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# UNIVERSITY OF CALGARY

Biomaterials for Intervertebral Disc Repair

by

Haeyeon Lee

A THESIS

# SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

## BIOMEDICAL ENGINEERING GRADUATE PROGRAM

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#### UNIVERSITY OF CALGARY

### FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "BIOMATERIALS FOR INTERVERTEBRAL DISC REPAIR" submitted by HAEYEON LEE in partial fulfilment of the requirements of the degree of MASTER OF SCIENCE.

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#### Abstract

Each intervertebral disc (IVD) in the spine has an inner gel-like nucleus pulposus (NP) surrounded by an outer annulus fibrosus (AF). IVD degeneration has been linked to low back pain, a medical condition that affects millions of people and has significant socioeconomic consequences. The goal of this study was to assess the properties of different biomaterials to determine their utility in IVD repair strategies. Different compositions of gellan gum were investigated for NP repair. Through optimization of gellan gum properties, it was found that 2% (w/v) low acyl gellan gum had the best mechanical properties while having a suitable gelling temperature for cell encapsulation. When gellan gum, fibrin, and chitosan/gelatin/glycerol phosphate hydrogels were compared for sealing defects in the AF, it was found none could withstand pressures as high as intact IVDs. Therefore, a triphasic prototype construct composed of Kryptonite bone cement, gellan gum, and reinforcing fibre was evaluated to determine if it could contribute to AF repair. Whereas, it was found that constructs with sutures had better tensile properties than those with electrospun fibres, overall the current generation of constructs was not sufficient for AF repair. This thesis represents an important step in understanding the use of biomaterials for IVD repair.

#### Acknowledgements

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Haeyeon Lee

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# List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
А	Area (mm <sup>2</sup> )
ACD	Annulus Closure Device
AF	Annulus Fibrosus
C/G/GP	Chitosan/Gelatin/Glycerol Phosphate
DMA	Dynamic Mechanical Analysis
E	Complex compressive modulus (kPa)
E'	Storage Modulus (kPa)
E"	Loss Modulus (kPa)
ECM	Extracellular Matrix
ePTFE	Expanded Polytetrafluoroethylene
F	Force (N)
FDA	Food and Drug Administration
GF	Growth Factor
HA	High Acyl
IVD	Intervertebral Disc
LA	Low Acyl
MSC	Mesenchymal Stem Cell
NP	Nucleus Pulposus
NPR	Nucleus Pulposus Replacement
PAA	Poly(acrylic acid)
PET	Polyethylene Terephthalate
PGA/HA	Polyglycolic Acid/Hyaluronic Acid
PLGA	Poly(lactic-co-glycolic acid)
PDLA	Poly-D, L-lactide
PVA	Poly(vinyl alcohol)
SIS	Small Intestinal Submucosa
TDR	Total Disc Replacement
TGF-β	Transforming Growth Factor Beta
UTS; MPa	Ultimate Tensile Strength (MPa)
δ	Phase Lag (radians)
3	Strain (mm/mm)
σ	Stress (kPa)

#### **CHAPTER ONE: THESIS OVERVIEW**

Degeneration of the intervertebral disc (IVD) can cause low back pain (LBP), which has significant socioeconomic consequences. Therefore, this thesis investigates the use of biomaterials for repairing the IVD.

In order to facilitate readers of this thesis, the next chapter, will provide a description of the anatomy and physiology of the IVD. In short, the IVD links adjacent vertebral bodies and provides stability, mobility, and load bearing capabilities to the spine. It is composed of an inner gel-like nucleus pulposus (NP) which is surrounded by an annulus fibrosus (AF) composed of concentric lamellae of collagen fibres. The cartilage endplates provide anchorage to the bone and allow for diffusion of nutrients.

The mechanism of IVD degeneration will also be explained in detail in the next chapter where IVD degeneration can occur due to abnormal loading, biochemical changes, reduced nutrition, and genetic predisposition. In IVD degeneration, structural failure of the AF can occur leading to herniation of the NP. These herniations can impinge on adjacent nerves causing LBP.

LBP is a major cause of disability having significant socioeconomic consequences. Current treatments for LBP such as discectomy and spinal fusion will also be explained in the next chapter. However, these treatments compromise the normal biomechanical function of the spine, which can lead to the degeneration of adjacent segments. Therefore, due to the inability of the current methods to reverse damage and regenerate the IVD tissue, alternative methods will be discussed. Amongst these methods are the ones described in this thesis.

In chapter three, procedures for the preparation of a variety of biomaterials are given. Also, experimental methods for characterizing the mechanical properties of these biomaterials are explained in this chapter.

In chapter four of this thesis, biomaterials will be evaluated for their use in tissue engineering applications for repairing the NP.

Removal of the degenerated NP or insertion of a new NP replacement requires an incision in to the AF but the AF cannot readily repair itself. Therefore, in chapters five and six of this thesis, different biomaterials and multi-phase constructs will be examined for their ability to fill in a defect in the AF.

In chapter seven, conclusions from chapters four to six are given as well as a discussion of the future studies that should be performed.

The goal of the research presented in this thesis was to investigate different strategies to repair damage to IVDs. The biomaterials that were tested in this project were gellan gum, fibrin, chitosan/gelatin/glycerol phosphate, and poly(vinyl alcohol)/poly(acrylic acid)) (PVA/PAA). Further information about these biomaterials is given in their respective chapters.

The first objective of this project was to determine the optimal concentration and composition of gellan gum for NP tissue engineering applications by performing dynamic mechanical analysis (DMA) and determining the gelling temperature of various concentrations and compositions of gellan gum.

The second objective of this project was to compare various biomaterials (gellan

gum, fibrin, chitosan/gelatin/glycerol phosphate, and PVA/PAA) in terms of their mechanical properties to determine the most suitable biomaterial for sealing defects in the AF. DMA, tensile tests, and pressure testing were performed to assess the mechanical properties and functionality of the biomaterials in sealing an AF defect.

The third objective of this project was to develop a prototype, using one of the biomaterials from the second objective, which will be able to provide a better adhesion when sealing a defect in the AF.

Together this thesis will provide a glimpse into the complexity associated with repairing degenerative damage to the IVD.

#### **CHAPTER TWO: INTRODUCTION**

#### 2.1 Intervertebral Disc Structure and Function

The intervertebral disc (IVD) links adjacent vertebral bodies providing stability, mobility, and load bearing capabilities to the spine [1]. It is composed of three distinct parts: the nucleus pulposus (NP), the annulus fibrosus (AF), and the hyaline cartilage endplates as seen in Figure 2.1 [2].



**Figure 2.1:** A) The IVD is composed of the nucleus pulposus (NP), the annulus fibrosus (AF), and the cartilage endplates. B) The central NP is surrounded by lamellae of AF. Picture reproduced from [3] with permission from the Company of Biologists.

The central gel-like NP contains a randomly organized network of mainly type II collagen fibrils submerged in a matrix with a high proteoglycan content of about 50% of the NP dry weight [4]. When fully hydrated, the NP has a high water content that is

approximately 80% water by weight [5]. The main proteoglycan in the IVD is aggrecan with attached negatively charged chondroitin sulfate and keratin sulfate glycosaminoglycans, which attract cations with water retention properties [5-7]. The aggrecan molecules aggregate along hyaluronan chains in the middle of the NP as seen in Figure 2.2 [4, 8]. The chondroitin sulfate chains are critical for hydrating the tissue by attracting and retaining water, producing osmotic pressure that gives the disc its compressive strength [9, 10]. Randomly oriented elastin is also found in the NP which returns the tissue to its original shape after deformation [4].



**Figure 2.2:** Proteoglycan aggregates are surrounded by collagen fibres in the NP. The sulfated glycosaminoglycan side chains (solid lines) are attached to the central core protein of aggrecans (open line). The aggrecan molecules aggregate along the hyaluronan chains (dashed line). Picture reproduced from [11] with permission from the Copyright Clearance Centre.

The AF that encircles the NP is mainly responsible for resisting tensile forces. The outer AF is composed of concentric lamella of mainly type 1 collagen fibers [12]. The collagen fibers are oriented at approximately 60° to the spinal axis in opposite directions in alternate layers as seen in Figure 2.3 [1, 4, 13]. The organization and composition of the AF is important for its biomechanical function [1, 14]. This anisotropic structure allows the AF to support joint mobility and stability in different directions of bending and rotation [3]. The inner layers of the AF are less organized with a gradual change to type II collagen and greater proteoglycan content [2, 3]. The collagen fibers in the outer layers of the AF connect to the vertebral body, while in the inner layers of the AF, they unite with the NP or insert into the endplates [3, 4, 15]. The elastin creates cross-bridges between the lamellae and lies parallel to the collagen fibers inside the lamellae [4, 15, 16]. Elastin is important for recoil in the AF and inhibiting the lamellae from separating during loading [15].

The hyaline cartilage endplates lie horizontally on either side of the disc, providing an interface between the disc and bone. They are usually less than 1 mm thick [5]. The centre of the endplate is permeable and connected to vascular buds to allow for diffusion of nutrients, while the sides are thicker and impermeable [4, 17].

The NP can support compressive loads that generate hydrostatic pressure as it is rich in proteoglycans that give the NP its high swelling ability and interstitial swelling pressure [3, 18]. The isotropic and gel-like properties of the NP also allow it to uniformly transfer the loads to the AF so there are no regions of high stress concentrations [18]. The hydrostatic pressure generated in the NP is restrained by the AF [3]. The AF experiences significant tensile stresses due to the swelling pressure generated by the NP and the bulging and deformation experienced by the AF during compressive loading [18]. The inner AF also directly supports compressive loads as it is rich in proteoglycans [3]. The IVD is viscoelastic, making it flexible under low loads but rigid under high loads and an increase in strain is seen when a constant stress is applied [4].



**Figure 2.3:** The collagen fibres in the AF are oriented at approximately  $60^{\circ}$  to the spinal axis (or  $\alpha$  is approximately  $30^{\circ}$ ) in opposite directions in consecutive layers. Picture reproduced from [19] with permission from the Copyright Clearance Centre.

#### 2.2 Intervertebral Disc Cells and Nutrition

The cells in the outer AF are like fibroblasts. They are elongated and lie parallel to the collagen fibres [4, 11]. The cells in the inner AF and the cartilage

endplates are like chondrocytes [4, 20]. The cells in the NP start off as notochordal cells in newborns, but their numbers decrease until they are depleted by around four to ten years of age [4, 20]. During this time, the cells in the NP are gradually replaced by smaller and less metabolically active chondrocyte-like cells that may have originated from the cartilage endplates [3, 20, 21]. Cell density in the IVD is lower than other tissues, with cells accounting for less than 1% of the total volume of the IVD [4]. The average cell density in the adult AF and NP is about 9000 cells/mm<sup>3</sup> and 4000 cells/mm<sup>3</sup> respectively [3]. In comparison, other tissues, such as heart tissue, have a much higher cell density of around 10<sup>8</sup> cells/cm<sup>3</sup> [22].

As an IVD is the largest avascular structure in the body, diffusion is the primary way in which cells within the IVD receive nutrients [4, 23]. Diffusion of nutrients into the IVD occurs through the permeable region in the centre of the cartilage endplates that are connected to the vascular buds in the vertebral bodies and through the sides of the AF that are surrounded by blood vessels as shown in Figure 2.4 [4]. Lumbar IVD cells can be as far away as 8 mm from the closest blood supply [4]. Hence the limited ability of the cells in the IVD to self renew is probably due in part to the IVD being avascular. The ability to self renew is believed to decrease over time due to calcification of the cartilage endplates, resulting from aging and diseases such as scoliosis, that causes a decrease in the nutrient supply [4].



**Figure 2.4:** Structure of the intervertebral disc. The cartilage endplates are bonded to the cortical bone of vertebral bodies and collagen fibres in the AF and NP. Diffusion of nutrients from surrounding blood vessels into the IVD through the permeable region of the cartilage endplates is indicated by arrows. Picture reproduced from [11] with permission from the Copyright Clearance Centre.

