Characterization of tissue-specific matrix-derived bioscaffolds for nucleus pulposus cell culture

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Abstract

Bioscaffolds derived from the extracellular matrix (ECM) have shown the capacity to promote regeneration by providing tissue-specific biological instructive cues that can enhance cell survival and direct lineage-specific differentiation. This study focused on the development and characterization of two-dimensional (2-D) and three-dimensional (3-D) cell culture platforms incorporating decellularized nucleus pulposus (DNP). First, a novel detergent-free protocol was developed for decellularizing bovine NP tissues that was effective at removing cellular content while preserving key ECM constituents including collagens and glycosaminoglycans. Culture studies showed that 2-D coatings derived from the DNP could support cell attachment but did not maintain or rescue the phenotype of primary bovine NP cells, which dedifferentiated when serially passaged in monolayer culture on tissue culture plastics. Similarly, the incorporation of DNP particles within methacrylated chondroitin sulphate hydrogels as a 3-D culture platform was insufficient to maintain or rescue the bovine NP cell phenotype based on gene expression patterns.

Keywords

Intervertebral disc degeneration, nucleus pulposus (NP), decellularization, tissue engineering, cell therapy, bioscaffolds, coatings, hydrogels, methacrylated chondroitin sulphate (MCS), extracellular matrix (ECM)
Summary for Lay Audience

Low back pain is the leading cause of disability worldwide. While the cause of back pain is not well understood, intervertebral disc (IVD) degeneration is thought to be a major contributor. IVD degeneration is thought to originate in the central region of the IVD, which is a gel-like tissue termed the nucleus pulposus (NP). Current therapies primarily focus on pain management rather than targeting the underlying degenerative condition. Surgical approaches often result in the degeneration of adjacent IVDs due to altered spine biomechanics. The lack of disease-modifying treatments has motivated the investigation of biomaterial-based therapies in order to regenerate the NP and restore mechanical function. In particular, extracellular matrix (ECM)-derived bioscaffolds present a promising approach for promoting tissue regeneration. The ECM is unique to each tissue and consists of proteins and sugars that provide tissue structure and biological cues that can guide cellular behaviour.

Tissue decellularization, a process aimed at removing the cellular content of a tissue, while preserving the cell-instructive ECM, can be applied to develop ECM-derived bioscaffolds capable of guiding cellular behaviour for use in cell culture and delivery platforms. The current study developed a detergent-free decellularization protocol that effectively extracted immunogenic cellular components from the tissue while preserving pro-regenerative ECM components such as collagens and glycosaminoglycans. Following validation of the decellularization protocol and the characterization of the decellularized NP (DNP), the DNP was applied to generate novel 2-D coatings and 3-D hydrogel bioscaffold platforms. As a first step towards testing the potential of these culture platforms, the behaviour of bovine NP cells grown on the 2-D DNP coatings or within the 3-D hydrogels containing DNP was assessed. The bioscaffolds were shown to support bovine NP cell attachment and viability but the incorporation of the tissue-specific ECM was insufficient to prevent undesired changes in NP-associated gene expression that are observed when the cells are cultured in 2-D on tissue plastics. Overall, these studies developed novel DNP-derived bioscaffolds that should be further investigated as cell culture or delivery platforms for applications in IVD regeneration.
Co-Authorship Statement

All experimental work in this thesis was completed by Marco Herrera, with the exception of the data presented in Figure 3.4 and Figure 3.5. More specifically, Dr. Pascal Morissette Martin performed the immunohistochemical staining and imaging shown in Figure 3.5 on the native and decellularized nucleus pulposus tissues using samples provided by Marco Herrera. Tim Goldhawk from the Western Nanofabrication Facility performed the SEM imaging shown in Figure 3.4.
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To my advisory committee, Dr. Silvia Penuela, Dr. Arghya Paul and Dr. Katherine Willmore, thank you for the thought provoking insightful feedback, and dedication to guiding the progression of this MSc project.

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<td>2-D</td>
<td>Two dimensional</td>
</tr>
<tr>
<td>3-D</td>
<td>Three dimensional</td>
</tr>
<tr>
<td>ABAM</td>
<td>Antibiotic-antimycotic</td>
</tr>
<tr>
<td>ACTA2</td>
<td>α-smooth muscle actin 2</td>
</tr>
<tr>
<td>ADAMTS</td>
<td>A disintegrin and metalloproteinase with thrombospondin motifs</td>
</tr>
<tr>
<td>AF</td>
<td>Annulus fibrosus</td>
</tr>
<tr>
<td>ASC</td>
<td>Adipose stromal cell</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CEP</td>
<td>Cartilaginous end plate</td>
</tr>
<tr>
<td>COL</td>
<td>Bovine tendon type I collagen</td>
</tr>
<tr>
<td>CS</td>
<td>Chondroitin sulphate</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DAT</td>
<td>Decellularized adipose tissue</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DMMB</td>
<td>Dimethyl methylene blue</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>DNP</td>
<td>Decellularized nucleus pulposus</td>
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<tr>
<td>dsDNA</td>
<td>Double stranded DNA</td>
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ECM  Extracellular matrix
EDTA  Ethylenediaminetetraacetic acid
ELISA  Enzyme-linked immunosorbent assay
EthD-1  Ethidium homodimer-1
FBS  Fetal bovine serum
FN  Fibronectin
FOXA1  Forkhead box A1
GAG  Glycosaminoglycan
GAPDH  Glyceraldehyde 3-phosphate dehydrogenase
HA  Hyaluronic acid
H&E  Hematoxylin and eosin
IHC  Immunohistochemistry
IL  Interleukin
iPSCs  Induced pluripotent stem cells
IVD  Intervertebral disc
KS  Keratan sulphate
LBP  Low back pain
LN  Laminin
MCS  Methacrylated chondroitin sulphate
MHA  Methacrylated hyaluronic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stromal cell</td>
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<tr>
<td>NP</td>
<td>Nucleus pulposus</td>
</tr>
<tr>
<td>OHP</td>
<td>Hydroxyproline</td>
</tr>
<tr>
<td>PAX1</td>
<td>Paired box 1</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonylfluoride</td>
</tr>
<tr>
<td>P/S</td>
<td>Penicillin/streptomycin</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>SB</td>
<td>Sulfo betaine</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
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<tr>
<td>sGAG</td>
<td>Sulphated glycosaminoglycans</td>
</tr>
<tr>
<td>SHH</td>
<td>Sonic hedgehog</td>
</tr>
<tr>
<td>SOX9</td>
<td>SRY-box 9</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with 0.1% Tween</td>
</tr>
<tr>
<td>TBXT</td>
<td>Brachyury</td>
</tr>
<tr>
<td>TCP</td>
<td>Tissue culture plastic</td>
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<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
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<td>UV</td>
<td>Ultraviolet</td>
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Chapter 1

1 Literature Review

1.1 The intervertebral disc

1.1.1 Anatomy and function of the intervertebral disc

Intervertebral discs (IVDs) are fibrocartilaginous structures situated between adjacent vertebral bodies along the spinal column. IVDs play a vital role in transferring and distributing compressive loads, allowing for multi-axial mobility\(^1\). In humans, there are 23 IVDs making up ~33% of the spinal height\(^2\). In adults, IVD size varies based on spinal region, with individual cervical IVDs having a height of ~3 mm and individual lumbar IVDs having a height of ~7-10 mm\(^3\). Each IVD consists of 3 morphologically distinct yet interdependent tissues: the inner gelatinous nucleus pulposus (NP), the outer annulus fibrosus (AF) and the superior and inferior cartilaginous endplates (CEP) (Figure 1.1AB).

