3, 6, and 12 months and showed that the chondrocyte-treated discs had significant amount of matrix formation at 3 months, and to a lesser extent at 6 and 12 months. The carrier only and MSC group, however, showed very little proteoglycan matrix and rather a type I/II collagen-enriched scar tissue. By contrast the chondrocyte group produced a matrix that was type II collagen rich, indicating that chondrocytes may be a more effective cell source for this type of IVD treatment via fibrin glue carrier.

One of the key issues with the use of fibrin and fibrin glues in tissue engineering applications is their lack of stability and poor mechanical properties. To alleviate this issue, crosslinking of fibrin with various materials has been investigated. Schek et al. (Schek et al., 2011) crosslinked fibrin gels with the natural crosslinking agent genipin and were able to achieve a gel that had a shear modulus in the range of native annular tissue. They also demonstrated compatibility *in vitro* with human disc cells when the ratio of

genipin:fibrin was 0.25:1 or less. The morphology of the cells however was more rounded in the crosslinked gels than in fibrin alone, and cell proliferation was slower. An additional benefit of crosslinking the gels with genipin was the production of an adhesive property between the fibrin gels and tissue, as shown by adhesive strength tests where crosslinked gels remained adhered to tissue at strains that exceeded physiological levels, failing at 15-30%. This study demonstrates a promising biomaterial that could be used as a sealant for small annular defects, although degradation rates, fatigue behaviours and long term biocompatibility have not yet been addressed.

Genipin-crosslinked fibrin gels have also been studied for articular cartilage regeneration by Dare et al. (Dare et al., 2009). This study found that genipin crosslinking at low concentrations didn't significantly affect cell viability of human articular chondrocytes and significantly increased the dynamic compressive and shear moduli of the hydrogels. Additionally, the ratio of collagen II versus collagen I gene expression increased more than 8-fold over a five week period, indicating that this material may be able to support the deposition of an articular cartilage-like tissue.

1.5.3 Composite Materials for Intervertebral Disc Tissue Engineering

There has been significant research into regenerating independent structures of the IVD such as the AF or NP, yet more recent studies have taken a holistic approach to attempt to tissue engineer a composite IVD replacement that contains distinct materials mimicking the structure and function of the native AF and NP. Mizuno et al. (Mizuno et al., 2004) developed a composite intervertebral disc implant that was implanted into mice for up to

twelve weeks. The annulus portion of the disc was fabricated from polyglycolic acid and polylactic acid with AF cells seeded into the scaffold. The NP cells were suspended into a 2% alginate gel, and injected into the centre of the AF portion of the construct. The implants were then placed into the subcutaneous space of mice for up to twelve weeks. It was found that the morphology and histology of the discs resembled that of the native disc, with type I collagen present in the AF portion of the construct and type II collagen predominant in the NP.

A later study by the same group evaluated mechanical properties of these constructs at 16 weeks and found the equilibrium compression modulus of the tissue engineered disc to be around 49MPa and hydraulic permeability of around $5x10^{-14}m^2/Pa$, values that are between those measured for NP and AF alone (Mizuno et al., 2006). These studies show an exciting step towards the possibility of engineering two distinct tissue structures within one composite disc implant.

Bowles et al. (Bowles et al., 2011) developed a composite disc composed of an alginate NP and collagen AF and seeded the construct with ovine AF and NP cells. The construct was implanted into the caudal spine of rats for up to six months and were found to maintain disc height, produce extracellular matrix containing cartilage-specific collagens and proteoglycans, and integrated into the spine. There are however, significant challenges in translating this work into a human IVD, and significant work will need to be conducted before these models are clinically feasible.

1.6 Business Plan Assessment of Project Feasibility for Commercialization

It is important for researchers working on clinical problems to keep in mind the context of their solutions while making design decisions. A business plan was developed based on the potential "product" of this overall project and submitted to the "Student Technology Innovation Challenge" (STIC), a student business plan competition hosted by Innovate Calgary to promote entrepreneurial thinking in students in Southern Alberta. The competition involved writing a 10 page business plan and making a 5 minute "pitch presentation" to pitch your idea to potential investors to convince them to invest in your "product". The business plan placed second in the competition for southern Alberta. The following is an excerpt from the business plan which discusses some critical elements that should be kept in mind when designing an engineering solution such as the one described in this thesis. This includes a market analysis, assessment of the competition, and marketing, sales and distribution strategies.

1.6.1 Market Analysis Summary

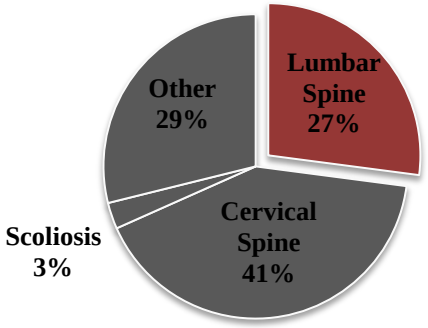
The global spine market has grown from $3.8 billion in 2004 to over $7.1 billion in 2010 with the number of spinal surgeries performed annually increasing in both number as well as cost. Spinal fusions, one of the most common treatment options for spinal disc degeneration, are among the top five surgeries performed in the United States each year, with an annual cost of $8.9 billion in 2007 (Sung et al., 2011). With over 300,000 spinal fusion surgeries and 250,000 discectomies (removal of the nucleus) performed in the United States each year, there is a significant market for an intermediate intervention such as our solution. This would likely increase the number of people who elect to have

surgery, due to the fact that the solution would be more effective at restoring mechanical function to the spine. Spinal fusions have limited clinical evidence proving their effectiveness compared to no surgery – for this reason the US government is considering withdrawing medical coverage for these surgeries. This would further enlarge the market available for patients and surgeons seeking a solution for spinal disc degeneration that has proven clinical results.

1.6.2 Market Segmentation

While the overall global spine market is large, there are many clinical reasons for carrying out a spinal fusion procedure. Our product will target only those spinal fusions that are conducted due to issues in the lumbar spine including degenerative disc disease and displacement, or herniation, of a disc. Below is a summary of a 2008 study conducted on spinal fusions in the United States according to the primary diagnosis of approximately 341,000 spinal fusions conducted. The lumbar spine segment of the market accounts for approximately 27% of all spinal fusions completed. This segment would contain patients who are ideal candidates for surgeries using our product, and we would expect to achieve approximately 2% penetration of this market in the first year, increasing to 10% of the market within 5 years due to the fact that this product will be the only alternative to spinal fusions for patients with this primary diagnosis.

Primary Diagnosis Leading to Spinal Fusion



1.6.3 Important Industry Considerations

1.6.3.1 Regulatory Environment

One of the major barriers to entry in this industry is the difficult and unpredictable regulatory environment. Prior to reaching market, this product would need to be approved by Health Canada and the Food and Drug Administration (FDA) for release in Canada and the United States respectively. These would be the two primary markets for the device. Secondary markets if success was achieved would include Europe (requiring a CE mark for approval), Asia and Australia. As the FDA is the most difficult regulatory body to get approval from, this analysis primarily focuses on the FDA's requirements.

Our product would be a categorized as a class III medical device, the highest class of medical devices and most difficult, expensive, and time consuming approval to obtain. To begin clinical trials, you must first obtain an Investigational Device Exemption (IDE) approving your device for investigational use in human subjects. This approval process to have your clinical trial protocols approved typically takes six months. After clinical trials have been conducted, an application for a Pre-Market Approval (PMA) must be

completed and approved prior to releasing the product. This process is lengthy and expensive requiring approximately $5-10 million and 4-5 years to complete, making it a significant barrier to entry for many companies.

1.6.3.2 Intellectual Property

Another important aspect to consider in the medical device industry is intellectual property (IP). In a competitive industry, it's critical to file for patent protection as soon as you have developed a device in order to prevent any other companies from producing the exact same product. IP also poses a barrier to entry for many companies as it is time consuming and expensive to obtain as patents must be filed in each country in which you require your idea to be protected. Once the patent is filed however, we will have twenty years of freedom to operate without anyone else being able to reproduce the invention. Prior to patenting our product, we will conduct a prior art search to ensure that our idea isn't infringing upon anyone else's inventions. Once the material selection and product design have been finalized, a patent lawyer will be hired to file our first patent application in the US. From this file date we would have one year to file a patent anywhere else we would like to market our product. The total cost for patenting in North America would be approximately $60,000 and may cost up to $500,000 for international patenting. Any litigation required if a company was found to be infringing upon our IP would incur additional costs.

1.6.4 Main Competitors

There are four major medical device companies with large spinal surgery divisions who would be existing competitors in the industry. While these companies have significantly

more capital at their disposal for research and development of new devices which makes them difficult to compete with, they also provide a viable exit strategy later in the commercialization process if we chose to sell or license our intellectual property instead of fully developing, producing, marketing and selling the product ourselves.

1.6.4.1 Medtronic

Medtronic is a world leader in medical device development with a large spinal and bone therapy division. In 2011 Medtronic reported a revenue of $15,933 billion (approximately 23% of which was from spinal products) with net earnings around $3.1 billion. Current products that would be competitive with our device is their "Prestige Cervical Device", a full prosthetic disc replacement. While this product is very successful, it would not be directly competing with our product as our solution is an earlier intervention to prevent further damage to the disc while the Prestige is a full disc replacement for discs that have been damaged too badly to be recovered. Also, Medtronic is currently not exploring cartilage repair, the target of our product, but rather bone in growth for full spinal fusions within their biologics department.

1.6.4.2 Stryker

Stryker Spine, the spinal division of Stryker, has a range of products from minimally invasive pain treatments to full disc replacements. Approximately 9% of Stryker's sales come from this division (Sung et al., 2011), however a majority of their products focus on vertebral repair and replacement and they do not currently provide any solutions for

degenerative disc disease that would directly compete with ours. Significantly smaller than Medtronic, Stryker's estimated revenues for 2011 were $8.2 billion.

1.6.4.3 Zimmer

Zimmer is a large medical company focused entirely on musculoskeletal health with approximately 5% of its sales coming from their spinal division. While it isn't as large as Medtronic, with estimated revenues for 2011 of just over $4.4 billion (Sung et al., 2011), Zimmer is one of our most prominent competitors. While they currently have no spinal products that would directly compete with our product, they do have a large biologics division specializing in bone and tissue grafts which could potentially expand to develop tissue engineered products such as ours. Due to Zimmer's smaller size and closely aligned product goals, I would expect Zimmer to be the company most interested in licensing our purchasing our product IP.

1.6.4.4 DePuy Sine (Johnson & Johnson)

DePuy is the spinal division of Johnson & Johnson, contributing approximately 2% of Johnson & Johnsons total annual revenue for 2010 of $61.5 billion (Sung et al., 2011). DePuy's products are primarily surgical supplies for fixation of spinal implants and full spinal disc replacements. Their biologics include bone graft replacements, but they have no products currently focused on cartilage repair in the intervertebral disc.

1.6.4.5 Academic Research

The main competition for the development of products similar to ours comes from within academia. There are approximately nine other major research groups across the world, primarily located within Canada and the United States, who are working on spinal disc replacement research. Of these groups, three are working on solutions for the Annulus Fibrosus (AF) (the same target as our product), two are primarily working on developing adhesive materials that will adhere to cartilage, two are working on replacement materials for the Nucleus Pulposus and two are focused on full disc replacements. While each group is focusing on quite different solutions (their research can be tracked through academic publications) the three groups focused on the AF are trying to solve the same problem that our device solves. For this reason these groups will be the greatest threat as competition and their progress should be monitored to ensure that we position our product appropriately in the market place should they develop any products within the same time frame as us.

1.6.5 Parties of Interest for Marketing

The key parties that our product must appeal to are the hospital and clinic administrators, the insurance companies who ultimately pay for the product, and the surgeons who use the product. In order for the product to be successful, it is critical that it appeals to each of these parties individually.

1.6.5.1 Insurance Providers

To appeal to insurance providers, both short term and long term gains will be highlighted. Payers are most interested in saving money up front, so it will be important to price our product so that it costs the same as a spinal fusion, with additional future benefits, or costs less than fusions. Fusions can have a total cost of $60,000 - $90,000 depending on the complexity of the procedure. The materials typically cost around $3500, with additional related expenses such as paying the surgeon, anaesthesiologist, and nurses. Therefore, our product will be positioned such that its price is in the lower range of fusion material costs, around $3,000 per device depending on the final materials selected and the resulting costs for production. In addition to costing less in the short term for the product itself, our positioning to insurers will be that by undergoing this type of procedure, patients will require fewer revision surgeries and experience less future degeneration than spinal fusions, ultimately preventing future costs from surgeries and time at work lost.

1.6.5.2 Hospital & Private Practice Administrators

Hospital and private practice administrators have different financial incentives than the insurance providers who pay for the procedures. While the insurers save money by having fewer revision surgeries, hospitals lose revenue. Another difficulty in convincing administrators to order a new product is that they typically have buying agreements with specific companies, and receive discounts for using their products, making it more difficult to get a new product to be adopted in these large centres. The key to appealing to administrators will be to highlight the way our product will save the hospital itself

money. Factors such as a shorter surgery and recovery time (saving the hospital costs for their anaesthesiologist, surgeon, and nurses) would be very appealing. Additionally, factors such as having the product come in a sterilized, disposable package so that they are not required to spend hospital funds for sterilization would create significant value added to the product for the hospital. These considerations will be kept in mind with the final design of the product as well as the manufacturing and sterilization procedures used in our operations.

1.6.5.3 Surgeons

The final party the product must appeal to is the surgeons that will be using it. Not only do surgeons have an advisory role in selecting products for clinics and hospitals, but they also sit on technology review boards that evaluate new technologies for both their clinical and economic merits and determine which new products will be adopted. Many surgeons use techniques or products simply because that was what they were trained on or because they have a special relationship with the manufacturer. Therefore, it will be a critical part of our marketing strategy to develop relationships with key users who will champion the promotion of our product. For the product to appeal to a surgeon, it must be easy to use, not require extensive additional training or personnel, and should shorten the required surgery times.

1.6.6 Marketing Strategy

Our marketing strategy will be to develop relationships with key surgeons who will use and promote our product. It will be important to get these surgeons on board prior to

clinical trials and to select people who have significant influence within the industry. These users will then spread knowledge of our product through the administration chain to those who directly purchase products. If surgeons begin to request our product, insurance companies will consider it for reimbursement – it is very rare for an insurer to cover a product at the request of the manufacturer.

In addition to developing these key relationships, we will attempt to get our product into teaching hospitals throughout North America as many surgeons simply don't adopt new technologies because they weren't trained on them. Early introduction to our product to the newest generation of surgeons will give us a significant competitive advantage amongst a group who are more open to the newest and best methods and products for surgeries. Finally, we will promote our product at medical device trade shows, conferences, and medical journals.

1.6.7 Distribution

Due to the cost and large personnel requirement required to build a sales force, we will be outsourcing our distribution to a medical sales company who already has a sales force within our target markets in Canada and the United States. A flat rate will be paid to a sales company initially to start selling our product, with an additional commission based incentive to ensure that our product is getting optimal promotion over any other competition. It will be necessary to align our interests with the interests of the sales staff to achieve maximal exposure for our product.

1.7 Future Directions for Tissue Engineered Repair of the Intervertebral Disc

Recent studies investigating the feasibility of developing a composite, tissue engineered disc to provide a full intervertebral disc replacement show the direction that tissue engineering can take in the repair of degenerated discs. These solutions, however, are in the early stages of development further research will be required before a full tissue engineered disc replacement is a clinical reality. The overall goal of this research project was to develop a tissue engineering solution that could patch a defect in the annulus fibrosus, resulting in a reduction in pain for patients and restoring functionality of degenerated discs.

In order to achieve this goal, ideally the nucleus material would need to be retained within the disc in order to maintain disc height and pressure, or be replaced with an equivalent material. There are many studies directed at this development with various materials such as collagen (Bron et al., 2009a), polyethylene glycol (Vernengo et al., 2007), alginate, chitosan, fibrin and hyaluronic acid. They could be implanted into the disc space after discectomy, or in some designs, injected through the annulus (Vernengo et al., 2010). While these solutions may provide a functional replacement for the NP, without appropriate closure methods for the annulus to maintain pressurization of the central region of the disc, these solutions will only achieve limited success (See et al., 2012).

Currently, sutures are the preferred surgical technique for annular closure after NP implantation and have been shown to be more effective than surgical adhesives alone for

closure stability, with improved performance when used in combination with a surgical adhesive (Heuer et al., 2008). In addition to sutures, there are many novel mechanical closure devices being developed. Such devices are typically anchor-like implants or devices such as the Xclose© that acts as a modified suture with anchors. Surgical mesh systems to support weak tissue are also being investigated (Murphy et al., 2010) and some like the Inclose©, are already commercially available (Bron et al., 2009b). These mechanical methods, however, are not ideal as they do not replace the lost annulus material or repair the biomechanical changes that caused the damage (Burke et al., 2007). For this reason, there is a significant need to develop a new method for annular closure and repair for herniated discs or small, concentric tears and fissures.

Achieving sufficient closure of the annulus fibrosus after rupture due to herniation or other injuries is a significant problem. One of the key aspects to consider when designing a regenerative solution for annulus repair is the material used to close the defect. There are many criteria that should be considered in selecting a biomaterial for annulus repair (Table 1.2). The biomaterial selected to close the annulus must be able to function mechanically immediately, provide sufficient adhesion and initial stability while new tissue is being generated, support growth and proliferation of encapsulated cells and provide guidance or signals for new matrix deposition. The ideal material would be biocompatible with non-toxic degradation products, produce a low immune response, have a degradation profile that would be inversely proportional to the speed at which new tissue is deposited, and have sufficient mechanical properties for the challenging environment in the disc. The goal of this study was to evaluate various commercial and

novel biomaterials for their suitability to be used for the repair of tears in the annulus fibrosus.

Table 1.2: Design Criteria for a Biomaterial used in Annulus Fibrosus Repair

Chapter Discussed In Thesis	Design Criteria	Property to be Tested	Benchmark Value	Reference
Chapter 4	Strong Adhesion	Yield Strain	15%	(Nerurkar et al., 2010)
	Similar Mechanical Properties	Elastic Modulus (Outer and Inner Annulus)	17.4 ± 14.3 MPa 5.6 ± 4.7 MPa	(Elliot and Setton, 2000)
	Cytocompatibility	Cell Viability	N/A	(Wang et al., 2007)
	Stability	Degradation Time	5 Weeks	(Eyrich et al., 2007)
Chapter 5	Promote Tissue Repair	Presence of fibrocartilage-specific proteoglycans (Collagen I, Collagen II, Aggrecan)	N/A	(Nesti et al., 2008)

To assess these biomaterials, this thesis was broken down into three specific aims. The first specific aim was to test commercially available surgical adhesives to determine if they would produce a sufficient bond to AF tissue to be used for repair of the AF (chapter 4). In order to be utilized for repairing the AF, repaired tissue would be required to stay bonded up to a tensile strain of 15% of the tissues initial length. Tensile testing was

conducted using bovine AF tissue to determine if such a bond could be produced between the adhesives and this tissue.

The second aim was to determine if a genipin-crosslinked fibrin gel could be seeded with human bone marrow mesenchymal stem cell and maintain cell viability as well as produce sufficient mechanical and chemical properties for AF repair (chapter 4). The biomaterial was compared to uncrosslinked fibrin gels and characterized by studying its degradation profile over 30 days, its mechanical stiffness under compression up to 15% strain, and its cell viability over 12 days.

The third specific aim was to examine the response of mesenechymal stem cells to implantation in bovine AF tissue to determine if it would promote an appropriate cell lineage commitment (chapter 5). An *in vitro* tissue integration model was developed where a reproducible, critical defect in bovine annulus tissue was filled with a cell-seeded biomaterial and loaded in tension. Histology and gene expression were used to study the cell response to fibrin and genipin-crosslinked gels over a 12 day time period.

Chapter Two: Challenges for Integration of Repair Strategies for Annulus Fibrosus Tissue Engineering – Development of an *In Vitro* Model

The following section is a review article which will be submitted for publication in the spring of 2013, analyzing the key challenges for achieving integrative repair of the annulus fibrosus.

2.1 Introduction

Disc degeneration is a common disorder, with an autopsy study showing that 97% of individuals over the age of 50 had disc degeneration (Kandel et al., 2008). Over 80% of the adult population is affected by low back pain (Shvartzman et al., 1992), with surgical procedures being performed on approximately 5% of those affected (Whatley et al., 2011). Degenerative disc disease has a large economic impact, with direct medical costs of $33 billion and indirect costs around $100 billion in the United States annually (Waddell, 1996). This economic burden is similar to that imposed by coronary heart disease and is greater than that of disorders such as diabetes, Alzheimer's disease, and kidney disease (Urban and Roberts, 2003). Back pain may be caused by a variety of disorders, but many of them are strongly associated with degeneration of the intervertebral disc (IVD) (Hadjipavlou et al., 2008; Kalichman et al., 2010; Luoma et al., 2000; Macnab, 1986; Millecamps et al., 2012; de Schepper et al., 2010; Urban and Roberts, 2003).

The IVD consists of three distinct regions: the annulus fibrosus (AF) is a thick outer ring of fibrous cartilage htat surrounds a gel like tissue known as the nucleus pulposus (NP). Type I collagen accounts for approximately 70% of the content in the AF along with a proteoglycan matrix, allowing it to withstand significant tensile stresses (Urban and Roberts, 2003). The NP contains high levels of aggrecan and collagen type II making it more gelatinous than the AF and allows the IVD to absorb compressive stresses (Masuda and Lotz, 2010). Two cartilage endplates sandwich the NP and provide a thin horizontal layer of hyaline cartilage that acts as an interface between the IVD and the vertebral bodies (Waddell, 1996). These three tissues, while distinct, are strongly interdependent and damage caused to one will create unequal loading throughout the disc resulting in injury and degradation to the other tissues (Roberts and Urban, 2011). In the healthy spine, only 5-10% of loads experienced are transmitted through the posterior portion of the spine, however with disc degeneration, this increases to 40%, thus increasing the risk of disc herniation in the posterior region (Papadakis et al., 2011).

The IVD is aneural and avascular with a low cell density. These characteristics result in poor regeneration and healing abilities in the disc. One of the primary causes of disc failure is rupture of the outer region of the disc, or disc herniation, in the posterior region of the disc (Papadakis et al., 2011). Currently there are no intermediate or early intervention treatment options available for patients with herniated discs. Current treatments are aimed at reducing pain and symptoms rather than repairing degeneration in discs (Kandel et al., 2008; Koepsell et al., 2010; Urban and Roberts, 2003). Most treatments are palliative and include the use of muscle relaxants, injection of

corticosteroids, local aesthetic and manipulation therapies (Urban and Roberts, 2003). Disectomies are a surgical practice directed at removing all or part of the damaged tissue. While these procedures often reduce pain, they do not restore the disc to its original height or load bearing capacity (Boyd and Andrew, 2006; Gotfried et al., 1986; Nomura et al., 2001).

Palliative treatments don't encourage regeneration or healing of the damaged tissues and surgical options reduce patient mobility and can lead to further degeneration in the future. For this reason, there is a significant need for a treatment option that can prevent leakage of the NP from a ruptured portion of the disc while allowing the disc to regenerate itself and achieve long term stability. Tissue engineering strategies may be a promising solution to develop repair modalities that will provide the initial structural support required to close a defect in the AF, preserve the NP tissue and maintain the disc height to retain normal biomechanical function of the disc. Biological aspects incorporated into these strategies could promote regeneration of new tissue to bridge the gap in the annulus and lead to a long term, regenerative solution for AF repair.

2.2 Repairing the Annulus Fibrosus

2.2.1 Current Annulus Fibrosus Closure

Spinal disc herniation can occur due to trauma or degradation of the disc, resulting in a rupture in the AF allowing the gelatinous NP to seep out. Back pain may result from the release of inflammatory chemicals or direct impingement of the NP on nerves, motivating over 250,000 discectomies annually in the US to partially or fully remove the NP and

alleviate this pain (Sung et al., 2011). While these procedures reduce pain, they alter the biomechanical loading in the spine and can ultimately lead to further degeneration in the disc or require multiple revision surgeries for recurrent herniation. A more effective solution would be to develop a method that would allow for complete closure of the AF, allowing the NP to be left in the disc space and maintain normal spinal biomechanics.

Many tissue engineering approaches for IVD repair look to regenerate or replace the NP, however without appropriate closure methods for the annulus to maintain pressurization of the central region of the disc, these solutions will only achieve limited success (See et al., 2012). Currently, sutures are the preferred surgical technique for annular closure after NP implantation and have been shown to be more effective than surgical adhesives alone for closure stability, with improved performance when used in combination with a surgical adhesive (Heuer et al., 2008).

In addition to sutures, there are many novel mechanical closure devices being developed. Such devices are typically anchor-like implants or devices such as the Xclose© that acts as a modified suture with anchors. Surgical mesh systems to support weak tissue are also being investigated (Murphy et al., 2010) and some like the Inclose©, are already commercially available (Bron et al., 2009b). These mechanical methods, however, are not ideal for use with disc tissue as it may cause further damage to the tissue and cannot be self-repaired (Burke et al., 2007).

2.3 Surgical Adhesives for Annulus Fibrosus Repair

Another possible method of annulus tissue repair that has been investigated is the use of existing, commercially available surgical adhesives. Stable fixation and host integration of a tissue implant is critical to achieving successful function of a tissue engineered IVD (Ramaswamy et al., 2006), however developing an adhesive to successfully achieve this is a challenge. Bioadhesives are either natural or synthetic materials that are able to repair soft tissue and have been utilized in surgeries for over a century (Vernengo et al., 2010). The ideal method for adhesion would achieve a tight bond with surrounding tissues and share properties of the host tissue, be easily and quickly applied clinically, would promote tissue repair and be biocompatible (Wang et al., 2007). The most widely used adhesives today are fibrin glues, cyanoacrylates, and glutaraldehyde-based adhesives.

2.3.1 Fibrin Glue

Fibrin glue was first used as a haemostatic agent over a century ago and has successfully been used an adhesive since 1940 (Patel et al., 2010). Fibrin glues most commonly work by converting fibrinogen to fibrin in a reaction catalyzed by thrombin, mimicking the end stages of the coagulation cascade (MacGillivray, 2003). Fibrin is an advantageous material for a medical adhesive as it is derived from human plasma proteins, making it highly biocompatible and biodegradable. It has not been associated with any types of inflammation, foreign body reactions or tissue necrosis and is commercially available (Jackson, 2001). Disadvantages include a low cohesive strength, poor adhesion (93.4 5

± 14.0gf/cm^2 (Otani et al., 1996)), and a risk of viral transmission due to its origins from human plasma (Burke et al., 2007; Vernengo et al., 2010).

Fibrin has been used with varying degrees of success in tissue engineering applications including on fibrocartilage in the knee and annulus fibrosus. Fibrin is commonly studied to facilitate autologous chondrocyte implantation to promote healing of deep articular cartilage defects and shows promising results in terms of cell proliferation and ECM deposition for this use (Wysocka et al., 2010). Initial results using fibrin glue as a scaffold for mesenchymal stem cells (MSCs), chondrocytes or cell growth factors to facilitate meniscal healing have showed initial results in small animal studies indicating that fibrin would be an appropriate cell delivery vehicle and promotes ECM deposition *in vivo* (Jung et al., 2010; Park, 2005; Scotti et al., 2009; Silverman et al., 2000). Some studies, however, provide contradictory evidence showing fibrin glue to have insufficient adhesion to provide adequate fixation of grafts (Reckers et al., 2009) with bonding strength values ranging from 3.4 gf/cm^2 to 93.4gf/cm^2 reported for adhesion of fibrin glue to various materials (Otani et al., 1996).