#### **2.3 Etiology of Intervertebral Disc Degeneration**

Various factors such as abnormal loading, biochemical changes, reduced nutrition, and genetic predisposition have all been linked to IVD degeneration [3, 4]. Abnormal mechanical loading is thought to be one of the factors that can lead to IVD degeneration. Compressive overloading of IVDs may result in degenerative changes such as structural failure and increased apoptosis [24, 25]. Injury to IVDs, such as AF tearing or surgical herniation, has also been shown to lead to IVD degeneration [26, 27].

It has also been reported that lumbar fusion, which alters the biomechanics in the spine, leads to degeneration of the IVDs adjacent to the fused segment lending further evidence to abnormal loading being one of the factors in IVD degeneration [28].

Lack of nutrient supply to IVDs is also thought to be one of the causes of IVD degeneration [29]. Cells need oxygen and nutrients such as glucose to function. However, calcification of the cartilage endplates leads to a decrease in the oxygen and nutritional supply in IVDs, which in turn leads to an acidic environment due to anaerobic respiration [4, 30]. Matrix synthesis rates have been shown to decrease when the environment is acidic and low in oxygen [31, 32] while cell viability also decreases at acidic pH and low glucose levels [33]. Hence, a deficiency in the nutrient supply to the IVD can eventually result in IVD degeneration.

Genetic predisposition may be another factor in IVD degeneration. Familial predisposition for IVD degeneration and herniation has been shown in previous studies [34, 35]. Also, collagen IX [36, 37], aggrecan [38], and vitamin D receptor [39] gene polymorphisms have been linked to a risk of developing IVD degeneration.

#### 2.4 Effects of Intervertebral Disc Degeneration

One of the changes that occur during IVD degeneration is the decrease in overall proteoglycan content, which leads to a loss of hydration and osmotic pressure in the NP [40]. This can, in turn, cause abnormal stresses in the IVD which have been linked to discogenic pain [41]. Due to degenerative changes in the NP, the compressive loads are not transferred to the AF uniformly [42]. This can result in structural failure of the AF,

such as circumferential and radial tears. In turn, this can sometimes lead to posterior radial bulges or herniations of the NP that can impinge on surrounding nerves causing pain [3]. Loss of disc height is also seen in IVD degeneration causing compression of adjacent structures, which can lead to pain [3].

Low back pain (LBP) is a major cause of disability in people between the ages of 20 and 50 and has significant socioeconomic consequences [43]. In the US, over 65 million people are afflicted with LBP and 12 million of these cases are thought to be due to degenerative disc disease [44]. Furthermore, about a third of the patients affected by LBP require extensive care and hospitalization [43]. A review of recent studies from 1995-2004 has estimated the total direct costs (which involve an exchange of money) and indirect costs (which reflect the economic consequences without a direct exchange of money, such as loss of employment productivity) of LBP to range from \$19.6 to \$118.8 billion per year [45].

#### 2.5 Current Treatments for Low Back Pain

The most common treatments for chronic LBP involve providing short term pain relief by the use of analgesics or physiotherapy [46]. When these options fail, the only other alternative is a surgical procedure, such as spinal fusion or discectomy [4].

Spinal fusion is performed when there is a large amount of instability in the spine [47]. During spinal fusion, the disc that is thought to cause LBP is removed and the adjacent vertebral bodies are fused using bone graft and sometimes spinal instrumentation for internal fixation is used to stabilize the spine [4, 47]. Spinal fusion

has been shown to be successful in alleviating LBP [4, 48, 49]. However, studies of patients that have undergone spinal fusion have shown further degeneration of adjacent IVDs and altered biomechanics of the spine, such as increased intradiscal pressure and stress responses in adjacent levels [28].

In discectomy, a part of the NP is removed, often without reconstructive surgery of the IVD. Short-term (two years after surgery) benefits of discectomy such as reduction of pain have been shown [50, 51]. However, this procedure results in a loss of disc height, a decrease in NP pressure, an increase in stresses and strains in the AF, and damage to the AF which can cause further degeneration of the IVD [50, 52]. Also, reoccurrence of herniations by the remaining NP may be seen after discectomy [53].

NP replacement (NPR) devices such as the Prosthetic Disc Nucleus device (PDN, Raymedica, Bloomington, MN), as shown in Figure 2.5, attempt to restore disc height and flexibility to original levels and lessen the forces transmitted to surrounding structures while preserving the remaining structures by using a minimally invasive surgical procedure [54, 55]. The NP is removed and replaced by the PDN. The hydrogel inside the polyethylene jacket absorbs fluid and swells while the jacket maintains the shape of the hydrogel. However, with NPR devices, there are problems related to migration of the implant [54, 56].

Total disc replacements (TDR) have been developed as an alternative to spinal fusion in order to maintain the mobility of the vertebral segment and decrease the risk of degeneration at adjacent IVDs while providing pain relief [57]. TDR devices such as Charite (DePuy Spine Inc., Warsaw, IN) and ProDisc (Synthes Spine Inc., West Chester, PA) (see Figure 2.6) when used clinically, have been shown to provide considerable pain relief in short-term studies of up to two years after surgery [58, 59]. However, the long-term effectiveness of these devices is still unknown [57]. Furthermore, there are potential problems with TDR devices such as migration, prolapse, and failure [46].



**Figure 2.5:** The NP replacement device, Prosthetic Disc Nucleus, is a surgical treatment for degenerative disc disease which attempts to restore disc height and flexibility to original levels and lessen the forces transmitted to surrounding structures. Picture reproduced from [60] with permission from the Copyright Clearance Centre.



**Figure 2.6:** Total Disc Replacement (TDR) devices such as Charite (left) and ProDisc (right) have been used to treat chronic back pain caused by degenerative disc disease. They have been developed as an alternative to spinal fusion. Left picture reproduced from [61] and right picture reproduced from [62] with permission from the Copyright Clearance Centre.

#### 2.6 Annulus Fibrosus Closure Methods

#### **2.6.1** Commercial Products

Structural defects can occur in the AF due to degenerative disc disease and from performing discectomy. It is important to seal the defect in the AF after discectomy to prevent reoccurrence of herniations or extrusion of the NP replacement device when the spine is loaded.

The Xclose Tissue Repair System (Anulex Technologies Inc., Minnetonka, MN), as shown in Figure 2.7, closes the incision in the AF by putting T-Anchors on the sides of the opening and suturing the incision [63]. Preclinical studies have shown that the incision remains closed and spinal flexibility is maintained when tested in cyclic loading through rotation, lateral bending, flexion, and extension [64]. However, previous findings have shown that suture methods do not help with the healing of the AF [64].

The Barricaid Annular Reconstruction Device (Intrinsic Therapeutics Inc., Woburn, MA), as shown in Figure 2.8, is inserted through the incision in the AF after discectomy to create a strong, flexible barrier between the AF and the NP [65]. This device is made from an expanded polytetrafluoroethylene (ePTFE) mesh with a titanium bone anchor [64, 65]. Initial use of the Barricaid device has shown that this device reduced the number of recurrent herniations, and maintained intradiscal pressure and height of the disc when compared to patients without this device [64].

The Inclose Surgical Mesh System (Anulex Technologies Inc., Minnetonka, MN), as shown in Figure 2.9, is designed as a barrier and a scaffold for the repair of the AF [63]. The mesh is made from a polyethylene terephthalate (PET) monofilament braid. The device is inserted in its closed form for a minimally invasive surgery on a disposable delivery tool and is expanded beneath the defect in the AF [63]. The implant is then anchored using nonabsorbable sutures. Preliminary studies have shown no reoccurrence of herniations and negligible effect on spine flexibility [64].



**Figure 2.7: A:** The Xclose Tissue Repair System closes the incision in the AF by suturing the incision. **B:** T-Anchors on the sides of the incision and sutures hold the incision together. Picture reproduced from [63] with permission from Anulex Technologies.



**Figure 2.8:** A: The Barricaid Annular Reconstruction Device is a commercial product for sealing defects in the AF. B: The Barricaid device is shown in its implanted position in the IVD. It has been inserted through an incision in the AF after discectomy to act as a barrier between the AF and the NP. Pictures removed due to copyright restrictions and can be found at [65].



**Figure 2.9:** The Inclose Surgical Mesh System acts as a barrier and a scaffold for the repair of the AF. Picture reproduced from [63] with permission from Anulex Technologies.

Although commercial products for sealing the AF are available, there is still research being performed to investigate different methods as suturing, as in the case of the Xclose Tissue Repair System, does not recreate the lost parts of the AF and long term consequences of the commercial products have not been characterized. Furthermore, none of these commercial products employ a direct biologically regenerative approach where cells can be delivered with the device in order to regenerate the damaged AF tissue.

#### 2.6.2 Current Research

Ahlgren *et al.* (2000) investigated the effect of repairing AF defects with sutures on the healing strength of the IVD after discectomy [66]. It was found that direct repair by suturing AF defects did not significantly change the healing in the IVD [66]. Heuer, *et al.* (2008) investigated the use of conventional closure methods of suturing, gluing with fibrin glue and cyanoacrylate glue, and a combination of both suturing and gluing to seal the AF [54]. Sealing the AF by suturing or using fibrin glue was found to fail the earliest while a combination of suturing and using cyanocacrylate glue was able to last the longest [54]. Although these conventional sealing methods were better than no sealing at all where the implant extrudes after a few cycles, none of these methods were able to last up to the maximum number of cycles showing that these methods were not good enough for the long-term sealing of the AF [54].

Ledet *et al.* (2009) investigated the use of small intestinal submucosa (SIS) in sheep for closure of defects in the AF after discectomy [67]. Biomechanical testing involved injecting a glycerol/crystal violet solution into the disc using an infusion pump until failure or the maximum pressure transducer value of 10 MPa was reached. For reference, normal pressures in the IVD range from 0.1 MPa while lying prone to 2.3 MPa while lifting a 20 kg weight [68]. Average maximum pressure before failure was found to be 5.05 MPa for SIS treatment, 2.00 MPa in anulotomy (which is an incision into the AF) -only treatment, and 7.68 MPa in exposure-only treatment. Only the exposure and anulotomy-only treatment were statistically different [67].

Histology showed greater tissue formation and integration with the AF in the SIS treated discs than the anulotomy-only discs. However, the new tissue was not organized like the native AF making this more of a healing response than regeneration. The insertion of screws into the vertebral body appeared to result in new bone formation which was augmented by the SIS patch. Therefore, the SIS patch needs to be refined to

improve AF repair while reducing bone formation [67].

Hegewald *et al.* (2009) investigated the use of a polyglycolic acid biomaterial lyophilized with hyaluronic acid (PGA/HA) for NP replacement and to seal defects in the AF after nucleotomy [69]. The spinal units were tested in flexion/extension and lateral bending. The three discs with unsealed defects showed herniation of the PGA/HA nucleus implant. While bulging was seen in the four discs that were sealed with the PGA/HA patch, none showed actual herniation of the nucleus implant during testing. However, future studies need to investigate the consequences of fatigue cyclic loading, shear loading, and the use of the patch on highly degenerated discs [69].

Bron *et al.* (2010) investigated the use of a novel annulus closure device (ACD) in a goat nucleus replacement model [70]. The polyethylene ACDs were cylindrical with barbed rings. After discectomy with an annulus defect of 3 mm in diameter, a collagen nucleus implant was inserted, and the ACD was inserted inside the defect until all the barbed rings were within the defect.

Biomechanical testing involved applying an axial compression load of up to a maximum of 5000 N until extrusion of the collagen nucleus implant or the ACD was seen. All ACDs were able to withstand at least 1000 N which corresponds to about 5 MPa. The insertion of ACD did not have a negative impact on the range of motion. The ACDs placed in two goats were still in place two weeks after surgery. However, six weeks after surgery, only two ACDs were still in place, seven were partially displaced, and seven were fully displaced. Severe plastic deformation was seen in all of the ACDs. In two cases, severe osteolytic reaction were seen most probably due to friction resulting

in endplate necrosis [70].