The NP is a highly hydrated proteoglycan-rich gel-like structure located centrally within the IVD. The NP is generally comprised of a randomly arranged loose network of type II collagen, containing proteoglycans, such as aggrecan. Aggrecan carries a net-negative charge that attracts and retains water within the NP through polar interactions. During compressive loading, the hydrated nature of the NP allows for the transfer of load, both radially to the AF, and axially to the adjacent vertebral body\(^4\). During embryonic development and at birth, the NP is populated by large vacuolated notochord cells which are progressively lost during the first 10 years of life and replaced by small chondrocyte-like cells (Figure 1.1C)\(^5,6\).

The NP is circumferentially constrained by the type I collagen-rich concentric lamellar rings that make up the AF\(^2\). The AF is highly organized, with each lamella composed of parallel collagen fibers arranged at an angle of 60° from the vertical axis\(^7\). Adjacent lamellar rings have alternating collagen fiber orientation to the left and right of the vertical axis\(^2,4\). This alternating oblique orientation allows the AF to resist pressure from the NP, limiting its expansion under compressive load\(^4\). The AF contains an inner and an
outer region, which differ slightly in their extracellular matrix (ECM) and cellular composition. The outer AF is largely comprised of type I collagen, while the inner AF is predominantly type II collagen. In addition, the proteoglycan content decreases gradually from the inner AF to the outer AF. In terms of the cells, the outer AF contains elongated fibroblast-like cells while the inner AF contains round chondrocyte-like cells, which are aligned between the collagen lamellae and surrounded by a glycosaminoglycan (GAG)-rich peri-cellular matrix. The outer AF is anchored to the adjacent vertebral body through type I collagen and elastin-containing extensions known as Sharpey’s fibers. The NP interfaces with the inner AF and both tissues are anchored to the vertebral bodies superiorly and inferiorly via the CEP.

The CEP is a thin layer of hyaline cartilage formed by chondrocytes, which plays a crucial role in nutrient regulation within the IVD. In human IVDs, the healthy NP is avascular throughout life, while the CEP and outer AF remain vascularized into adulthood. As such, the CEP functions to permit the passive diffusion of nutrients, metabolic by-products, and oxygen from vertebral capillaries to the NP and inner AF. Under compressive load, water is extruded from the NP, carrying metabolic waste away from cells of the IVD and towards vessels within the vertebral body and outer AF. As the load is attenuated, water is drawn back into the NP, carrying nutrients and oxygen to the cells of the IVD.

The synergistic interactions of the healthy NP, AF, and CEP are crucial for the maintenance of proper IVD function and avoidance of pathologies including IVD degeneration. IVD degeneration is characterized by cellular, biochemical, and structural changes disrupting the healthy interaction of IVD structures. Such changes, are thought to originate within the central region of the IVD, the NP. As such, there is interest in developing novel therapies targeting the degenerative changes in the NP as an alternative treatment strategy for IVD degeneration.
Figure 1.1. Schematic illustration of intervertebral disc (IVD) anatomical structure and cellular composition. The IVD is located between vertebral bodies within the spinal column. It is comprised of three distinct tissues: **(A)** the inner gel-like nucleus pulposus (NP), surrounded by the concentric lamella of the annulus fibrosus (AF), **(B)** both of which are flanked superiorly and inferiorly by the cartilaginous end plates (CEP). **(C)** At birth, the NP consists largely of notochord cells which are progressively lost and replaced by mature NP cells up to ~10 years of age in humans.

### 1.2 The nucleus pulposus

#### 1.2.1 Extracellular matrix composition and organization

The ECM is a three-dimensional network of proteins, proteoglycans, and other macromolecules, making up the non-cellular component within a tissue\(^6\). The tissue-
specific ECM composition and organization of the NP is critical for the proper mechanical function of the IVD. In a healthy IVD, water makes up the large majority of the NP, accounting for ~80% of the total NP wet weight\(^2,17\). The NP ECM predominantly consists of proteoglycans enmeshed within an irregular network of type II collagen and elastin fibers\(^7\). Various ECM components contribute to the mechanical properties and modulate cellular function within the NP including proteoglycans, collagens, and non-collagenous proteins.

**Proteoglycans:** Proteoglycans are a class of glycosylated proteins composed of a core protein covalently linked to GAG chains such as chondroitin sulphate, keratan sulphate, or heparan sulphate\(^18\). The NP ECM contains various small proteoglycans, including biglycan, decorin, fibromodulin, and lumican, as well as large proteoglycans including aggrecan and versican, with aggrecan being the most abundant\(^17\). Proteoglycans play an important role in NP hydration, and constitute approximately 65% of the NP ECM dry weight\(^17\). Aggrecan is formed by a central core protein covalently linked to numerous highly sulphated GAG (chondroitin sulphate and keratan sulphate) chains. Multiple aggrecan monomers are covalently crosslinked to hyaluronic acid to form a large aggregating proteoglycan\(^19\). Chondroitin sulphate and keratan sulphate are the most abundant GAGs found within the native NP\(^7\). Chondroitin sulphate and keratan sulphate are innately anionic, thus providing aggrecan with a net negative charge that promotes water influx into the NP\(^15\). The high water content generates swelling pressure that contributes to the maintenance of the IVD height and proper transmission of compressive loads\(^14,20\). GAGs also serve to sequester growth factors, thereby increasing their local concentration within the cellular microenvironment of the NP\(^21\).

**Collagens:** Collagens are structural proteins consisting of a right-handed triple helical structure, which contribute to tissue structural integrity and tensile strength\(^22\). The ECM of the NP incorporates a randomly arranged network of collagen types I, II, III, V, VI, IX, XI, XII, and XIV, with the relative abundance of each type varying depending on health and age\(^15\). Within the healthy NP, type II collagen is the most abundant collagen, making up ~15-20% of the ECM dry weight\(^17\).
Non-collagenous proteins: Non-collagenous proteins account for ~20% of the ECM dry weight within the NP, and consist largely of adhesive glycoproteins and elastin\textsuperscript{23}. Glycoproteins are proteins, which through post-translational modifications are covalently linked to saccharide chains. Generally, glycoproteins play an important role in mediating cell-ECM interactions, including cell adhesion\textsuperscript{23}. Within the NP, laminin and fibronectin are involved in cell signaling through integrin cell surface receptors\textsuperscript{17}. Elastin is a vital protein found within various connective tissues that provides resilience and elasticity to tissues\textsuperscript{24}. Within the NP, elastin makes up ~10% of the ECM dry weight, and plays an important role in the elasticity and resilience of the tissues following deformation\textsuperscript{23}.