2.3.2 Cyanoacrylates

Cyanoacrylates are a family of fast-acting adhesives that polymerize in the presence of water or blood to form solid, impermeable plastics *in situ*. While there are some commercially available glues, they're primarily applied in periodontics (Vernengo et al., 2010). Cyanoacrylates have superior adhesive strength over other adhesives (Chivers, 1997) but are known to be cytotoxic, are associated with acute and chronic inflammation

and tissue necrosis, and are recommended for superficial application only (Jackson, 2001). Biomechanical studies with histoacryl glue, in the family of cyanoacrylates, showed a superior fixation over conventional vertical sutures in repairing meniscal tears (Ayan et al., 2007). However, a study using a commercially available cyanoacrylates adhesive, Dermabond™, found that the inflammatory response created in a rabbit model made the use of this adhesive for meniscal applications inappropriate (Reckers et al., 2009).

2.3.3 Glutaraldehydes

Glutaraldehydes are aliphatic organic molecules containing aldehyde groups at each end. These dialdehydes are readily able to react with the amines on proteins of the tissue extra-cellular matrix making it an ideal adhesive for tissue applications (Vernengo et al., 2010). This adhesive is commercially available in the form of BioGlue (CryoLife) and is one of the leading glues for cardiovascular surgery in the world. Limited studies have been carried out using this type of adhesive on fibrocartilage, although a high bonding strength of 65 kPa was found in one study using glutaraldehyde to repair articular cartilage (Englert et al., 2007).

A majority of these commercially available tissues adhesives either have poor biocompatibility or poor adhesion for use in fibrocartilage applications [8, 10-12]. The lack of strategies for implanting and fixing tissue engineered constructs has hindered the clinical application of many tissue engineering solutions that have been developed (Bron et al., 2009b).

2.3.4 Biomimetic Tissue Adhesives

There have been many recent attempts to use biomimicry to create a new tissue adhesive based on powerful adhesives that occur in nature, particularly in wet conditions, such as marine mussels (Cha et al., 2008; Choi et al., 2010; Choi et al., 2011; Hwang et al., 2007; Lee et al., 2007; Ninan et al., 2007) and the hairs on geckos feet (Geim et al., 2003; Lee et al., 2007).

Marine mussels secrete adhesives to attach themselves in marine environments and adhere to surfaces through a bundle of strong filament threads called a bysuss. At the end of each thread is an adhesive plaque that contains water-resistant proteins. These proteins are known to be one of the most powerful adhesives in the world (Cha et al., 2008). Marine adhesive proteins (MAPs) are ideal for biomedical applications as they are flexible and elastic and are able to maintain adhesion in a wet environment. They're also biodegradable, environmentally friendly and biocompatible. Therefore, significant research is underway to characterize and apply these proteins in various cartilage tissue engineering applications (Cha et al., 2008).

Six adhesive proteins have been identified, and some are now commercially available in the form of Cell-Tak (BD Bioscience Clontech) and MAP (Swedish Bioscience Lab). Due to the low production yields and high cost of producing these MAP products, they're typically only available in quantities small enough for lab scale applications (Hwang et al., 2007). Recombinant hybrids of these proteins are also available from Kollodis Bioscience, providing a method of obtaining greater production yields. Limited studies

have been carried out using these proteins in an adhesive, however they have been shown to have superior adhesive properties over several cyanoacrylates glues when used to adhere porcine small intestinal submucosa (Ninan et al., 2007).

Geckos are able to adhere to wet and dry surfaces by the adhesive force of millions of hairs acting together through the action of van der Waals forces and/or capillary interactions. Individually, each hair is only capable of a miniscule amount of adhesive force, about 10^{-7} N, but when acting together they adhere with a strength of about 10 N cm^{-2}. Microfabrication techniques using electron-beam lithrography have been attempted to create a "gecko tape" that mimics this type of adhesion. The gecko tape was able to retain its adhesive properties over several detachment-attachment cycles but did experience degradation of its adhesive properties (Geim et al., 2003). Another attempt to fabricate such an adhesive was able to adhere underwater with a strength of 9N per cm^2 of surface area, which approximates the strength of gecko adhesion in dry conditions (Lee et al., 2007). Currently, however, this technique is extremely expensive (Geim et al., 2003) and not feasible for applications in medical repair within a reasonable timeframe.

2.4 Defining a New Direction for Annulus Repair

Current closure methods for the AF do not provide a satisfactory solution. While suturing provides sufficient mechanical closure strength, especially when supplemented with surgical adhesives, it causes damage to the fibrocartilaginous tissue that has no ability to repair itself, ultimately leading to further degeneration in the future. Tissue adhesives

have been extremely successful in many topical (Qureshi et al., 1997), cardiovascular (Raanani et al., 2001), and orthopaedic (Ahn et al., 1997) applications, there are no commercially available tissue adhesives today that successfully address the issue of adhering to a fibrocartilaginous tissue with high water content. While there may be research in the area of new tissue adhesives, particularly those in the area of biomimetics, they currently are too expensive to be practical for applications in developing tissue adhesives to repair the AF. For this reason, there is a significant need to develop new, tissue engineering strategies to attempt to form a patch or plug in an AF defect that has the ability to deposit new, functional fibrocartilaginous tissue to permanently seal the tear in the annulus, providing a long term, regenerative solution for ruptured AFs.

There have been many approaches developing such tissue engineered constructs from a variety of materials for AF repair, but they fail to define what the key requirements are to achieve repair of the annulus and address the feasibility of developing a biological based patch/plug type of device that has the ability to biologically repair a defect. One of the key issues that leads to the failure of a majority of tissue engineered constructs is the lack of ability of a tissue engineered construct to successfully integrate with the host tissue. Poor integration can lead to uneven distribution of loads throughout the tissue, ultimately resulting in degeneration of the tissue (Zhang et al., 2005).

The following section will address what they key requirements are to achieve successful integration of a tissue engineered construct for annulus fibrosus repair and assess the clinical feasibility of repairing the AF by such methods based on literature to date.

2.5 Keys to Integration of Tissue Engineered Constructs

2.5.1 Presence of Viable Cells at the Interface

One of the key factors required for successful integration of tissue engineering constructs is viable cells at the cartilage-cartilage or cartilage-biomaterial interface (DiMicco and Sah, 2001; Khan et al., 2008; Obradovic et al., 2001; Zhang et al., 2005). Tissue repair and wound healing generally requires cell migration, adhesion and proliferation as well as ECM deposition and remodeling to successfully occur (Ahsan and Sah, 1999), and having a large number of viable cells at the integrative interface facilitates this process.

It has been shown that when pieces of explanted cartilage tissue are arranged in a lap joint configuration in culture media containing FBS, integrative repair begins to occur. This integration was dependent on viable cells at the interface between the opposing pieces of cartilage (DiMicco and Sah, 2001), and the chondrocyte's ability to synthesise and deposit new proteoglycans was directly related to the adhesive strength (Reindel et al., 1995). Viable cells are required at the interface and one study showed that chondrocytes on one of the two interfaces was sufficient for integration (DiMicco and Sah, 2001). The ability of these cells to migrate is also critical to the successful integration of a tissue engineered construct, with one study showing that in constructs that integration was considered to have occurred, chondrocytes had migrated up to 1.5mm from the interface into the host cartilage (Theodoropoulos et al., 2011). Another study showed that chondrocyte migration was required between the tissue interfaces for cartilage repair by implanting a collagen scaffold seeded with chondrocyte at a tissue interface for a 40 day culture period. Histology after this period showed a continuous

cartilage-scaffold interface and cells were found to have migrated to the surrounding mature tissue, driving the remodelling of the ECM (Pabbruwe et al., 2009).

The requirement for cellular migration and ECM deposition has also be indicated for regeneration of tendons (Koob and Hernandez, 2002), ligament (Deehan and Cawston, 2005), and meniscus (Ionescu et al., 2011); all tissues which have also been identified as requiring integration to prevent long-term failure of repair (Lu et al., 2010)(Deehan and Cawston, 2005; Lu, 2006; Spalazzi et al., 2006). A study on the repair of the anterior cruciate ligament (ACL) looked at the effect of mimicking the transition naturally occurring at the ACL-bone interface in terms of the cell population present at each level. A triphasic scaffold was created to support a multilineage cell culture containing fibroblasts, chondrocytes, and osteoblasts. The stratified scaffold was able to support multi-tissue regeneration and may provide an innovative method to regenerate the interface between soft tissue and bone transition regions by surrounding an ACL graft with phase-relevant cells (Deehan and Cawston, 2005).

In tissue engineering the annulus, there has been significant debate over the best source of cells for successful repair with chondrocytes, AF cells, and mesenchymal stem cells (MSCs) being the most commonly studied. Chondrocytes are cells found in healthy articular cartilage within a hydrated extracellular matrix containing sulfated glycosaminoglycans (GAGs) and type II collagen (Shao and Hunter, 2007) and are believed to be able to maintain their phenotype and proliferate in the disc while producing a cartilage specific matrix (Almqvist et al., 2001). One study has shown that

the use of autologous disc chondrocytes, when expanded in culture and injected into a degenerated intervertebral disc, reduced back pain (Meisel et al., 2007). While they show promise for their ability to generate a cartilage-like matrix in various materials, the source of chondrocytes for tissue engineering applications is a challenge. Using autologous chondrocytes can result in morbidity or degeneration at the harvest site (Cabraja et al., 2012) and it can be difficult to get sufficient cell numbers from both autologous and allogenic cell sources.

The same issue is true of human AF cells, which have also been considered for tissue engineering of the disc. In the healthy, non-degenerated AF, there is a sparse cell population at a density of approximately 9,000 cells/mm^3 (Maroudas et al., 1975), making it very challenging to obtain a high enough cell number for tissue engineering applications. While these cells have the advantage of being native to the location for repair and have been shown to be able to be cultured in various scaffold materials including collagen (Bron et al., 2012; Composite et al., 2010), nanofibrous polycaprolactone (PCL) (Koepsell et al., 2011; Nerurkar et al., 2008), and fibrin (Schek et al., 2011), the challenge of obtaining a clinically relevant number of healthy, viable cells without causing damage to the donor site makes this cell source less feasible for tissue engineering strategies (Nerurkar et al., 2009; See et al., 2012)

Human bone marrow- or adipose-derived mesenchymal stem cells have also been more recently studied as a potential source of cells for AF tissue engineering. MSCs have the ability to differentiate towards cartilage, bone, adipose tissue, tendon, muscle or stromal

connective tissue (Freshney et al., 2007), however due to the fact that IVD cells have no specific markers or genes that differentiate them from other lineages (Kandel et al., 2008), it is unknown if they can differentiate towards IVD-like cells. AF cells have their own distinct morphology and synthesize a distinct ECM (Bron et al., 2009b) and cells of the inner AF are described to have a similar profile to that of articular chondrocytes while the outer AF cells have a profile resembling fibroblasts (See et al., 2012). One study found that when analyzing the phenotypes of human IVD cells, MSCs adopted a gene expression profile that more closely resembled IVD cells than articular chondrocytes when cultured with TGF-β (Steck et al., 2005).

MSCs can have a similar phenotype, they have been shown to have a decrease in proliferative ability and gene expression of matrix proteins in a niche that mimics the chemical microenvironment of the IVD (low nutrient conditions, low oxygen and acidic pH) (Wuertz et al., 2008). This may suggest that while MSCs have the ability to differentiate into IVD-like cells, they may not have optimal function when placed into the harsh environment of a degenerative IVD. Another issue to consider with undifferentiated MSCs is the possibility of their migration and deposition in undesired locations. For example one study looked at the fate of allogenic bone marrow MSCs injected into a degenerated disc rabbit model and found the presence of large anteriolateral osteophytes after 3 and 9 weeks (Vadala et al., 2011). Another study differentiated human adipose-derived MSCs into chondrocytes prior to mixing them with fibrin glue and injecting them into the back of nude mice. It was found that after 4, 8 and 12 weeks, collagen-like tissue was formed with gene expression of aggrecan, type II

collagen and SOX-9 (Jung et al., 2010). Therefore, MSCs could be an appropriate cell source based on their ease of accessibility and the possibility of using an autologous cell sample, and then differentiated into a chondrocyte lineage prior to use in tissue engineering scaffolds to avoid undesired migration and differentiation.

Regardless of the cell source studied, many approaches to developing tissue engineering solutions for the annulus fibrosus have acknowledged the importance of incorporating a cellular component into their constructs to enhance the potential for integration with the host tissue. A recent study attempted to simulate an IVD-like assembly using rabbit bone marrow MSCs cultured in cell sheets on silk scaffolds for AF regeneration. Cell sheets have been shown to improve the number of cells that are retained over cell suspensions at the interface by maintaining the cell adhesion proteins within the construct (Li et al., 2001). The study found that after 5 weeks of culture, the cells within the assembly were viable and metabolically active, with an ECM similar in composition to the inner AF generated (See et al., 2012). While the authors showed that that the cells could be grown on the silk scaffolds in cell sheets and generate an AF-like ECM, they have not shown how this type of construct will interact with host tissue once implanted. Another study, however, using cell sheets for osteochondral defects showed significant integration with surrounding cartilage and subchondral bone. Cell sheets were added to poly-(lactic-co-glycolic acid) (PLGA)/MSC scaffolds and implanted in osteochondral defects in rabbits. The cell sheet scaffolds were found to have larger amounts of hyaline cartilage and higher histological scores compared to those scaffolds containing only PLGA and free MSCs and showed significant integration (Qi et al., 2012).

Addition of adhesive molecules to silk constructs has also been studied to enhance initial cell adhesion. Silk has been used to make a porous scaffold to support AF cell attachment and ECM accumulation and a study by Chang et al (Chang et al., 2007) showed that while attachment of RGD to the scaffolds didn't enhance cell attachment, it may be possible to use these scaffolds to modify the phenotype of the cells – something that may be beneficial when trying to mimic the transition in cell phenotype between the inner and outer regions of the annulus. Crosslinking silk fibroin fibres with chondroitin sulfate (CS) has also been attempted to impart superior biological functionality, but was found to produce an insignificant difference in terms of collagen or GAG content (Bhattacharjee et al., 2012).

The effect of the microstructure of electrospun PCL nanofibrous scaffolds on AF cell proliferation and ECM production has also been studied, acknowledging the importance of finding a scaffold microstructure that mimics natural ECM conditions to allow for cells to proliferate and generate a native ECM. A study found that when comparing scaffolds containing random, aligned, and round-ended PCL nanofibres, scaffolds with oriented fibres strongly affected the orientation of cells which would align in the prevailing fibre direction, in concurrence with previous studies (Koepsell et al., 2011). The innovative round-ended fibre scaffold substantially improved the cell adhesion compared to the other scaffolds and may be key to facilitating initial cell attachment, however there was no difference in DNA content after 14 days on any of the scaffolds (Koepsell et al., 2010). This study shows that adjusting the microstructure of a scaffold

can have a positive effect on the number of cells which would be present at the interface upon implantation, indicating this may enhance integration.

A final study showing the importance of live cells at the interface used the treatment of articular cartilage with necrosis inhibitor (Necrostatin-1) or apoptosis inhibitor (Z-VAD-FMK) at the wound edge to prevent cell death. The study found that ultimately cartilage-cartilage integration was enhanced with these pre-treatments, particularly in the case of Z-VAD-FMK (Gilbert et al., 2009), further supporting the need for live cells at the tissue interface region.

2.5.2 Removal of Inhibitory Molecules to Enhance Integration

One issue that has been cited as limiting the ability of a tissue engineered construct to achieve integration with host tissue is the presence of proteoglycans or lubricating molecules in joints which are known to inhibit cells ability to adhere, infiltrate, and proliferate *in vivo*. To overcome this issue, many approaches have been taken including enzymatic pre-treatments of tissues prior to implantation of engineered constructs to allow for cells to actively enhance cell recruitment, synthesis, proliferation and cartilage remodeling (Obradovic et al., 2001).

One study which used enzymatic pre-treatment with chondroitinase prior to addition of a chondroitin sulphate–methacrylate-aldehyde (CS-MA-ald) adhesive solution found that this protocol resulted in a larger transition region as indicated by MRI analysis, indicating a greater extent of tissue integration as compared to other control groups (Ramaswamy et

al., 2006). Another study used a novel method to covalently crosslinking a hydrogel (polyethylene glycol diacrylatel) to cartilage tissue by utilizing "tissue-initiated" photopolymerization. This process required a pre-treatment of the tissue by enzymatic digestion with chondroitinase ABC to remove proteoglycans from the integrative surface followed by an oxidative pre-treatment to create free tyrosyl radicals which were used to covalently attach the hydrogel to the collagen in the tissue (Wang et al., 2004). In this process if either the enzymatic or oxidative pre-treatments were removed, no hydrogel formation could occur.

Obradovic et al. (Obradovic et al., 2001) hypothesized that the integrative properties of their tissue engineered constructs could be controlled by both the duration of cultivation in their bioreactor as well as by trypsin treatment of adjacent cartilage to remove GAGs at the surface. Their study found that the highest adhesive strength at the integration interface occurred with the use of composites made from immature constructs and trypsin treated explants. The increase in cartilaginous tissue that was formed during the study was attributed to a dense population of cells in the young constructs that were able to actively remodel and proliferate due to the removal of the GAGs.

Enzymatic treatment with hyaluronidase and collagenase have also been studied in chondral defects to determine if pre-treatment could enhance the number of viable cells at the wound edge, resulting in the production of collagens and increased biomechanical bond strength. Histology showed that there were significantly more cells present at the wound edge in tissues that were enzymatically treated with an average tissue integration

(measured as a percentage of the total interface length that was connected) of 83% in enzymatically treated groups and only 44% in non-treated tissues. Immunohistochemical staining showed presence of limited type I collagen and abundant col II at the interface, however there was no significant difference between the treated and non-treated groups (Van de Breevaart Bravenboer et al., 2004). While there was no difference in ECM composition, the enzymatically treated groups displayed enhanced integration as shown by a 58% increased adhesion strength at the interface in push out tests compared to control groups (1.32 ± 0.15 MPa versus 0.84 ± 0.14 MPa). While enhanced adhesion was observed, the interfacial strength was still 7-fold less than that of intact cartilage (8.8 ± 0.52 MPa), indicating significant improvements upon this method must be achieved.

Another study, recognising the importance of promoting cell migration, proliferation and matrix deposition, developed an innovative method of delivering the collagenase by incorporating it into PEO nanofibres that released the enzyme upon hydration. The *in vitro* results showed that there was partial digestion of the tissue at the wound edge which increased the cell density at that location 2-fold and led to a 2-fold increase in integrative tissue repair after 4 weeks as compared to controls (Qu et al., 2013).

There are other molecules in addition to proteoglycans that have been shown to inhibit integration including β-Aminopropionitrile (Ahsan et al., 1999) and lubricin (Englert et al., 2005; Schaefer et al., 2004) β-Aminopropionitrile has been shown to inhibit lysyl oxidase, a catalyst of enzyme-mediated collagen crosslinks which occur early in growth

and may contribute to cartilage repair. In *in vitro* studies, β-Aminopropionitrile inhibited the formation of enzyme-mediated collagen crosslinks and inhibited the integration of repair cartilage (Ahsan et al., 1999). Lubricin (PRG4), a mucinous glycoprotein found in synovial fluid, has also been found to reduce integrative repair, reducing adhesive strength of cartilage explants by more than 10-fold (Schaefer et al., 2004). While the mechanisms of inhibition are now known for these molecules, no studies have addressed the issue of blocking their action in order to enhance integration, particularly for cartilage in synovial joints.

Many other enzymes and growth factor delivery methods have been studied for enhancement of integration in meniscus and ligament engineering including addition of transforming growth factor beta 3 (TGF-β3), which increased integration strength in a meniscus construct from approximately 50kPa to 100kPa over an 8 week culture period (Ionescu et al., 2012).

2.5.3 Adhesion and Initial Boundary Stability for Integration

Although enzymatic treatment at the surface has been shown to increase cell viability and numbers at the integrative interface, it does not address the issue of adhesion of the tissue engineered construct to the host tissue. Strong adhesion is required to reinforce the boundary between the construct and host tissue and initial stability must be achieved to acquire successful integration of tissue engineered solutions (Allon et al., 2012). Many

methods of adhering cartilage constructs to host tissue have been studied including the use of surgical adhesives (Singh et al., 2011)(Reckers et al., 2009), chondroitin sulfate (CS)-based adhesives (Wang et al., 2007)(Strehin et al., 2010), and enzymatic treatments (Allon et al., 2012).

Wang et al. (Wang et al., 2007) showed that by functionalizing CS with two arms – a methacylate group to bond to an acrylate based polymer and an aldehyde group to bond with amines on tissue – allowed the CS to act as an adhesive bridge between the two materials. The study showed that the method using a polyethylene glycol diacrylatel (PEGDA) gel was shown to integrate with cartilage and after a 5 week culture period and had an ECM that was rich in cartilage-specific collagen and proteoglycans. *In vivo* studies showed that the adhesive was required to maintain the hydrogel in place in chondral defects in rabbits and when implanted in goat femoral condyles, the cartilage defects treated with the adhesive hydrogel showed significantly greater repair than the empty, untreated defects after 6 months. These results support the theory that strong adhesion is required to enhance integration of implants with host cartilage.

Tissue transglutaminase (tTG), a calcium dependent enzyme that catalyzes crosslinking between lysine and glutamine residues (Jones and Messersmith, 2007), has also been widely studied as a means by which adhesion to host cartilage can be achieved. An adhesive bond has been shown to develop between cartilage surfaces when brought into contact in the presence of tTG, with the adhesive strength increasing linearly with increasing concentrations of tTG. Adhesive strength could be further improved by as

much as 40% by combining enzymatic pre-treatment with chondroitinase ABC or hyaluronidase to remove GAG chains prior to treatment with tTG (Jurgensen et al., 1997). Jones et al. (Jones and Messersmith, 2007) showed that it could be used to enzymatically couple peptide-polymer conjugates to cartilage proteins under mild conditions through the formation of isopeptide bonds. Peptide-polymers conjugated to hydrogels by tTG crosslinking was also tested as an adhesive on guinea pig skin and found to have similar or better adhesion as fibrin glue (Hu and Messersmith, 2005) producing an injectable, *in situ* forming adhesive that is biodegradable.

Another study by McHale et al. (McHale et al., 2005) synthesized covalently crosslinked elastin-like polypeptide hydrogels (ELPs) by genetically encoding amino acid residues that could be crosslinked via tTG. The hydrogels, seeded with chondrocytes, were injectable and underwent a temperature-induced phase transition and crosslinked *in situ* via tTG. The hydrogels were shown to support cell infiltration as well as cartilage matrix synthesis and accumulation. tTG has also been used to coat biomaterial surfaces (composed of PCL and coated with fibronectin to enhance cell attachment) and was found to have a large effect on cell morphology. Cells on PCL scaffolds had a rounded morphology, however the addition of tTG as a coating caused cells to change to a spread morphology and was concluded to possibly enhance stabilization of a biomaterial implant at the integration surface (Heath et al., 2002).

Microbial transglutaminase (mTG), a less expensive and calcium independent enzyme analogous to tTG, was used to develop a biomimetic adhesive based on the factor XIIIa

crosslinking of fibrin that occurs in the blood coagulation cascade. In this novel solution applied to retinal reattachment, gelatin was used in place of fibrin and the mTG was used in place of the calcium-dependent transglutaminase factor XIIIa to bind bovine retinal tissue under wet conditions (Chen et al., 2006). The study found that the lap-shear strength of the adhesive formed was around 15-45 kPa, which is in the same range of other soft-tissue adhesives. While these adhesives have been shown to provide insufficient adhesion for applications in cartilage to date (Reckers et al., 2009), if adhesive strength using this method could be increased this may be a feasible biomimetic approach for adhesion.

2.5.4 Integration of Collagen Fibrils

Other studies have suggested that adhesive strength is not only dependent on initial adhesion of the material to the host tissue, but is also correlated to collagen deposition (DiMicco and Sah, 2001). Integration of collagen fibrils from the engineered and native tissue is one of the greatest challenges to overcome in order to achieve tissue integration.

DiMicco et al. (DiMicco and Sah, 2001) conducted a study in which mechanical integration between two cartilage explants, cultured in apposition, were tested after two weeks of incubation in both a live-live and live-dead tissue conformation. The study found that after two weeks, the mechanical integration of the cartilage explants was related to the collagen deposition, but more specifically to the extent incorporation of [^{3}H]Proline. Hydroxylation of proline, an α-amino acid, increases the stability of collagen and is a critical biochemical process for the maintenance of connective tissues.

The study found that the level of incorporated [^{3}H]Proline was lower in the overlapping region of the live-live samples, indicating that limitation of transport processes may have an effect on integration. The collagen deposition was greater in live tissue when it was adjacent to a dead block of tissue, possibly due to improved nutrient transport. [^{3}H]Proline incorporation was also improved in samples that were cultured in 20% FBS. This correlation between integration of [^{3}H]Proline and the development of adhesive strength suggests that collagen synthesis, deposition, and processing is critical for integrative cartilage repair.

Integration of collagen fibres is also critical in the repair of tendons, ligaments and meniscus, but similarly to the AF, it is difficult to achieve due to their complex structures (Kew et al., 2011). Tendons are composed of linear fibre bundles of highly crosslinked collagen, and currently there is no way of rejoining broken collagen fibres in a mechanically stable way (Koob and Hernandez, 2002). Since synthetic collagen fibres typically have a lower tensile strength due to a lack of crosslinking when developed *in vitro*, many crosslinking methods have been used including glutaraldehyde and carbodiimide crosslinking, both which have been shown to increase tensile strength and stiffness to near native levels (Kato et al., 1991). One study investigated the effect of collagen diameter on tensile strength and found that the thinner the diameter, the greater tensile strength, in the range of 75-110 MPa (Dunn et al., 1993). However, while crosslinking collagen has been shown to increase its strength, it may lower the number of cell attachment sites (Kew et al., 2011) creating a trade-off of having sufficient cells for ECM deposition and remodeling for mechanical integrity.

2.5.5 Achievement of Biomechanical Parity in Repair Tissue Constructs

The biomechanical properties of a tissue engineered construct is required to match that of the native tissue to prevent abnormal loading across the boundary (Van de Breevaart Bravenboer et al., 2004). One method of achieving mechanical parity is by mimicking the microarchitecture of the native annulus tissue to achieve functionally similar tissues. The AF contains 10-20 sheets of collagen lamellae that are tightly packed together in the circumferential direction around the periphery of the disc. The lamellae are stiff and have the ability to sustain significant tensile hoop stresses, but the arrangement of the collagen fibres allows for bending and movements between the vertebral bodies. Within each of the lamellae, the fibres are arranged in parallel and pass obliquely from one vertebrae to the next at alternating +/- 65° angles (Adams et al., 2006). This angle-ply laminate structure of the AF is very difficult to replicate, but is critical to its mechanical function.