Therefore, conventional closure methods of suturing and gluing and the novel polyethylene ACDs have not been shown to be effective for long-term sealing of the AF. Also, the SIS and PGA/HA patches do not provide a way to deliver cells with the sealing patch for a regenerative approach to repairing the AF defect. For this reason, there is increased research focusing on finding tissue engineering based approaches to repairing the IVD.

#### 2.7 Tissue Engineering

The goal of IVD tissue engineering is to restore the biomechanical function of the IVD through tissue regeneration. Tissue engineering is dependent on three major components: the cells, the scaffold, and the signals.

#### 2.7.1 Cells

Typically when engineering a particular tissue, the cells are sourced from that tissue. However, finding the appropriate cell source for IVD tissue engineering is difficult due to the low availability of cells in the disc. There is only a low yield of NP cells that can be harvested from the IVD and they have a low proliferative capacity when they are being cultivated [4]. Studies have investigated the use of mesenchymal stem cells (MSCs) for IVD tissue engineering where they have been transplanted or differentiated into cell types of the IVD [71-73]. This is an attractive option as autologous MSCs can be obtained by using a bone marrow puncture procedure on the

#### patient [4].

#### 2.7.2 Scaffolds

In tissue engineering, the scaffold plays an important role in imitating the extracellular matrix (ECM) in the body. The scaffold is also useful as a means of delivering cells to the intended site and could potentially protect them from the unfavourable implantation environment [4]. Important considerations in the design of a scaffold include mechanical properties, biocompatibility, biodegradability, and delivery of the scaffold into the implantation site [74]. Some of the biomaterials that have been investigated as a scaffold for IVD tissue engineering include collagen [75], hyaluronan [75], alginate [76], and poly-D, L-lactide (PDLA) [77]. However, disadvantages with these scaffolds have been reported. While collagen/hyaluronan scaffolds were able to maintain functional disc cells, the proteoglycan content in the scaffolds only reached 10% of the amount in the native NP [75]. Alginate scaffolds could not form a mechanically functional matrix [76] while cells did not adhere to PLDA beads [77].

#### 2.7.3 Signals

As there is reduced cell activity and nutrient availability in degenerated IVDs, signals such as growth factors (GFs) could be necessary to make IVD tissue engineering more effective [4]. GFs are bioactive molecules that are essential in moderating tissue formation and maintenance by acting through the endocrine, paracrine, or autocrine systems [4]. Most IVD tissue engineering approaches have used GFs from the
transforming growth factor beta (TGF- $\beta$ ) superfamily, such as TGF- $\beta$ 1 or bone morphogenic proteins [4].

Mechanical signals applied to cells can also trigger pathway reactions, gene expression, and secretion of ECM. As cells in the native IVD experience hydrostatic pressure, compression, tension, fluid flow, and osmotic pressure, a review has reported studies where external mechanical loads were applied [74]. External mechanical loads such as cyclic stress and hydrostatic pressure have been shown to increase ECM synthesis by IVD cells [78].

#### **CHAPTER THREE: MATERIALS AND METHODS**

# 3.1 Gellan Gum Hydrogel Preparation

Low acyl gellan gum hydrogels were prepared by dissolving 1, 2, 3, 4, 5, and 6% (w/v) low acyl gellan gum powder (G1910, batch #108K0054, Sigma-Aldrich, ON, Canada) in distilled water. Low acyl/high acyl gellan gum blend hydrogels were prepared by dissolving 2% (w/v) low acyl and high acyl gellan gum powder (Kelcogel CG-HA, lot #9F6035A, CP Kelco US, Inc., DE, USA) in ratios of 50:50 and 75:25 (w/w) in distilled water. The solution was heated to 90°C and kept at this temperature while stirring until the gellan gum had fully dissolved. To this solution, 0.03% (w/v) CaCl<sub>2</sub> (C8106, batch #108K1294, Sigma-Aldrich, ON, Canada) was then added. The solution was removed from heat and left to cool in a Petri dish at room temperature until the gellan gum solution had set into a gel.

#### **3.2 Fibrin Gel Preparation**

Fibrinogen (F8630, lot #029K7636V, Sigma-Aldrich, ON, Canada) was dissolved in PBS to obtain a final concentration of 80 mg/mL. Thrombin (T4648, lot #070M7351V, Sigma-Aldrich, ON, Canada) was dissolved in a 40 mM CaCl<sub>2</sub> solution in distilled water to obtain a final concentration of 50 U/mL. Equal volumes of the fibrinogen and thrombin solution were used to form the fibrin gel. The thrombin solution was first drawn up into a syringe. Then a new needle was used to draw up the fibrinogen solution into the same syringe. The mixed solution was quickly injected in

liquid form into a mould or directly into the defect in a bovine tail IVD after which it formed a gel.

#### 3.3 Chitosan/Gelatin/Glycerol Phosphate Hydrogel Preparation

To make 15 mL of the chitosan/gelatin/glycerol phosphate (C/G/GP) solution, 2.5% (w/v) chitosan (MW 100,000 – 300,000; degree of deacetylation  $\geq$  90%, 34905, lot #A0281394, Acros Organics, NJ, USA) and 1% (w/v) gelatin (G1890, lot #070M0081V, Sigma-Aldrich, ON, Canada) were dissolved in 12 mL of 0.1 M acetic acid (A6283, batch #10610MH, Sigma-Aldrich, ON, Canada).  $\beta$ -glycerophosphate disodium salt hydrate ( $\beta$ -GP, 10% (w/v), G5422, batch #020M5443, Sigma-Aldrich, ON, Canada) was dissolved in 3 mL of distilled water. The  $\beta$ -GP solution was chilled to 4 °C by leaving it in the fridge and added dropwise to the chitosan/gelatin solution while stirring at around 100 rpm to prevent immediate precipitation. The solution was then transferred to a Petri dish and put into the incubator at 37 °C until the solution gelled.

#### **3.4 PVA/PAA Hydrogel Preparation**

PVA/PAA hydrogels were prepared by dissolving 10% (w/v) PVA (MW 31,000-50,000, 363138, Sigma-Aldrich, ON, Canada) and 2.5% (w/v) PAA (MW 450,000, 181285, Sigma-Aldrich, ON, Canada) in distilled water. The PVA/PAA solution underwent six cycles of freezing for 21 hours at -20 °C and thawing for 3 hours at room temperature to form a gel. The PVA/PAA hydrogel is formed through physical crosslinking due to formation of crystallites during repeated freeze-thaw cycles [79].

# 3.5 Kryptonite Construct

An AF construct is a physical structure that could be used to seal defects in the AF. In the work presented here, a construct to repair defects in the AF was envisioned which contained three regions – a solid region at each end, with a flexible central region (shown in Figure 3.1). The construct would span the distance between the two adjacent vertebral bodies on either side of the disc. The solid region at each end would adhere to each of the vertebral bodies, thereby affixing the construct in place. The flexible central region would span the AF, thereby providing a means to seal, and promote repair, of the damaged AF. It was decided to use Kryptonite <sup>TM</sup> Bone Cement for the two ends of the construct, and 2% (w/v) low acyl gellan gum for the central region. To increase the tensile properties of the construct, either sutures or electrospun poly(lactic-co-glycolic acid) (PLGA) fibres were used. The ends of each fiber were embedded in the ends of the construct and spanned the flexible central region.



**Figure 3.1:** The envisioned Kryptonite construct is adhered to the vertebral bodies through the ends made from Kryptonite<sup>TM</sup> Bone Cement while the gellan gum is attached to the defect site in the AF. Sutures or fibres are embedded in the middle of the construct.

# 3.5.1 Preparation of Construct Moulds

The moulds were made by cutting out the desired shape of the Kryptonite construct in a rubber sheet and the placing that sheet on top of a new rubber sheet, which were clamped together using binder clips. The same moulds were used for all samples. The mould was dumb-belled shaped as shown in Figure 3.2 in order to perform tensile testing. The ends were wider so the sample would break in the middle instead of where it was clamped. Dimensions were w = 19 mm, L = 115 mm, w<sub>n</sub> = 6 mm, and L<sub>n</sub> = 33 mm according to the American Society for Testing and Materials (ASTM) standard for tensile testing.



**Figure 3.2:** Dumbbell shaped moulds for the Kryptonite constructs. This figure is not drawn to scale.

# 3.5.2 Preparation of Kryptonite<sup>TM</sup> Bone Cement

Kryptonite<sup>TM</sup> (Doctors Research Group, Inc., CT, USA) is a commercially available product that comes as three separate components (component A – prepolymer, B – polyol, and C – calcium carbonate) which must be mixed to form the bone cement. The details regarding why Kryptonite was chosen for this application will be discussed in Chapter 6. The three components were mixed together by hand.

# 3.5.3 Preparation of Fibres

In this study, both sutures and electrospun fibres were evaluated in the construct. The sutures were coated vicryl polyglactin 910 sutures (diameter:  $4-0 / \sim 0.2$  mm, length: 18", J386, Ethicon, Inc., USA). The electrospun fibres were made from PLGA and produced in, and provided by, the laboratory of Dr. Anastasia Elias from University of Alberta. Briefly, the electrospun fibres were made by mixing 8% (w/v) PLGA in 3:1 tetrahydrofuran:N,N-Dimethylformamide overnight. This solution was pumped at a rate of 50 µL/min by using a syringe pump. The distance from the tip of the needle to the

rotating collector was 5.4 cm and the voltage differential was 6.17 kV.

# 3.5.4 Preparation of Gellan Gum

The 2% (w/v) low acyl gellan gum was prepared according to the method stated in section 3.1.

# 3.5.5 Preparation of the Kryptonite Construct

The Kryptonite mixture was poured into the ends of the rubber mould halfway to the top while still in its liquid form. The mixture stayed in place without requiring fixation as it was quite viscous.

Next, either four lines of sutures or three layers of electrospun PLGA fibres were placed lengthwise on top of the Kryptonite mixture poured into the mould. More Kryptonite mixture was then poured on top of the sutures or fibers until it had reached the top of the mould. The construct was then left to set overnight at room temperature. Once the construct had set, 2% (w/v) low acyl gellan gum poured into the space left in the middle of the construct. The completed Kryptonite construct is shown in Figure 3.3.



**Figure 3.3:** The Kryptonite constructs were made in rubber moulds. It was embedded with electrospun PLGA fibres and contained 2% (w/v) gellan gum in the middle section. The arrow indicates where the PLGA fibres have been embedded inside the gellan gum.

# **3.6 Dynamic Mechanical Analysis**

Dynamic mechanical analysis (DMA) was performed to characterize the compressive mechanical properties of the hydrogels. Complex compressive moduli were calculated to compare the values with those of native tissues. Storage and loss moduli were calculated to determine elastic and viscous properties of the hydrogels.

DMA was performed using a Bose Electroforce test frame (ELF-3200, Bose, MN, USA) at room temperature on the day the gellan gum, fibrin, and C/G/GP hydrogel samples were created. Five cylindrical samples were tested for each type of hydrogel. The samples were created using a 6 mm diameter biopsy punch. The samples went through five compression cycles at each of the frequencies 0.01, 0.1, 1, and 10 Hz with constant amplitude displacements of 3% of the sample height. These are typical values

for DMA tests [80]. The force and displacement data were recorded at a rate of 100 points/sec. Calibration for the Bose Electroforce test frame had been performed by Bose technicians.

Complex compressive modulus (E; kPa) represents the bulk sample behaviour or its viscoelasticity and is the sum of the storage and loss moduli. It was calculated by using the equation:

$$E = \frac{\sigma}{\varepsilon} \tag{1}$$

where  $\sigma$  (kPa) and  $\epsilon$  (mm/mm) are the average of three peak-to-peak stress and strain amplitudes respectively.