1.2.2 Cells of the nucleus pulposus

The NP is formed by a heterogenous cell population that changes depending on age and health of the IVD\textsuperscript{5,6}. Early in human life, the NP is primarily comprised of large vacuolated cells arising from the embryonic notochord\textsuperscript{25}. During early embryogenesis, the notochord, a rod-like structure, forms along the posterior/anterior axis within the embryo midline\textsuperscript{10}. The notochord plays a critical role in patterning surrounding tissues (neural tube, sclerotomes), through the expression of key signalling molecules, including sonic hedgehog (\textit{SHH})\textsuperscript{10}. Following formation of the axial skeleton, the notochord disappears at the site of vertebrae development, yet is maintained at the site of IVD formation and eventually gives rise to the NP\textsuperscript{26}. Key transcription factors including brachyury (\textit{TBXT}) and forkhead box A1 (\textit{FOXA1}) play a vital role in the proper formation of the NP from the embryonic notochord\textsuperscript{26}. Additionally, through SRY-box 9 (\textit{SOX9}) and paired box 1 (\textit{PAX1}) transcription factors, the notochord regulates gene expression, required for the differentiation of adjacent mesenchymal cells to give rise to the AF, CEP, and vertebral bodies, forming the axial skeleton\textsuperscript{10,26}.

Notochord cells are also responsible for the deposition of GAG-rich ECM during IVD formation, encapsulating themselves into a network of interconnected cell clusters\textsuperscript{5}. Their ability to synthesize large amounts of proteoglycans contributes to maintenance of the hydrated nature of the NP\textsuperscript{27}. In addition, notochord cells are thought to confer a cytoprotective effect on mature NP cells through the secretion of soluble factors promoting cell proliferation and matrix synthesis\textsuperscript{24}. While some vertebrates, such as mice
and rabbits, maintain their notochord cell population into adulthood, other species such as humans and cows undergo a progressive loss of notochord cells in the NP shortly after birth up to 10 years and 3 years of age, respectively\textsuperscript{25}. As the notochord cell number decreases, the NP becomes populated by small, round chondrocyte-like NP cells\textsuperscript{5,6}. Lineage tracing studies in mice have shown NP cells to be of notochordal origin\textsuperscript{30}. As such, notochord cells are believed to become terminally differentiated, giving rise to the mature NP cells\textsuperscript{10,30}. The loss of notochord cells has been associated with the initiation of IVD degeneration\textsuperscript{31}.

1.3 Low back pain and intervertebral disc degeneration

According to the most recent Global Burden of Disease study, low back pain (LBP) is the leading cause of disability worldwide\textsuperscript{32}. It is predicted that as much as 84\% of the general worldwide population will suffer from LBP at some point in their lives\textsuperscript{33}. Episodes of acute LBP (lasting less than 4 weeks) are most common, and are generally resolved through conservative treatments such as activity modification, chiropractic manipulation, or physical therapy\textsuperscript{34}. It is estimated that 10-15\% of acute LBP cases will progress to chronic LBP (lasting more than 3 months)\textsuperscript{33,34}. The global lifetime prevalence of chronic LBP is approximately \textasciitilde{}20\%\textsuperscript{33}. As such, LBP is one of the most prevalent musculoskeletal conditions afflicting Western society\textsuperscript{35}. Individuals suffering from LBP tend to experience higher societal and psychological burdens such as work absence, poor work productivity, dysfunctional relationships, social isolation, anxiety, and depression\textsuperscript{36–38}. In Canada, the debilitating effects of back pain lead to one of the highest medical resource usage costs when compared to other chronic conditions\textsuperscript{39}, with direct medical cost associated with LBP ranging from 6 to 12 billion dollars annually\textsuperscript{40}. In the United States, direct and indirect costs of LBP have been estimated to exceed 100 billion dollars annually\textsuperscript{40,41}. Since LBP predominantly affects individuals between 40 to 80 years of age, the economic burden is predicted to increase as the Canadian population shifts towards an older demographic\textsuperscript{42}.

The cause of chronic LBP has been debated, but is considered to be multifactorial in nature\textsuperscript{33,43,44}. Studies have examined the anatomical structures from which chronic LBP may originate, including the IVDs, facet joints, sacroiliac joint, vertebral bodies,
paraspinal muscles, ligaments, and nerve roots. Such studies have found the IVD, sacroiliac joint and the facet joints to play large roles in chronic LBP, with the IVD being the most common source of chronic LBP. Approximately 40% of individuals with chronic LBP present with IVD degeneration. IVD degeneration is thought to occur as part of the aging process; however, factors such as genetics, trauma, and altered mechanical loading can result in earlier and more rapid onset of degeneration.

1.4 Intervertebral disc degeneration

The pathogenesis of IVD degeneration remains poorly understood, as it is difficult to distinguish the biological changes that lead to degeneration from those of aging or adaptive remodeling. IVD degeneration is marked by structural, cellular, morphological, molecular, and mechanical changes. Within the IVD there is a shift towards greater ECM catabolism, altered matrix synthesis, cell senescence, cell apoptosis, inflammation, and in-growth of vasculature and nociceptive nerves into the NP. These changes result in a loss of the NP-AF boundary, decreased NP hydration, fibrosis of the NP, and an inability of the IVD to withstand compressive loads. IVD degeneration can be accelerated by genetic factors, altered mechanical loading, or injury, leading to altered spine biomechanics, spinal instability, and discogenic pain.

1.4.1 Degenerative changes in the nucleus pulposus

The etiology of IVD degeneration has been attributed to progressive cellular and microenvironment changes originating within the central NP. In humans, the NP undergoes progressive loss of notochord cells throughout the first decade of life. Loss of notochord cells has been associated with the onset of IVD degeneration, likely linked to their role in secreting factors that stimulate NP cells to synthesize proteoglycans. Notochord cells show increased proteoglycan synthesis when compared to mature NP cells in vitro. Further, in vitro studies have demonstrated that notochord-cell conditioned media has both anabolic and cytoprotective effects on NP cells following cytokine exposure. These effects have been associated with the secretion of the anabolic factors, including connective tissue growth factor (also known as CCN2) by notochord cells.
The NP ECM is maintained by cells in a state of dynamic equilibrium, constantly undergoing protein turnover. Constant remodeling allows the ECM to accommodate tissue growth and repair damage incurred through daily ‘wear and tear’. Degeneration is marked by an increase in cellular senescence and apoptosis, thereby diminishing the capacity of the NP to undergo matrix turnover and repair, leading to further degeneration. The ECM within a degenerative NP is characterized by a shift from type II collagen to type I collagen (fibrosis), and a decreased proteoglycan/GAG content.