Nerurkar et al. (Nerurkar et al., 2009) developed a nanofibrious biological laminate composed of planar sheets of PCL and bovine MSCs that replicated the functionality of the AF by developing a strategy that mirrored the natural multi-scale organization of the extracellular matrix in the tissue. The study found that after 10 weeks of *in vitro* culture, the engineered bi-lamellar tissues reached mechanical equivalence to the AF. The scaffolds were also able to direct the deposition of organized, collagen-rich ECM that mimicked the microarchitecture of the AF, indicating that mirroring the natural angle-ply structure of the AF may assist in developing an ECM that is more analogous to that of native cartilage. While the tissue formed would have potential for repairing a defect in

the annulus, a method of fixing the construct in place and having it integrate with the native tissue would be required for success.

Similarity in stiffness has also been shown to contribute to repair of tendon defects. A study that used a collagen based tissue engineering construct combined with MSCs found that when it was implanted in the central-third patellar tendon defect in rabbits, the construct was able to produce a tissue with a tangent stiffness the same as normal patellar tendon up to 32% of the failure force (Juncosa-Melvin et al., 2006). Implantation of this stiffer construct was positively correlated with repair tissue stiffness after 12 weeks.

2.6 Challenges in Achieving Tissue Integration in the Annulus Fibrosus

The causes of degenerative disc disease are largely unknown, making it very challenging to prevent and repair. Many factors have been cited as contributing to DDD, including the avascularity of the disc, mineralization of or trauma to the cartilage endplates, mechanical factors, vertebral body microfracture, loss of cells or low cell activity, aging, lifestyle and genetics (Anderson and Tannoury, 2005; Battie and Videman, 2006; Setton and Chen, 2006). Significant changes occurring in a degenerative disc from such a large range of factors presents a unique challenge for tissue engineering solutions in the IVD.

While significant headway has been made in the area of achieving integration in articular cartilage, tendons, and ligaments, there have been very few studies that assess integration in the AF or IVD. The AF is a fibrocartilage, with unique challenges for integration compared with articular cartilage. The following section will assess the key issues that

make achieving tissue engineering and integration of tissue engineered constructs within the AF particularly challenging.

2.6.1 Anatomical Complexity

The anatomical structure of the IVD is complex, particularly the AF. Within each lamellae of the AF the fibres are arranged in parallel and pass obliquely from one vertebrae to the next. This alternating arrangement of fibres within the annulus is critical to disc integrity as it prevents a cleavage plane through which the NP could seep through and provides resistance to tension in multiple directions (Adams et al., 2006). The outermost fibres of the AF are considered to be the "ligamentous" portion of the AF with fibres attaching directly to the bone, providing the greatest contribution to resisting movement. The inner fibres insert into the vertebral endplates (Adams et al., 2006).

There are also regional variations within the AF which affect the ECM composition. The inner AF acts as a transitional region between the outer AF and the NP, experiencing greater amounts of hydrostatic pressure than the outer annulus, which primarily experiences tensile and compressive forces (Guterl et al., 2013). The inner AF is composed primarily of collagen type II, which decreases towards the outer portion of the disc. Collagen type I is more predominant in the outer portion of the disc, and reduces towards the inner portion of the disc. This transition of ECM composition is difficult to mimic and is largely a result of the mechanical forces experienced in each region (Bron et al., 2009b).

2.6.2 Chemical Microenvironment

In addition to the complex angle-ply structure and transitional ECM components, the chemical microenvironment within the AF provides a challenge for cellular based tissue engineering therapies. The AF is largely avascular, limiting its ability for self-repair due to low oxygen and nutrient transport (Guterl et al., 2013). Blood supply to the disc is limited to the cartilaginous endplates and the borders of the AF, relying on diffusion of nutrients which can be dependent on the direction of strain (Jackson et al., 2008). A majority of nutrients diffuse through the disc from capillaries that originate in the vertebrae, with cells that may lie as far as 8mm from the nearest blood vessels, resulting in significant cell death and contributing to disc degeneration (Urban et al., 2004). If calcification of the endplates has occurred as part of the degenerative process, this can further block nutrient supply to cells (Kandel et al., 2008), causing a significant challenge for tissue engineering solutions in the disc.

There are also large gradients of oxygen concentration throughout the disc, with the centre of the disc having as low as 1% O_2 present in a large disc. It has been shown that cell activity and matrix synthesis can be significantly reduce in low oxygen concentrations, and while cells from the AF seem to require relatively little O_2, they produce energy primarily through glycolysis (Bibby and Urban, 2004). Cells of the AF use large amounts of glucose and produce a lot of lactic acid, resulting in a low pH due to the accumulation of this waste product (Bibby and Urban, 2004). With a high glucose demand, cell death can begin to occur at glucose levels below 0.5mM.

2.6.3 Mechanical Demands

The IVD, and more specifically the AF, are subject to complex loading which is difficult to replicate *ex vivo* for testing and even more difficult to engineer solutions for *in vivo*. The spine undergoes motion in 6 degrees of freedom, creating a complex loading environment for the AF. Due to the highly pressurized NP, hoop stresses are placed on the inner AF while tensile and compressive stresses resulting from spinal movement are prominent in the outer AF (Guterl et al., 2013). Even under static conditions the lumbar disc can experience loads around 0.2 MPa (Nachemson, 1960; Wilke et al., 1999) with loads increasing 3-5 fold during spinal movement.

In addition to the normal mechanical demands experienced in the AF, degenerative changes which affect the mechanics of the spine must also be taken into account. In a degenerative disc, there is a trend of increasing compressive and shear modulus (Iatridis et al., 2006) with compressive stresses increasing by 160%.

2.6.4 Clinical Presentation

In addition to the tissue engineering challenges associated with a complex tissue such as the annulus, the lack of unified diagnostic criteria to identify, diagnose and treat damage to the annulus creates an additional challenge to implementing tissue engineered solutions. Chronic low back pain is the leading cause of work-related disability in the population under 45 years of age, however only 15% of those with pain have underlying nerve-root compression and 85% have non-neurogenic low back pain, making it very difficult to diagnose the exact cause of their back pain (Sehgal and Fortin, 2000).

Internal disc disruption (IDD) was originally defined by Crock as the loss of a normal distinction between the NP and AF and extensive disorganization and fissuring in the AF while still maintaining the external disc contour and appearance with no nerve root compression (Crock, 1970). The ideal time to treat and repair annular fissures would be at this stage, prior to the formation of full thickness focal tears, however tears at this stage are often present in patients who are asymptomatic (Ernst et al., 2005) and therefore are not identified early enough for preventative treatments.

IDD is present in 30-50% of patients with chronic low back pain and patients present with symptoms ranging from a dull ache and deep-seated burning to a sensation of a weak or unstable back, pain in the hips and lower limbs and an inability to sit for extended periods of time (Schwarzer et al., 1995). A clinical diagnosis based on patient history and a physical examination alone is difficult (Sehgal and Fortin, 2000), however the American College of Physicians does not recommend the use of (Sehgal and Fortin, 2000)imaging techniques which can more accurately diagnosis issues in patients who have only presented symptoms for 4-6 weeks unless there are red flags of serious underlying conditions.

Imaging techniques, while useful tools in diagnosing many pathologies that result in back pain such as disc herniation, spinal stenosis, fracture or osteoporosis (Becker and Stumbo, 2013), have limited ability to identify annular tears at early stages. X-rays and CT scans often appear normal, although CT scans may identify low-density zones in the AF region that may suggest annular pathologies. On MRI, well hydrated discs have a

high signal intensity while degenerated discs will have a lower intensity, however a normal MRI doesn't necessarily rule out the presence of IDD.

The most reliable method of diagnosing IDD is discography in which a needle is inserted into the disc and contrast is injected. The disc stimulation will typically reproduce pain in patients with chronic back pain while not inducing pain in asymptomatic individuals, even if they have disc degeneration (Sehgal and Fortin, 2000). Typically, for a diagnosis to be made, there are three criteria that must be met. Provocation discography must reproduce the patients pain in the affected discs, disc stimulation to 1-2 other discs must fail to reproduce the pain (to avoid false positive tests), and a CT discogram must identify the presence of fissures in the AF extending to the outer third of the annulus (Becker and Stumbo, 2013). Fissures on the inner third of the AF are rarely painful as only the outer third of the disc is innervated in a healthy back (Aoki et al., 2004), however it is believed that in painful discs, micro-blood vessels and nerve endings grow into areas of the AF that are normally avascular (Freemont et al., 2002).

Due to the lack of clear clinical presentation of annular tears, it is difficult to implement tissue engineering solutions at early stages prior to presentation of full thickness focal defects that impinge on nerves. With 90% of patients experiencing full relief of low back pain symptoms with minimal intervention (Becker and Stumbo, 2013), it's also difficult to justify the implementation of solutions that are any more invasive than current solutions. For this reason, tissue engineering solutions in the disc should focus on

repairing issues such as herniation which have a clearer clinical presentation and diagnosis.

2.7 Development of an *In Vitro* Model to Study Tissue Integration in the Annulus Fibrosus

Due to the limitations discussed above, a more detailed understanding of the mechanism of integration in fibrocartilage in a complex environment such as the AF is of critical importance for the further development of tissue engineered constructs for repair in the IVD. While critical strides have been made in the development of various biomaterials that replicate the form, function, extracellular matrix and mechanical properties of the AF, there is very little understanding about how these factors must interact in order to achieve successful integration of a tissue engineered construct.

In vitro testing methods have been described for studying integration for articular cartilage. In one model, cartilage explants and isolated chondrocytes were anchored to agarose gel on a culture plate, with the agarose sealing the two components together upon gelation. Isolated chondrocytes were added to the confined defect created in the explant and after 4 weeks of *in vitro* culture, neocartilage with proteoglycans and type II collagen were formed, but no integration was found (Enders et al., 2010). Another study created annular explants of articular cartilage which were filled with either fibrin, agarose or poly(glycolic acid) scaffolds and cultured over a period of 20 or 40 days. It was found that the presence of native tissue had a significant effect on the DNA, GAGs, and hydroxyproline content (Hunter and Levenston, 2004), implying that the presence of a

living tissue culture is an important factor to study when considering the type of ECM deposited by cells in a scaffold intended for implantation adjacent to living tissue.

While there have been methods developed to study integration *in vitro*, no models have been described that allow for a repeatable, critical defect to be created in a tissue and loaded under conditions that mimic some aspect of physiological function to determine how loading can affect integration of a tissue engineered construct. Many studies have described tissue engineering solutions that could be implanted to bridge a defect in the annulus fibrosus, with the assumption that new tissue will be able to form and integrate with the native tissue. However, few of these studies consider the implications of having these scaffolds under load as they would be *in vivo* after implantation, and there is no current way to investigate how various materials would integrate with native tissue in a way that can be compared across various models. For that reason, our lab has developed an *in vitro* model that allows for the study of integration of a biomaterial in a small, repeatable defect under simple mechanical loading.

The model was developed for a custom loading device previously developed in our lab (Dussault, 2002). The loading device applies a cyclic tensile strain of 5% to six bovine annulus specimens, held between two clamps within plates in the device. Fresh bovine tissue was obtained immediately after slaughter and dissected to obtain live tissue samples. A uniform, critical defect was created in each specimen using a 4mm biopsy punch which can subsequently be filled with a variety of biomaterials and cellular therapies and loaded over the desired period of time. This device allows for the study

between the interface of the tissue and the biomaterial filling the defect after it has been loaded for a specified period of time to determine whether it is possible for a biomaterial to integrate with the native annulus tissue while under load.

2.8 Conclusion

Back pain is a significant problem in North America, with one of the leading causes of pain resulting from herniation of the intervertebral disc. Currently, treatments to repair herniated IVDs include palliative treatments or surgical removal of the inner NP, neither of which address the long-term regeneration and stability of the tissue. Current tissue engineering techniques have identified many materials that could be used to produce constructs to implant into an annulus defect and close the gap in the AF, allowing for the NP to be retained in the disc and maintain the natural disc height and biomechanics. However, these tissue engineering solutions currently do not adequately address the issue of integration of the constructs with the native tissue, a critical aspect to achieving long term success for such an implant.

There are several factors that are critical to achieving integration of a tissue engineered construct with native tissue including the presence of viable cells at the tissue interface, removal of inhibitory molecules such as proteoglycans, integration of collagen fibres with the construct, and development of a construct and new tissue that has mechanical parity with the native tissue. While there have been many studies investigating the mechanisms behind these keys for integration, there have been few studies that combine investigating

these factors with development of a novel biomaterial for repair of a defect in the annulus fibrosus.

Our lab has developed a custom loading device that has the ability to study a repeatable, critical defect under simple loading conditions so that various biomaterials can be tested to determine their effectiveness at achieving integration with a piece of bovine annulus tissue *in vitro*. A model such as this is critical for the success of future tissue engineering solutions for the annulus as the mechanisms behind integration can be studied in a simplified model, providing insight into what may hinder or promote tissue integration.

Further research into the mechanisms behind integration of cartilage, specifically in a fibrocartilage such as the annulus fibrosus, will need to be conducted and modeled with various biomaterials that show promise for repairing annular defects. If integration mechanisms can be better understood, it may help to expedite the translation of some existing tissue engineering methods into clinical solutions.

Chapter Three: Methods & Materials

The following chapter describes the methods and materials used to culture human bone marrow mesenchymal stem cells, fabricate surgical adhesives and fibrin gels, and characterize and analyze genipin-crosslinked fibrin gels seeded with human mesenchymal stem cells. Many of these methods have been previously developed in our lab and are standard for the culture and maintenance of stem cells as well as for analysis of gene expression and histology.

3.1 Mesenchymal Stem Cell Culture

3.1.1 Culture Media Preparation & Cells

For studies in Chapter 4, cell culture medium containing 10% fetal bovine serum (FBS) was prepared by mixing 500mL of 1% low glucose Dulbecco's Modified Eagle Medium (DMEM) (11330-032; Gibco) with 55mL of FBS (12483-012; Gibco) in a biosafety cabinet. The media (also referred to as 10% FBS DMEM) was supplemented with 5mL of 100x antibiotic-antimycotic (15240-062; Gibco) containing 1000 units/mL of penicillin, 1000µg/mL streptomycin and 25µg/mL Fungizone® (amphotericin B). All media for these studies used FBS lot #582725 and human bone marrow mesenchymal stem cells (MSCs) were obtained from Cambrex Bio Science Walkersville, Inc. (Lot 060630B).

For studies in Chapter 5, human bone marrow MSCs were obtained from Lonza (Lot 0000296578) and cultured with Lonza's recommended mesenchymal stem cell growth medium. The media was delivered as a kit and prepared in a biosafety cabinet by combining 440mL basal medium (12-707F; Lonza), 50mL of mesenchymal growth supplement (MCGS) (PT-4106F; Lonza), 10mL of L-Glutamine (PT-4107E; Lonza), and 0.5mL GA-100 (gentamicin sulfate and amphotericin-B) (PT-4505E; Lonza).

3.1.2 Cryopreservation

Freezing medium for cryopreservation was prepared by combining 80% (v/v) culture medium with 10% (v/v) FBS and 10% (v/v) dimethyl sulfoxide (DMSO). Cells were trypsinized and collected at a density of $1x10^6$ cells/mL, then centrifuged and resuspended in 1mL of freezing medium. 1 mL of the cell suspension was transferred to "Cryotubes Stor-It" cryovials (391-8342; VWR) then left at -80°C for up to one month or moved to liquid nitrogen for storage until future use.

3.1.3 Thawing Cells

As soon as cells were received, the cells were thawed for 2 minutes then transferred to 5mL of temperature equilibrated culture medium and centrifuged at 1000rpm for 5 minutes. The supernatant was removed and the cell pellet re-suspended in 1mL of medium. A cell count was carried out using a haemocytometer. To conduct cell count, 10µL of the cell suspension was placed in a tube with 10µL of Trypan Blue Solution (15250-061; Life Technologies). Cells were counted in all 4 chambers then equations 3.1 and 3.2 were used to calculate total number of cells:

$$Cells\ Per\ mL = Average\ Live\ Cell\ Count\ Per\ Square\ x\ Dilution\ Factor\ x\ 10^4 \quad (3.1)$$

$$\text{Total Cells} = \text{Volume of Suspension x Cells Per mL} \quad (3.2)$$

Cells were then seeded at a density of 5000 cells/cm^2 in a 75cm^2 tissue culture flask (82050-856; VWR).

3.1.4 Monolayer Culturing

All cell culture and expansion was carried out in 75cm^2 tissue culture flasks (T-75). T-75s were subcultured when cells were approximately 85-90% confluent, approximately every 6 days. The media was removed from cell culture flasks and discarded. Each T-75 was then rinsed with 2 x 5 mL of 1X phosphate-buffered saline (PBS) (10010-023; Invitrogen). 1X PBS is the only type of PBS used for all experiments. 4mL of Trypsin EDTA (25200056; Invitrogen) was added to each flask, allowed to sit for 3-4 minutes at 37^{o}C then neutralized with 5mL of 10% FBS DMEM. The cell suspension was removed and transferred to a 50mL conical tube. The flasks were again rinsed with 2 x 5mL PBS wash and added to the conical tubes, then spun down at 1000rpm for 5 minutes. The supernatant was then aspirated from the conical tubes and the cell pellet re-suspended in 1mL of 10% FBS DMEM per T-75 flask into which the cells would be expanded. Cell counts were then performed as described and cells were re-seeded at a density of 5000 cells/cm^2 in each T-75 with 12mL of culture medium.

For all cell culture, 50% media changes were performed every 3-4 days with 50% of the media being removed and discarded then replaced with fresh media.

3.2 Screening of Surgical Adhesives

3.2.1 Fabrication of Surgical Adhesives

All surgical adhesives were obtained by donation of samples from various sales representatives and fabricated according to the manufacturer's instructions. The following are brief descriptions of the fabrication process for each surgical adhesive tested.

3.2.1.1 BioGlue

The BioGlue delivery system contains a syringe, syringe plunger, and applicator tips. The syringe was removed from its packaging and held upright, then tapped until any bubbles were located at the top of the syringe. The locking collar was rotated to the "open" position and the tip connected to the syringe, then rotated back to the locked position to lock the tip in place. The plunger was then inserted into the syringe system and gently depressed to remove bubbles from the system and prime it for use. BioGlue is most effective when applied to a dry surface, so tissue was blotted dry prior to application of BioGlue.

3.2.1.2 FloSeal

The FloSeal gelatin matrix system contains a gelatin component and a thrombin component. The gelatin component contains a 5mL syringe containing the gelatin matrix, a 5 mL syringe connector, a bowl for thrombin, and applicator tips. The thrombin component contains a vial of human thrombin (containing 2500 units thrombin, 225-275 mg total protein, and 40-60mg sodium chloride), a 5mL vial of 40µmol/mL

calcium chloride solution and a 5mL syringe with needle. The thrombin solution was prepared by using the 5mL syringe and needle to transfer the 5mL of calcium chloride solution to the vial containing the lyophilized thrombin. The thrombin was dissolved and transferred to the sterile bowl in the gelatin component system. The thrombin was aspirated into the 5mL syringe with connector then connected to the syringe containing the gelatin matrix. The plunger on the syringe for the thrombin was fully depressed to pass the thrombin solution into the syringe containing the gelatin matrix. The solution was then transferred back and forth between the syringes approximately 20 times to thoroughly mix the solutions. The applicator tip was then attached and the solution allowed to sit for 30 seconds prior to use to obtain the optimal consistency.

3.2.1.3 Tisseel

The Tisseel sealant kit contains a sealer protein concentrate and thrombin in a dual chambered syringe as well as a fibrinolysis inhibitor solution and a calcium chloride solution. To prepare the glue, the sealer protein concentrate was reconstituted using the fibrinolysis inhibitor solution and the thrombin reconstituted in the calcium chloride solution at 37^{o}C within the dual syringe system. The solutions were allowed to dissolve prior to use.

3.2.2 Tensile Testing of Surgical Adhesive

3.2.2.1 Preparation of the Tissue

To test the adhesive strength of the surgical adhesives to bovine annulus tissue, frozen cow tails were obtained from a local butcher shop and dissected to remove the

intervertebral discs. The annulus was then sliced vertically to open the disc and the nucleus was removed, leaving a long strip of annulus. The annulus was then cut into pieces that were approximately 2cm long (Figure 3.1).

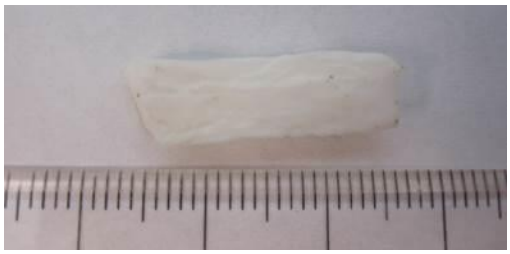


Figure 3.1: Bovine annulus fibrosus tissue after removal of nucleus and trimming into a flat section (scale is ruler in cm).

Control samples of annulus alone were left in this configuration, with sandpaper super-glued to the ends to act as grips in the tensile testing device. Samples to be tested in a lap joint configuration with various adhesives were then cut in half so each half was approximately 1.5cm in length, then glued together in a lap joint configuration with an overlap region of approximately 0.5cm (Figure 3.2a) to test for apparent shear strength (according to ASTM standard F2255-05). Samples were also prepared in a butt joint configuration to test for wound closure strength (according to ASTM standard F2458-05) where the pieces were glued end to end in their original positions (Figure 3.2b). Pilot studies were conducted using super glue to test the protocol prior to experiments with surgical adhesives. Sandpaper grips were super-glued to the outer edges of the samples to allow for easy gripping within the tensile testing machine.

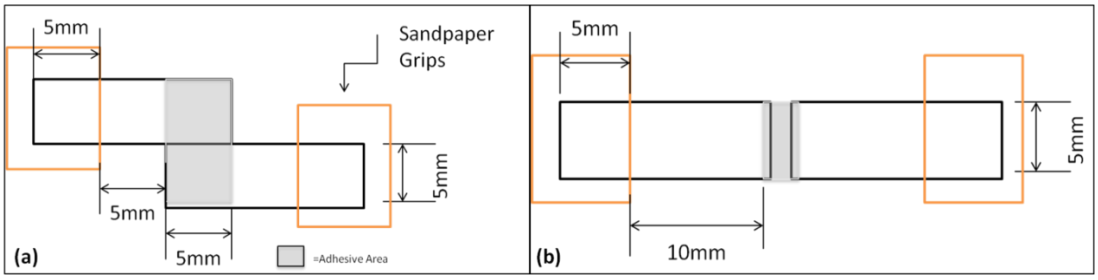


Figure 3.2: Specimen template in (a) lap joint and (b) butt joint configuration for tensile testing.

3.2.2.2 Tensile Testing

Tensile tests were conducted at the Bose Centre on the University of Calgary campus using Bose Electroforce 3200 testing device and WinTest software. ASTM Standard F2255-05 (Testing protocol for strength properties of tissue adhesives in lapshear by tension loading) was followed for these experiments. The tissue was kept moist at all times using PBS to condition the tissue prior to testing. The samples were wrapped in PBS soaked gauze and placed in a Ziploc bag, then left in a water bath at 37^{o}C for one hour. The adhesive to be tested was then prepared and the tissue samples were removed from the Ziploc bag and the surface was patted dry with fresh gauze. The tops and sides of the samples which were not to be bonded were covered with petroleum jelly to prevent bondage beyond the desired region. A sufficient amount of adhesive was added to the region of overlap for the specimens to be prepared in a lap joint or butt joint configuration and the two sides of the test specimens were fixed together, applying a small amount of force for about 30 seconds to allow the bond to set. The width and length of the adhesive bond area were measured and recorded. The specimens were then

re-wrapped in PBS soaked gauze and placed in a water bath at 37°C for one hour to be conditioned prior to testing.

After conditioning, the test specimens were stabilized to the testing room temperature for 15 minutes prior to testing, while being kept moist with PBS to prevent shrinking. The test specimens were loaded into the grips of the testing machine such that the applied load coincided with the long axis of the specimen. The specimens were then loaded to failure at a constant cross-head speed of 50mm/min. The bond area was recorded and the apparent shear strength was calculated using equation 3.3:

$$Apparent\ Shear\ Strength\ (MPa) = \frac{Maximum\ Load\ Applied}{Bond\ Area} \quad (3.3)$$

3.3 Genipin-Crosslinked Fibrin Gel Fabrication

3.3.1 Preparation of Solutions

100 mg/mL fibrinogen (F8630-5G; Sigma) was prepared by warming PBS to 37°C and sprinkling fibrinogen over the surface of the PBS and allowing it to slowly dissolve before continuing to add more fibrinogen until all the fibrinogen had dissolved. Genipin (078-03021; Cedarlane) was prepared at 0.02 mg/mL, 0.050 mg/mL and 0.100 mg/mL for various experiments by dissolving genipin in DMEM. 0.0294g calcium chloride ($CaCl_2$) (C1016-100G; Sigma) was dissolved in 500mL H_2O to yield a 40mM calcium chloride solution. 500 units of thrombin (T9549-500UN; Sigma) was dissolved in 5mL PBS for a thrombin solution that was 100 UN/mL. To prevent degradation by

fibrinolysis, 200 mg/mL 6-aminohexanoic acid was dissolved in H_2O and 10µL/mL was added to all media during incubation periods for a final concentration of 2 mg/mL 6-aminohexanoic acid in the culture medium. All solutions except fibrinogen were sterile filtered using a 0.45µm syringe top filter (28145-481;VWR) prior to use. Fibrinogen was prepared in a biosafety hood to maintain maximum sterility, however due to the viscosity of the solution and sensitive nature of the protein, it could not be further sterilized.

3.3.2 Fabrication of Fibrin Gels

Fibrin gels were fabricated in 24 well plates as controls for all experiments to be compared to the genipin-crosslinked fibrin gels. Fibrin gels were prepared by adding 10µL $CaCl_2$ and 60µL Thrombin to the well plate then mixing to combine the solutions. 600µL of fibrinogen was added and quickly pipetted up and down approximately 5 times to mix the solutions well prior to polymerization. Gels were left to polymerize in an incubator at 37°C, 5% CO_2 and 95-98% humidity for one hour, then were removed from the 24 well plate and either used or transferred to 6 well plates and submerged in 8mL of 10% FBS-DMEM with 2 mg/mL (final concentration) 6-aminohexanoic acid to be left in culture for various time periods.

3.3.3 Genipin-Crosslinked Fibrin Gels

Genipin-crosslinked fibrin gels were fabricated in 24 well plates using one of three methods for degradation experiments in chapter 4 in which fabrication methods were compared. For all subsequent experiments, gels were fabricated using a combination of the two methods, method #3.

3.3.3.1 Fabrication Method #1

In fabrication method #1 (Table 3.1), gels were fabricated in 24 well plates by combining 10µL $CaCl_2$, 60µL of thrombin (100 UN/mL) and 600µL of fibrinogen (100mg/mL) and allowing them to polymerize in an incubator at 37°C, 5% CO_2 and 95-98% humidity for one hour. 400µL of genipin (0.1mg/mL) was then added on top of the gels and allowed to permeate into the gel and crosslink for 24 hours. Gels were then removed from the 24 well plate and either used or transferred to 6 well plates and submerged in 8mL of 10% FBS-DMEM to be left in culture for various time periods.

3.3.3.2 Fabrication Method #2

In fabrication method #2 (Table 3.1), 968µL fibrinogen, 22 µL thrombin and 10µL of genipin were all mixed at once and left to crosslink for one hour at 37°C, 5% CO_2 and 95-98% humidity. Gels were then removed from the 24 well plate and either used or transferred to 6 well plates and submerged in 8mL of 10% FBS-DMEM to be left in culture for various time periods.