Storage modulus (E'; kPa) represents the elastic component of the gel. The loss modulus (E"; kPa) represents the viscous component of the gel. The storage and loss moduli were calculated from the equations:

$$E' = \frac{\sigma}{\varepsilon} \cos \delta \tag{2}$$

$$E'' = \frac{\sigma}{\varepsilon} \sin \delta \tag{3}$$

where  $\delta$  (radians) is the phase lag between stress and strain.

The target compressive modulus for the biomaterial to be used for NP repair is that of the native NP, which is 310 kPa [81]. The target compressive modulus for the biomaterial to be used for AF repair is that of the native AF, which has been reported to be 52 kPa [82], 380 kPa [83], 560 kPa [81], and 740 kPa [84] by various studies.

# 3.7 Gelling Temperature of Gellan Gum

The gelling temperatures of low acyl and blends of low acyl/high acyl gellan gum were determined using a Fisher Scientific Traceable® Ultra<sup>TM</sup> digital thermometer. After the gellan gum powder was dissolved and CaCl<sub>2</sub> was added, the solution was removed from heat and the temperature was checked until a gel had formed. It was determined that the gellan gum had formed a gel when the solution could no longer flow when the beaker was tipped.

Gelling temperature was determined for 1, 2, 3, 4, 5, and 6% (w/v) low acyl gellan gum with the addition of 0.01, 0.03, 0.05, and 0.07% (w/v) CaCl<sub>2</sub>. Gelling temperatures of 1, 2, and 3% (w/v) low/high acyl gellan gum blends in ratios of 0:100, 25:75, 50:50 and 75:25 (w/w) with 0.03% (w/v) CaCl<sub>2</sub> were also measured. The gelling temperature tests were performed together with Stephanie Fisher (another MSc student in the laboratory).

#### **3.8 Tensile Testing**

The 2% (w/v) low acyl gellan gum hydrogel, finbrin hydrogel, and entire Kryptonite construct samples for tensile testing were created using dumbbell shaped rubber moulds as show in Figure 3.2. The dimensions of the samples are listed in Table 3.1. The dimensions of the Kryptonite construct samples were created according to the American Society for Testing and Materials (ASTM) standard D638-10 Type IV (non-rigid plastics) [85]. The dimensions of the hydrogel samples were created to be 2/3 of the Kryptonite construct size as it was difficult to create fibrin hydrogels of such a large

size due to the fast gelling time of these hydrogels (less than 30 seconds). The Kryptonite constructs without gellan gum hydrogel in the middle section were rectangular in shape with a total dimension of 20 mm in width and 60 mm in length. The Kryptonite ends were square in shape with a dimension of 20 mm by 20 mm.

**Table 3.1:** Sample dimensions for tensile testing according to the ASTM standard D638-10 Type IV (non-rigid plastics) for tensile testing.

Sample	<b>w</b> ( <b>mm</b> )	l (mm)	w <sub>n</sub> (mm)	$l_n (mm)$
Hydrogel	13	17	4	22
Kryptonite Construct	19	115	6	33

A Bose Electroforce test frame was used to pull the samples at a rate of 0.1 mm/sec until failure according to the ASTM standard for tensile testing or the maximum displacement limit of the test frame was reached. Five samples were tested for each of the hydrogel conditions and two samples were tested for the suture Kryptonite constructs and one sample for the electrospun fibre Kryptonite constructs. Due to supply issues regarding the Kryptonite and the electrospun PLGA fibres, only a limited number of samples of the Kryptonite constructs could be tested. Tensile testing could not be performed on C/G/GP hydrogels as they were too weak to be clamped by the grips.

A dot tracking system was employed to determine internal strain in the samples. Five dots were drawn on the gellan gum and fibrin hydrogel samples with a marker. The dots were drawn in the middle of the narrow section of the samples as shown in Figure 3.4. A camera system was used to track the movement of the five dots to calculate the internal strain in the samples by determining the change in the distance between the dots. Internal strain was calculated as the hydrogels slip out of the grips and so the displacement recorded by the Bose Electroforce test frame is not the actual displacement experienced by the sample and cannot be used in the calculation of strain. The dot tracking system was not used when performing tensile tests for the Kryptonite constructs as the grips could clamp firmly onto the Kryptonite ends of the samples. The force, displacement, and strain data was recorded at a rate of 100 points/sec.



Figure 3.4: A) Placement of five dots on the samples for tracking internal strain during tensile testing. B) An example of a sample that had undergone a tensile test. These figures are not drawn to scale.

The ultimate tensile strength (UTS; MPa) is the maximum stress the sample can withstand when being stretched. The target tensile strength is that of the native AF, which is between 3.9 and 8.6 MPa [86]. It was calculated using the equation:

$$UTS = \frac{F}{A} \tag{4}$$

where F (N) is the maximum force and A (mm<sup>2</sup>) is the cross-sectional area of the narrow section.

#### **3.9 Bovine Tail Sample Preparation**

Bovine tail IVDs were used in order to determine the effectiveness of various biomaterials used to seal defects in the AF. A discussion on why bovine tails were a good model for this application is provided in Chapter 5. A defect was created in the bovine tail IVD and sealed using a biomaterial. Pressure testing was performed on this sample in order to determine how well the biomaterial could seal the defect.

Bovine tails were purchased from a local butcher (XL Beef, AB, Canada). All the soft tissue was removed from the bovine tails while they were intact using scalpels. Only the soft tissue around the top three discs out of five were removed as the lower discs were too small for testing. The dissected bovine tails were wrapped in saline soaked paper towels, placed in plastic bags, and stored at 4 °C with the exception of the frozen IVD condition until the time of the IVD pressure testing. For the frozen IVD condition, the bovine tails were stored at -20 °C for seven days without the use of cryoprotectants and thawed overnight at 4 °C prior to the IVD pressure testing.

## 3.10 Intervertebral Disc Pressure Testing

## 3.10.1 Equipment Description

A 16G BD Precision Glide Needle was inserted into the right lateral side of an IVD as shown in Figure 3.5. Crystal violet (0.05% (w/v), C3886, Sigma-Aldrich, ON, Canada) dissolved in PBS was pumped into the IVD using a hand pump. Efforts were taken to ensure the needle was inserted into the centre of each IVD by holding the tip of the needle above the centre of the IVD and placing a mark on the needle with a pen to indicate the depth to which it should penetrate into the IVD. A DC power supply (model 1760A, B&K Precision, CA, USA) was used to provide a 10 V excitation voltage to the pressure transducer (model PX905-1.5 KGV, Omegadyne, Inc., OH, USA). The pressure data was recorded in mV at a rate of 1 point/sec using a portable handheld data logger (model OM-DAQPRO-5300, OMEGA Engineering, Inc., CT, USA). The IVD pressure testing setup is shown in Figure 3.6



**Figure 3.5:** The needle was inserted into the right lateral side of the IVD in the bovine tail during the pressure testing. The AF defect was on the opposite side of where the needle was inserted.



**Figure 3.6:** IVD pressure test setup. The needle was inserted into the right lateral side of the IVD and the hand pump was used to pump crystal violet solution into the IVD until failure. The pressure in the IVD was measured at the point where the pressure transducer was inserted.

# 3.10.2 Method for Pressure Testing

The maximum burst pressures of frozen and non-frozen intact IVDs were determined through pressure testing. Each of the discs to be tested was randomly assigned a treatment (either frozen IVD or non-frozen IVD).

This test was also performed to compare the differences of the maximum burst pressures of intact IVDs, IVDs with a standard hole punched in them, and IVDs that had

a standard hole that had been patched using gellan gum, fibrin, or C/G/GP hydrogels. For all conditions except the intact condition, a hole was created in the left lateral side of the bovine tail IVD using a 6 mm biopsy punch. With the exception of the hole only condition, the hole was then patched with gellan gum, fibrin, or C/G/GP hydrogel by injecting the solution of the gels until the hole was completely filled using a syringe while they were still in liquid form. To ensure consistency between samples and that the hydrogels had fully gelled, the discs were loosely covered with saline soaked paper towels and tested 30 minutes after the hydrogels were injected.

The test was ended when either leakage of the crystal violet solution was seen from the IVD or the maximum pressure limit of the test setup was reached. If the solution leaked from the needle insertion site, this data was not included in the analysis. Six samples were tested for each condition in the frozen intact vs. non-frozen intact IVD pressure test.

For the pressure testing of the hydrogels, the test was repeated using a new sample in the case where the solution leaked back out of the place where the needle was inserted until the maximum burst pressures of six samples for each condition were obtained. The pressure testing procedure was based on an accepted published protocol [67].

The calibration of the pressure transducer was certified up to three years after the time of purchase and all pressure testing experiments were performed within that time frame. A calibration curve of the reading from the pressure transducer (mV) vs. the actual pressure (kPa) was created using the calibration data provided with the pressure

transducer. The calibration curve was then used to convert the pressure recorded in mV into kPa. The calibration of the pressure transducer was checked by placing a pressure gauge at the point where the needle should be. The pressure shown in mV in the data logger was compared to the pressure gauge (model EN 837-1, Bourdon Haenni, France) reading at a range of 500-3500 kPa in increments of 500 kPa. This check was performed five times.

# **3.11 Statistical Analysis**

The DMA data were analyzed using two-way ANOVA and Tukey's post-hoc test. Normal Probability Plots were used to evaluate the normality assumption; no evidence of significant non-normalities was found.

The gelling temperature data were analyzed using ANOVA. The normality assumption was verified using Normal Probability Plots and by cross-referencing the GLM results to Kruskal-Wallis tests. In cases where the F-test was significant, Bonferroni's post-hoc tests were conducted to compare pairwise conditions.

The tensile test data and the frozen/non-frozen intact IVD pressure testing data were analyzed using the Mann-Whitney test. The hydrogel patched IVD pressure testing data was analyzed using the Kruskal-Willis test followed by a Dunn's post test. Significance was taken as p < 0.05.

# **CHAPTER FOUR: OPTIMIZATION OF GELLAN GUM PROPERTIES**

# 4.1 Introduction

Various biomaterials have been investigated for IVD tissue engineering applications such as synthetic polymers, like polyglycolic acid and polylactic acid [87, 88], or natural polymers, like collagen and alginate [71, 75]. However, there are limitations such as the toxicity of crosslinking agents and photoinitiators [89, 90], inadequate mechanical strength [91], loss of strength during degradation [92], and insufficient characterization of its mechanical properties [15].

A hydrogel contains a network of hydrophilic polymer chains with a large water content. Gellan gum is an attractive hydrogel for tissue engineering applications as it can be injected as a liquid into a defect area using a minimally invasive method, take the shape of the defect, and form a gel at normal body temperature. This is possible as gellan gum solution only forms oriented bundles that are linked together when the temperature is lowered to its gelling point. Moreover, there is evidence to suggest that it is effective for encapsulating cells for tissue engineering purposes as it is nontoxic, can be processed without harsh reagents, and can maintain cell viability at 1% (w/v) concentration [80, 93, 94].

Gellan gum is a linear anionic polysaccharide composed of repeating tetrasaccharide units of  $\beta$ -D-glucose,  $\beta$ -D-glucuronic acid,  $\beta$ -D-glucose, and  $\alpha$ -L-rhamnose as shown in Figure 4.1 [95]. It is produced by the bacteria *Sphingomonas paucimobilis* from glucose as an extracellular polysaccharide and is widely used in the

food industry as a thickener and stabilizer [93]. It has also recently been studied for tissue engineering applications including IVD tissue engineering applications [80, 96]. The acetylated high acyl gellan gum, as shown in Figure 4.1A, which is the natural form, is soft and elastic while the deacetylated low acyl gellan gum, as shown in Figure 4.1B, is firm and brittle [80, 97]. When these two forms are combined, gels of intermediate properties can be produced [97].