Pro-inflammatory cytokines produced by resident NP cells modulate matrix catabolism, promote cellular senescence and apoptosis, and stimulate chemokine production. Cytokines including tumour necrosis factor-α (TNF-α), interleukin 1β (IL-1β), IL-1α, IL-6, IL-17, IL-8, IL-2, IL-4, IL-10, and interferon-γ have all been implicated in IVD degeneration, of which IL-1β and TNF-α have been the most studied. For example, exposure of human NP cells to IL-1β in vitro induced the expression of catabolic proteinases including matrix metalloproteinase (MMP)-3, MMP-13, and the aggrecanase a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-4. Similarly, exposure of human NP to TNF-α induced the expression of ADAMTS-4 and ADAMTS-5. TNF-α has also been implicated in the promotion of neuronal ingrowth to the IVD, a potential cause of discogenic pain in patients with LBP.

ECM catabolism following MMP and ADAMTS upregulation decreases proteoglycan content within the NP, thereby lowering water retention and hydrostatic pressure. This impacts the ability of the IVD to transmit, resist, and redistribute compressive loads. The resulting aberrant loading across the joint potentiates further degeneration. Loss of NP hydration also reduces the ability of nutrients, oxygen, and cellular waste to diffuse across the NP, increasing cellular stress. As such, the reduction in hydration decreases the pH of the NP, resulting in a further decrease in proteoglycan synthesis and increased production of catabolic enzymes such as MMPs by NP cells.
1.5 Clinical treatments for intervertebral disc degeneration

Current treatments for IVD degeneration are aimed towards pain management rather than targeting the underlying disease pathobiology. Treatments vary from non-invasive procedures such as physiotherapy to invasive surgical approaches. Surgical treatments include the immobilization of the IVD through spinal arthrodesis (spinal fusion), or the removal of the degenerated IVD (discectomy) followed by insertion of an artificial IVD (total disc replacement). Surgical treatments often reduce pain and restore mobility in the short term; however, they often accelerate degeneration of adjacent IVDs due to altered spine biomechanics. As such, surgical intervention might be ineffective for the long-term treatment of IVD degeneration. For this reason, novel cell-based therapies that aim to address the aberrant biochemical and cellular changes within the IVD have garnered significant attention and require further investigation.

1.6 Regenerative therapies targeting the nucleus pulposus

Since IVD degeneration is thought to originate within the NP, most studies to date have focused on engineering regenerative strategies targeting the NP. Regenerative therapies aim to restore the native NP biomechanical function through replenishing the altered biochemical composition. An attractive therapeutic approach is the combined use of scaffolds and pro-regenerative cell populations. In theory, a NP-mimicking bio-instructive scaffold could be used as a cell delivery platform and/or to support cell-mediated tissue repair through de novo ECM synthesis by resident cell populations.

Previous studies have investigated cell-based approaches both with or without scaffolds. Although cell-only therapies have shown promise in vitro, long-term cell survival may be impacted by the harsh microenvironment within the degenerated NP, limiting their therapeutic efficacy in vivo. Moreover, cell injections into the IVD often result in cell leakage at the injection site, leading to poor cell retention. A potential solution is the encapsulation of cells within a scaffold, which may lead to higher cell survival and retention in vivo. As a result, a considerable amount of research has focused on exploring the effects of various biomaterial scaffolds on regenerative cell populations. Previous studies demonstrated that culture of NP cells in a three-
dimensional hydrogel microenvironment more effectively maintains cell phenotype compared to monolayer culture, as seen by increased ECM production and NP gene expression\textsuperscript{71}. Cell encapsulation within scaffolds may also act as a barrier, temporarily protecting cells from the adverse inflammatory microenvironment associated with degeneration\textsuperscript{72,73}. Current scaffolds investigated in NP tissue-engineering applications can be broadly categorized based on their biomaterial composition as either natural (e.g., hyaluronan, collagen, decellularized NP) or synthetic\textsuperscript{72}.

The efficacy of any cell-based therapy will be dependent on the capacity of the pro-regenerative cells to adopt/maintain the NP-specific phenotype, as seen by the maintenance of NP-associated gene markers, in addition to producing NP-specific ECM including proteoglycans and type II collagen\textsuperscript{75,76}. Various cell types have been investigated, including mature NP cells\textsuperscript{77}, chondrocytes\textsuperscript{78}, notochord cells\textsuperscript{6}, mesenchymal stromal cells (MSC)\textsuperscript{79}, adipose-derived stromal cells (ASC)\textsuperscript{80}, and induced pluripotent stem cells (iPSCs)\textsuperscript{81}. Mature NP cells have been widely studied due to their ease of sourcing from bovine and porcine NP sources, which closely resemble the cellular makeup of human NP\textsuperscript{6}. Although a good candidate for NP regeneration studies, expansion of these cells \textit{in vitro} results in a loss of the differentiated cell phenotype, limiting their potential for expansion\textsuperscript{82}. Sourcing of healthy human NP tissues is also a barrier to the translation of therapies involving the delivery of mature NP cells\textsuperscript{83}. Alternatively, the role which notochord cells play in the formation and maintenance of the young NP has led to their study. However, availability and sourcing are even more challenging due to their loss at an early age in humans and in bovines, which are commonly used as a model system\textsuperscript{5,6}. Based on their similarity to NP cells in terms of proteoglycan and type II collagen matrix production, chondrocytes have been studied as another alternative cell source for NP regeneration\textsuperscript{84}. However, their translational potential is limited as chondrocytes have a distinct phenotype and produce ECM associated with hyaline cartilage which differs from that of the NP\textsuperscript{78}.

The limitations of various terminally differentiated adult cell populations have motivated the investigation of cell therapies involving multipotent and pluripotent cell populations that have the capacity to contribute directly or indirectly to NP regeneration. Multipotent
MSCs reside in various tissues, and can differentiate \textit{in vitro} to display markers of connective tissue cell types including muscle, tendon, bone, cartilage, and adipose cells\textsuperscript{76}. Studies have reported their capacity to differentiate into an NP-like phenotype capable of producing type II collagen and proteoglycans, such as aggrecan\textsuperscript{85}. Although these studies have shown promise, further studies assessing NP specific markers are required to differentiate the chondrocyte phenotype from the NP phenotype\textsuperscript{84}. Although not extensively studied in the context of NP regeneration, ASCs have been shown to produce NP-associated ECM components and restore disc height when incorporated into hydrogels containing decellularized NP tissue\textsuperscript{80}. Since NP cells are of notochordal origin, MSCs and ASCs may be limited in their ability to fully differentiate into NP cells, and further studies investigating cell phenotype are thus required. In contrast to the restricted potential of MSCs, iPSCs derived autologously from adult patients theoretically have the capacity to differentiate into any cell type in the body. As such, there is growing interest in the development of iPSC-based therapies for NP regeneration. Studies to date have largely focused on the development of protocols to differentiate human iPSCs towards an NP phenotype\textsuperscript{81} and a notochord phenotype\textsuperscript{86}; further studies are required to assess their behaviour \textit{in vitro} and \textit{in vivo}.