3.3.3.3 Fabrication Method #3

In fabrication method #3, gels were fabricated in 24 well plates by combining 10µL $CaCl_2$, 60µL of thrombin and 400µL of genipin and mixing the solutions well. Fibrinogen was then added to the solution and quickly pipetted up and down approximately 5 times to mix the solution well prior to polymerization. Gels were left to polymerize in an incubator at 37°C, 5% CO_2 and 95-98% humidity for one hour then

were removed from the 24 well plate and either used or transferred to 6 well plates and submerged in 8mL of 10% FBS-DMEM with 2 mg/mL (final concentration) 6-aminohexanoic acid to be left in culture for various time periods.

Table 3.1: Summary of Genipin-Crosslinked Fibrin Gel Fabrication Methods to produce gels with a final volume of 1.07mL

Method #1	60µL of thrombin + 600µL fibrinogen → Polymerize for 1 hour →400µL of genipin on top of gel → 24 hour crosslinking.
Method #2	10µL of genipin + 60µL of thrombin → Mix Well → 968µL fibrinogen → Polymerize for 1 hour
Method #3	10µL $CaCl_2$ + 60µL of thrombin + 400µL of genipin → Mix Well → 600µL fibrinogen → Polymerize for 1 hour

3.3.4 Seeding Mesenchymal Stem Cells in Gels

Human bone marrow MSCs were seeded in gels fabricated using method #3 for all experiments that utilized cells. After collecting cells from flasks, the cell pellet was re-suspended at a density of 500,000 cells in 20µL culture medium. The cell suspension was added to the gel solution after $CaCl_2$, thrombin, and genipin were combined and mixed prior to adding fibrinogen to polymerize the gels. Gels were left to polymerize in an incubator at 37^{o}C, 5% CO_2 and 95-98% humidity for one hour then were removed from the 24 well plate and either used or transferred to 6 well plates and submerged in

8mL of 10% FBS-DMEM with 2 mg/mL (final concentration) 6-aminohexanoic acid to be left in culture for various time periods.

3.4 Gel Characterization

3.4.1 Degradation & Cell Mediated Degradation

To determine the degradation profiles of genipin-crosslinked fibrin gels compared to fibrin gels, wet weight was used as an indication of their stability over time. Gels were fabricated as described above then placed in 6 well plates with 8mL of 10% FBS DMEM and 6-aminohexanoic acid at a final concentration of 2 mg/mL. The well plates were left in an incubator at 37°C, 5% CO_2 and 95-98% humidity and wet weight measurements were taken every 2-3 days in a biosafety cabinet to maintain their sterility. Prior to measuring their wet weight, the gels were gently blotted with tissue to remove any excess media. Weight measurements were recorded and the change in weight over time was plotted. Percent weight change over time was calculated using equations (3.4) and (3.5):

$$\%\ Change\ in\ Weight = \frac{(W_T - W_{T0})}{W_{T0}}\ x\ 100\% \qquad (3.4)$$

$$\%\ of\ Original\ Weight = 100\% - \%\ Change\ in\ Weight \qquad (3.5)$$

where: W_T = Weight at the specified time point, W_{T0} = Weight of gel at time=0.

3.4.2 Cell Viability

A Molecular Probes LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells (L3224; Invitrogen) was used to determine cell viability within gels. The kit contains 4mM calcein AM in anhydrous DMSO and 2mM ethidium homodimer-1 in DMSO/H_2O 1:4 (w/v). A solution containing 4µM Calcein and 4µM ethidium homodimer-1was prepared by combining 8µL of the 4mM calcein solution into 8mL of PBS. The solution was mixed then 16µL of the 2mM ethidium homodimer-1 solution was added to the calcein and PBS solution. To avoid loss of fluorescent activity, all vials were kept wrapped in aluminum foil and exposed to light for as little time as possible during mixing.

To stain the gels, gels were removed from media and placed in 6 well plates (1 gel per well) and rinsed with 3x 5mL of PBS, allowing the gels to soak for 10 minutes with each rinse. All the PBS was removed and the gels were then submerged in the calcein and ethidium solution. Well plates were wrapped in aluminum foil and placed in an incubator at 37°C, 5% CO_2 and 95-98% humidity for 1 hour. After that time period, the calcein and ethidium solution was removed from the gels and they were again rinsed with 3x 5mL washes of PBS, allowing the gels to soak for 10 minutes with each rinse.

After staining, gels were imaged using a Zeiss Axio Examiner Z1 confocal microscope with Zen software. The microscope was set to take images using a 20x objective and 488nm and 543nm lasers. The image area was kept constant at 303.34µm x 303.34µm. A minimum of 3 images were taken per gel, from random locations within the gel. Live

cells were stained green and dead cellos were stained red, allowing the live and dead cells to be counted. The percent cell viability was calculated using equation (3.6) from the average of all of the live and dead cells counted in each gel (n=3):

$$\%\, Viable\ Cells = \frac{Number\ of\ Viable\ Cells}{Total\ Number\ of\ Cells\ Counted}\ x\ 100\% \tag{3.6}$$

3.4.3 Confined Compression Testing

Compression testing was carried out using a Bose Electroforce 3200 testing device with WinTest software. Gel samples were prepared in 96-well plates for testing as this provided an appropriately sized sample for the compression chamber. Samples were loaded into the compression chamber, with the porous plug placed on top of the gel. Measurement of the initial thickness of the gel was taken by placing the plunger inside the compression chamber and measuring the length of the plunger height outside of the chamber using calipers. Equation (3.7) was used to determine the initial height of the sample:

$$Height\ of\ Sample = (h_{P+S}) - (h_P + h_{PP}) \tag{3.7}$$

where: h_{p+s} = height of plunger outside of chamber with sample in place, h_p = height of plunger outside of chamber (no sample in place), and h_{pp} = height of porous plug.

The samples were preloaded with the weight of the plunger which produced a load of 0.384N for 5 minutes. The compression chamber was then loaded into the testing device and the platen was lowered until 0.1N of force was registered depressing the plunger into the compression chamber. A second pre-load of -0.5N was applied to the sample for 5 minutes prior to testing. For compression testing, a ramp to level (based on % strain) program was used in which the sample was compressed at a rate of 0.02mm/s (1.3mm/min) until it reached a defined displacement for the desired strain rate. The resulting force and displacement was converted to stress and strain values using equations (3.8) and (3.9):

$$Stress\ (kPa) = \frac{Force\ (N)}{Sample\ Area\ (mm^2)} * 1000 \qquad (3.8)$$

$$Strain\ (\%) = \frac{L - L_0}{L_0}\ x\ 100\% \qquad (3.9)$$

where: L = Final height of the specimen (mm) and L_O = Original height of specimen(mm)

The collected stress and strain data were plotted as a stress-stain curve and the slope of the linear region used to determine the aggregate modulus. Compressive forces are negative while tensile forces are positive, however for simplicity, all compression data was plotted on a positive axis.

3.5 Annulus Damage Tissue Culture Model

3.5.1 Model Development

3.5.1.1 Preparing Annulus Fibrosus Tissue

Fresh tails from female cows less than 28 months of age were received within 2-3 hours of the animals being slaughtered and immediately dissected for use. Intervertebral discs were excised and the nucleus was removed, leaving only the annulus fibrosus. 3cm sections of annulus were cut such that the lamellae were aligned parallel to the length-wise direction of the sample specimen (Figure 3.3).

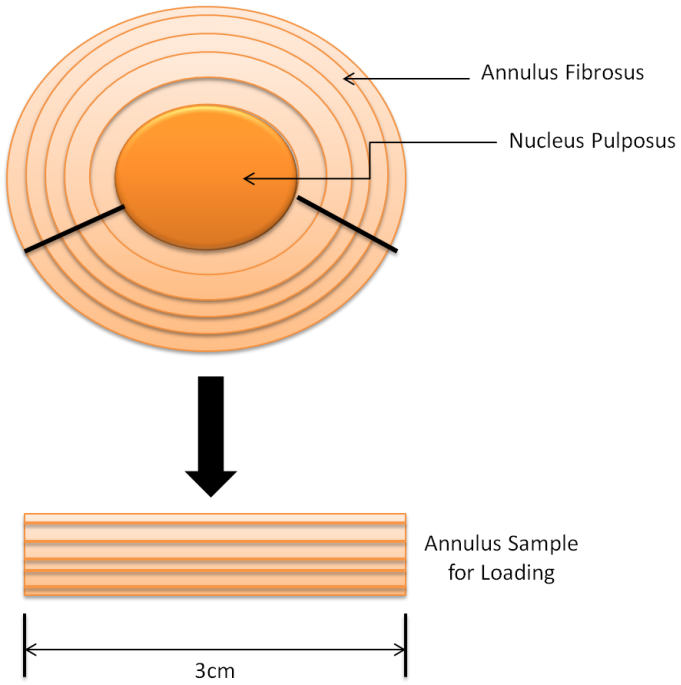


Figure 3.3: Full intervertebral discs were obtained from female cow tails and sections of annulus removed to yield a ~3cm piece of circumferential annulus.

3.5.1.2 Creating and Filling a Defect

Once the tissue was prepared, a 4mm biopsy punch was used to create a defect in the centre of the 3cm section of annulus, cutting sagittal through the transverse plane of the annulus. The defects were then stretched for approximately 2 minutes using tweezers to allow for more flexibility to insert a gel into the defect. Genipin-crosslinked gels were prepared using method #1 and were used immediately after they had polymerized for one hour. A 6mm biopsy punch was used to make small gel plugs (Figure 3.4a) from the full gel samples and were inserted into the 4mm biopsy hole in the annulus such that they fit snugly (Figure 3.4b), but would not be squeezed out upon application of a small amount of tension.

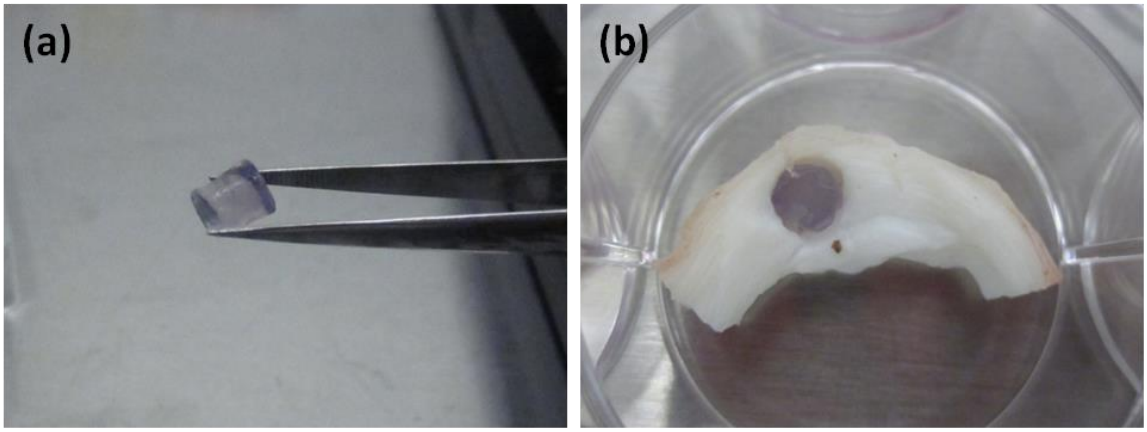


Figure 3.4: (a) Genipin-crosslinked fibrin gel (0.1 mg/mL genipin) plug made using 6mm biopsy punch from full gel sample and (b) 6mm biopsied gel inserted into 4m biopsied defect in a piece of bovine annulus.

3.5.2 Loading Device & Loading Regime

3.5.2.1 Well Plates

A custom loading device was previously developed in our lab (Dussault, 2002) which allows for the cyclic tensile loading of six specimens simultaneously. Each specimen loaded into the device is contained within a well plate shown in Figure 3.5. The well plates and lids, as well as clamps used to hold the specimens, are made of plastic which can be sterilized by spraying with ethanol then exposed to UV light in a biosafety cabinet for one hour. The lids and wells were re-designed based on previous studies conducted to accommodate a 2cm gap between the clamps once they were inserted into the specimen holders.

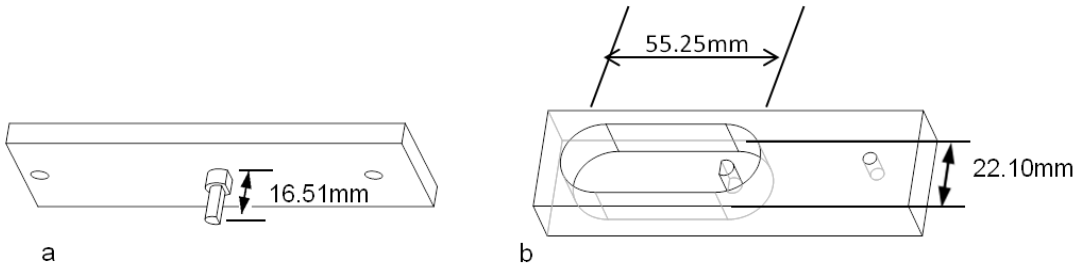


Figure 3.5: Design for (a) well plate lid and (b) well plate for the cyclic loading device with dimensions. Peg diameter is 5mm.

3.5.2.2 Specimen Holders

The specimen holders for the device, shown in Figure 3.6, consist of a well plate which is fixed to the loading device platform and contains a peg protruding from the bottom. A lid, also containing a peg protruding inwards to the well when in place, fits snugly over top of the well.

The annulus specimen is held at either end by tissue clamps which contain holes that fit over top of the pegs on the lid and well, holding the tissue specimen in tension. With the well being fixed, a linear motor moves a platform which is attached to the lids and results in the lid being displaced and a cyclic tensile loading being transmitted to the specimen.

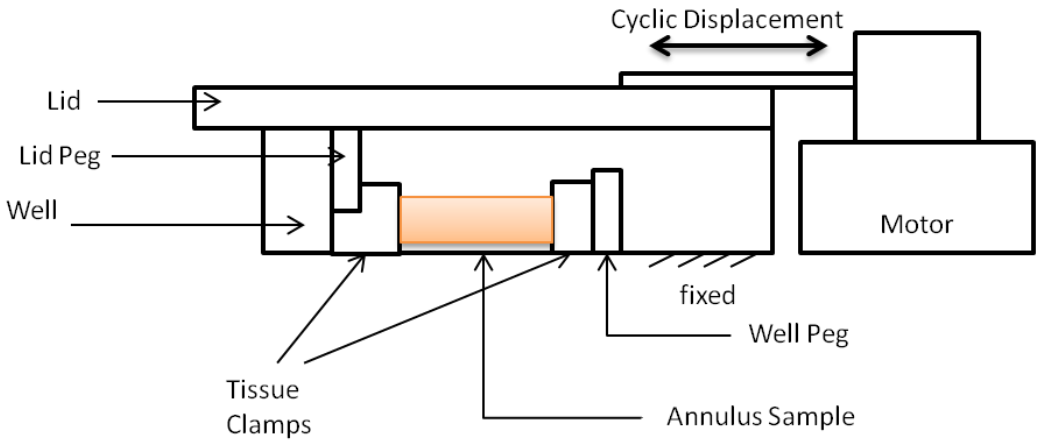


Figure 3.6: Side-view schematic of a single well plate in a custom loading device. The tissue sample is held by two tissue clamps which fit over top two pegs - one attached to the well which is fixed and one attached to the lid which is displaced cyclically.

3.5.2.3 Full Cyclic Loading Device

The loading device, shown in the incubator in Figure 3.7, consists of a motor box with a moving arm that attaches to the gliding plate on the main device platform. There are six locations in which the well plates are placed and are kept fixed to the device base. When the lids are attached, they are clamped in place which fixes them to the gliding plate. When the motor is turned on, the moving arm displaces the gliding plate, and

subsequently the well plate lids. This displaces the specimens as well which are attached to the lid via the lid peg, applying a cyclic, tensile load.

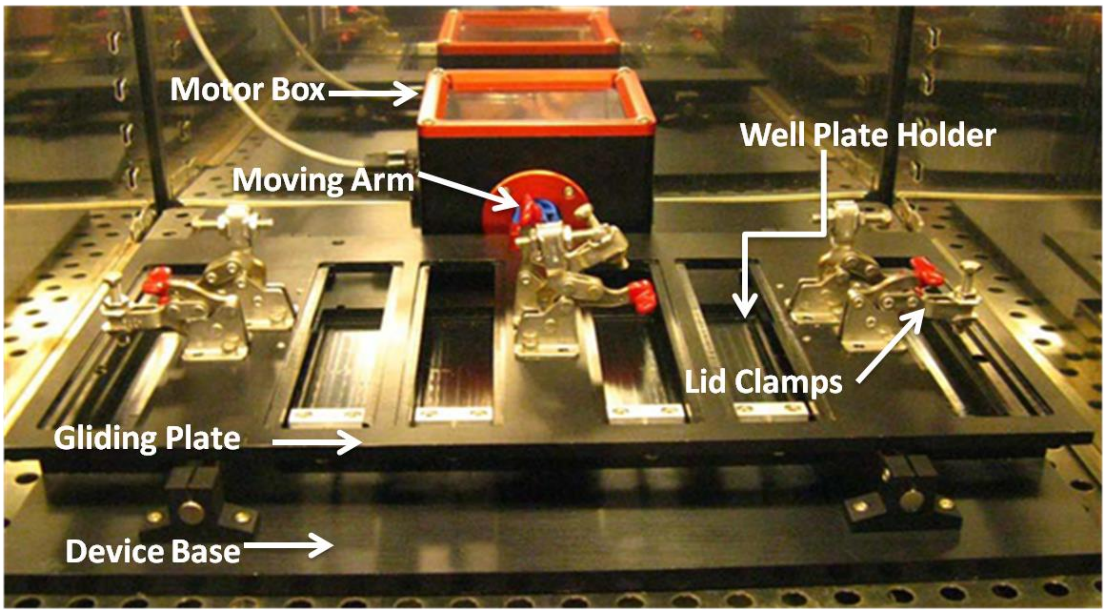


Figure 3.7: Full cyclic loading device in incubator consisting of an arm controlled by a motor that moves the gliding plate. Wells are placed in plate holders and lid clamps hold lids in place on gliding table to allow for cyclic tensile loading.

3.5.2.4 Loading Regime

Once samples were placed in the loading device, the lids were clamped to the gliding plate of the loading device to hold them in their starting position. A preload of 0.5N was applied to the specimens by hanging a 50g free weight from the lids of the well plates for 10 minutes. Once the pre-loading was complete, the device was moved from the sterile biosafety cabinet to the incubator at 37°C, 5% CO_2 and 95-98% humidity where it remained for the duration of the experiment for time points of either 1, 7, or 12 days. The specimens were loaded to 5% strain of the initial length at 1 Hz for four hours, and then

rested for 16 hours. This cycle was repeated for the duration of the loading experiment at each time point.

3.6 Mesenchymal Stem Cell Gene Expression

Gene expression of human mesenchymal stem cells seeded in fibrin or genipin-crosslinked fibrin gels (0.100 mg/mL genipin) was studied at days 1, 7, and 12. All samples were normalized to a cell only control. Gene expression at each time point was compared in gel samples that were left in 10% FBS DMEM for the culture period, gel samples that were implanted into a bovine tissue defect that was non-loaded and left in a 6 well plate with 10% FBS DMEM for the culture period, and samples that were implanted into a bovine tissue defect and loaded in tension to 5% strain in well plates containing 10% FBS DMEM for the culture period.

3.6.1 Trizol RNA Extraction

3.6.1.1 Homogenization

The entire work surface was sprayed down with RNase Zap (AM9780; Invitrogen) prior to starting RNA extraction. To homogenize gels, they were harvested in a 1.5mL RNA free microcentrifuge tube (14231-062; VWR) and samples were crushed with an 18 gauge needle. The samples were centrifuged at 14,000rpm for 3 minutes and any liquid found after centrifugation was removed. 1mL of Trizol (15596-018; Invigrogen) was added. The gels were broken up with multiple passes through an 18 gauge then a 21 gauge needle to homogenize the gels.

For cell only controls, 500,000 cells were placed into an RNA free tube and spun down at 1000rpm for 3 minutes after which any remaining liquid was removed. 1mL of Trizol was pipetted into the tube and pipetted up and down to mix well.

3.6.1.2 Phase Separation and RNA Precipitation

RNA was extracted according to the manufacturer's instructions (Invitrogen). The tubes with homogenized samples were left at room temperature for 5 minutes to complete dissociation then 200µL of chloroform (for every 1mL of Trizol) was added to solubilize the lipids and proteins. The mixture was pipetted up and down to mix well then vortexed for 15 seconds and left at room temperature for 2-3 minutes to complete dissociation. The samples were then centrifuged at 12,000g at 4°C for 15 minutes. After centrifugation, two phases were visible within the tube. The upper phase (RNA and DNA) was transferred to a new tube without touching the bottom phase (which contains proteins – contamination with proteins results in RNA degradation). 0.5mL of isopropanol per 1 mL of Trizol was added to precipitate the RNA and pipetted up and down vigorously. The solution was mixed and stored at room temperature for 10 minutes, then centrifuged at 12,000g at 4°C for 10 minutes. At this point, the RNA pellet was visible on the side of the tube.

The supernatant was discarded and 1mL of 75% ethanol was added and pipetted up and down to mix well, then further mixed by inverting the tube. Samples were vortexed for 3 seconds then centrifuged at 7,500g at 4°C for 5 minutes. Any liquid left in the tube was carefully removed by pipette and left to air dry on ice for 5-7 minutes. 30µL of ultra-

pure double distilled water (10977-015; Gibco) was added and pipetted up and down, vortexed and quickly spun down in the centrifuge before being placed back on ice.

3.6.1.3 RNA Quantification

After RNA isolation, each sample was mixed and vortexed. A GE NanoVue was used to quantify the RNA. The NanoVue was blanked using 2µL of ultrapure water then 2µL of each RNA sample was pipetted onto the sensor to determine the RNA concentration. The RNA yield, and the 280/260 and 260/230 ratios were recorded to ensure purity of the RNA from protein and ethanol contamination respectively. Samples were then immediately used for cDNA preparation or frozen in the -80°C for future use.

3.6.2 cDNA Extraction

Reverse transcription of RNA to cDNA was conducted using a cDNA Reverse Transcription Kit (4368814; Applied Biosystems). The components of the cDNA Reverse Transcription Kit were placed on ice then 50µL reactions were prepared containing 2µg of total RNA. Total RNA required for a 50µL reaction was calculated (with 2 µg total RNA per 50µL total reaction). A 25µL master mix was prepared for each 50µL reaction along with the RNA in H_2O according to the table below:

Table 3.2: Preparation for a 50µL Reaction

Component	Volume	Final Concentration
10x RT Buffer	5 µL	1x
25x dNTP Mix (100mM)	2 µL	0.08mM
10x RT Random Primers	5 µL	1x
Multiscribe Reverse Transcriptase	2.5 µL	50 U
Nuclease-free H_2O	10.5 µL	-
2 µg RNA + RNase Free ddH_2O	25 µL	-

The required amount of master mix for the desired number of reactions was prepared in RNASE free tubes (with 1-2 extra reactions prepared to account for pipetting error). The master mix was placed on ice and gently mixed. 25 µL of the master mix was pipetted into individual tubes followed by 25 µL of RNA/H_2O, pipetting up and down two times to mix well. The tubes were sealed then briefly vortexed to eliminate any air bubbles then loaded into the BioRad C1000™ Thermal Cycler. The reaction was carried out under the following conditions:

Table 3.3: BioRad C1000 Thermal Cycler Reaction Program for RNA Reverse Transcription

Stage	Time	Temperature
1	10 minutes	25°C
2	120 minutes	37°C
3	5 minutes	85°C
Cool Down	-	4°C

3.6.3 Real Time Quantitative Polymerase Chain Reaction (RT-PCR)

For real time PCR experiments (rt-PCR), a master mix for each gene was prepared containing the reagents shown in Table 3.4. The appropriate volume of master mix was prepared for each primer and vortexed well.

Table 3.4: Reagents used to prepare rt-PCR master mix for gene expression studies (volumes shown for each individual reaction).

Reagent	Volume
Taq Man Universal PCR Master Mix (4324018; AB)	10 µL
Primer	1 µL
H_2O	7 µL

For all rt- PCR experiments, the following primers were used:

Table 3.5: Primers used in gene expression studies of human bone marrow MSCs in fibrin and genipin gels.

Primer	Primer Type	Assay ID
Type I Collagen	Target	Hs00164004_m1
Type II Collagen	Target	Hs00264051_m1
Aggrecan	Target	Hs00153936_m1
Sox9	Target	Hs01001343_g1
18S Ribosomal	Endogenous Control	Hs03003631_g1 18S

All primers were obtained from Applied BioSciences and each sample was run in triplicate with each primer. The master mix was then pipetted into a 96-well plate, vortexing each sample prior to pipetting it and mixing up and down with the pipette tip frequently. After the master mix was added, 2 µL of cDNA was added to each well. For the negative control, 2 µL of H_2O was added to the well instead of cDNA. An optical adhesion cover was placed on top of the 96-well plate and the plate was mixed on a Fisher Microplate Mixer for 15 seconds then centrifuged at 1000rpm for a few seconds. The plate was then placed into an Applied Biosystems 7900HT Fast Real-Time PCR system. The reaction initiated for 10 minutes at 95°C before running through 40 cycles of 10 seconds at 95°C followed by 1 minute at 60°C.

3.6.4 Analysis of Real Time Polymerase Chain Reaction Data

To analyze the rt-PCR data, the comparative Cycle Threshold (Ct) method was used (Schmittgen and Livak, 2008). In this method, the Ct values of the samples of interest are compared with a control (non-treated sample). The non-treated sample for these experiments was confluent human bone marrow MSCs in monolayer culture. Both of these samples are also normalized to an endogenous control sample (18S housekeeping gene). To calculate the comparative Ct, or ΔΔCT values, equation 3.10 is used.

$$\Delta\Delta CT = \Delta Ct_{Sample} - \Delta Ct_{Reference} \qquad (3.10)$$

Where: ΔCt_{Sample} = Ct value of any sample normalized to the endogenous control gene

$\Delta Ct_{Reference}$ = Ct value for the control normalized to the endogenous control gene.

The fold induction for the gene expression was calculated using equation 3.11 and gene expression data was plotted in GraphPad Prism 6 software as fold induction increase for each gene in each sample.

$$Fold\ Induction = 2^{-\Delta\Delta Ct} \quad (3.11)$$

3.7 Histology

3.7.1 Fixation & Paraffin Embedding of Gels & Tissue

Samples to be used for histology were fixed in 4% paraformaldehyde (PFA) for 3-4 days then placed in a Leica TP1020 tissue processor for fourteen hours to be infiltrated with paraffin. The following program was followed in the processor to infiltrate samples:

Table 3.6: Processing times in various reagents for paraffin infiltration of samples used for histology.

Stage	Reagent	Time
1	H_2O	1 hr
2	80% Ethanol	1 hr
3	95% Ethanol	1 hr
4	95% Ethanol	1 hr
5	100% Ethanol	1 hr

6	100% Ethanol	1 hr
7	100% Ethanol	1 hr
8	Xylene Subs	1 hr
9	Xylene Subs	1 hr
10	Xylene Subs	1 hr
11	Paraffin	2 hr
12	Paraffin	2 hr

Samples were removed from the processor and embedded in paraffin wax then sliced using a microtome in 10µm sections and placed on glass microscope slides. Samples were then stored at room temperature until they were ready to be stained for histology.