**Figure 4.1:** Molecular structures of A) high acyl gellan gum and B) low acyl gellan gum.  $M^+$  indicates the molecular ion or the one-electron oxidation product. Picture reproduced from [98] with permission from the Copyright Clearance Centre.

A gellan gum solution will undergo a gellation process when the temperature of the solution is lowered to its gelling point causing the molecule to transition from a coil conformation to a double helix. The double helices then self-assemble into oriented bundles which are linked together by regions of untwined polysaccharide chains as shown in Figure 4.2 [99]. Cations must be present in order to form a stable gel as they are used to cross-link the double helices by forming direct bridges by site binding between double helices [93].



**Figure 4.2:** Mechanism for the formation of gellan gum gels. Picture reproduced from [98] with permission from the Copyright Clearance Centre.

Gellan gum was specifically chosen for investigation as a scaffold in NP tissue engineering applications because it is an injectable hydrogel allowing for minimally invasive procedure, can support cell encapsulation, can be processed without harsh reagents, has mechanical properties comparable to many other hydrogels, and is already used in ophthalmic applications for humans [80, 98].

The objective of this study was to determine the optimal concentration and composition of gellan gum for use as a scaffold in NP tissue engineering applications. The optimal target properties of the gellan gum would be to have a modulus similar to the native tissue of around 200-300 kPa and have a gelling temperature that is around 37°C

and 42°C to allow for cell encapsulation. Other design criteria are that it should be flexible, have a high water content, and should be able to support cell growth and survival.

## 4.2 Results

# 4.2.1 Dynamic Mechanical Analysis

Dynamic mechanical analysis (DMA) is a technique used for characterizing materials, especially its viscoelasticity. DMA was performed on 1-6% (w/v) low acyl gellan gum (in increments of 1%) and 2% (w/v) low/high acyl gellan gum (in weight ratios of 75/25 and 50/50) with 0.03% (w/v) CaCl<sub>2</sub>. Concentrations of 1% (w/v) gellan gum and 0.03% (w/v) CaCl<sub>2</sub> were the starting point as it had been investigated by Oliveira *et al* [94]. Higher concentrations were tested in hopes of finding a concentration of gellan gum that would have a compressive modulus that was closer to that of the native NP of 310 kPa [81]. The total polymer concentration of 2% (w/v) for the low/high acyl gellan gum blend was chosen as a lower concentration would make the gel too weak for this test setup. Higher concentrations were not tested as the gelling temperatures of the 2% (w/v) blends were already very high. The complex compressive modulus, storage modulus, and loss modulus of 1-6% (w/v) low acyl gellan gum were calculated from the DMA results and are shown in Figures 4.3, 4.4, and 4.5 respectively.

With an increase in the concentration of low acyl gellan gum, there was also an increase in the complex compressive modulus and storage modulus. The storage modulus was much higher than the loss modulus for all concentrations of low acyl gellan





**Figure 4.3:** Complex compressive moduli (E) of 1-6% (w/v) low acyl (LA) gellan gum. Five samples were tested for each of the conditions and values have been graphed as means  $\pm$  SEM. Picture reproduced from [98] with permission from the Copyright Clearance Centre.



**Figure 4.4:** Storage moduli of 1-6% (w/v) low acyl (LA) gellan gum. The storage moduli values are slightly lower than the complex compressive moduli values. Five samples were tested for each of the conditions and values have been graphed as means  $\pm$  SEM. Picture reproduced from [98] with permission from the Copyright Clearance Centre.



**Figure 4.5:** Loss moduli of 1-6% (w/v) low acyl (LA) gellan gum. Five samples were tested for each of the conditions and values have been graphed as means  $\pm$  SEM. Picture reproduced from [98] with permission from the Copyright Clearance Centre.

Each gellan gum concentration was significantly different from the other concentrations with p<0.001 for the complex moduli. This was also the case for the storage moduli and loss moduli for all gellan gum concentrations except between the loss moduli of 4% and 5% (w/v) low acyl gellan gum concentrations.

The complex compressive modulus, storage modulus, and loss modulus of 2% (w/v) total polymer gellan gum blends are shown in Figures 4.6, 4.7, and 4.8 respectively. All gellan gum moduli values are given in Tables A.1, A.2, and A.3 in the Appendix. The addition of high acyl gellan gum to the low acyl gellan gum resulted in a decrease in the complex and storage moduli. The moduli values for the low acyl/high acyl gellan gum blends could not be obtained for the frequency of 10 Hz as they were too soft for this test setup so the force response from the DMA tests did not appear sinusoidal. While no quantifiable measures were made, the gels formed by pure low acyl gellan gums.

The blend condition and the frequency were both significant factors with p<0.001. The interaction between the blend condition and the frequency was not significant for the complex or storage moduli with p=0.45 and 0.62 respectively, but was significant for the loss modulus with p<0.001. Each blend condition was significantly different from each other with p<0.001.



-- 2% LA Gellan Gum -- 2% LA/HA Gellan Gum 75/25 -- 2% LA/HA Gellan Gum 50/50

**Figure 4.6:** Complex compressive moduli of 2% total polymer blends of low acyl/high acyl (LA/HA) gellan gum. The storage and loss moduli values could not be obtained for the gellan gum blends at 10 Hz as they were too soft for this test setup. Five samples were tested for each of the conditions and values are shown as means  $\pm$  SEM. Picture reproduced from [98] with permission from the Copyright Clearance Centre.



-- 2% LA Gellan Gum -- 2% LA/HA Gellan Gum 75/25 -- 2% LA/HA Gellan Gum 50/50

**Figure 4.7:** Storage moduli of 2% total polymer blends of low acyl/high acyl (LA/HA) gellan gum. The storage and loss moduli values could not be obtained for the gellan gum blends at 10 Hz as they were too soft for this test setup. The storage moduli values are slightly lower than the complex compressive moduli values. Five samples were tested for each of the conditions and values are shown as means  $\pm$  SEM. Picture reproduced from [98] with permission from the Copyright Clearance Centre.



-- 2% LA Gellan Gum -- 2% LA/HA Gellan Gum 75/25 -- 2% LA/HA Gellan Gum 50/50

**Figure 4.8:** Loss moduli of 2% total polymer blends of low acyl/high acyl (LA/HA) acyl gellan gum. The storage and loss moduli values could not be obtained for the gellan gum blends at 10 Hz as they were too soft for this test setup. Five samples were tested for each of the conditions and values are shown as means  $\pm$  SEM. Picture reproduced from [98] with permission from the Copyright Clearance Centre.

# 4.2.2 Gelling Temperature

Gelling temperatures of 1-6% (w/v) low acyl gellan gum (in increments of 1%) with the addition of 0.01-0.07% (w/v) CaCl<sub>2</sub> (in increments of 0.02%) were mapped out as shown in Figure 4.9. The CaCl<sub>2</sub> concentration of 0.03% (w/v) was chosen as it had

been previously investigated by Oliveira *et al* [94]. Other CaCl<sub>2</sub> concentrations around 0.03% (w/v) were investigated to determine the effect of CaCl<sub>2</sub> concentration on the gelling temperature. The gelling temperature increased with an increase in both the gellan gum concentration and CaCl<sub>2</sub> concentration. As the temperature range suitable for cell encapsulation while allowing for *in vivo* gellation is between 37°C and 42°C, only 1% (w/v) low acyl gellan gum with 0.05% (w/v) CaCl<sub>2</sub> and 2% (w/v) low acyl gellan gum with 0.01 or 0.03% (w/v) CaCl<sub>2</sub> fit this criteria.

Both the gellan gum and  $CaCl_2$  concentrations were significant factors on the gelling temperature with p<0.001. Pairwise comparison of gellan gum concentrations indicated that all gellan gum concentrations tested were significantly different. Pairwise comparison of CaCl<sub>2</sub> concentrations indicated that all CaCl<sub>2</sub> concentrations were significantly different except for 0.01% vs. 0.03% (p=0.11) and 0.03% vs. 0.05% (p=0.13).

The gelling temperatures of 1, 2, and 3% (w/v) total polymer low acyl/high acyl gellan gum blends in weight ratios of 75/25, 50/50, 25/75, and 0/100 with the addition of 0.03% (w/v) CaCl<sub>2</sub> is shown in Figure 4.10. Both the total gellan gum concentration and the ratio of the low acyl/high acyl gellan gum did not have a significant effect on the gelling temperature with p=0.121 and p=0.299 respectively.



**Figure 4.9:** Gelling temperatures of 1-6% (w/v) low acyl (LA) gellan gum containing 0.01-0.07% (w/v) CaCl<sub>2</sub>. The temperature was monitored using a Fisher Scientific digital thermometer until the gellation point. One sample was tested for each condition. This experiment was performed with Stephanie Fisher, another MSc student in the laboratory. Picture reproduced from [98] with permission from the Copyright Clearance Centre.



**Figure 4.10:** Gelling temperatures of 1, 2, and 3% (w/v) total polymer blends containing low acyl (LA) and high acyl (HA) gellan gum with HA gellan gum w/w % of 25, 50, 75, and 100%. N = 2 for the 1% (w/v) gellan gum with 25 (w/w %) HA gellan gum measurement. This experiment was performed with Stephanie Fisher, another MSc student in the laboratory. Picture reproduced from [98] with permission from the Copyright Clearance Centre.

# 4.3 Discussion

#### 4.3.1 Dynamic Mechanical Analysis

DMA tests were performed on various concentrations and blends of gellan gum in order to determine the concentration and blend that would have a modulus close to that of the native NP. DMA tests of low acyl gellan gum showed that the stiffness of the gellan gum increased with increasing concentrations of gellan gum with moduli values between 25 and 400 kPa. The storage moduli were generally 4-5 times greater than the loss moduli and very close to the complex compressive moduli showing that the gellan gum hydrogels were highly elastic, which allows the hydrogel to return to its original state. The large decrease in the loss moduli values compared to the complex compressive and storage moduli was in part due to the scale of the loss moduli graph being smaller than the other two graphs making the effect more exaggerated. Also, it is likely to be a property of the hydrogel itself as viscoelasticity is dependent on the rearrangement of the polymer chains when stress is applied.

The compressive modulus of the NP has been reported to be 310 kPa [81]. While this value of the NP modulus was obtained from confined compression experiments, this is the closest method that could be found for comparison. From this compressive modulus value of the NP, the 5% (w/v) low acyl gellan gum with a complex compressive modulus of  $324.1\pm6.3$  kPa may be a good candidate for use as a scaffold in NP tissue engineering applications. However, the gelling temperature of the gellan gum at these concentrations is too high for cell encapsulation purposes with a gelling temperature that is around 55°C. Therefore, 5% (w/v) low acyl gellan gum cannot be used for cell encapsulation purposes.

DMA testing of the low acyl/high acyl gellan gum blends with a total polymer concentration of 2% (w/v) showed that the stiffness of the blends decreased by approximately 50% when high acyl gellan gum was added. The texture of the hydrogels formed by the gellan gum blends were more desirable for NP tissue engineering as they

are not as brittle as the gel formed by the pure low acyl gellan gum. However, the gellan gum blends had a much lower stiffness than the native NP compressive modulus of 310 kPa and higher gelling temperature compared to the pure low acyl gellan gum making it unsuitable for this purpose.

# 4.3.2 Gelling Temperature

As the gelling temperature of the gel is dependent on the gellan gum and CaCl<sub>2</sub> concentrations [100, 101], the gelling temperatures of various concentrations of low acyl gellan gum and CaCl<sub>2</sub> were measured in order to determine the optimal concentration for cell encapsulation purposes when it is used as a scaffold for NP tissue engineering. The optimal combined concentrations of gellan gum and CaCl<sub>2</sub> should result in a gelling temperature that is approximately that of normal body temperature. Both the low acyl gellan gum concentration and CaCl<sub>2</sub> concentration were found to significantly influence the gelling temperature. Only the low acyl gellan gum concentrations of 2% (w/v) or less had a gelling temperature that was suitable for cell encapsulation purposes.