\section{1.7 Tissue decellularization}

Tissue decellularization is a process, which aims to remove immunogenic cellular components from tissues, while preserving the native ultrastructure and cell-instructive biochemical composition of the ECM as much as possible\textsuperscript{87}. As each tissue has a unique distribution of cells and ECM, decellularization protocols must be tailored to each tissue type, with almost every tissue in the body having an established protocol\textsuperscript{88,89}. Promisingly, various allogenic and xenogeneic decellularized tissue scaffolds are commercially available for clinical use, including from tissues such as dermis, heart valve, and small intestinal submucosa\textsuperscript{87,90}.

\subsection{1.7.1 Decellularization techniques}

Tissues are typically decellularized through a combination of treatments specifically selected based on the physical, biochemical, and cellular composition of the tissue-of-
interest, with the goal of maximizing decellularization while retaining the complex ECM composition. In general, treatment strategies can be broadly classified into three distinct categories: chemical, physical, or biological treatments, all of which strive to reduce cellular content\textsuperscript{87,88,91}.

**Chemical treatments for tissue decellularization:** Chemical treatments include the use of detergents (ionic, non-ionic, zwitterionic), acids and bases, hypo-/hypertonic solutions, chelating agents, and alcohols\textsuperscript{87,88,91}. Various chemicals can disrupt or solubilize the cell membrane, and help to separate nuclear and cellular content from the ECM\textsuperscript{87}. Chemical treatments can be effective at removing DNA content; however, their use often disrupts the complex structure and composition of the ECM\textsuperscript{87}. Anionic detergents, such as sodium dodecyl sulphate (SDS), although effective at solubilizing cellular membranes, can also denature matrix proteins and pose a cytotoxic risk if not adequately removed from the decellularized scaffold\textsuperscript{87}. Treatment with acids and bases can eliminate growth factors and disrupt collagen fibril crosslinking, thus affecting the biological cues and mechanical properties of the ECM\textsuperscript{87}. Hypo- and hypertonic solutions are effective at lysing cells, but require further treatments to remove residual cellular debris\textsuperscript{91}. Chelating agents, such as ethylenediaminetetraacetic acid (EDTA), work by sequestering divalent cations (such as Ca\textsuperscript{2+} and Mg\textsuperscript{2+}), which play a key role in mediating cell adhesion to the ECM\textsuperscript{91}, to allow easier cellular extraction.

**Physical treatments for tissue decellularization:** Physical treatments include the use of freeze-thaw cycles, pressure, agitation, and sonication\textsuperscript{91}. Freeze-thaw cycles involve the freezing of tissue resulting in intracellular ice crystal formation, leading to cell membrane disruption and cell lysis\textsuperscript{91}. Agitation and sonication are often used in tandem with chemical treatments as they assist with cell lysis and cell clearance\textsuperscript{91}. In general, physical decellularization strategies lyse cellular membranes, but typically fail to remove the residual nuclear and cellular material from within the tissues\textsuperscript{87,88,91}. As such physical treatments need to be used in combination with chemical or biological treatments to extract the remaining cellular content\textsuperscript{88}.
Biological treatments for tissue decellularization: Biological treatments primarily involve the use of enzymes that target specific cellular components. For example, enzymes can be used to target adhesion proteins in order to disrupt cell-cell and cell-ECM interactions (e.g., proteases, trypsin), clear unwanted ECM material, or cleave residual nuclear content (e.g., deoxyribonuclease (DNase), ribonuclease (RNase)) following cell lysis. However, during the decellularization process, disrupted cells may release unwanted proteases, leading to degradation of the ECM. As such, protease inhibitors such as phenylmethylsulfonylfluoride (PMSF) are often used in combination with chemical and physical treatments to help preserve the ECM composition and structure.

1.7.2 Characterization of decellularized tissue

Following decellularization, it is vital to confirm the removal of cellular content and to assess the retention of the ECM composition and its ultrastructure. No established criteria exist for the residual cellular content that can safely remain following decellularization. However, minimum guidelines have been proposed by P.M. Crapo and colleagues, including quantitative and qualitative analysis of cellular content post-decellularization. More specifically, they propose that tissues are sufficiently decellularized when there is: (1) <50 ng double stranded DNA (dsDNA) per mg ECM dry weight, and (2) a lack of visible nuclei in tissue sections stained with 4’,6-diamidino-2-phenylindole (DAPI) or hematoxylin and eosin (H&E). These guidelines were based on in vivo findings assessing the host immunogenic response to various decellularized tissue scaffolds. However, due to the large variations in the structure and cellular density/composition between tissues and species, further research is required to establish tissue-specific criteria for the allowable residual cellular material that will avoid a negative host response.

Analysis of cellular clearance: In order to assess the presence of residual cells and cellular debris, both qualitative and quantitative approaches are often used in conjunction. Qualitative analysis can be done using fluorescent nuclear stains, of which DAPI or Hoechst are commonly used. Various other histological and immunohistochemical (IHC) approaches can be carried out to visualize cell content, such as H&E staining or
staining for cytoskeletal elements (e.g., actin). Decellularization is typically quantitatively assessed through analysis of dsDNA content, via the Quant-iT™ PicoGreen® dsDNA kit.

**Analysis of ECM retention:** To visually assess the retention, spatial distribution, and composition of specific ECM components, histological staining (e.g., H&E, PicroSirius Red, Toluidine Blue, Masson’s trichrome) and IHC techniques can be employed. To complement the staining results, biochemical assays are often performed to quantify specific ECM constituents. For example, sulphated GAGs and total collagen content can be quantified via the dimethyl methylene blue (DMMB) and hydroxyproline (OHP) assays, respectively. In addition, in-depth characterization of the ECM composition can be carried out through techniques such as mass spectrometry and enzyme-linked immunosorbent assays (ELISAs). Mass spectrometry is advantageous in its unbiased approach and high-throughput capacity, leading to a more comprehensive characterization of the ECM constituents. In addition, ELISA is a useful approach to quantify specific proteins-of-interest within the decellularized tissues relative to native tissue controls. To complement the compositional analyses, transmission electron microscopy (TEM) and scanning electron microscopy (SEM) techniques are commonly employed in order to visualize the ECM ultrastructure.

### 1.7.3 Nucleus pulposus decellularization

Previous studies have begun to investigate decellularized NP tissue as a potential bioactive material for NP regeneration. Due to the limited availability of healthy human NP tissue, past studies have validated decellularization protocols using porcine or bovine NP tissues. The NP tissues from these species more closely resemble the cellular and ECM composition of the human NP as compared to other preclinical animal models such as the mouse or rabbit.