3.7.2 Safranin O Staining

For Safranin O staining, samples were deparaffinised in Slide Brite (Sasco Chemical Group, Inc.) and hydrated in distilled water. Samples were stained in hematoxylin for 10 minutes to stain the nuclei black/blue followed by 10 minutes of washing in running tap water. They were then stained with fast green solution for 5 minutes, rinsed quickly in 1% acetic acid solution for no more than 10 seconds, then were stained in 0.1% Safranin O solution for 5 minutes. Slides were then dehydrated and cleared with 95% ethyl alcohol, absolute ethyl alcohol, and xylene using 2 changes each, 2 minutes each. Slide covers were mounted using resinous medium and samples observed under a light microscope at 5x, 10x and 40x magnification.

3.8 Statistics

Unless otherwise stated, all values are displayed as the mean ± standard error with error on graphs being shown as standard error. The data was analyzed in GraphPad Prism 6 with a Bonferonni Multiple Comparison Test used in cases where the F-test was significant. Significance was shown with a p-value of less than 0.05 ($p<0.05$).

Degradation studies and cell viability were analyzed using two-way ANOVA with time and the type of gel being the independent variables. Compression studies were analyzed using one-way ANOVA. Gene expression studies were analyzed using two-way ANOVA. Three gels were made for each condition (n=3) and each sample was run in three to six replicates (9-18 total measurements). The measurements for each sample were averaged, and the variance between the samples calculated using ANOVA.

Chapter Four: Material Selection & Characterization

This chapter presents the characterization results of a genipin-crosslinked fibrin gel to determine if it had suitable properties to be utilized in the repair of the annulus fibrosus. The initial studies aimed to determine if an existing, commercially available surgical adhesive could provide sufficient adhesion to annulus and be modified to enhance its mechanical properties for use in a tissue which undergoes complex loading. The results determined these materials were not satisfactory and fibrin gels, crosslinked with genipin, were characterized to determine if they could be seeded with human bone marrow mesenchymal stem cells for annulus repair.

4.1 Introduction

Surgical adhesives have been used in many cardiovascular and orthopaedic applications. An adhesive that would have the ability to secure a fibrocartilage to a focal defect site without the need for sutures would be an attractive solution for annulus repair. Stabilization of the transplanted tissue would promote integration with the host tissue, thereby overcoming some critical issues associated with tissue transplantation. The most widely used surgical adhesives today are fibrin, glutaraldehyde, cyanoacrylate, or hydrogel-based adhesives. Their clinical performance is dependent upon their physical properties such as their elasticity, tensile strength, and adhesive strength to biological tissues (Kull et al., 2009).

Cyanoacrylate adhesives rapidly solidify upon contact with weak bases such as water or blood (Petersen et al., 2004). There are several commercially available adhesives including Histoacryl (Braun, Michigan, USA) and Glubran (GEM, Italy) but only one available adhesive, Dermabond (Ethicon,USA), has FDA approval for topical wound closure. While cyanoacrylate glues have been shown to have superior adhesion over other types of surgical adhesives (Kull et al., 2009), due to their low biocompatibility and toxic degradation products (Jackson, 2001; Reckers et al., 2009), this family of adhesives was not investigated in this study.

Fibrin adhesives are formed through the conversion of fibrinogen to fibrin upon interaction with thrombin, mimicking the end stages of the blood coagulation cascade (Figure 4.1). Thrombin also activates fibrin stabilizing factor (factor XIII) which crosslinks the fibrin polymer in the presence of calcium resulting in strong, covalent bonds (Petersen et al., 2004). Fibrin adhesives are utilized in various surgical applications for tissue adhesion, suture support, hemostasis, and wound care (Petersen et al., 2004). This is an attractive class of adhesives for internal applications because they are biocompatible, fully resorbable, and produce no inflammatory response, foreign body reaction or tissue necrosis (García Páez et al., 2004; Jackson, 2001). While these adhesives are elastic, they have poor tensile and adhesive strength, must be applied to a dry surface (Kull et al., 2009) and have been shown to provide insufficient fixation for meniscus repair, a tissue with similar biological and mechanical properties to the AF (Reckers et al., 2009).

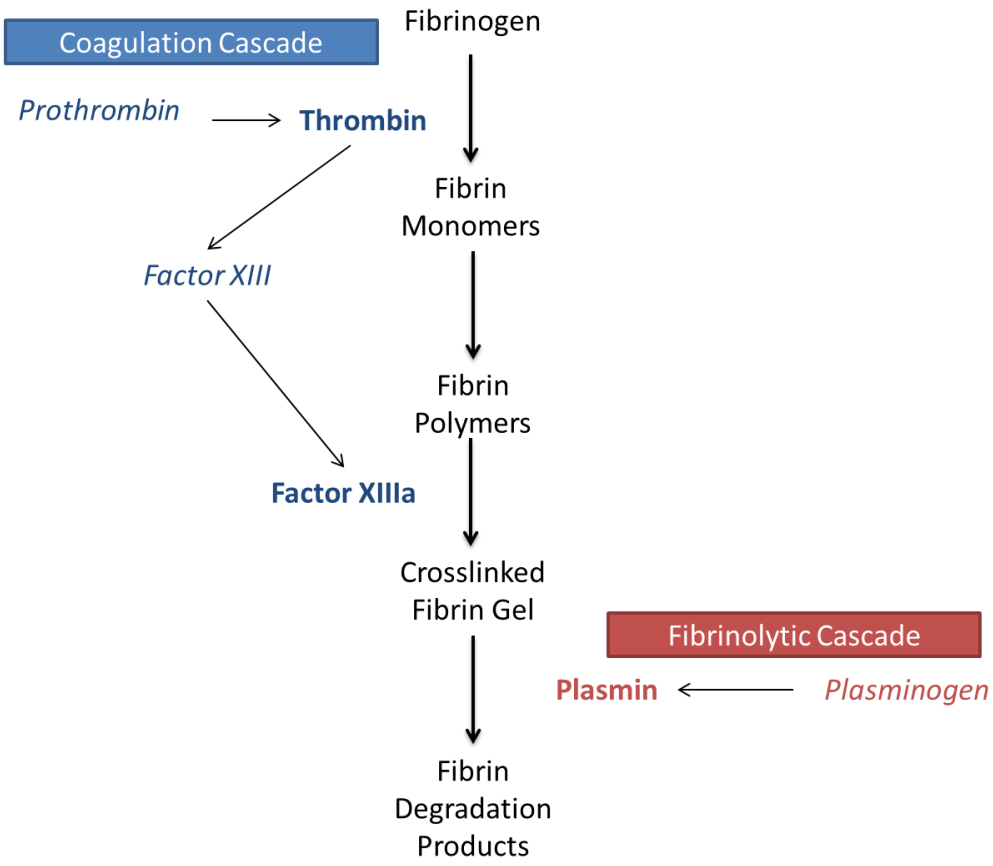


Figure 4.1: End stage of blood coagulation cascade where fibrinogen is converted to a gel via crosslinking with thrombin and degraded by plasmin.

Tisseel is a fibrin adhesive produced by Baxter (Illinois, USA) and is indicated for use as a sealant to be applied topically as an adjunct to hemostasis in surgeries. Tisseel is comprised of two components, a sealer protein solution and a thrombin solution. The sealer protein solution contains fibrinogen (67-106 mg/mL) as well as a fibrinolysis inhibitor to prevent degradation. The thrombin solution contains 400-625 UN/mL of human thrombin as well as 34-44 µM/mL calcium chloride.

Glutaraldehyde based adhesives typically contain an albumin component and adhesion is achieved via a condensation reaction between the two components. The glutaraldehyde reacts with amine groups in the albumin protein, specifically by binding lysine molecules together, resulting in a cross-linked network (Dumitriu and Popa, 2013). Bioglue (CryoLife International Inc, Georgia, USA) is a glutaraldehyde based adhesive composed of 10% glutaraldehyde and 45% bovine serum albumin solution (BSA). It is dispensed through a double chambered system where the glutaraldehyde molecules from one chamber crosslink the bovine serum albumin molecules dispensed through the other simultaneously with the tissue proteins at the repair site to create a flexible, mechanical seal (Passage et al., 2002). Bioglue begins to polymerize within 20-30 seconds and reaches its full bonding strength within two minutes. It is indicated by the manufacturer as an adjunct to standard methods of achieving hemostasis (such as sutures or staples) in adult patients in open surgical repair of large vessels, but has not been approved for neurosurgery, including as a dural sealant, as it hasn't been evaluated for its safety and effectiveness in these applications.

FloSeal (Baxter, Illinois, USA) is a gelatin matrix from the extraction of collagen which is followed by gelation, crosslinking and stabilization with glutaraldehyde (Oz et al., 2003). When the gelatin granules are exposed to blood, they swell and reduce the blood flow. The adhesive then functions through a similar mechanism to fibrin adhesives whereby fibrinogen in the blood is exposed to thrombin in the adhesive which converts it to a fibrin polymer, physically entrapping the granules and resulting in a clot composed of the FloSeal collagen granules (Oz et al., 2003).

Surgical adhesives have potential as a starting material for annulus fibrosus repair if they could seal a tear or rupture in the annulus directly and be modified to enhance their mechanical properties to match those of the AF. However, adhesives have been cited as either having insufficient mechanical properties or producing a significant inflammatory response, which may make them inappropriate for this application. For this reason, fibrin gels were also investigated as a biomaterial for repair of the AF and characterized in this chapter.

Fibrin gels, similar in their formation to the clots in fibrin adhesives, have been widely studied as materials for cell delivery in tissue engineering (Fussenegger et al., 2003; Jung et al., 2010; Osathanon et al., 2008; Park, 2005). Fibrin gels on their own, however, have insufficient mechanical properties to repair a tear in the AF. For this reason, recent studies have investigated the use of crosslinkers to help stabilize mechanically weak gels such as fibrin and increase their stiffness (Schek et al., 2011).

Genipin ($C_{11}H_{14}O_5$) is a naturally derived crosslinking agent extracted from the plant *Gardenia jasminoides Ellis*. It has been used in herbal medicine to reduce inflammation, fever, and promote the discharge of bile (Yao et al., 2004). Genipin has anti-inflammatory effects *in vivo* and has been shown to be 10,000 times less toxic than glutaraldehyde to 3T3 fibroblasts (Sung et al., 1999), making it an attractive alternative to other commonly used crosslinking agents. While the exact mechanism of the reaction is not well understood, genipin functions by crosslinking functional groups to amino acids

on adjacent proteins (Yao et al., 2004) with two possible mechanisms proposed by Butler et al. (Butler et al., 2003).

To determine if genipin-crosslinked fibrin gels had suitable properties for tissue engineering applications in the annulus, genipin-crosslinked gels have been characterized and compared to fibrin gels alone to see if they had comparable cell compatibility while increasing their stiffness (compressive moduli) and stability (degradation time).

4.2 Results

4.2.1 Tensile Testing of Commercially Available Adhesives

To determine if existing, commercially available surgical adhesives have sufficient bond strength for applications in annulus repair, three adhesives were selected to test their adhesive strength to explanted pieces of bovine annulus tissue. Tisseel (Fibrin Glue, Baxter), Bioglue (Glutaraldehyde, Cryolife) and FloSeal (Collagen, Baxter) were prepared according to the manufacturers' instructions and used to adhere two pieces of annulus tissue in a lap joint configuration to test apparent shear strength or butt joint configuration to test for wound closure strength according to ASTM standards F2255-05 and F2458-05 respectively.

Pilot studies were carried out using super glue (cyanoacrylate) to prepare specimens (Figure 4.2) and develop the testing protocol on the Bose Electroforce tensile testing device.

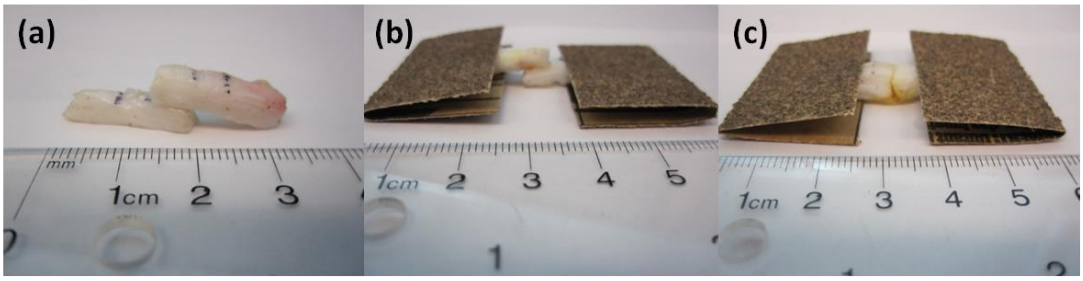


Figure 4.2: Samples prepared for pilot tests using superglue. (a) Annulus in a lap joint configuration, (b) lap joint configuration with sandpaper grips, and (c) butt joint configuration with sandpaper grips.

Samples were prepared using FloSeal, BioGlue and Tisseel for testing in a lap joint configuration. The FloSeal, once reconstituted, was a very light and porous material (Figure 4.3) that did not produce any adhesion to the annulus tissue. Upon application and placing the two pieces of annulus tissue in apposition, the FloSeal seeped out between the pieces of tissue and did not form any type of bond. With no bond formed, tissue samples were not tested in the tensile testing device for this adhesive.

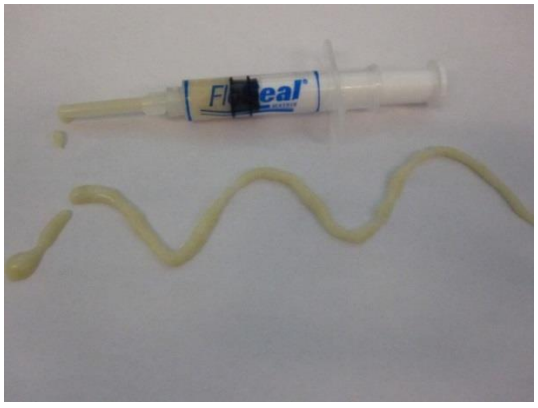


Figure 4.3: FloSeal applicator syringe and the porous material produced after reconstitution of the components as per the manufacturer's instructions.

Using the manufacturer's instructions, BioGlue was then reconstituted and samples were prepared for application of the adhesive. Upon application of the adhesive to the tissue, the glue was very difficult to work with. The samples were insufficiently glued together and the adhesive bond area was not confined to the desired area, even with boundaries covered in petroleum jelly to limit the bonding. After samples were prepared, the adhesion was very poor and all of the samples broke during handling, indicating that the adhesive bond formed by the BioGlue was insufficient, and none of the samples were mechanically tested.

Tisseel was successfully fabricated according to the manufacturer's instructions and samples were prepared in both a lap joint and butt joint configuration. The samples were loaded in the axial direction, and the glue could be seen visibly stretching in strings while still adhered to the tissue and slowing peeling away from the tissue. The results of the tensile test with Tisseel were analyzed, however the amount of force required to break the samples was so low that it could not be distinguished from experimental noise using a 450N load cell, with an accuracy of 0.5% (2.25N).

Due to the lack of success with creating a strong adhesive bond to the bovine annulus specimens with any of the commercial adhesives (Table 4.1), no further tensile testing was conducted with surgical adhesives.

Table 4.1: Summary of tensile testing results with commercially available surgical adhesives and bovine annulus tissue.

Commercial Adhesive	Type of Adhesive	Result
Tisseel (Baxter)	Fibrin Glue	Insignificant amount of force required to break sample (<2.25N).
BioGlue (Cryolife)	Glutaraldehyde	Samples broke while loading into the tensile testing machine (force of gravity)
FloSeal (Baxter)	Collagen-Based Hydrogel	No adherence to samples.

4.2.2 Characterization of Genipin-Crosslinked Fibrin Gels

Due to the ineffectiveness of the tested surgical adhesives for producing an adhesive bond to bovine annulus tissue, fibrin gels crosslinked with genipin and seeded with human bone marrow MSCs were studied as a potential material for annulus repair. Fibrin gels alone are known to have poor mechanical properties and stability, they have previously been crosslinked with genipin to enhance their mechanical properties (Dare et al., 2009; Schek et al., 2011) to make them appropriate for tissue engineering applications in annulus repair. The fibrin gels were crosslinked with low concentrations of genipin and characterized to determine if MSCs could be seeded within the gels while maintaining viability and sufficient mechanical properties.

4.2.2.1 Fabrication of Genipin-Crosslinked Fibrin Gels

Two methods for fabricating genipin-crosslinked fibrin gels were modified from the literature and compared. For fabrication method #1 (Lien et al., 2008), the fibrinogen (100 mg/mL) and thrombin (100 UN/mL) were mixed and allowed to polymerize for one hour before adding genipin (0.1 mg/mL) on top of the gels. The genipin was allowed to permeate through the gels and crosslink over a 24 hour time period. In fabrication method #2 (Schek et al., 2011), genipin (0.1 mg/mL), thrombin (100 UN/mL), and fibrinogen (100 mg/mL) were initially mixed together, allowing the genipin to crosslink the fibrinogen as it polymerized.

To determine which of the methods produced a more stable gel, a degradation study was conducted in which gels crosslinked using method #1 or method #2 were cultured in 8mL of 10% FBS DMEM in an incubator at 37°C, 5% CO_2 and 95-98% humidity. Fibrin gels that were not crosslinked were used as a control to determine if crosslinking the gels using either method produced a more stable gel. Regular wet weight measurements were taken to determine the change in mass over time and plotted to show degradation profiles (Figure 4.4).

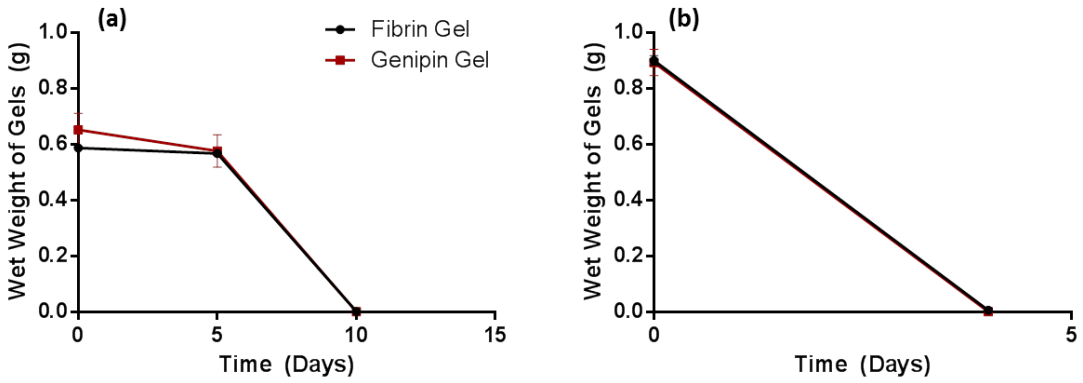


Figure 4.4: Degradation profiles of fibrin and genipin-crosslinked fibrin gels (genipin gels) over time. Gels were fabricated using (a) method #1 and (b) method #2 (n=3; mean ± standard error).

Due to the rapid degradation of gels fabricated using method #2, this method was not investigated any further. However, even with fabrication method #1, the gels fully degraded within 10 days which is unacceptable for a gel to be used for a tissue engineering repair solution in the annulus. Further studies were conducted to determine if the degradation time could be reduced. The fabrication method was further refined and fabrication method #3 was developed. In this method, thrombin, $CaCl_2$, genipin, and fibrinogen were all combined at the same time and allowed to polymerize for one hour. To further stabilize the gels, an antifibrinolytic agent was added to the media. It has been shown that the addition of chemicals that inhibit fibrinolysis, the breakdown of fibrin, can increase the stability of gels and decrease the degradation time (Fussenegger et al., 2003). To reduce the degradation time of the gels, 6-aminohexanoic acid at a final concentration of 2 mg/mL was added to the 10% FBS DMEM in which the gels were cultured as this

has been shown to be effective at increasing gel stability to over five weeks (Dare et al., 2009).

Another degradation study was conducted and the change in wet weight over time compared between the fibrin and genipin gels (Figure 4.5).

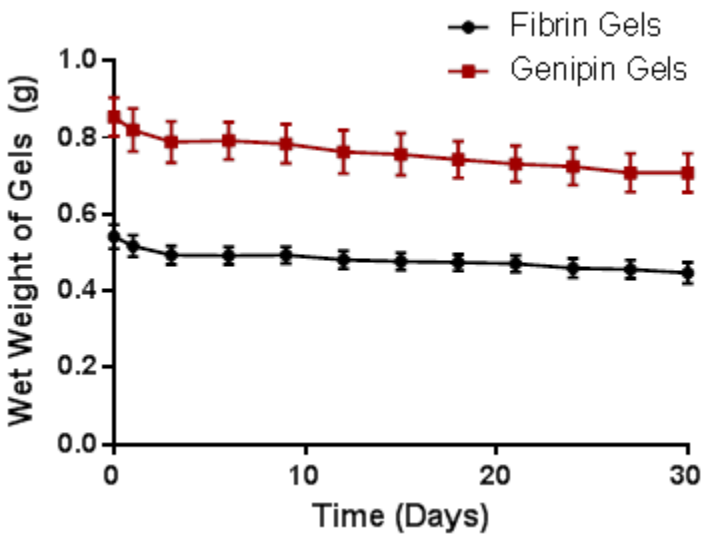


Figure 4.5: Degradation profiles of fibrin and genipin-crosslinked fibrin gels (genipin gels) fabricated using method #3 (n=3; mean ± standard error).

Fabrication method #3 produced gels that were stable over a 30 day time period and did not significantly change in weight over the 30 days (two-way ANOVA, $p<0.05$). The new fabrication method combined with 6-aminohexanoic acid had a significant, positive effect on stabilizing both the fibrin and genipin-crosslinked gels over a 30 day time period and therefore was utilized for all subsequent experiments.

4.2.2.2 Cell-Mediated Degradation

A cell-mediated degradation study was conducted to determine if the addition of cells would affect the stability of the gels over time. Due to the significant difference in initial weight of fibrin and genipin gels produced using method #3, the degradation was plotted as a percent change in the gels original weight (Figure 4.6).

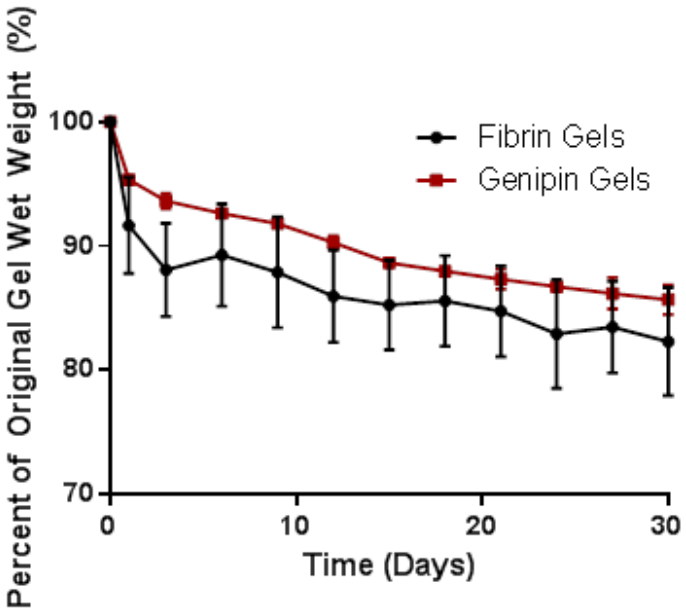


Figure 4.6: Degradation profile of fibrin and genipin-crosslinked fibrin gels (genipin gels) containing $5x10^5$ MSCs over time (n=3; Mean ±Standard Error).

Two-way ANOVA ($p<0.05$) showed the genipin and fibrin gels both had significant changes in weight over the 30 days, however even after this time period they both retained 80% of their original wet weights. There was no significant difference in the change in gel weight between the fibrin and genipin gel groups at any time point,

indicating that both are stable over 30 days with or without cells seeded in them. The results of all the degradation studies are summarized in Table 4.2.

Table 4.2: Summary of degradation study study findings comparing various methods of fabricating fibrin and genipin-crosslinked fibrin gels. Total time until dissolution or percent of original wet weight remaining at the end of degradation study for each fabrication method is shown.

Fabrication Method	Type of Gel	Days Until Dissolution
Fabrication Method #1 (No Cells)	Fibrin	4
	Genipin	4
Fabrication Method #2 (No Cells)	Fibrin	10
	Genipin	10
Fabrication Method #3 (No Cells)	Fibrin	Stable at 30 Days *(82.8 % of Original Wet Weight)*
	Genipin	Stable at 30 Days *(82.8% of Original Wet Weight)*
Fabrication Method #3 (Cells)	Fibrin	Stable at 30 Days *(82.3% of Original Wet Weight)*
	Genipin	Stable at 30 Days *(85.7 % of Original Wet Weight)*

4.2.2.3 Confined Compression Testing

Genipin-crosslinked fibrin gels have previously been shown to maintain a cell viability around 50% when chondrocytes were encapsulated in gels containing 0.045 mg/mL of genipin (Dare et al., 2009). However, in order to increase the mechanical stiffness of the gels, higher concentrations of genipin may be required. A confined compression study was conducted using genipin-crosslinked fibrin gels with genipin concentrations of 0.025 mg/mL, 0.050 mg/mL and 0.100 mg/mL to determine whether different concentrations of genipin crosslinker would significantly affect the mechanical stiffness of the gels, while maintaining a concentration in a range that would likely not have a detrimental effect on cell viability. Fibrin gels that were not crosslinked were used as a control to determine if various crosslinking concentrations increased gel stiffness.

Compression testing was conducted on a Bose Electroforce 3200 testing device (Figure 4.7b) to determine the aggregate modulus of fibrin and genipin-crosslinked fibrin gels. Fibrin and genipin gels were tested immediately after removing them from a 24 well plate, as the gels were an appropriate size for the confined compression chamber (12.8mm) (Figure 4.7a).

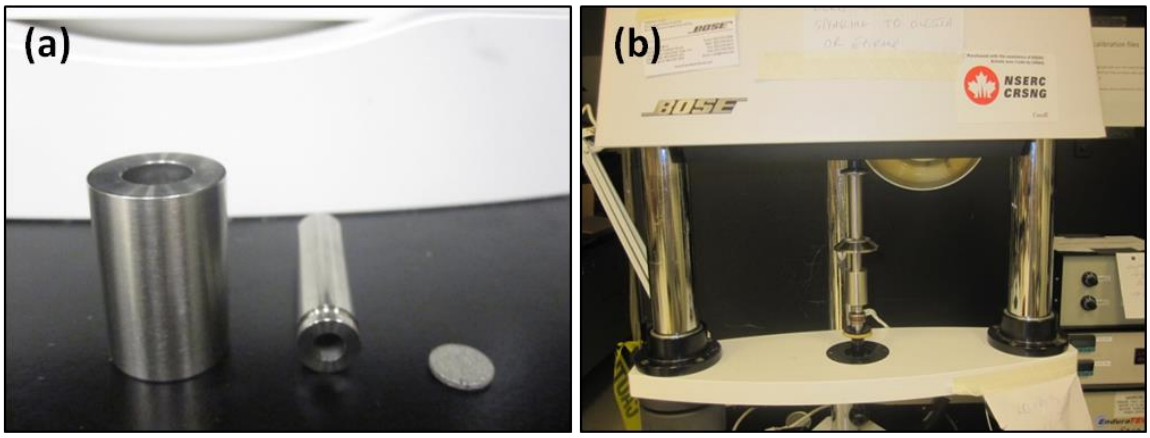


Figure 4.7: Confined compression experimental set up with (a) compression chamber, plunger and porous plug and (b) Bose Electroforce testing device with compression chamber ready for loading.