The gelling temperature of the low acyl/high acyl gellan gum blend with a 75/25 ratio was much lower than any of the other measurements. This may be because the high acyl gellan gum transitions into a double helix at a much higher temperature than the low acyl gellan gum [102], but with a larger concentration of the low acyl gellan gum, the gelling temperature was affected more by the low acyl gellan gum.

Further studies have been done by Stephanie Fisher [103] where MSCs were seeded into 2% (w/v) low acyl gellan gum scaffolds that had TFG- $\beta$ 1 releasing

nanoparticles incorporated into it in order to encourage differentiation of the MSCs into NP cells. However, it did not enhance the differentiation into NP cells as less TFG- $\beta$ 1 was released than expected.

It is important to fully characterize the gelling behaviour of gellan gum in order to determine the composition and concentration that will result in a hydrogel with the desired properties for the intended application. From this study, the optimal gellan gum concentration and composition was determined to be the 2% (w/v) pure low acyl gellan gum for use as a scaffold in NP tissue engineering. This composition had the highest compressive modulus while still having a gelling temperature between 37-42°C.

# CHAPTER FIVE: COMPARISON OF BIOMATERIAL MECHANICAL PROPERTIES FOR ANNULUS SEALING

#### **5.1 Introduction**

#### 5.1.1 Biomaterials

A series of biomaterials were tested to evaluate which biomaterial would be most appropriate for use in sealing defects in the AF. The design criteria for a biomaterial used to seal holes in the AF for this project are: i) it should be flexible, ii) it should have a compressive modulus around 400-700 kPa so it would have a modulus that is close to that of the native AF [83, 84], iii) it should have a tensile strength around 4-8 MPa so it would have a similar tensile strength as that of the native AF [86], iv) it should adhere well enough to be able to withstand pressures up to 2.3 MPa as this was the greatest intradiscal pressure when various activities were performed [68], and v) it should be able to support cell growth and survival.

Fibrin is a fibrous biopolymer formed by the polymerization of fibrin monomers that have undergone enzymatic conversion from fibrinogen catalyzed by thrombin [104]. It plays an important role in blood clotting, the inflammatory response, and wound healing [105]. Fibrin has been investigated for various applications in tissue engineering as it is biocompatible, biodegradable, can contain cells and growth factors, and has a nano-fibrous structure that imitates the ECM [104-106]. Fibrin was chosen to be tested as it is an injectable hydrogel allowing for a minimally invasive procedure in sealing the defect, is biocompatible, and can support cells allowing for eventual
regeneration of the AF tissue. It has also been shown to promote cell proliferation, stable tissue morphology *in vitro*, cartilaginous tissue formation, and glycosaminoglycan production of AF cells [107].

Chitosan is a polysaccharide that is produced by deacylation of chitin which is a part of the exoskeleton of arthropods [108]. It has been used in pharmaceutical and tissue engineering applications as it is biocompatible, biodegradable, non-toxic, and has a low cost [109, 110]. The addition of a disodium salt ( $\beta$ -glycerol phosphate (GP)) causes chitosan solutions to become thermosensitive and gel at 37°C [110, 111]. The addition of gelatin to the chitosan/GP solution has been shown to increase the mechanical strength of the hydrogel [109, 110]. Gelatin is biocompatible, biodegradable, and of low cost [109]. C/G/GP was one of the biomaterials chosen as it is also an injectable hydrogel gelling at body temperature, biocompatible, has improved mechanical properties compared to chitosan with the addition of gelatin, and has previously been investigated in tissue engineering applications. It has also been investigated for NP tissue engineering where NP cells had normal cell viability, proliferation, and increased glycosaminoglycan and aggrecan production [109].

Poly(vinyl alcohol) (PVA) has been investigated for biomedical applications such as cartilage repair or drug delivery systems as it is biocompatible, elastic, and contains a high amount of water [112, 113]. PVA can be physically cross-linked through freeze-thaw cycles which allows for the production of PVA hydrogels without the presence of chemical crosslinkers [112]. Poly(acrylic acid) (PAA) is a biocompatible material that can be added during the freeze-thaw process to improve the lubricity of the hydrogel [112]. This material was chosen to be tested before deciding to try injectable hydrogels. It was chosen because it is biocompatible, can be moulded during the freeze-thaw cycling process into a specific shape (it was envisioned as a type of plug shaped like a dumbbell so it would not slip out easily from the defect site), and has been proposed for use as an artificial cartilage due to its biocompatibility, high water content, comparable mechanical characteristics of thermal annealed PVA/PAA hydrogels to native cartilage, and surface lubricity [112].

Gellan gum was chosen as one of the biomaterials to be tested for its effectiveness in sealing defects in the AF because it is an injectable hydrogel, is noncytotoxic allowing it to be combined with cells to allow for regeneration of the AF, and has been optimized for tissue engineering applications in the previous chapter allowing for cell encapsulation.

# 5.1.2 Bovine Models

As the functionality of the AF sealing method that will be developed through this project must be tested, the use of animal tissues must be considered. Animal tissues are often used in research due to some problems with human tissues. The disadvantages in using human tissues are that fresh human spines are difficult to obtain and that there are large variations in geometry and mechanical properties [114]. Animal tissues are easier to obtain and their geometry and mechanical properties are more consistent.

While the use of quadruped models has been questioned due to their spines lying horizontally, from a biomechanical standpoint, both quadruped and human spines are

loaded by axial compression along the long axis [115]. Quadruped spines rely on tensile forces from muscles and ligaments to maintain the posture of the spine which results in the spine being mainly loaded by axial compression [115]. However, quadruped spines are likely to experience greater compression loads than human spines as suggested by higher vertebral bone densities in quadrupeds [115, 116]. Tail IVDs are mainly loaded by muscle contraction and tension of passive structures. Therefore, loading on the tail IVD will increase with an increase in muscle contraction and tension of passive structures [116].

Bovine IVDs are similar to human IVDs in that there are some notochordal cells at birth that decrease over time [116]. However, there are anatomical differences between the human and bovine IVDs as shown in Figure 5.1. Bovine tail IVDs are smaller and more circular than human IVDs [116, 117]. While there are differences in mechanical properties, when it is normalized by disc area, the compressive stiffness and range of motion is not significantly different between bovine tail and human IVDs [117]. There were also no significant differences in the glycosaminoglycan and water content between each of the NP, inner AF, and outer AF of bovine tail IVDs and human IVDs are similar suggesting that the compressive stress in both the bovine tail and human IVDs have similar magnitudes [116].

The objective of this study was to compare the mechanical properties of gellan gum, fibrin, C/G/GP, and PVA/PAA hydrogels to determine the most appropriate biomaterial to seal defects in the AF. The most appropriate biomaterial will be determined by finding one that has compressive and tensile properties close to that of the native AF and is able to adhere well at the defect site in the AF.



**Figure 5.1:** Anatomical differences between a human lumbar IVD (left) and a bovine tail IVD (right). Picture reproduced from [116] with permission from the Copyright Clearance Centre.

# 5.2 Results

## 5.2.1 Dynamic Mechanical Analysis

DMA was performed on 2% (w/v) low acyl gellan gum, fibrin, C/G/GP, and PVA/PAA hydrogels. The complex compressive moduli, storage moduli, and loss moduli of the four kinds of hydrogels were calculated from the DMA results and are shown in Figure 5.2, Figure 5.3, and Figure 5.4 respectively. All moduli values are given in Tables A.4, A.5, and A.6 in the Appendix. By finding the compressive moduli

of the hydrogels, their compressive mechanical properties can be compared to that of the native tissue. It is important to find a biomaterial with a comparable compressive mechanical property as otherwise it would not be able to withstand the stresses applied when it is implanted.



**Figure 5.2:** Complex compressive moduli of 2% (w/v) LA gellan gum, fibrin, C/G/GP, and PVA/PAA hydrogels. Values could not be obtained for the PVA/PAA, fibrin, and C/G/GP hydrogels at 10 Hz as they were too soft for this test setup. Five samples were tested for each of the conditions and values have been graphed as means  $\pm$  SEM.



**Figure 5.3:** Storage moduli of 2% (w/v) LA gellan gum, fibrin, C/G/GP, and PVA/PAA hydrogels. Values could not be obtained for the PVA/PAA, fibrin, and C/G/GP hydrogels at 10 Hz as they were too soft for this test setup. Five samples were tested for each of the conditions and values have been graphed as means  $\pm$  SEM.



**Figure 5.4:** Loss moduli of 2% (w/v) LA gellan gum, fibrin, C/G/GP, and PVA/PAA hydrogels. Values could not be obtained for the PVA/PAA, fibrin, and C/G/GP hydrogels at 10 Hz as they were too soft for this test setup. Five samples were tested for each of the conditions and values have been graphed as means  $\pm$  SEM.

Out of the four kinds of hydrogels tested, 2% (w/v) low acyl gellan gum had the highest complex compressive and storage moduli values, followed by PVA/PAA, then fibrin, and C/G/GP hydrgels. Values could not be obtained for fibrin, C/G/GP, and PVA/PAA hydrogels at 10 Hz as they were too soft for this test. Therefore, the force

response from the DMA tests did not appear to be sinusoidal, making the analysis of the results difficult. The type of hydrogel and frequency were significant for all three moduli with p<0.01 and the interaction between the hydrogel type and frequency was only significant for the loss moduli with p<0.001. The complex compressive moduli and storage moduli of all hydrogels were significantly different from each other with p<0.001 with the exception of fibrin vs. C/G/GP hydrogels. Only the loss moduli values of the 2% (w/v) gellan gum was significantly different from each of the other hyrogels with p<0.001.

### 5.2.2 Tensile Testing

Tensile testing was performed on 2% (w/v) low acyl gellan gum and fibrin hydrogels. The tensile strength and the tensile moduli of the hydrogels were calculated from the tensile test data and are shown in Figure 5.5 and Figure 5.6 respectively. Two out of the five gellan gum samples broke at the bottom of the narrow region and three out of the five gellan gum samples broke at the top of the narrow region during the tensile testing. However, none of the fibrin samples broke before the tensile test concluded.

There was no significant difference between the tensile strength of fibrin and 2% (w/v) low acyl gellan gum. The tensile strength values for fibrin and gellan gum were  $34.7 \pm 2.4$  kPa and  $36.7 \pm 3.0$  kPa respectively.

There was a significant difference between the tensile modulus of fibrin and 2% (w/v) low acyl gellan gum with p<0.01. The tensile moduli for fibrin and gellan gum were  $1.05 \pm 0.05$  kPa and  $1.82 \pm 0.23$  kPa respectively.



**Figure 5.5:** Tensile strength of fibrin and 2% (w/v) LA gellan gum. Five samples were tested for each hydrogel type and values have been graphed as means  $\pm$  SEM.



**Figure 5.6:** Tensile modulus of fibrin and 2% (w/v) LA gellan gum. Five samples were tested for each hydrogel type and values are shown as means  $\pm$  SEM. \*\* signifies p<0.01.

# 5.2.3 Pressure Testing

Pressure testing was performed on frozen and fresh bovine tail IVDs by injecting fluid into the IVD. This test was performed to investigate if gradually freezing the IVDs without cryoprotectants at -20 °C for 7 days had an adverse effect on the maximum pressure that can be withstood by the IVDs as it is more convenient to use frozen bovine tails since they can last longer. The maximum pressures measured within the intact frozen and fresh bovine IVDs are shown in Figure 5.7.



Figure 5.7: Maximum pressures of intact frozen and fresh bovine tail IVDs. Four samples were included for each condition and values have been graphed as means  $\pm$  SEM. \* signifies p<0.05.