Mercuri and colleagues were the first group to publish a decellularization protocol specific for porcine NP tissue. This study used various detergents (Triton X-100 & deoxycholic acid), ultrasonication, and nuclease to decellularize the porcine NP. Despite the high efficacy of cellular removal (~98%), the protocol resulted in matrix
disruption, with a decrease in both the collagen and GAG content (~45% GAG retention)\textsuperscript{99}. A subsequent study used bovine NP tissue to test 13 different decellularization protocols which varied in detergent concentrations and incubation times\textsuperscript{97}. Briefly, the protocols included sonication, EDTA, Triton X-100, deoxycholic acid, sodium azide, and DNase/RNase treatment, and their finalized methods resulted in a significant reduction in DNA content (92%) and GAG content (~30% retention)\textsuperscript{97}.

Illien-Jünger et al. developed and tested various bovine NP decellularization protocols, which involved 5 freeze-thaw cycles and tissue milling followed by 3 different treatment combinations: (1) sodium deoxycholate and DNase treatment, (2) sodium deoxycholate, SDS and DNase treatment, and (3) sodium deoxycholate, SDS, Triton X-100, and DNase treatment\textsuperscript{98}. Protocol 1, which minimized detergent use, was most effective in reducing the dsDNA content (~86%) and maintained the highest GAG content (~16% retained)\textsuperscript{98}.

Wachs et al. used a combination of detergent treatments (sulfobetaine (SB)-10, Triton X-200, SB-16,) and enzymatic treatments (DNase/RNase) to generate a decellularized porcine NP scaffold\textsuperscript{96}. Their protocol resulted in a significant reduction in both cellular content (~96%) and GAG content (~45% retention) within their decellularized NP.

Various other groups have tested detergent-based NP or IVD decellularization protocols for their efficacy to remove cellular content while preserving the biochemical composition of the NP\textsuperscript{80,96–98,101–103}. However, the use of detergents has limitations, including the potential for alterations in the ECM structure and composition, and cytotoxicity concerns associated with the presence of residual detergent. As such, there would be value in exploring detergent-free decellularization approaches, which have been applied effectively for other tissue types, including adipose tissue\textsuperscript{89} and cartilage\textsuperscript{104}.

1.7.4 Cell-instructive effects of the ECM

Although ECM macromolecules are generally highly conserved between species, every tissue in the human body has a unique ECM composition and architecture, which play important roles in defining the tissue structure and function. On a cellular level, the tissue-specific ECM provides biochemical and biomechanical cues that can direct cellular function and phenotype\textsuperscript{51}. Numerous studies have sought to capitalize on the intrinsic
bioactivity of the ECM by incorporating specific ECM components within biomaterial scaffolds to support cell attachment or growth. For example, in the context of IVD regeneration, Francisco et al. showed that incorporating laminin within polyethylene glycol (PEG) scaffolds enhanced NP cell retention following intradiscal injection relative to delivery of NP cells in unmodified PEG scaffolds within rat tail IVDs\textsuperscript{105}.

Expanding on this concept, there is a growing body of the evidence that the complex ECM composition within decellularized tissues may be more effective than incorporating single ECM components within biomaterials for directing cell fate and function towards the goal of promoting tissue regeneration\textsuperscript{106}. More specifically, numerous studies have suggested that tissue-specific ECM can be harnessed to direct the lineage-specific differentiation of stem or progenitor cells in culture\textsuperscript{106-110}. For example, research in the Flynn lab has demonstrated that bioscaffolds derived from decellularized adipose tissue (DAT) can promote the adipogenic differentiation of human ASCs in culture\textsuperscript{110} and can also stimulate host adipose tissue regeneration in vivo\textsuperscript{111}. Similar tissue-specific compositional effects on differentiation have been reported with other decellularized tissue sources, including bone\textsuperscript{107}, cartilage\textsuperscript{112}, liver\textsuperscript{113}, lung\textsuperscript{114}, and myocardium\textsuperscript{115}.

In addition to biochemical composition, the biomechanical and structural properties of the ECM can also modulate cell function\textsuperscript{116}. Cells can bind to the ECM via specialized receptors, including integrins, which form focal adhesions that transmit forces to the actin cytoskeleton\textsuperscript{117}. Similar to biochemical signaling, integrin-mediated mechanotransduction allows cells to sense and respond to mechanical stimuli by activating downstream signaling cascades that can modulate cell behaviour\textsuperscript{51}. Studies have suggested that the lineage-specific differentiation of stem or progenitor cells can be augmented by culturing the cells on substrates that mimic the Young’s modulus of the target tissues\textsuperscript{106,118}. In the context of NP regeneration, Xu et al., found that the viability, proliferation, and ECM production of rat NP cells were improved when the cells were encapsulated within softer (~45 kPa) gelatin hydrogels as compared to stiffer (~85 kPa) hydrogels\textsuperscript{116}.
1.7.5 Methods for fabricating ECM-derived scaffolds

Decellularized ECM can be applied in its intact form as a 3-D bioscaffold or further processed to generate more application-specific scaffold formats that incorporate decellularized ECM as a bioactive component. Previously-investigated scaffold formats include ECM-derived hydrogels, porous foams, microcarriers, and coatings. To fabricate these alternative formats, decellularized tissues are typically further processed through mechanical (e.g., cryo-milling, mincing) and/or enzymatic means (e.g., α-amylase, pepsin) to obtain ECM powders, suspensions, or solutions that incorporate a complex composition that can mimic the native tissue source.

In most strategies to date, pepsin digestion has been used to generate ECM-derived peptide solutions that can be manipulated to form hydrogels. However, the non-specific proteolytic cleavage that occurs during pepsin digestion can negatively impact the bioactivity of the ECM, along with the stability of the resultant scaffolds. In an alternative approach, the Flynn lab has developed strategies using α-amylase digestion in place of pepsin to generate ECM suspensions that can be used to make various scaffold formats including microcarriers, foams, and coatings. In contrast to pepsin, α-amylase cleaves glycosidic linkages in the telopeptide regions of collagens, allowing for the preservation of collagen fibrils. In developing this new digestion strategy, Shridhar et al. compared the effects of culturing human ASCs on coatings synthesized from either pepsin-digested or α-amylase-digested DAT. The α-amylase-digested DAT coatings were softer, thicker, more stable, and maintained a fibrous ultrastructure that was not observed in the pepsin-digested coatings. Importantly, only the α-amylase-digested coatings showed bioactive effects relative to uncoated tissue culture plastic, enhancing both ASC proliferation and adipogenic differentiation.

In the context of NP regeneration, Wachs et al. applied pepsin digestion to generate DNP hydrogels that were capable of in situ gelation at body temperature. Human NP cells encapsulated within the DNP hydrogels remained viable throughout a 21-day in vitro culture period, with GAG production increasing over time. In another approach, Zhou et al. combined micronized DNP with additional chondroitin sulphate to counteract the
loss of GAG that was observed with their detergent-based decellularization protocol and crosslinked the materials with genipin to form hydrogels that could be used to encapsulate cells. The incorporation of the chondroitin sulphate was shown to promote the expression of NP-associated gene markers and the synthesis of type II collagen compared to cells cultured in DNP alone hydrogels or in monolayers on tissue culture plastic. In vivo testing showed that their composite scaffolds could partially restore the ECM content and disc height within a degenerated NP rabbit model.