Three gels at each concentration were tested in compression at a constant rate of 0.02mm/s to 15% strain. The gel was then released and immediately compressed to 15% strain again. Three compressions in succession were used to ensure that full contact was being made and to remove excess liquid from the gel in the initial run, which ensured that more accurate properties of the material were being tested in the second run and third runs. Due to failure of the gel in several samples on the third run, the data from the second run of each compressed sample was used for analysis.

The force and displacement raw data from the testing device was converted into a stress strain curve, which yielded a curve that had a relatively linear region from approximately 0-10% strain. The curves for each of the three runs for each sample were plotted as shown Figure 4.8 with the fibrin gel sample below. Typically, the first run exhibited a

shallower slope with a lower peak stress which then increased in the second and third runs.

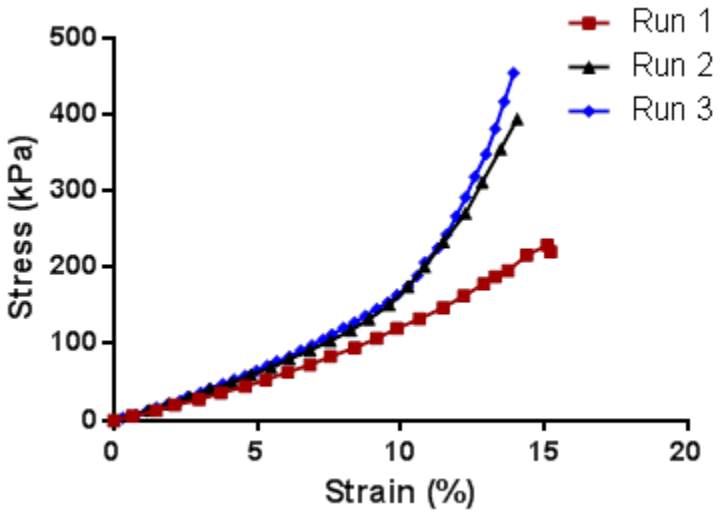


Figure 4.8: Stress strain curve of a sample fibrin gel for each of three runs by compressing samples from 0-15% strain at 0.02mm/s.

To determine the aggregate modulus, the slope of the linear region between 0-10% strain was obtained for the second runs for all of the samples (n=3) and averaged (Figure 4.9). There was a trend of increasing compressive modulus with an increase in genipin concentration. Only the 0.100 mg/mL genipin-crosslinked gels had a significantly greater aggregate moduli (17.17± 0.677MPa) than the fibrin gels (1320± 95kPa) when compared using one-way ANOVA ($p<0.01$).

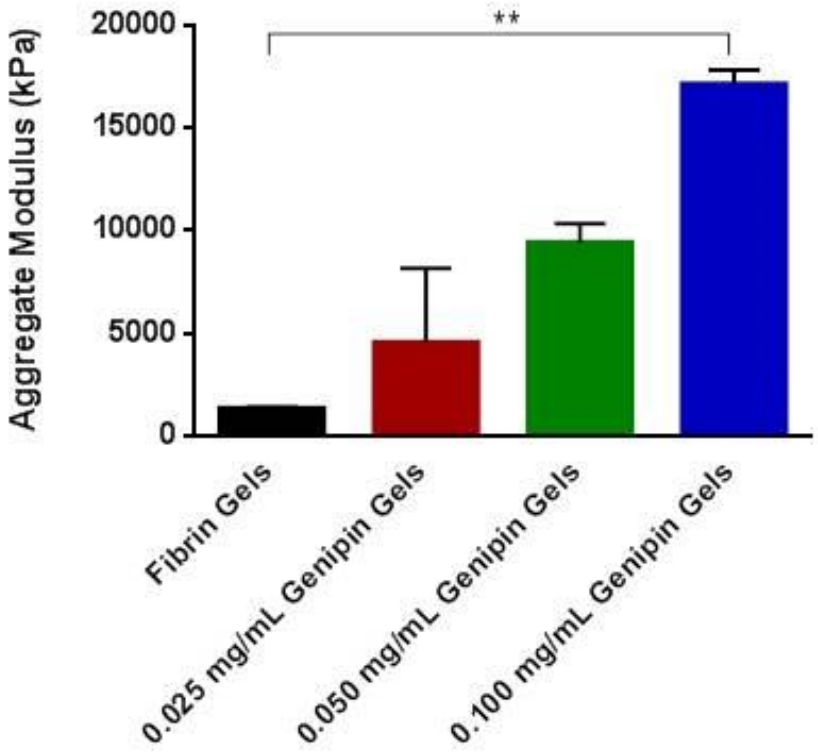


Figure 4.9: Aggregate modulus of fibrin and genipin gels (n=3) at 0-10% strain crosslinked with various concentrations of genipin (mean + standard error). Genipin gels compared to fibrin gel using one-way ANOVA ($p<0.05$)

4.2.2.4 Cell Viability

Cell viability within the gels was determined by staining the gels with calcein and ethidium homodimer-1 and imaging them under a confocal microscope. For this study, cell viability was studied over an extended period of time in the gels. Both fibrin gels and gels crosslinked with 0.100 mg/mL genipin were cultured for one, eight, or twelve days. Gels were then stained with calcein and ethidium homodimer-1 and immediately imaged on a confocal microscope.

Calcein AM permeates into live cells and is enzymatically converted from non-fluorescent Calcein AM to a calcein which fluoresces green. Ethidium homodimer-1 enters cells with a damaged membrane and fluoresces red upon binding with nucleic acids, marking dead cells (Invitrogen, 2013). Figure 4.10 shows a higher percentage of cells stained green than red and there doesn't appear to be a significant change in cell numbers or viability over time. Both live and dead cells were counted to determine the percent cell viability and plotted over time (Figure 4.11).

There was a general trend of decreasing cell viability over time, but only the change in viability from day 1 to day 12 for the fibrin gel group was significant by two-way ANOVA analysis ($p<0.05$). There was no significant change in cell viability over the twelve day time period for the genipin-crosslinked gels or in cell viability between the fibrin and genipin cells at any time point.

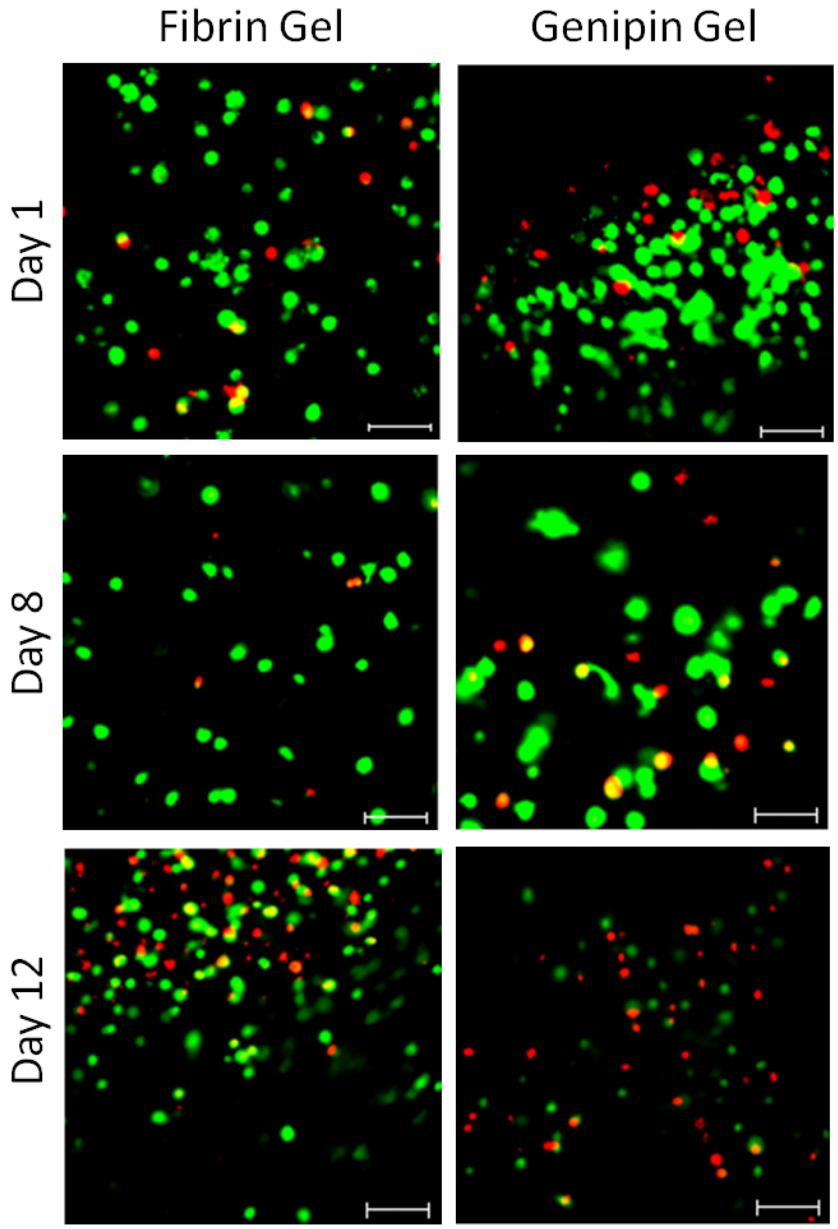


Figure 4.10: Live-dead cell images taken on confocal microscope (single slice) showing live (green) and dead (red) cells in genipin and fibrin gels over time. Images taken at various locations within gels. Scale bar is 50µm.

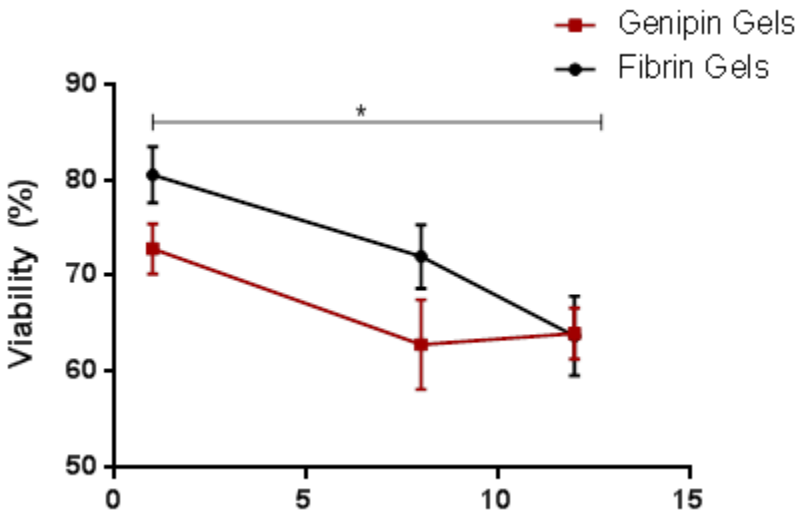


Figure 4.11: Percent cell viability calculated in a region of interest, randomly located within fibrin and genipin gels over time (n=3; mean ± standard error). A minimum of six images were analyzed for each sample. Significance level * = $p<0.05$ for two-way ANOVA compared viability in the fibrin gels over time.

4.3 Discussion

Commercially available surgical adhesives were investigated for their potential to repair the annulus fibrous. Initial pilot studies using a cyanoacrylate-based super glue showed that it was possible for an adhesive material to produce a strong bond to bovine AF tissue for the tensile tests. The samples failed at the interface between the two adhered pieces of AF tissue, indicating that the strength of the tissue itself was greater than that of the adhesive (data not shown). The commercial surgical adhesives tested in this study produced little or no adhesion to bovine AF tissue and are therefore insufficient for applications in repairing the AF. The FloSeal matrix produced no bond with the material

whatsoever, which was not surprising due to the fact that the adhesive functions in the presence of blood by forming a "clot" that encapsulates the collagen matrix (Oz et al., 2003), and there was minimal amounts of blood present in the bovine tissue. In applications with a fibrocartilage that is highly avascular, FloSeal is an inappropriate adhesive due to its mechanism of reaction.

BioGlue did appear to achieve a minimal amount of bonding with the annulus, however even after allowing it to fully polymerize, the adhesive bond was extremely fragile and minimal amounts of handling were sufficient to break the test samples, regardless of whether they were prepared in a lap joint or butt joint configuration. Although BioGlue is not indicated for use in dural repair, it has been used for this application successfully in the lumbar spine to seal the dura (Yuen and Kaye, 2005). Due to the fact that BioGlue was successfully used in repairing this stiff, inflexible, connective tissue, it was expected that it would produce a stronger bond to a fibrocartilage. Its insufficient bond strength, however, makes it inappropriate for further investigation in a loadbearing, elastic tissue such as the AF.

Fibrin glue was the only surgical adhesive tested that produced a strong enough bond to be loaded and successfully tested in tension, however the force required to break the bond was insignificant. This result is consistent with other findings that fibrin glues and gels, while possessing desirable properties for biocompatibility, produce insufficient bond strength for annulus repair (Reckers et al., 2009). Due to its high cell compatibility and

ability to guide ECM deposition (Jung et al., 2010), however, fibrin as a biomaterial for AF repair was deemed to warrant further investigation.

Crosslinking is widely used to improve the tensile strength of a wide variety of materials, particularly in tendon and ligament engineering (Alfredo Uquillas et al., 2012). While many traditional crosslinking agents have been investigated for use in cartilage tissue engineering, including glutaraldehyde, due to their high cytotoxicity alternative methods of crosslinking must be explored. Recently, genipin has been studied (Dare et al., 2009; Kim et al., 2010; Lien et al., 2008; Schek et al., 2011; Zhang et al., 2010) as an alternative crosslinking agent to traditional methods due to its comparatively low cytotoxicity (Sung et al., 1999).

Using a high concentration of genipin, 400 mg/mL in a 0.25:1 ratio with fibrinogen, to crosslink fibrin gels has been shown to enhance their mechanical properties and the material in lap tests adhered to AF tissue up to strains of 15-30% before failing (Schek et al., 2011). Cells seeded on gels with high concentrations of genipin, however, had slow proliferation, indicating that they would not be ideal for developing a reparative solution, but rather as an adhesive for small annular defects.

Integration is a critical element for success of a tissue engineering repair strategy. If a construct designed to bridge a gap in the annulus doesn't have the ability to maintain cell viability and encourage proliferation and deposition of ECM, there is little chance the solution will ever integrate with the host tissue and therefore wouldn't produce a long term, regenerative solution for AF repair. As the goal of this project is to achieve

regeneration of tissue within a biomaterial implanted in an AF defect, the ability to support cellular functions within the construct was essential. Therefore lower concentrations of genipin crosslinker were investigated to determine if they would have a significant effect on gel stiffness while still maintaining high cell viability and stability over time.

Two fabrication methods previously described in the literature were modified and compared in a degradation study to determine if one method would produce more stable gels than the other. The gels fabricated using method #2 completely dissolved within 4 days, making them an inappropriate choice for a tissue engineering construct. The gels fabricated using method #1 were more stable, degrading completely after 10 days. Due to the ineffectiveness of either of these solutions, further literature review and troubleshooting yielded an improved method for fabrication, method #3, which was used for all subsequent experiments.

One of the major detriments to utilizing fibrin gels for tissue engineering applications is their rapid degradation rate (Ahmed et al., 2010). Degradation of fibrin occurs via fibrinolysis, whereby enzymes such as plasmin breakdown fibrin fibres and digest fibrin (Fussenegger et al., 2003). Plasminogen, the precursor for plasmin, may be present in low levels in fetal bovine serum (FBS) that is added to the culture media (Tuan and Grinnell, 1989) or may be activated by plasminogen activators released by cells within the gels (Radosevich et al., 1997). Crosslinking of fibrin hydrogels with genipin has been previously shown to increase their stability (Dare et al., 2009), however it has also been

shown that even crosslinked fibrin gels may still be unstable and the addition of fibrinolytic inhibitors may be required in order for them to maintain stability over time (Eyrich et al., 2007).

Many fibrinolytic inhibitors have been investigated with fibrin gel constructs with the most common being aprotinin and epsilon-aminocaproic acid (6-aminohexanoic acid). Stabilization of fibrin-chondrocyte constructs was achieved by the addition of aprotinin and tranexamic acid into scaffolds that remained stable over a four week *in vitro* culture (Fussenegger et al., 2003). These constructs were then successfully implanted into articular cartilage defects in rabbits. Previously in our lab, 6-aminohexanoic acid has been successfully used to stabilize fibrin constructs (Dussault, 2002), and a final concentration of 2 mg/mL of this antifibrinolytic was utilized for all further studies.

Another degradation study was conducted with fabrication method #3 and 2 mg/mL of 6-aminohexanoic acid added to the culture mediuim. Both fibrin and genipin gels fabricated using this method were stable over a 30 day time period with no significant change in weight.

Another degradation study was conducted investigating the effects of the addition of cells to the fibrin and genipin-crosslinked gels. Human bone marrow mesenchymal stem cells were selected as a cell source for these experiments due to their clinical potential to provide an autologous cell source. It has been shown that with increasing density of cells within fibrin gels, the degradation rate increases (Meinhart et al., 1999), however these gels were stable over 30 days, even with the addition of hb-MSCs.

Wet weight can be a good indication of whether a gel is degrading or not over time, however with hydrogels, changes in wet weight can also be caused by increases or decreases in the water (for example if they swell or contract) and don't necessarily show that the material has degraded. To confirm that the decreases in wet weight over time are a result of degradation and not contraction of the gel, dry weight measurements could also be taken of the gels by lyophilizing them and weighing the dry material remaining at each time point. This requires a very high number of samples, however, and since the gels were shown to be stable over 30 days with minimal changes in weight, whether by degradation or contraction, they have shown sufficient properties for further investigation.

In addition to being chemically stable over time, gels utilized for AF repair must be able to withstand significant mechanical forces *in vivo*. The annulus experiences high circumferential tensile and shear forces in the outer region, with a tensile modulus of 20MPa and a shear modulus of 0.1MPa required of the annulus to withstand the forces (Stokes and Iatridis, 2005). Radial compression is also experienced in the inner annuls. This complex loading is difficult to mimic *ex vivo*, however it is important that a biomaterial meet at least minimal mechanical requirements to be considered for further investigation as an annular repair material.

Confined compression tests were conducted to determine the compressive modulus of the material to obtain an indication of gels stiffness. Confined compression was selected as it most closely mimics the *in vivo* loading conditions of the restricted IVD (Nerurkar et al.,

2010). Since fibrin gels crosslinked with genipin at a concentration of 0.045 mg/mL have previously been shown to support viable chondrocytes (Dare et al., 2009), concentrations within this range were used for the compression study to determine how great of an effect the genipin concentration had on the stiffness of the gels.

For this study, fibrin gels were compared to genipin gels containing 0.025 mg/mL, 0.050 mg/mL and 0.100 mg/mL of genipin crosslinker. There was an increase in the aggregate moduli of the gels with increasing genipin concentrations, with gels crosslinked with 0.100 mg/mL of genipin producing the highest modulus of 17.17±0.677 MPa. This was an significant ($p<0.05$) increase over the modulus of the fibrin gels (1320± 95kPa). The native annulus has been shown to have an aggregate modulus in the range of 440-750 kPa in confined compression (Iatridis et al., 1998) indicating that the gels tested in confined compression would have sufficient stiffness in compression to match the native annulus. Since gels crosslinked with genipin at a concentration of 0.100 mg/mL showed a significant increase in stiffness, this concentration of genipin was used for all subsequent studies.

The aggregate modulus gives a good indication of the stiffness of the material, however there are many other mechanical properties including the tensile and shear moduli which are also critical factors to the function of the annulus. The stiffness of the gel is sufficient to show that the material warrents further investigation, as the goal of this study was to improve the stiffness and stability of fibrin gels which have shown to be weak. However further studies could be conducted to characterize the gels fully in terms of their mechanical properties.

To determine if a concentration of 0.100 mg/mL of genipin would retain sufficient cell viability in the gels, a viability study was conducted with fibrin and genipin-crosslinked gels at this concentration. Fibrin and genipin gels were seeded with $5x10^5$ human bone marrow MSCs and were cultured for 1, 8, or 12 days prior to being stained with calcein and ethidium homodimer-1 and imaged to determine cell viability within the gels.

At each time point, there was no significant difference in cell viability between the fibrin gels and the genipin gels, with consistent viability between 60% - 85%. There was a significant difference in cell viability in the fibrin gel group between days 1 and 12, with the viability dropping from 80.52% on day 1 to 63.72% on day 12. Cell viability appeared to be generally higher in fibrin gels than in those crosslinked with 0.100 mg/mL of genipin, however the difference was not significant, indicating that gels crosslinked with 0.100 mg/mL of genipin would be an appropriate scaffold material for MSCs.

There are challenges in obtaining accurate live/dead cell counts from materials in which the cells cannot be removed, such as these gels. While taking several images from multiple locations within the gel allows for a good indication of where the cells are and what the viability is, it involves some human error in which a region of interest may be selected because of a high cell number or viability due to investigator bias. Additionally, there's human error in counting the cells using Image J, where inconsistencies in what is classified as a cell and what is not can lead to variation in the results. These biases were minimized by taking images at random locations in the gels and by conducting all of the cell counts in one sitting. The brightness threshold was adjusted to the same level on all

of the images to reduce the background, and a consistent method of counting cells was developed, so even if the numbers may vary from the true viability, the method for counting cells in all conditions was consistent.

4.4 Conclusions

The surgical adhesives tested produced insufficient bond strength to bovine AF tissue and do not warrant further investigation for use as a biomaterial for the repair of defects in the AF. Fibrin glue was the only adhesive to produce a bond with the tissue at all, indicating that fibrin gels may have adequate mechanical and adhesive properties to repair a defect in the annulus if they were crosslinked with a cytocompatible crosslinking agent such as genipin.

Degradation of the fibrin and genipin-crosslinked gels was a significant problem, and as a result, 2 mg/mL of 6-aminohexanoic acid was added to the culture medium to reduce the effects of fibrinolysis and decrease the degradation rate. With the selected fabrication method and addition of the antifibrinolytic agent, the gels were stable over a 30 day time period with and without cells, indicating that the material would have sufficient stability for further investigation.

Genipin gels crosslinked using 0.100 mg/mL of genipin had significantly higher compressive moduli than fibrin gels that were not crosslinked and using genipin at this concentration may produce gels that have appropriate mechanical properties while maintaining cytocompatibility. At a concentration of 0.100 mg/mL, the genipin produced

a gel that had comparable cell viability at 1, 8, and 12 days to fibrin gels that weren't crosslinked.

Overall, fibrin gels crosslinked with 0.100 mg/mL of genipin have sufficient biological compatibility and mechanical stiffness for use in annular repair and should be investigated further as a repair material in fibrocartilage tissue engineering applications.

Chapter Five: Annulus Fibrosus Damage Tissue Integration Model

Genipin-crosslinked fibrin gels have been identified as a material for the repair of defects in the annulus fibrosus (Dare et al., 2009; Schek et al., 2011). While the properties of the material have been characterized, the effects of culturing mesenchymal stem cells (MSCs) within these gels are unknown. It is desirable for cells to differentiate within a biomaterial and start to deposit new extracellular matrix to promote integration with host tissue.

This chapter discusses the results of gene expression and histology experiments conducted to investigate the effects of culturing human bone marrow MSCs in fibrin gels compared to genipin-crosslinked fibrin gels. The effect on gene expression was also studied in genipin-crosslinked fibrin gels used to plug a defect in bovine annulus tissue, under a tensile load and unloaded.

5.1 Introduction

The differentiation fate of mesenchymal stem cells is affected by many factors including their biochemical and mechanical environments. MSCs have the ability to differentiate into a variety of cell lineages to produce bone, cartilage, fat, muscle, and tendons (Ilie et al., 2012). The most commonly studied lineages are chondrogenic, osteogenic, and adipogenic differentiation of MSCs. Confirmation of differentiation along a specific lineage can be determined by identifying phenotypic markers and matrix components that

are associated with various tissue types. Early markers of chondrogenic, osteogenic and adipogenic differentiation are summarized in Table 5.1.

Table 5.1: Early markers for lineage commitment of human bone marrow mesenchymal stem cells (Delaine-Smith et al., 2011)

Cell Lineage	Cell Morphology	Gene Expression	Matrix Proteins
Chondrogenic	Large, spherical cells	SRY-box 9 (Sox9) Collagen Types II, X, and XI; Aggrecan, Cartilage oligomeric matrix protein	Collagen type II, Aggrecan
Osteogenic	Cubisoidal shape cells	Runt-related transcription factor 2, Osterix	Collagen type I, Osteocalcin, Osteonectin, Osteopontin
Adipogenic	Large, rounded cells	Peroxisome proliferator-activated receptor-gamma, lipoprotein lipase, collagen type VI.	Lipid Vesicles

Cells in the inner AF are considered to be "chondrocyte-like" while the outer AF cells more closely resemble fibroblasts (Guterl et al., 2013). Ideally, for applications in the repair of annulus, MSCs would be differentiated into a fibroblastic or chondrogenic lineage. Chondrocytes are the cell population native in cartilage (Parsons, 1998) and have been utilized as a cell source for AF tissue repair (Dare et al., 2009; Wan et al., 2008). Inclusion of MSCs in a tissue engineered construct commonly form a fibrous repair tissue more closely resembling a fibrocartilage than an articular cartilage (Acosta

et al., 2011; Li et al., 2009; Zellner et al., 2010), which would be appropriate for repair in the AF.

Fibroblasts are the principal cellular constituent in connective tissues and are present in almost every tissue in the body (Sorrell and Caplan, 2009) making them a diverse population of cells. Currently there are no specific molecular markers or gene expression profiles that can be used to definitively characterize fibroblasts (Riley, 2008) although genes such as collagen I, tenascin-C, lumican, decorin, biglycan, fibromodulin and cartilage oligomeric matrix protein have been suggested to indicate a tendon-fibroblastic lineage (Taylor et al., 2009). Generally, fibroblasts are defined morphologically by their elongated spindle shape (Sorrell and Caplan, 2009). Due to the lack of definitive markers for fibroblastic differentiation, chondrogenesis was the focus of the gene expression studies.

Chondrogenesis requires a three dimensional environment and is typically promoted through addition of exogenous anabolic factors such as transforming growth factor beta (TGF-β), which has been shown to induce chondrogensis and discourage adipogenic and osteogenic differentiation (Jian et al., 2006; Zhou et al., 2004), or addition of bone morphogenic protein 7 (BMP-7) (Richardson et al., 2010).

Formation of chondrocytes during embryogenesis occurs through three stages. Mesenchymal stem cells undergo pre-cartilage condensation followed by chondrocyte

proliferation and differentiation, and finally chondrocytes enter into hypertrophy and ossification (Figure 5.1).