There was a significant difference between the maximum pressures of intact frozen and intact fresh bovine tail IVDs with p<0.05. The maximum pressures of intact fresh and frozen bovine tail IVDs were  $3.49 \pm 0.15$  MPa and  $1.69 \pm 0.44$  MPa respectively.

Pressure testing was performed on fresh bovine tail IVDs that (i) were intact, (ii) had a hole created using a biopsy punch, and (iii) with holes that had been created using a biopsy punch and then filled using either C/G/GP, fibrin, or 2% (w/v) low acyl gellan gum hydrogels to test how well the biomaterials can adhere to the defect site in the AF.

The average maximum pressures of each of these conditions are shown in Figure 5.8.



Figure 5.8: Maximum pressures of fresh bovine tail IVDs that were intact, had a hole only, or had holes that had been patched using C/G/GP, fibrin, or 2% (w/v) LA gellan gum. Six samples have been included for each condition and values are shown as means  $\pm$  SEM. \* signifies p<0.05.

There was only a significant difference between the maximum pressures of the intact and hole only conditions with p<0.05. The intact condition had the highest maximum pressure with a value of  $3.31 \pm 0.51$  MPa and the hole only condition had the lowest maximum pressure with a value of  $0.58 \pm 0.23$  MPa. The maximum pressures of

IVDs that had been patched with C/G/GP, fibrin, or gellan gum were  $1.11 \pm 0.55$  MPa,  $0.70 \pm 0.16$  MPa, and  $1.49 \pm 0.78$  MPa respectively.

#### **5.3 Discussion**

#### 5.3.1 Dynamic Mechanical Analysis

DMA tests were performed on 2% (w/v) low acyl gellan gum, fibrin, C/G/GP, and PVA/PAA hydrogels to determine the biomaterial that would be most suitable for AF sealing by having a modulus that is closest to the compressive modulus of the native AF. Out of the four hydrogels, the 2% (w/v) gellan gum had the highest modulus and fibrin and C/G/GP having the lowest modulus. The storage moduli were around 4-5 times higher than the loss moduli, and close to the complex compressive moduli values indicating that the hydrogels were highly elastic. PVA/PAA hydrogels probably had the highest variability in the moduli values as the top surfaces of the samples were rather uneven compared to the other hydrogels.

While the 2% (w/v) gellan gum had the highest compressive modulus of around 80 kPa, it is still not as high as most of the AF compressive moduli values that have been reported. The compressive moduli values of the AF have been stated to be 52 kPa [82], 380 kPa [83], 560 kPa [81], and 740 kPa [84] by various studies. The differences in values could be due to differences in species and test protocols. While these values were obtained from confined compression experiments, these values are the closest method for comparison. It is important for the compressive strength to be comparable to the native AF as the biomaterial should be able to withstand the forces inside the body.

#### 5.3.2 Tensile Testing

Tensile tests were performed on 2% (w/v) low acyl gellan gum and fibrin to determine the material that had tensile properties closest to that of the native AF. PVA/PAA was excluded from further testing since it is not injectable as it forms a gel through freeze-thaw cycling. Tensile tests could not be performed on C/G/GP hydrogels as they were too weak to be held by the clamps available for the Bose Electroforce test frame.

There was no significant difference between the tensile strength of fibrin and 2% (w/v) gellan gum with values of  $34.7 \pm 2.4$  kPa and  $36.7 \pm 3.0$  kPa respectively. However, both these values are not close to the AF tensile strength values that have been reported in the literature. The axial tensile strength of the outer anterior AF and the outer posterior AF have been reported to be  $3.9 \pm 1.8$  MPa and  $8.6 \pm 4.3$  MPa respectively [86]. While it is difficult to say what the exact ultimate tensile strength of the fibrin hydrogel would be as none broke before the displacement limit of the Bose Electroforce test frame was reached, it is unlikely that it would be close to the tensile strength values of the AF as the fibrin hydrogels could be broken by hand. However, tensile tests were performed in order to have a quantitative measurement.

There was a significant difference between the tensile modulus of fibrin and 2% (w/v) low acyl gellan gum with tensile moduli values of  $1.05 \pm 0.05$  kPa and  $1.82 \pm 0.23$  kPa respectively. However, both these values are again not close to the tensile modulus of the AF. The axial tensile modulus of the outer anterior AF and outer posterior AF have been reported to be  $16.4 \pm 7.0$  MPa and  $61.8 \pm 23.2$  MPa respectively [86].

#### 5.3.3 Pressure Testing

Pressure testing was performed on fresh and frozen intact bovine tail IVDs to assess the effect of frozen storage of the bovine tails on the burst pressure. The maximum pressure of the fresh intact bovine tail IVD was significantly higher than the frozen intact IVD with maximum pressures of  $3.49 \pm 0.15$  MPa and  $1.69 \pm 0.44$  MPa respectively.

Previous studies have reported that freezing does not have a significant effect on the biomechanical properties of human IVDs such as AF tensile properties [118]; NP hydrostatic pressure [119]; spinal motion segment full load displacements during shear, rotation, and lateral bending [120]; spinal motion segment compressive stiffness [121]; and IVD creep response [122].

However, freezing was shown to have an effect on the biomechanical properties of porcine IVDs such as IVD compressive creep response [123], spinal motion segment ultimate compressive load [122], and some of the spinal motion segment motion parameters [124]. The difference may be due to the lower water content of the human IVDs as it decreases ice crystal formation and nuclear expansion during freezing. This can result in a decreased risk for damage to the surrounding AF and endplates [122]. Hence further testing was performed of fresh bovine tail IVDs.

Pressure testing was performed on fresh intact IVDs, IVDs with a hole punched by a biopsy punch, and IVDs with a hole patched by either 2% (w/v) low acyl gellan gum, fibrin, or C/G/GP in order to determine the biomaterial that would be most suitable for sealing defects in the AF. These biomaterials were chosen as they are injectable and gel at body temperature allowing for a minimally invasive procedure when sealing the defect in the AF. Furthermore, as mentioned before, these biomaterials have been previously investigated for tissue engineering purposes showing biocompatibility. This will allow the biomaterial to be used as a scaffold in order to regenerate the AF tissue in the future.

Results from this study showed that there is only a significant difference between the intact IVD and the hole only case. The maximum burst pressure of the intact IVD was  $3.31 \pm 0.51$  MPa which is comparable to 2.17 MPa obtained by Ahlgren *et al* [66]. However, Ledet *et al* reported a value of 7.68 MPa [67]. This difference is probably due to a higher maximum pressure limit in their equipment setup as in some cases, the maximum pressure limit in this project was reached before any leakage was seen from the intact IVDs. The maximum burst pressure of the hole only case was 0.58  $\pm 0.23$  MPa, which is comparable to values of 0.33 MPa [66] and 2.00 MPa [67] obtained from different studies.

The 2% (w/v) gellan gum had the highest maximum burst pressure out of the three biomaterials. However, it is not a statistically significant difference from the other biomaterials. The maximum burst pressure of 2% (w/v) gellan gum was  $1.49 \pm 0.78$  MPa. This is higher than the maximum burst pressure of 0.39 MPa for box defect repaired using a muscle fascia graft and sutures as reported by Ahlgren *et al* [66]. However, it is lower than the value of 5.05 MPa reported by Ledet *et al*, where they repaired the AF defect using a small intestinal submucosa patch anchored by titanium bone screws. Wilke *et al* reported that the intradiscal pressure of IVDs was between 0.1 MPa while lying down to 2.3 MPa while lifting a 20 kg weight [68]. Although it is not

a statistically significant difference, the holes patched by any of the three biomaterials performed better than the hole only case. However, it is still not as good as the intact IVD case and is slightly lower than the intradiscal pressure measured when lifting a weight. Hence, a different way to adhere the biomaterials to the AF, or a different biomaterial altogether, or a different approach than just filling a hole with one biomaterial must be considered.

# CHAPTER SIX: KRYPTONITE CONSTRUCTS PROPOSED FOR ANNULUS FIBROSUS SEALING

#### **6.1 Introduction**

As the results of the previous chapter showed that none of the three biomaterials tested (2% (w/v) low acyl gellan gum, fibrin, and C/G/GP hydrogels) could seal a defect in the AF such that it would be as good as the intact IVD case, an alternative Kryptonite construct prototype was developed.

Kryptonite Bone Cement is created by combining a prepolymer and polyol derived from castor oil with calcium carbonate [125, 126]. It is non-toxic and has mechanical properties similar to bone [126]. It also results in a stable bond with bone within 24 hours and has a porous structure which enables osteointegration with bone [127]. Kryptonite has been approved by Health Canada and US Food and Drug Administration (FDA) for use in repairing cranial defects [125].

As Kryptonite forms a stable bond with bone, the Kryptonite ends of the construct can be attached to the vertebral bodies on either ends of the IVD using Kryptonite. The 2% (w/v) gellan gum was placed in the middle of the construct to seal an AF defect as it is flexible unlike Kryptonite and had the highest compressive modulus out of the biomaterials that had been tested in the previous chapter. The sutures or the electrospun fibres ran through the entire length of the construct to provide better tensile properties and was necessary to hold the construct together as Kryptonite does not bind to gellan gum.

The electrospun fibres were another component of the Kryptonite constructs. Electrospinning has gained interest in the tissue engineering field as it can produce nanofibrous scaffolds that can mimic the structure of the ECM [128]. Electrospun scaffolds have a high porosity and surface area to volume ratio that allows for better cell attachment and mass transfer properties [129].

PLGA is biocompatible, biodegradable, and has been approved by the FDA [128]. Electrospun PLGA scaffolds produce spaces during degradation that allow for cells to penetrate the scaffold and have been investigated for various tissue engineering applications such as cartilage and cardiac tissue engineering [129, 130].

The objective of this study was to develop a prototype construct using Kryptonite, gellan gum, and a fibrous component to seal defects in the AF and assess its mechanical properties. This study was performed with the biomaterials on hand to determine if the concept of a Kryptonite construct had merit.

#### **6.2 Results**

Tensile tests were performed on Kryptonite constructs that were joined by either coated vicryl polyglactin sutures or electrospun PLGA fibres only. Tensile strength and tensile modulus of these constructs were calculated from the tensile test data and are shown in Figure 6.1 and Figure 6.2 respectively.

The tensile strength and modulus of the suture only Kryptonite construct were  $638.0 \pm 3.1$  MPa and  $2960 \pm 376$  MPa respectively. For the Kryptonite constructs made with sutures, in one construct, two out of the four sutures had been pulled out of the

Kryptonite block while in the other construct, none of the sutures had been pulled out or broken but they had been stretched. The tensile strength and modulus of the PLGA fibre only Kryptonite construct were 0.44 MPa and 118 MPa respectively. The PLGA fibres broke approximately in the middle during the tensile test.



**Figure 6.1:** Tensile strength of Kryptonite constructs joined by sutures or electrospun PLGA fibres only. The tensile strength of the Fibre Only condition is 0.44 MPa so it cannot be seen on this graph. Two samples were tested for the suture only condition and one sample was tested for the fibre only condition. Suture only value is represented as mean  $\pm$  SEM.



Figure 6.2: Tensile modulus of Kryptonite constructs joined by sutures or electrospun PLGA fibres only. Two samples were tested for the suture only condition and one sample was tested for the fibre only condition. Suture only value is represented as mean  $\pm$  SEM.

Tensile tests were also performed on entire Kryptonite constructs consisting of both 2% (w/v) gellan gum and a fibrous component (either sutures or PLGA fibres). Tensile strength and tensile modulus of these constructs are shown in Figure 6.3 and Figure 6.4 respectively.

The tensile strength and modulus of the entire Kryptonite construct with 2% (w/v) low acyl gellan gum and sutures were  $807.9 \pm 11.2$  MPa and  $5184 \pm 241$  MPa respectively. For these Kryptonite constructs made with sutures, in one construct, three out of the four sutures had been broken while the fourth had been stretched out. In the

other construct, none of the sutures had snapped or pulled out but they were all stretched out. The tensile strength and modulus of the entire Kryptonite construct with 2% (w/v) gellan gum and PLGA fibres were 0.444 MPa and 180 MPa respectively. The PLGA fibres had broken at one end of the Kryptonite and gellan gum interface.