1.8 Hydrogel scaffolds

Hydrogel scaffolds are hydrophilic, three-dimensional polymer networks that contain a high water content. Hydrogels are of great interest for NP regeneration in part due to their water absorption and retention properties that can mimic the native NP, in addition to their tunable mechanical properties and their capacity to facilitate oxygen and nutrient diffusion. Hydrogels can be made up of naturally-derived and/or synthetic polymers, and are held together by either chemical (covalent bonds) or physical (non-covalent) crosslinking. Crosslinking can be induced through different modalities, such as ultraviolet (UV) light, heat, divalent cations, or through pH changes. In general, UV- or thermally-initiated chemical crosslinking results in polymer networks with superior mechanical properties and increased stability in comparison to physically-crosslinked networks that form through chain entanglement.

Synthetic polymers are advantageous in terms of their more tunable mechanical properties and their well-defined composition that allows greater reproducibility, with less batch-to-batch variation as compared to many naturally-derived materials. However, synthetic polymers are often limited by their lack of innate biomimetic and bioactive properties, and can have challenges associated with low cell attachment, cytotoxicity, and limited biodegradability in vivo. Synthetic polymers previously investigated in NP regeneration include PEG, poly(N-isopropylacrylamide), polyurethanes, and poly-ε-caprolactone based scaffolds. Synthetic polymers are often functionalized with ECM components such as hyaluronic acid or laminin to generate composite hydrogels that combine robust mechanical properties with bioactive cues to enhance cell attachment and viability. However, these strategies typically fail to
recapitulate the complex ECM composition of the native NP microenvironment, which may be important for directing cell function.

Naturally-derived hydrogels are appealing due to their innate biocompatibility and capacity to degrade through natural processes (e.g., proteases) into non-toxic by-products, reducing cytotoxicity concerns\(^7\). Reactive groups such as acrylates, methacrylates, and acrylamides are often conjugated to polysaccharides to allow for polymer crosslinking\(^\text{125}\). Conjugation of these reactive groups allows for the modulation of the hydrogel properties through control over the degree of crosslinking, in addition to creating a stronger, more resilient natural hydrogel\(^\text{125,130}\). Various naturally-derived hydrogels, including chitosan, alginate, hyaluronan, collagen, gelatin, and agarose, have been studied for NP regeneration\(^7\). As constituents of the native NP, collagen- and hyaluronan-based hydrogels are commonly used in NP regeneration studies. Type II collagen hydrogels have been shown to promote the differentiation of ASCs towards an NP-like phenotype, resulting in upregulation of aggrecan and type II collagen gene expression and protein production compared to cells cultured in monolayer\(^\text{131}\). Hyaluronan-based hydrogels have been used to encapsulate NP cells promote the synthesis of GAGs\(^\text{132}\).

**1.8.1 Chondroitin sulphate-based hydrogels**

Chondroitin sulphate (CS) is a promising natural polymer for NP tissue engineering. More specifically, CS is an anionic linear polysaccharide comprised of repeating disaccharide units of D-glucuronic acid and N-acetyl galactosamine, which are sulphated at C\(_4\) and C\(_6\)\(^\text{130}\). As previously discussed, CS is a native ECM constituent in various tissues and is most prevalent in load-bearing tissues enriched in aggrecan such as cartilage and the NP\(^7,\text{133}\). As such, it has previously been used to generate natural hydrogels for use in regenerative therapies targeting these tissues\(^\text{104}\).

To form stable hydrogels, polymerizable reactive functional groups must be conjugated to the CS to allow for chemical crosslinking\(^\text{130,134}\). For example, methacrylate groups are commonly conjugated to the hydroxyl group on polysaccharides to allow for covalent crosslinking and rapid hydrogel formation\(^\text{125,135}\). More specifically, CS can be reacted with methacrylate anhydride to conjugate methacrylate to the CS hydroxyl groups,
forming MCS polymers\textsuperscript{130}. Such polymers can then be crosslinked via photo-crosslinking (UV light), thermal-crosslinking, or pH-induced crosslinking, allowing for the formation of hydrogels\textsuperscript{130,135}. In the resulting hydrogels, the degree of methacrylation can be modulated, allowing for control over mechanical properties\textsuperscript{135}. Previous studies have demonstrated that MCS hydrogels can be used to successfully encapsulate chondrocytes\textsuperscript{134} and human ASCs\textsuperscript{107} and support their viability in culture.

1.8.2 Composite hydrogel scaffolds

To generate scaffolds for cell encapsulation that possess favourable mechanical properties and provide cell-instructive cues to direct cell viability, proliferation, and differentiation, hydrogels can be combined with cryomilled tissue-specific decellularized ECM\textsuperscript{87}. This strategy has been applied to generate tissue-specific hydrogel composites incorporating ECM from a variety of tissues, including adipose tissue, bone, myocardium, and bladder\textsuperscript{107,136}. As a foundation for the project, the Flynn lab has previously incorporated cryo-milled decellularized adipose tissue and decellularized trabecular bone within cross-linkable MCS hydrogels to develop 3-D cell culture platforms that were shown to direct the lineage-specific differentiation of encapsulated human ACSs\textsuperscript{104,107}.

Interestingly, previous work suggests that the size of the cryo-milled particles can have an effect on the cellular response. The cryo-milling process can be tailored through alteration of the processing time and speed or through the use of sieves to obtain particles of specific size ranges. In previous work in the Flynn lab, Brown \textit{et al.} characterized the effects of varying DAT particle sizes on the proliferation and adipogenic differentiation of human ASCs encapsulated within methacrylated chondroitin sulphate (MCS) hydrogels\textsuperscript{137}. Overall, the incorporation of smaller particles (38±6 µm) was shown to augment ASC differentiation in comparison to hydrogels incorporating larger sized particles (278±3 µm) and MCS hydrogels without DAT\textsuperscript{137}. 
1.9 Project overview

1.9.1 Rationale

Current clinical treatments for IVD degeneration are focused on pain management and the alleviation of symptoms through surgery and physical therapy\textsuperscript{19,63}. With no disease-modifying therapeutics available, focus has shifted towards the development of novel cell-based strategies for NP regeneration. Towards this goal, this project focused on investigating the effects of tissue-specific ECM on the phenotype of NP cells within 2-D and 3-D cell culture platforms. Due to the key role of the ECM in mediating cell fate and function, it was hypothesized that DNP-based bioscaffolds would provide the biochemical cues necessary to support cell viability and maintain the phenotype of NP cells cultured \textit{in vitro}.

To date, the majority of studies expand NP cells on rigid 2-D tissue culture plastics (TCP). Although capable of supporting cell attachment and proliferation, primary NP cells cultured in monolayer on TCP undergo a loss of their native differentiated phenotype with serial expansion\textsuperscript{82,138}. Addressing this limitation, the current thesis sought to explore whether 2-D coatings or 3-D hydrogels fabricated from DNP could maintain or rescue the NP phenotype of serially-passaged NP cells based on analysis of gene expression.