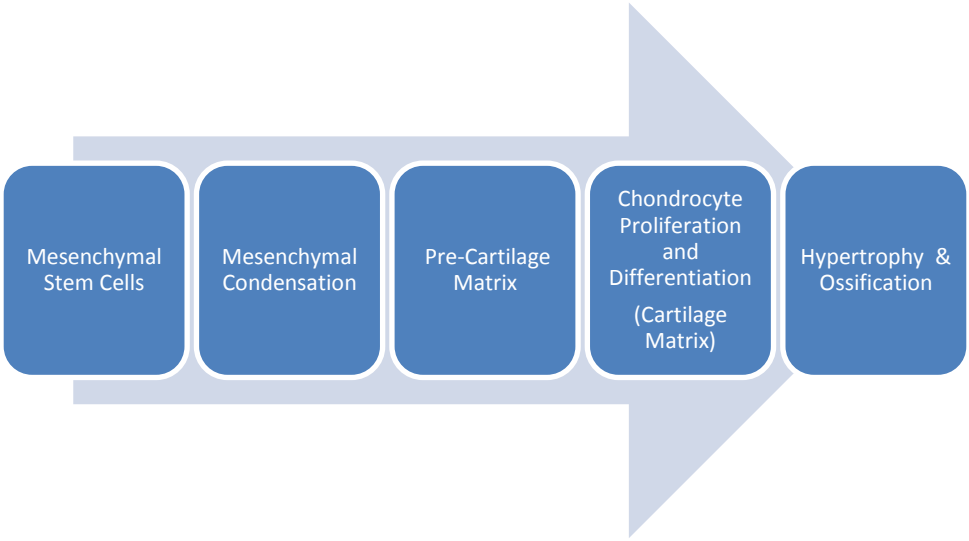


Figure 5.1: Stages of Chondrogenesis in Embryogenesis

Pre-chondrogenic MSCs form condensed cell aggregates and cell morphology changes from a fibroblastic spread morphology to more rounded (Singh and Schwarzbauer, 2012). During this condensation, fibronectin and expression of cell-cell adhesion proteins are rapidly upregulated, however after aggregation of the MSCs, these levels drop off. ECM proteins collagen I and versican are also found during mesenchyme condensation (Singh and Schwarzbauer, 2012). The pre-cartilage matrix will see lower levels of collagen I and versican, as well as low levels of tenascin-c and fibronectin. As the cells enter into chondrogenic differentiation and proliferation, collagens II and IX as well as aggrecan and versican are found in the ECM. The changes in ECM components during chondrogenesis are summarized in Table 5.2.

Table 5.2: Changes in Extracellular Matrix Components During Chondrogenesis

(Singh and Schwarzbauer, 2012)

ECM Protein	Mesenchyme Condensation	Pre-Cartilage Matrix	Cartilage Matrix
Collagen I	✓	✓	×
Collagen II	×	×	✓
Collagen X	×	×	✓
Fibronectin	✓	✓	✓
Tenascin-C	×	✓	×
Aggrecan	×	×	✓
Versican	✓	✓	✓

Mechanical loading, which would be a large factor for *in vivo* application of a tissue engineering construct, may also have an effect on the differentiation fate of MSCs. Both tensile and compressive loading regimes have been investigated for their potential in directing chondrogenic and osteogenic lineage commitment (Connelly et al., 2010; Jagodzinski et al., 2004; McMahon et al., 2008). The cellular response to loading can be measured by studying the regulation of genes or secretion of hormones a few hours or days after loading, or on a longer timescale of a few weeks, by observing their matrix deposition (Delaine-Smith et al., 2011).

In order to study the potential of genipin-crosslinked fibrin gels to integrate with AF *in vivo*, a simplified *in vitro* model was developed. A small, repeatable defect was created in a piece of bovine annulus using a 4mm biopsy punch and a gel seeded with human

bone marrow MSCs was used to plug the defect. Samples in the model were loaded in a custom loading device that applied a tensile strain of 5% of the original sample length to samples for a four hour period of time, followed by a sixteen hour period of rest. Gene expression and histology were used to assess the response of the cells in the gels in loaded and non-loaded conditions when implanted in the tissue to gain insight into the effects of implanting the gel in a piece of live tissue. Differentiation medium was not used in order to simplify the model to study the effects of the material and the loading on the cells without attempting to direct differentiation down a specific lineage. Due to difficulty in identifying specific fibroblastic gene markers, chondrogenic gene markers (Sox9, aggrecan and collagen II) were investigated in addition to collagen I which would identify a more fibrochondrocyte-like phenotype.

5.2 Results

5.2.1 Mesenchymal Stem Cell Gene Expression

5.2.1.1 Non-Loaded Fibrin and Genipin Gels

Gene expression in non-loaded fibrin gels and non-loaded genipin-crosslinked gels was compared to determine if one of the gels would have a greater effect on promoting a chondrogenic lineage commitment. Undifferentiated mesenchymal stem cell from the same lot in monolayer culture were used as a baseline for each gene, which were further normalized to 18S rRNA housekeeping gene and analyzed using the ΔΔCt method, then were plotted as fold induction of gene expression (Figure 5.2) compared to an undifferentiated mesenchymal stem cell control in static culture.

There was almost no aggrecan expression in the fibrin or genipin gels at days 1 and 7 with an increasing being observed at day 12, but no significant difference observed being the groups. Sox9 expression was significantly higher in the genipin group than in the fibrin group at day 12,

Col I expression was up-regulated at days 1 and 7 in both the fibrin and genipin groups, with the genipin gels having a significantly higher Col I expression at day 1 than the fibrin group (a 12-fold increase compared to a 7-fold increase). Over time, the decrease in Col I expression in the fibrin gel group was significant from day 1 to day 12. In the genipin gel group, the decrease was significant from day 1 to day 7.

There was increased expression of Col I in both groups at days 1 and 7, with the expression being significantly higher in the genipin group than the fibrin group at day 7. At day 12, there was no expression of Col II in either of the groups.

5.2.1.1 Genipin Gels in Tissue: Non-Loaded

To study the effect of the non-loaded gels being implanted in a tissue on MSC differentiation, genipin gels (non-loaded genipin gel group) were compared to genipin gels that were biopsied with a 6mm biopsy punch and placed in a defect in bovine annulus tissue (non-loaded tissue group) (Figure 5.3).

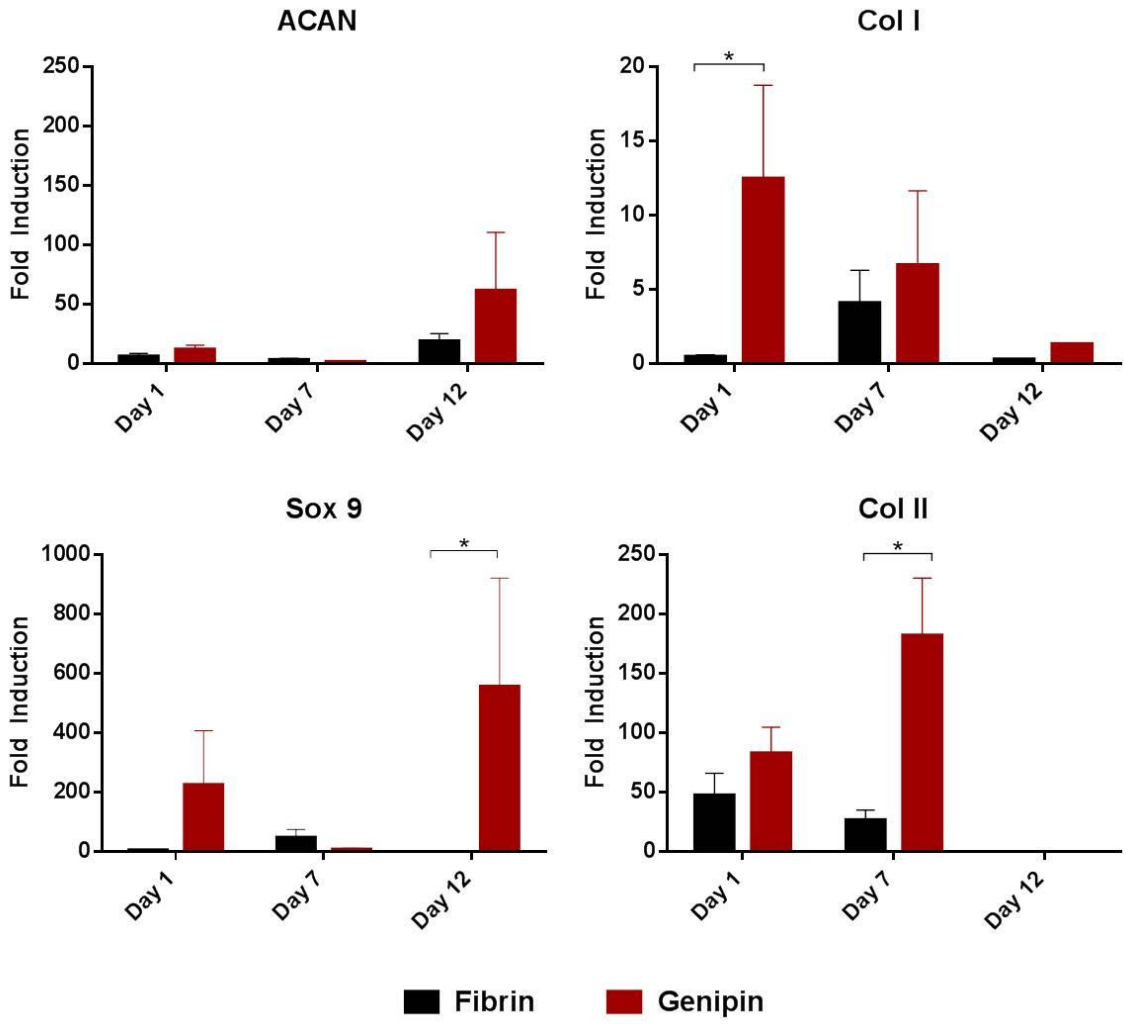


Figure 5.2: rt-PCR data showing fold induction compared to confulent mesencymal stem cells in monolayer culture and normalized to 18S housekeeping gene. Expression of aggrecan, collagen I, collagen II, and Sox9 in fibrin and genipin gels (n=3) containing MSCs at days 1, 7, and 12 (mean + standard error). Two-way ANOVA was used to compare gene expression in fibrin and genipin gel groups ($p<0.05$).

Gene expression of aggrecan was upregulated in both groups at days 1 and 12, with a greater increase at day 12, but there was no significant difference in expression between the two groups. Sox 9 was also upregulated at days 1 and 12 in the genipin group and at day 1 in the genipin group, with a significantly greater expression in the non-loaded tissue group at day 12 with a 63-fold increase.

There was an increase in Col I expression at day 1 in both groups, which decreased at day 7 and showed very low expression by day 12. There were no significant differences in expression between the two groups. Col II was upregulated at both days 1 and 7, with a 182-fold increase in the genipin gel group that was significantly higher than in the tissue group.

5.2.1.1 Genipin Gels in Tissue: Loaded in Tension

To determine the effect of tensile loading on MSC differentiation in the gels, the genipin gels in tissue were loaded in the custom loading device (loaded tissue group) over a 1 or 7 day time period (Figure 5.4). The non-loaded tissue was left in static culture for the full time period, while the loaded tissue group was loaded in tension to 5% strain for four hours followed by a sixteen hour rest period.

Aggrecan expression was upregulated equally in the loaded and non-loaded tissue groups on day 1, but on day 7 the expression decreased in the non-loaded tissue groups, but significantly increased by 128-fold in the loaded tissue group.

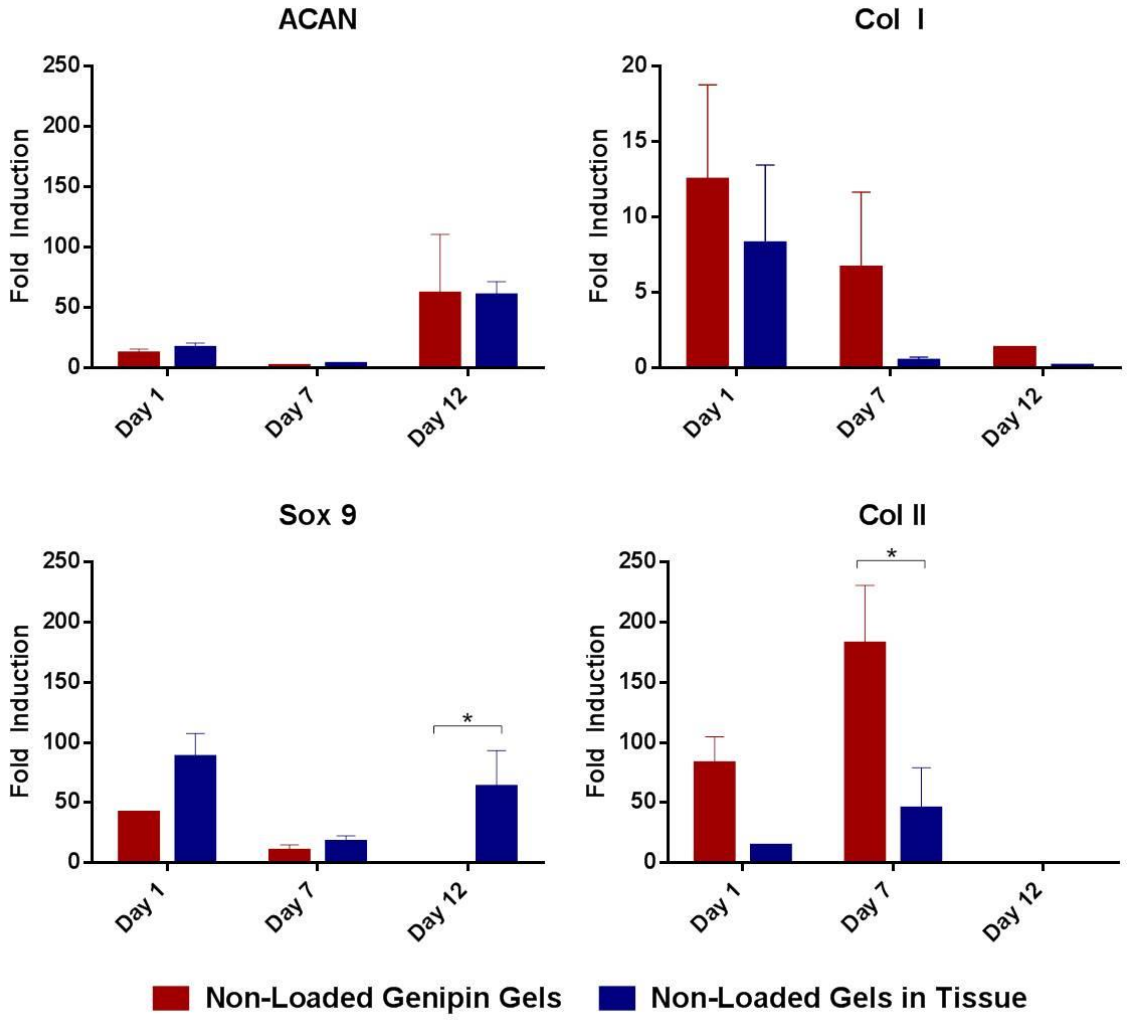


Figure 5.3: rt-PCR data showing fold induction compared to confulent mesencymal stem cells in monolayer culture and normalized to 18S housekeeping gene. Expression of aggrecan, collagen I, collagen II, and Sox9 in non-loaded genipin gels and non-loaded genipin gels in tissue (n=3) containing MSCs at days 1, 7, and 12 (mean + standard error). Two-way ANOVA was used to compare gene expression in non-loaded genipin gels and non-loaded gels in tissue ($p<0.05$).

Sox9 was also upregulated in both groups on day 1, with an 88-fold increase in the non-loaded tissue group and by 57-fold in the loaded tissue group with an insignificant difference between the two groups. The expression was reduced significantly in both groups by day 7, with no significant differences in expression between the loaded and non-loaded samples.

Col I was upregulated in both groups at day 1, with no significant difference between them, and showed almost no expression at day 7, a significantly decrease from day 1. Col II was upregulated to similar levels n both groups with a 14-fold increase in the non-loaded tissue group and a 19-fold increase in expression in the loaded tissue group at day 1. Col II expression was maintained in the non-loaded tissue group, but was significantly higher in the loaded tissue group.

5.2.1 Histology

Fibrin and genipin gels containing $5x10^5$ hb-MSCs/mL were stained with Safranin O and haematoxylin to identify the cell morphology and distribution within the gels as well as to determine if any glycosaminoglycans had been deposited by the cells at days 1, 7, or 12 (Figure 5.5).

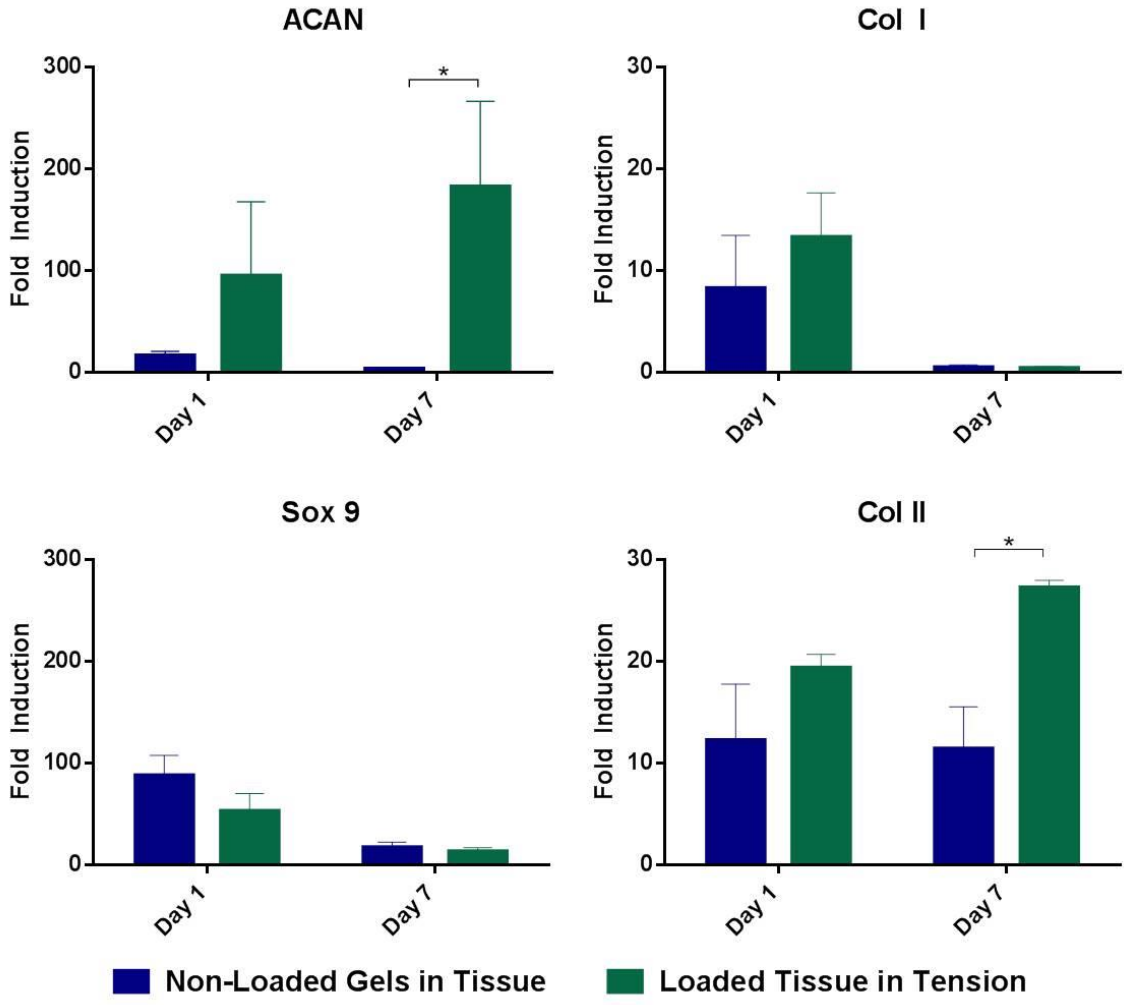


Figure 5.4: Real time PCR data showing fold induction compared to confulent mesencymal stem cells in monolayer culture and normalized to 18S housekeeping gene. Expression of aggrecan, collagen I, collagen II, and Sox9 in non-loaded genipin gels in tissue and genipin gels in tissue loaded in tension to 5% strain (n=3) containing MSCs at days 1, and 7 (mean + standard error). Two-way ANOVA was used to compare gene expression in non-loaded and loaded groups ($p<0.05$).

In general, in both the fibrin and genipin gels, nuclei can be observed as stained dark blue by the haematoxylin. The Safranin O stains glycosaminoglycans (GAGs) orange to red, but as can be observed in Figure 5.5, there was no noticeable Safranin O staining around the cells, indicating no GAG accumulation in or around the cells. Any observed pink to red background in the images appeared to be retention of some of the stain by the gel, not actual GAG accumulation. It is also notable that around many of the cells, holescan be observed (examples indicated by arrows on Figure 5.5).

In the day 1 fibrin gels, some of the cells appeared to be stained fully dark blue, with no distinguishable nuclei. There were large holes around many of the cells, which tended to clump together and were not very evenly distributed within the gels. It appeared in many cases that the cells seemed to be clumped together in large pockets or holes. This trend was also observed in the day 7 fibrin gels and can be seen in Figure 5.6a. Some of the cells instead of forming in clumps, also formed into long, strings of cells as shown in Figure 5.6b. These long strings of cells were also observed in the genipin gels on days 1 and 7. The day 7 gels in general appeared to have fewer cells, and some of the holes appeared to be empty, with no cells in them while others had cells which began to develop processes extending out through the holes and into the gels.

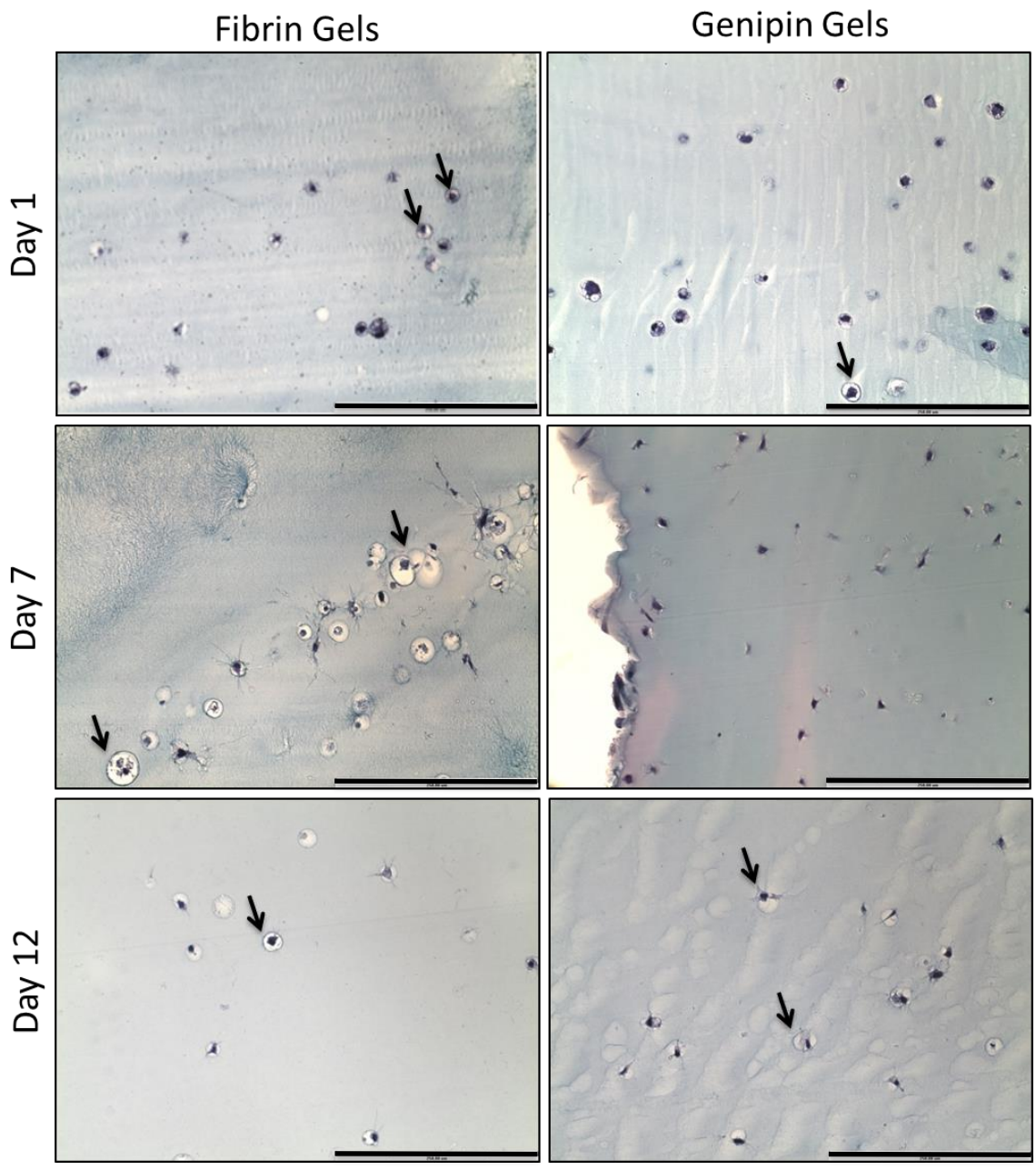


Figure 5.5: Safranin-O staining with hematoxylin on fibrin and genipin gels at days 1, 7, and 12. Arrows indicate examples of cells within holes or lacunae. Scale bars are 250µm.

The cells within the day 1 and day 7 genipin gels also formed into clumps and strings in a few locations, but in general seemed to be more evenly distributed Figure 5.7. There were some holes observed around the cells in the day 1 genipin gels, but they appeared smaller and fewer than in the day 1 and day 7 fibrin gels. By day 7, the holes in the genipin gels were more comparable to those in the fibrin gels, and cell processes were also beginning to develop.

The cells in both day 1 gels appeared to have a similar, rounded morphology, but in the day 7 genipin gels, the cells had a more spread morphology and appeared more fibroblast-like than a majority of the cells in the day 7 fibrin gels. While both day 7 fibrin and genipin gels started to have cell processes extending outwards, they were more noticeable in the genipin gels.

Both the day 12 fibrin and genipin gels appeared to have a more even cell distribution throughout the gel and were no longer localized in clumps or strings together. A majority of cells that were observed in the day 12 gels had large cell processes, some extending from outside of lacunae, others appeared to be suspended in the gel without holes. Figure 5.8 shows a very similar cell morphology between the cells in the day 12 fibrin gels (Figure 5.8a) and the day 12 genipin gels (Figure 5.8b). The cells had very clearly stained nuclei and a spread, fibroblast-like cell morphology with cell processes extending well outside of the lacunae. The fibroblastic morphology was more pronounced within the genipin gels at both day 7 and day 12 compared to the fibrin gels.