**Figure 6.3:** Tensile strength of Kryptonite constructs with 2% (w/v) LA gellan gum and sutures or electrospun PLGA fibres. The tensile strength of the Fibre Construct condition is 0.444 MPa so it cannot be seen on this graph. Two samples were tested for the suture construct and one sample was tested for the fibre construct. Suture Construct value is represented as mean  $\pm$  SEM.



**Figure 6.4:** Tensile modulus of Kryptonite constructs with 2% (w/v) LA gellan gum and sutures or electrospun PLGA fibres. Two samples were tested for the suture construct and one sample was tested for the fibre construct. Suture Construct value is represented as mean  $\pm$  SEM.

## 6.3 Discussion

As the results of the previous chapter showed that none of the three biomaterials tested (2% (w/v) low acyl gellan gum, fibrin, and C/G/GP hydrogels) could alone seal a defect in the AF when compared to the intact IVD case, an alternative Kryptonite construct prototype was developed.

Tensile testing was performed on Kryptonite constructs joined by either sutures or electrospun PLGA fibres in order to determine the force required to pull out or break the sutures or fibres. Tensile testing was performed on Kryptonite constructs with 2%(w/v) low acyl gellan gum and either sutures of PLGA fibres in order to determine the tensile properties of the constructs. In both the Kryptonite constructs with and without gellan gum, the constructs made with sutures had a greater tensile strength than ones with PLGA fibres.

As mentioned previously, the axial tensile strength of the outer anterior AF and the outer posterior AF have been reported to be  $3.9 \pm 1.8$  MPa and  $8.6 \pm 4.3$  MPa respectively [86]. The Kryptonite PLGA fibre constructs had a tensile strength that was lower than the values reported for the AF. The Kryptonite suture constructs had a higher tensile strength than the values reported in the literature for AF and hence it would be preferable to use sutures over PLGA fibres. The PLGA fibres were also rather fragile and difficult to handle. However, these were still tested as electrospun fibres can direct cell alignment and ECM deposition and it was hoped that using multiple layers of the fibres and embedding it in gellan gum would be enough to support the Kryptonite construct.

The axial tensile modulus of the outer anterior AF and outer posterior AF have been reported to be  $16.4 \pm 7.0$  MPa and  $61.8 \pm 23.2$  MPa respectively [86]. The tensile modulus of all the constructs were higher than the values reported in the literature for the AF indicating that they are stiffer than the AF. While it is important for the biomaterial to be flexible when it is sealing the AF as it needs to be able to withstand large deformations, it is probable that the flexible nature of the gellan gum placed in the AF defect will have a greater impact than the sutures or fibres. Therefore, as gellan gum is quite flexible, it should be able to withstand the deformations experienced when implanted. While the Kryptonite constructs no longer offer a minimally invasive surgical procedure as with the injectable biomaterials discussed previously, it will allow for better adhesion by bonding to the vertebral bodies. Kryptonite is a good choice as it is non-toxic and has mechanical properties similar to bone. Sutures were better than the electrospun PLGA fibres as the fibres were much more fragile than the sutures. The use of gellan gum will allow for cell encapsulation, which will eventually lead to the regeneration of the AF at the site of defect.

A successful construct for sealing defects in the AF should be able to adhere well to the defect so it does not loosen from the defect site. It should have a flexible component where it enters the AF section as it needs to be able to withstand large deformations. It should also have mechanical properties that are comparable to that of the native AF in order to not break down once it has been implanted. It would also be ideal to be able to support cell encapsulation so the defect area can be eventually regenerated by the cells.

The current Kryptonite construct may be able to be improved by finding a way to either modify gellan gum so it would be able to adhere better to the Kryptonite or possibly finding a replacement biomaterial that is still flexible, will allow for cell encapsulation, and has mechanical properties similar to that of the native AF while being able to adhere to Kryptonite. Sutures should be used instead of the fibres in order to provide better tensile strength. Also, in order to ensure that the sutures do not pull out of the Kryptonite, it should be ensured that the sutures are embedded right in the middle of the construct.

# **CHAPTER SEVEN: CONCLUSIONS AND FUTURE STUDIES**

# 7.1 Optimization of Gellan Gum Properties

The mechanical properties and gelling temperatures of low acyl gellan gum and low/high acyl gellan gum blends were investigated for their potential use as a scaffold in NP tissue engineering applications. While the compressive modulus of the 5% (w/v) low acyl gellan gum was the closest to that of the NP, the gelling temperature of the gellan gum at this concentration was too high for cell encapsulation purposes. As the gelling temperature of low acyl gellan gum concentrations of 2% (w/v) or less was suitable for cell encapsulation, the most optimal concentration of low acyl gellan gum was found to be 2% (w/v) in terms of stiffness and gelling temperature. Low/high acyl gellan gum blends were found to be unsuitable as it resulted in a decrease in stiffness and the gelling temperature was much too high for cell encapsulation. Therefore, the objective of determining the optimal gellan gum composition and concentration for NP tissue engineering applications was met.

## 7.2 Comparison of Biomaterial Mechanical Properties

Mechanical properties of 2% (w/v) low acyl gellan gum, fibrin, C/G/GP, and PVA/PAA hydrogels were assessed in order to determine the biomaterial that would be most suitable for sealing defects in the AF. DMA tests showed that gellan gum had a compressive modulus that was closest to that of the AF. Tensile tests showed that gellan gum and fibrin did not have significantly different tensile strength.

Pressure testing was performed on IVDs that had holes patched by 2% (w/v) low acyl gellan gum, fibrin, or C/G/GP hydrogels. While it was not a statistically significant difference, none of the IVDs patched using the three hydrogels had a maximum pressure that was as high as in the intact IVD case. Therefore, the objective of finding a single material to seal a defect in the AF was not met. A different way to adhere the biomaterial to the AF must be developed and 2% (w/v) gellan gum was determined to be the best starting point as it had the best mechanical properties out of the biomaterials tested.

#### 7.3 Kryptonite Constructs Proposed for Annulus Fibrosus Sealing

Prototypes for sealing defects in the AF were created using Kryptonite bone cement, 2% (w/v) low acyl gellan gum, and either sutures or electrospun PLGA fibres. Tensile tests were performed on these prototypes. Kryptonite constructs made with sutures had higher tensile strength than ones made with PLGA fibres. Kryptonite constructs made with sutures were deemed more suitable for sealing defects in the AF as they had a higher tensile strength and the PLGA fibres were too fragile and difficult to handle. Unfortunately, these constructs no longer provide a way for a minimally invasive surgery as with the injectable biomaterials but is probably necessary in order to provide a better way of fixation. The objective in creating a prototype for sealing of AF defects was met. However, improvements to the current Kryptonite construct can be made.

# 7.4 Future Studies

Future studies should be carried out to investigate cell encapsulation in 2% (w/v) low acyl gellan gum and differentiation into AF cells if MSCs are to be used. This is important as it would allow for regeneration of the AF tissue at the site of the defect. Mechanical properties of the gellan gum should also be determined when the cells have been encapsulated to determine the effect of gellan gum degradation while new tissue is being regenerated. The degradation rates should be studied so that new tissue can be regenerated before the scaffold has lost its mechanical integrity and degradation by-product effects should also be studied in order to determine its safety. Also, other mechanical tests such as shear tests and large amplitude studies should be performed in the future to fully characterize the mechanical properties of the gel. Future studies should continue to investigate gellan gum especially for NP tissue engineering applications as it is an injectable hydrogel that gels around body temperature, is gel-like, has a high water content, can support cell growth and survival, and had the highest compressive modulus out of the biomaterials tested in this project.

Also, future work should involve fatigue testing of the Kryptonite constructs and investigate how well they fare when used in an actual IVD. Due to the positive attributes discussed, gellan gum should continue to be investigated unless a biomaterial that has better mechanical properties, can withstand large deformations, is biocompatible, and allows for cell encapsulation can be found. There is also no clear way for the gellan gum section of the construct to adhere to the native AF so different ways of attaching the gellan gum to the AF must be investigated. Also, if the Kryptonite constructs are to be made with gellan gum, as it had the best mechanical properties out of all the biomaterials investigated in this project while allowing for cell encapsulation for tissue engineering purposes, better attachment between the Kryptonite and gellan gum should be investigated.

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## APPENDIX: VALUES FOR DYNAMIC MECHANICAL ANALYSIS RESULTS

Freq (Hz)	1% LA Gellan Gum	2% LA Gellan Gum	3% LA Gellan Gum	4% LA Gellan Gum	5% LA Gellan Gum	6% LA Gellan Gum	2% LA/HA Gellan Gum 50/50	2% LA/HA Gellan Gum 75/25
0.01	31.628	94.658	142.26	245.16	324.14	407.86	40.79	49.058
0.1	29.682	82.592	137.82	249.6	303.18	384.46	33.922	42.564
1	27.242	78.814	137.6	261.36	310.72	395.1	28.478	37.04
10	20.982	68.6	124.14	228.04	272.48	346.92		

**Table A.1:** Chapter 4 Complex Compressive Modulus (E; kPa) Values

**Table A.2:** Chapter 4 Storage Modulus (E'; kPa) Values

Freq (Hz)	1% LA Gellan Gum	2% LA Gellan Gum	3% LA Gellan Gum	4% LA Gellan Gum	5% LA Gellan Gum	6% LA Gellan Gum	2% LA/HA Gellan Gum 50/50	2% LA/HA Gellan Gum 75/25
0.01	30.102	93.014	139.38	235.46	319.28	399.92	40.52	48.132
0.1	29.486	82.256	137.14	247.98	301.86	382.38	33.788	42.344
1	27.1	78.508	137.12	260.42	309.84	393.84	28.308	36.96
10	20.736	67.546	123.04	226.98	271.38	345.68		

Freq (Hz)	1% LA Gellan Gum	2% LA Gellan Gum	3% LA Gellan Gum	4% LA Gellan Gum	5% LA Gellan Gum	6% LA Gellan Gum	2% LA/HA Gellan Gum 50/50	2% LA/HA Gellan Gum 75/25
0.01	9.6932	17.654	28.418	68.214	55.902	79.608	4.622	9.4716
0.1	3.3894	7.4126	13.472	28.436	28.308	39.978	2.71628	4.2964
1	2.7058	6.8354	10.9714	22.14	23.1	31.23	2.74006	1.71022
10	2.32592	10.9322	16.182	21.832	24.14	29.484		

**Table A.3:** Chapter 4 Loss Modulus (E"; kPa) Values

**Table A.4:** Chapter 5 Complex Compressive Modulus (E; kPa) Values

Freq (Hz)	C/G/GP	Fibrin	2% LA Gellan Gum	PVA/PAA
0.01	13.868	16.406	94.658	43.228
0.1	12.286	14.024	82.592	38.524
1	9.3552	11.5934	78.814	32.63
10		7.8586	68.6	

Freq (Hz)	C/G/GP	Fibrin	2% LA Gellan Gum	PVA/PAA
0.01	13.442	16.002	93.014	42.185
0.1	12.0614	13.916	82.256	41.425
1	9.0054	11.391	78.508	32.482
10		6.3638	67.546	

**Table A.5:** Chapter 5 Storage Modulus (E'; kPa) Values

 Table A.6: Chapter 5 Loss Modulus (E"; kPa) Values

Freq (Hz)	C/G/GP	Fibrin	2% LA Gellan Gum	PVA/PAA
0.01	3.318	3.6226	17.654	1.9055
0.1	2.086	1.7016	7.4126	3.086
1	2.08178	1.71162	6.8354	2.674
10		4.1292	10.9322	