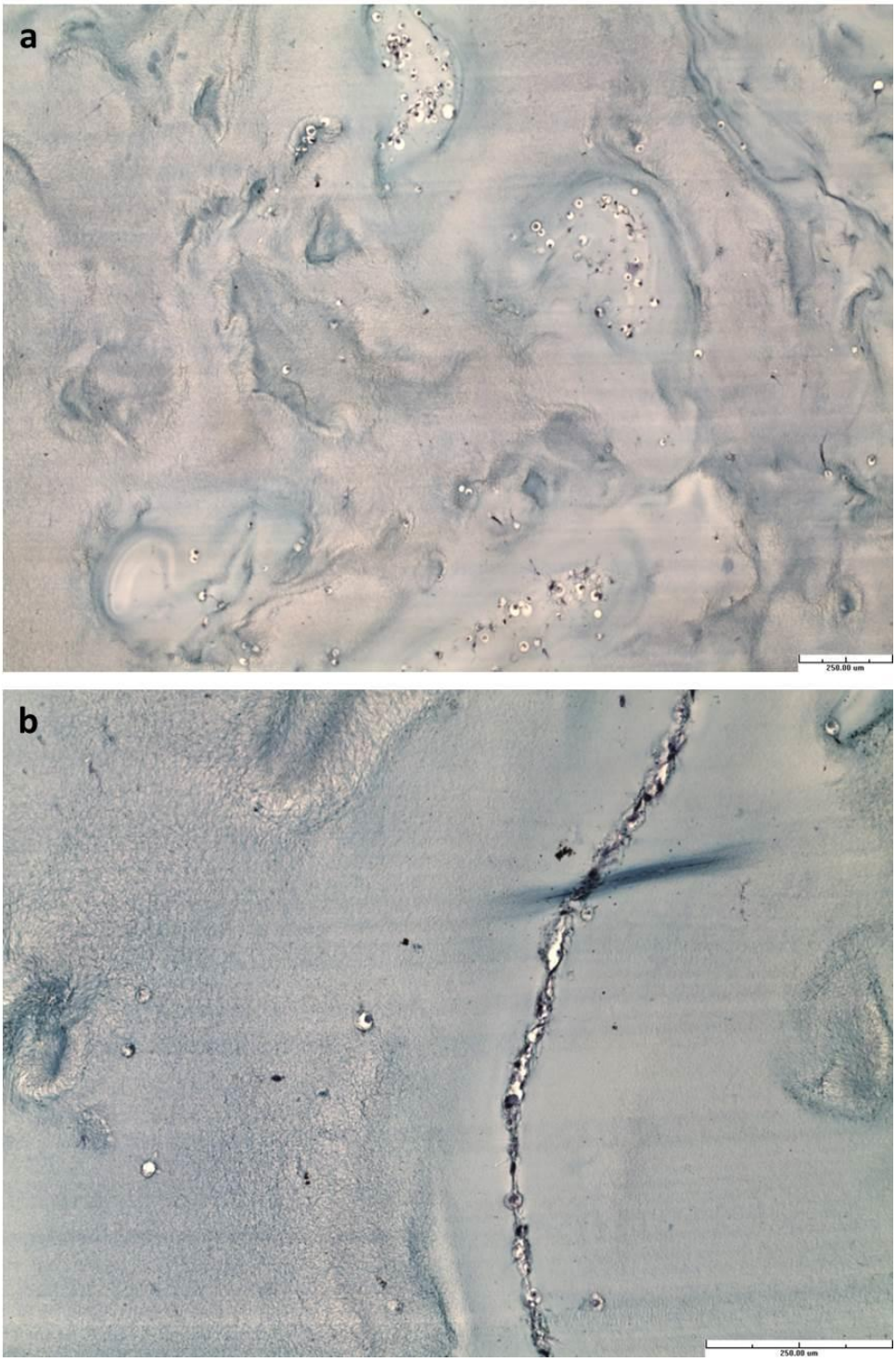


Figure 5.6: Day 7 fibrin gel showing (a) small groups of cells dispersed throughout the gel at 5x magnification and (b) long strings of cells formed in some locations within gels at 10x magnification. Scale bars are 250µm.

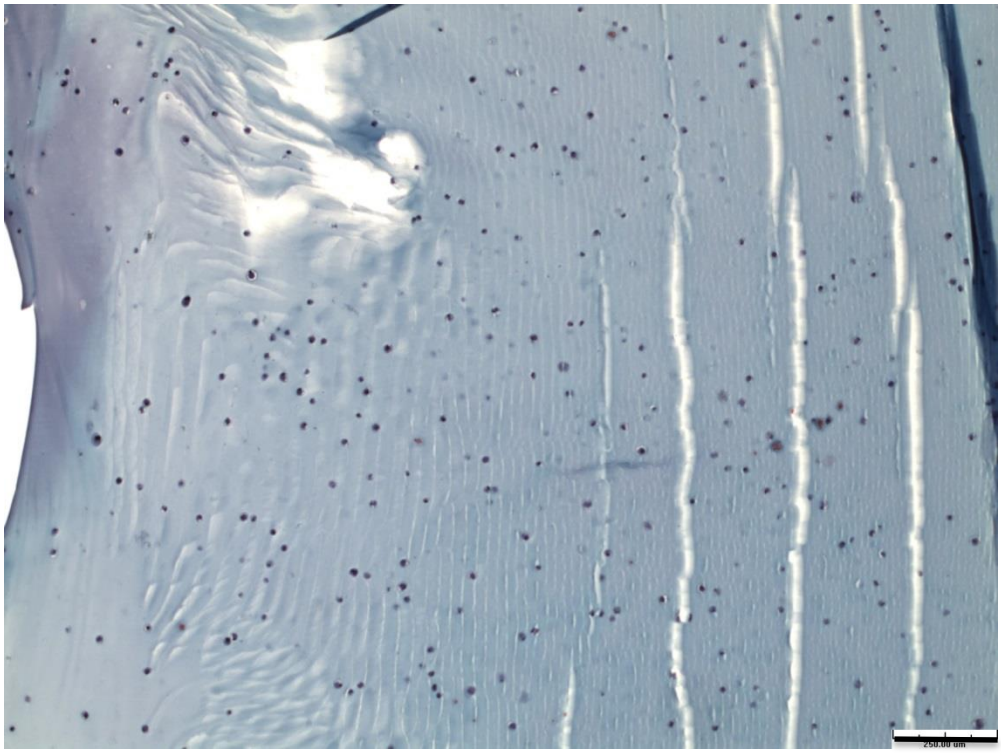


Figure 5.7: Day 1 fibrin gel gel showing even distribution of cells throughout the gel with minimal aggregation. 5x magnification. Scale bars are 250µm.

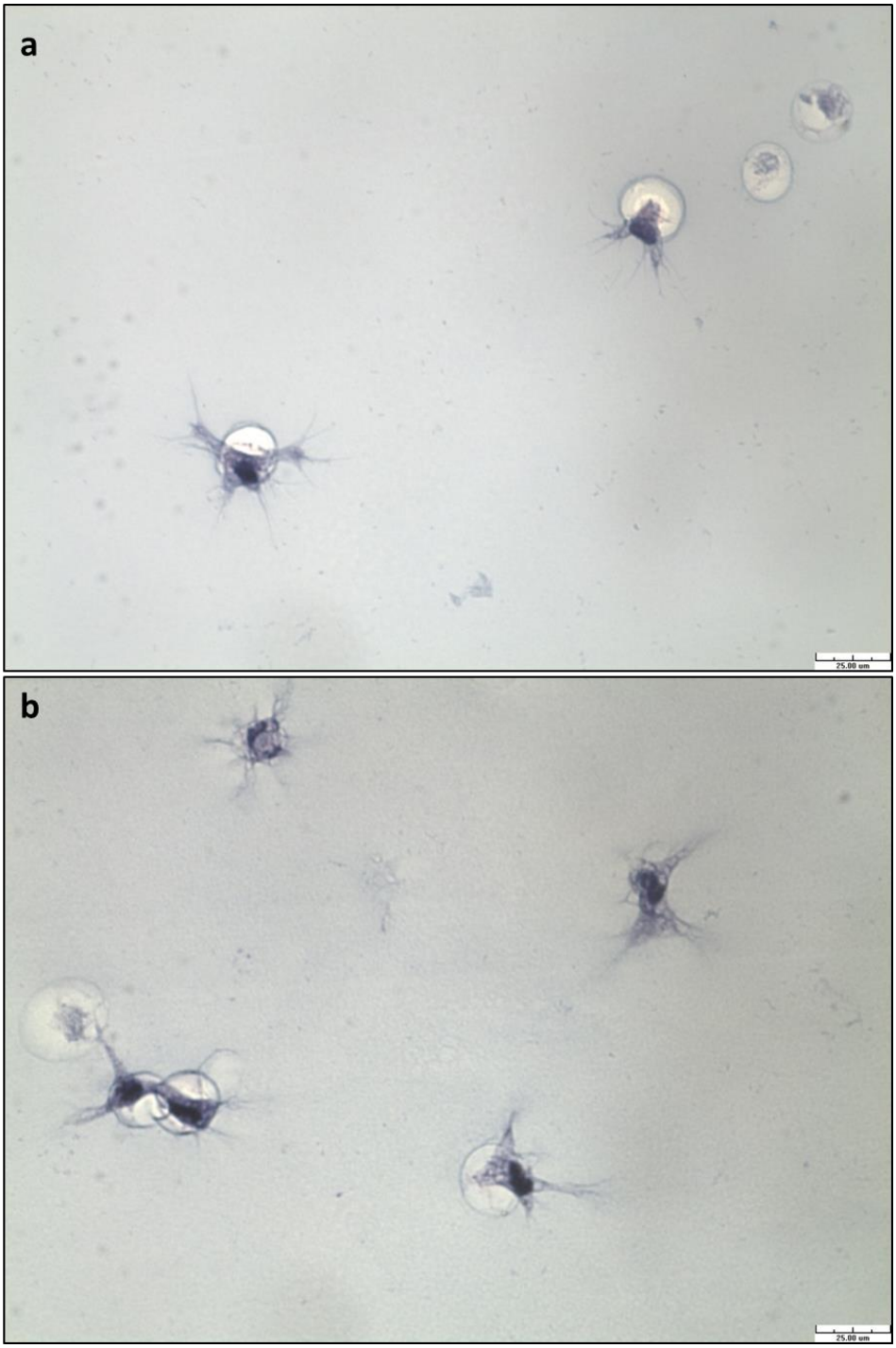


Figure 5.8: (a) Fibrin and (b) Genipin gels with hb-MSCs after 12 days in culture stained with Safranin O at 40x magnification. Scale bars are 25 µm.

5.3 Discussion

Human bone marrow mesenchymal stem cells were encapsulated in both genipin and fibrin gels, and rt-PCR was used to assess the effect of these materials on the gene expression and differentiation of the cells. Sox9 is one of the first markers expressed during chondrogenesis (Singh and Schwarzbauer, 2012) and is a transcription factor for type II collagen and aggrecan (Bi et al., 1999). Sox9 expression was highly upregulated at days 1 and 12 in genipin gels, with a significantly higher expression at day 12 than fibrin gels which indicates that the genipin gels may provide a more suitable environment for stimulating differentiation down a chondrogenic lineage.

An increase in Sox9 expression was also found when the gels were placed in non-loaded tissue samples, with an 88-fold increase experienced in this group at day 1 and a 63-fold increase at day 12. The expression of Sox9 was significantly higher in the tissue group than the genipin gels at day 12. This suggests that the cells altered their gene expression when in the tissue defect, which could be due to a variety of reasons. The samples placed into the tissue were created using a 6mm biopsy punch and placed into a 4mm biopsy defect in the tissue to ensure a snug fit. Consequently, the cells may have experienced some compressive forces that affected their gene expression. Additionally, since the unloaded genipin gels or the unloaded tissue samples were cultured in the same 6-well plates with the same volume of media, the cells would have experienced differences in oxygen tension and nutrient transport in culture. The unloaded genipin gels were a full sized gel, approximately 12.8mm in diameter while the gels that were placed into the tissue defect were biopsied from these full sized gels using a 6mm biopsy punch.

Oxygen tension has been implicated as a key factor that influences chondrogenic differentiation (Kim et al., 2011). Articular chondrocytes have been shown to be able to survive in high and low oxygen conditions, however they were metabolically less active and had lower mRNA expression in low oxygen (Grimshaw and Masoin, 2000).

Aggrecan and collagen are both key components in the annulus, with collagen type I being more prominent in the outer annulus and collagen II more prominent in the inner annulus. Collagen II is typically used as a marker for chondrogenic differentiation while collagen I often indicates osteogenic changes, however type I collagen is present in the early stages of chondrogenesis in the precartilage condensation, typically being expressed before collagen II and aggrecan (Singh and Schwarzbauer, 2012). Collagen I is also present in tendon fibroblasts and may be indicative of a fibrochondrocyte phenotype. Collagen I was present in a majority of the sample conditions at day 1 in the greatest amounts, and decreased over time, further supporting that chondrogenic differentiation may have been initiated early in these gels. Collagen I expression was significantly higher in the genipin gels compared to the fibrin gels at day 1, further supporting the genipin gels as a more appropriate material for promoting chondrogenic cell differentiation.

In both sets of gels, the collagen II expression decreased to very low levels at day 12 or weren't expressed at all at this time point. The genipin gels that were not in tissue showed a significantly greater expression of Col II at day 7, however when the samples were loaded in tension, this significantly increased Col II expression over non-loaded

samples at this time point. These trends support the possibility that the cells were undergoing a precartilage condensation stage and moving towards differentiation into mature chondrocytes (Singh and Schwarzbauer, 2012).

As Sox9 and collagen I decrease, it would be expected in chondrogenic differentiation that aggrecan and collagen II expression would increase. Type II collagen expression underwent an 82-fold increase at day 1 and 182-fold increase at day 7 in genipin gels which was significantly higher than fibrin gels at day 7. While the increase in collagen II expression implies that cells may have been undergoing chondrogenesis, the lack of aggrecan expression contradicts this, and collagen II was not expressed at all in fibrin or genipin gels on day 12. Aggrecan has been shown to have variable results in gene expression studies, often being expressed constituently across all time points (Mwale et al., 2006). Other studies investigating chondrogenic differentiation of MSCs showed that aggrecan showed a more gradual increase in expression, even in the presence of TGF-β (Barry et al., 2001; Xu et al., 2008; Zhang et al., 2012). The limitations of aggrecan as a marker for chondrogenesis indicate that gene expression data alone may not be sufficient to draw conclusions about changes in aggrecan expression, and protein expression should also be investigated.

While initial increases were observed, collagen II expression in the genipin gels was undetected at day 12. Other studies investigating whether a biomaterial alone has the ability to stimulate a chondrogenic lineage commitment have observed similar results in which fibrin was shown to have a significant effect on modulating MSC differentiation

markers at a transcriptional level, however after a 12 day time period mature, lineage-specific markers could not be identified (Huang et al., 2010). This may indicate that, as concluded in other studies, the presence of appropriate growth factors or induction media are required to achieve full differentiation into a mature, chondrogenic lineage (Barry et al., 2001). Alternatively, higher cell densities, the inclusion of cell aggregates, and a longer culture period may be able to fully differentiate the cells as was found with a type I collagen hydrogel (Zhang et al., 2012).

In the non-loaded and loaded tissue groups, aggrecan had similar expression at day 1, however at day 7, the aggrecan expression was very low in the non-loaded group, but jumped to a 182-fold increase in the loaded group. This was matched by a 11-fold and 27-fold increases in collagen II at day 7 for the non-loaded and loaded tissue groups respectively, suggesting that the loading may have had a positive effect at the later time points in stimulating chondrogenic differentiation. To induce chondrogenic differentiation, compressive loading is more commonly studied than tensile loading (Delaine-Smith et al., 2011), with compression of MSCs yielding varying results. It's generally agreed that the timing of the load applied and the media conditions are critical to the outcome of differentiation induced by compression. There have been some studies investigating tensile loading and MSC differentiation that have shown that short term loading in fibrin hydrogels can enhance protein and proteoglycan synthesis, but after 12 days, only protein synthesis was enhanced. Collagen I expression was affected by the loading, but aggrecan and collagen II were unaffected, indicating that this type of loading

may induce a fibrochondrocyte-like differentiation of the MSCs (Connelly et al., 2010), which could yield effective matrix deposition for annulus fibrosus tissue engineering.

Together, this gene expression data gives an indication that the cells within the genipin gels may be undergoing chondrogenic differentiation. The low expression of chondrogenic markers in the fibrin gels indicate that they may not be sufficient for supporting chondrogenic cell differentiation and the cells may respond more favorably in a stiffer scaffold such as the genipin-crosslinked gels. The cells responded to being placed in the non-loaded tissue samples, altering their gene expressions, however it's difficult to elucidate a trend from the data to determine whether placement in the defect enhanced chondrogenic differentiation or not.

Due to the fact that samples in the loading device could not be loaded past seven days without fungal contamination, the gene expression of the loaded samples were only studied at the day 1 and day 7 time points. Loading the samples resulted in a significant increase of aggrecan and collagen II over the non-loaded groups, indicating that loading may help promote chondrogenesis further down the differentiation pathway than the non-loaded samples. Future studies should also investigate the effects of higher cell seeding densities and/or the addition of cell aggregates, which have been shown to induce a chondrogenic lineage commitment without the addition of exogenous factors (Zhang et al., 2012).

The histology showed that there were not very significant differences in the morphology and GAG accumulation between the genipin and fibrin gels. Safranin O staining revealed that there was no GAGs deposited in or around the cells in either the fibrin or genipin gels at any of the time points, indicating that up to twelve days, the cells were likely not differentiated and therefore were not depositing a mature matrix that would lead to the deposition of cartilage. This is supported by the gene expression data that indicates that up until the later time points, there was minimal gene expression of advanced chondrogenic markers aggrecan and collagen II. This result is not surprising, however, as other studies that used fully differentiated chondrocytes in fibrin constructs observed only minimal Safranin O staining around cells after 1 week, with significant staining being observed at 5 weeks (Scotti et al., 2010).

Large holes were observed around many of the cells in all the gels at all time points, which has previously been described in the literature (Gruber, 2004; Meinhart et al., 1999; Scotti et al., 2010; Zhang et al., 2012). While some literature refers to these holes as "cartilage lacunae" (Gruber, 2004), they are likely the result of plasminogen activator being released by the cells (Radosevich et al., 1997) that results in fibrinolytic breakdown of the gels around the cells. The holes appeared to be smaller and form less rapidly in the genipin gels, indicating that the genipin crosslinking successfully stabilized the gels and reduced the effects of rapid degradation typically experienced in fibrin gels. However, the presence of the holes by day 12 indicates that while the crosslinking and 2 mg/mL of 6-aminohexanoic acid stabilized the gels, additional antifibrinolytic agents or increased concentrations may be required to prevent the cells from degrading the gels.

In the literature, 8,500 KIU/mL of aprotinin and 15 mg/mL of tranexamic acid were shown to sufficiently stabilize gels over 4 weeks. Effective concentrations and combinations of antifibrinolytic inhibitors could be further investigated to enhance the performance of these gels over time, however the effect on cell viability and proliferation would have to be observed to ensure that a negative effect was not found.

Gene expression and histology only give a limited picture of what has occurred with the differentiation of the cells. While gene expression can show that transcription is occurring and changes are occurring in various conditions, protein expression in conjunction with gene expression gives a better indication of what changes are actually expressed as proteins. Furthermore, studying the composition of the matrix that is deposited by the cells is a more relevant measure as regardless of what changes the cells go through over time, if the end result is a functional, fibrocartilaginous matrix, then the objective of the study has been achieved.

Histology is able to give a good indication of cell morphology and distribution, however it cannot identify whether cells are alive or not, and changes in viability have a significant effect on matrix deposition. Protocols have to be developed for each individual material and type of cells that are being studied, which can be a time-consuming process and appropriate positive and negative controls have to be verified before results can be validated. The use of immunohistochemistry would also help to further identify the composition of the matrix at later time points, to determine what functional matrix components are being excreted by the cells.

5.4 Conclusions

Gene expression of human bone marrow MSCs in fibrin gels and genipin-crosslinked fibrin gels were investigated over a twelve day time period. A low expression of chondrogenic-specific markers in the fibrin gels indicate that they do not provide a sufficient environment on their own to induce chondrogenesis. The gels are also less stable than the genipin-crosslinked gels as indicated by histology in which large holes are found around the cells in the fibrin gels. While the holes were also observed in the genipin gels, they were smaller and appeared more slowly indicating that the fibrin gels are may have been degraded more quickly by the cells than in the genipin gels.

Placement of the gels into a biopsied defect in bovine annulus tissue yielded an increase in some chondrogenic-specific gene expression in the gels, with this gene expression in most samples being increased when the tissue was loaded in tension. While the loading experiments could only be conducted to seven days, the aggrecan gene expression results indicate that loading may have a positive effect on inducing a fibrochondrogenic phenotype of the cells in genipin gels.

The initial results of these experiments have shown that genipin-crosslinked fibrin gels have the ability to support viable MSCs and promote expression of chondrogenic-specific markers. Further studies over longer time periods will be required to determine if genipin-crosslinked fibrin gels have the ability to support chondrogenic differentiation and deposition of new cartilage matrix by human bone marrow MSCs. These initial studies showed no deposition of GAGs, indicating that a longer time frame is required to

study the ECM deposited by the cells to determine if integration with bovine annulus tissue would be possible. These initial results, however, show that genipin-crosslinked fibrin gels have potential for use in the repair of annular tears, and further studies should be conducted to determine their potential for integration.

Chapter Six: Conclusions & Recommendations

Tears and ruptures in the annulus fibrosus of the intervertebral disc as a result of disc degeneration are a significant clinical problem that often present as herniated discs, resulting in significant pain and disability for patients. Lower back pain creates a large economic burden of $33 billion in direct medical costs and $100 billion in indirect costs each year in the United States (Waddell, 1996). A tissue engineering solution that has the ability to repair a defect in the AF and promote generation of new tissue to permanently seal tears or repair ruptures would be an ideal solution for AF repair.

In order to develop a solution for repairing a defect in the AF, design criteria were identified which would be the minimum requirements for an adhesive or repair strategy in the disc. The material would have to provide strong adhesion to the native tissue, able to undergo strains in the range of 15% and would require similar mechanical properties (compressive moduli of 1320 kPa). It would also have to be cytocompatible, stable for a time period that allows for new ECM deposition, and promote tissue repair and integration with the host tissue.

In this study, it was determined that commercially available surgical adhesives did not provide sufficient adhesion to bovine AF tissue. Fibrin gels crosslinked with genipin, a natural crosslinking agent, and seeded with human bone marrow mesenchymal stem cells were characterized and investigated for their potential in AF repair.

Degradation studies comparing fibrin and genipin gels determined that the addition of an antifibriniolytic agent, 6-aminohexanoic acid, was required to maintain the gels stability over a 30 day time period, after which both the fibrin and genipin gels still retained over 80% of their original weight. The mechanical properties of fibrin gels were significantly enhanced by the addition of genipin crosslinker, with a 0.100 mg/mL concentration of genipin producing a significant increase in compressive moduli over fibrin gels. Since 0.100 mg/mL genipin gels showed no significant difference in maintaining cell viability over 12 days compared to fibrin gels, 0.100 mg/mL concentrations of genipin were determined to be the best selection of concentration as they had the greatest effect on enhancing the mechanical stiffness of the gels.

Gene expression studies determined that fibrin gels may not be sufficient for promoting chondrogenic lineage commitment from MSCs, however the genipin-crosslinked gels showed enhanced gene expression of chondrogenic-specific markers, which indicate genipin gels may have the ability to promote this type of cell differentiation. Histology showed that at days 7 and 12, genipin gels promoted a more fibroblastic type of morphology than fibrin gels. There were larger holes found around the cells in the fibrin gels, indicating that they were degrading the gel to a greater extent than the genipin gels, and the genipin crosslinking is required to produce a more stable gel over time. No glycosaminoglycan accumulation was found up to a 12 day time point in either type of gel.

The effect of placing MSC-seeded genipin gels in bovine annulus tissue was also investigated, with the tissue loaded in tension to 5% strain or non-loaded. It was determined that cells responded to being placed in a defect in tissue however it was difficult to elucidate a trend as to whether placement in a defect increased chondrogenesis or not. Loading the samples significantly increased expression of late chondrogenic differentiation markers, indicating that it may help promote chondrogenic differentiation. Longer-term studies are required to determine the effects of loading on the MSC differentiation, however initial results show that loading did not have a detrimental effect on the cells up to 7 days.

Overall, the genipin-crosslinked fibrin gels have sufficient chemical and mechanical properties to be used as a tissue engineering material for annulus fibrosus repair and should be investigated further. Future studies should focus on the effects of cell seeding density and/or addition of cell aggregates to enhance chondrogenic differentiation within the gels without the need for exogenous growth factors. Additionally, other cell sources should be considered such as synovial mesenchymal cells and fully differentiated chondrocytes. Synovial mesenchymal stem cells may have the ability to form cartilage without the requirement of aggregating, which may be more successful at developing new tissue in a stiff material such as genipin-crosslinked gels. The use of fully differentiated chondrocytes would reduce the time in culture within the gels before glycosaminoglycan accumulation occurs, ultimately resulting in deposition of new fibrocartilaginous tissue more quickly. Further studies should also investigate more specific promotion of a fibrochondrogenic lineage similar to native AF cells.

Longer term studies are required to see the effects of loading on the lineage commitment of the MSCs. In order to successfully conduct longer-term loading studies, modifications will have to be made to the custom loading device to improve its sterility and prevent fungal or bacterial contamination of the samples. This could be achieved by developing a system that provides a steady flow of media through the tissue plates, or by creating a hole in the well plate lid with a plug that can be removed to facilitate media changes.

Future studies should also include analysis of gene expression on the protein level, to give a more clear picture of how the cells are differentiating. Further histology and immunohistochemistry should also be utilized to assess the matrix that is developed in longer term studies and determine if it has a similar biochemical makeup as native annulus tissue.

In addition to studying the gels over longer time periods, additional genes will have to be investigated to ensure that the cells haven't started down other lineages, particularly osteogenic and adipogenic. Genes such as tenascin-c that are indicative of a tendon fibroblast phenotype should also be investigated to determine if a more fibrochondrogenic phenotype is expressed by the cells. Other considerations for improving the ability of mesenchymal stem cells to differentiate down a chondrogenic lineage would include investigating the porosity of the gels, which may be limiting the nutrient transport to the cells and inducing endochondral ossification. Different loading regimes (duration of loading, increase in tension etc.) could also be investigated to further enhance the differentiation potential of MSCs down a fibrochondrogenic pathway.

While the genipin-crosslinked fibrin gels have sufficient mechanical, chemical and cytocompatible properties for application in annulus fibrosus tissue engineering, longer-term studies are required to better understand their effect on MSC differentiation and to determine if they have the ability to direct extracellular matrix deposition that has the ability to integrate with host tissues.

Overall, this study has achieved seeding mesenchymal stem cells into a genipin-crosslinked fibrin gel for the first time. A fabrication method which utilized low concentrations of genipin was developed to maintain high cell viabilities, while still producing a stiffer material than un-crosslinked fibrin gels. Degradation studies were conducted and the composition and culture conditions were modified until a gel which was stable over a 30 day time period could be produced.

A new *in vitro* tissue damage model was developed using a custom-loading device that was previously developed in our lab. This model will allow for the study of many factors affecting tissue repair and integration in the future including studying the effects of different loading regimes on stem cell differentiation, studying newly developed materials under *in vivo* loading conditions, and studying the interface between the biomaterial and tissue to develop methods that will facilitate tissue integration.

This study also showed for the first time that mesenchymal stem cells could be supported in a genipin-crosslinked fibrin hydrogel and further, that the material may help promote chondrogenic differentiation of human bone marrow MSCs.

This study has contributed to existing literature studying genipin-crosslinked fibrin gels by showing the capability of seeding a new cell line into the gels which has never been investigated before, and further defining the design space for this biomaterial, further characterizing the trade-offs between material properties and stiffness and high cell viability.

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