In the present thesis, a detergent-free decellularization protocol was established for bovine NP and the resultant DNP was characterized. The DNP was then used to generate DNP coatings or incorporated within MCS hydrogels, to generate 2-D and 3-D culture platforms, respectively. Primary bovine NP cells were cultured on DNP coatings or purified type I collagen (COL) coatings as a non-tissue-specific control to assess the effects of the NP ECM on cell survival and cell phenotype within a 2-D culture platform. Finally, composite MCS hydrogels incorporating DNP or COL particles were developed that could be used to encapsulate bovine NP cells with high viability and applied as a 3-D culture platform to assess the effects of the ECM on the phenotype of the encapsulated cells.
1.9.2 Hypothesis

The incorporation of DNP within 2-D and 3-D culture platforms will enhance the expression of NP-associated markers in primary bovine NP cells and restore the expression of NP-associated markers in dedifferentiated NP cells.

1.9.3 Specific aims

**Aim 1**: To validate a detergent-free decellularization protocol for bovine nucleus pulposus.

**Aim 2**: To characterize the effects of culture on coatings derived from DNP or COL on the phenotype of primary bovine NP cells at early (P0) and later (P3) passages.

**Aim 3**: To assess the effects of incorporating DNP or COL particles on the viability and phenotype of primary bovine NP cells at early (P0) and later (P3) passages encapsulated within MCS hydrogels.
Chapter 2

2 Materials and Methods

2.1 Bovine nucleus pulposus decellularization

Bovine tails from animals between 9-30 months-of-age were acquired from the Mount Brydges Abattoir within 2 hours post-mortem. Tails were stored at -20 °C until use and then thawed overnight at 4 °C prior to dissection. The musculature and soft connective tissues surrounding the vertebrae were removed under aseptic conditions. The absence of overt intervertebral disc (IVD) injury or degeneration was confirmed visually by the absence of vascular ingrowth or tissue abnormalities. The annulus fibrosus (AF) and nucleus pulposus (NP) of the IVD were isolated from the 5-6 most proximal vertebral levels by transverse cuts at the superior and inferior intervertebral surfaces (Figure 2.1A). IVDs were placed in phosphate-buffered saline (PBS; 1x) and the AF tissue was removed. Pooled NP were trimmed of any remaining AF and minced into 2 mm³ pieces using a scalpel and 2 mm biopsy punch.

For decellularization, all incubation steps were performed in 300 mL tubs with ~15 g of NP / 100 mL of solution, at 37 °C under agitation on an orbital shaker (125 rpm) (Figure 2.1B). Minced NP was placed in deionized water (dH₂O) supplemented with 1 v/v% antibiotic-antimycotic (ABAM) (Gibco®, Invitrogen, Burlington, Canada) and 0.27 mM phenylmethylsulfonyl fluoride (PMSF), then frozen at -80 °C overnight and subsequently thawed at 37°C over 3 hours. NP tissue was then incubated in Sorensen’s phosphate buffer digest solution [SPB digest; 0.55 M sodium phosphate dibasic heptahydrate (Na₂HPO₄·7H₂O), 0.17 M potassium phosphate (KH₂PO₄), 0.049 M magnesium sulphate heptahydrate (MgSO₄·7H₂O), (pH 7.3)] supplemented with 15,000 U DNase Type II (from bovine pancreas), 12.5 mg RNase Type III (from bovine pancreas) and 1 v/v% ABAM for 5 h. The resultant decellularized nucleus pulposus (DNP) samples were washed for 3 x 30 min in PBS (1x) prior to freezing at -80 °C in dH₂O and lyophilized using a Labconco Freezone 4.5 Lyophilizer (Labconco, Kansas City, United States) for 72 h.
Figure 2.1. Schematic representation of nucleus pulposus excision and decellularization protocol. (A) The bovine tail was dissected and nucleus pulposus extracted. (B) Work flow of detergent-free decellularization protocol. Abbreviations: SPB=Sorensen’s phosphate buffer, ABAM=antibiotic-antimycotic, PMSF=phenylmethylsulfonylfluoride.

2.2 Preparation of DNP/COL coatings

DNP and commercially-sourced bovine type I collagen (COL) (Advanced Biomatrix, Cat # 5164-5GM, Carlsbad, United States) were cryo-milled for synthesis of DNP and COL suspensions using previously-established methods\textsuperscript{107,110}. In brief, cryo-milling was performed by transferring 1 g of lyophilized DNP or COL into a Retsch 25mL milling chamber with two 10 mm stainless steel milling balls. The chamber was sealed, submerged in liquid nitrogen for 3 min, then milled for 3 min at 30 Hz (Retsch Mixer Mill MM 400 milling system). This cycle was repeated a total of 3 times. Cryo-milled particles were sieved through a 125 µm stainless steel mesh filter, and the particles that passed through the filter were collected and stored in a desiccator until further use.

To generate the suspensions, the DNP or COL particles were added at a concentration of 25 mg/mL to 0.3% (w/w) α-amylase in 0.22 M NaH\textsubscript{2}PO\textsubscript{4} (pH 5.4). The samples were digested under continuous agitation on an orbital shaker at 300 rpm for 72 h at room
temperature, followed by centrifugation at 1500 x g for 10 min. The processed DNP was rinsed twice with 10 mL of 5\% (w/v) NaCl solution and once in dH\textsubscript{2}O under agitation (300 rpm) at room temperature for 10 min. The digested DNP and COL particles were then resuspended in 0.2 M acetic acid at a concentration of 25 mg/mL (based on the initial dry mass) and incubated under agitation at 120 rpm overnight at 37 °C. The DNP and COL suspensions were then homogenized using a PRO250 benchtop homogenizer (PRO Scientific, Oxford, United States) and stored at 4 °C until further use.

To fabricate the coatings, DNP and COL suspensions were incubated at 37°C for 1 h to reduce their viscosity. The suspensions were then applied to coat tissue culture plastic (TCP) well plates at a concentration of 125 µL/cm\textsuperscript{2} and left overnight in a biological safety cabinet to dry. In preparation for culture, the coatings were disinfected with a series of ethanol rinses. More specifically, the coatings were rinsed 3 times for 30 min in 70% ethanol at room temperature. To gradually rehydrate the coatings, the samples were incubated for 30 min in 35% ethanol and then rinsed 3 times with PBS (1x) and 2 times in NP media prior to cell seeding.

2.3 Preparation of composite MCS ± DNP/COL hydrogel scaffolds

2.3.1 Methacrylation of chondroitin sulphate

The methacrylation of chondroitin sulphate was performed following previously-established methods\textsuperscript{104}. Synthesis of methacrylated chondroitin sulphate (MCS) was done under light protection in order to avoid potential crosslinking. Briefly, chondroitin sulphate (CS) (~50 kDa, LKT Laboratories Inc., St. Paul, United States) was dissolved at 0.2 g/mL in 0.2 M sodium phosphate monobasic (NaH\textsubscript{2}PO\textsubscript{4}, pH 5.4) buffer. A volume of 60 µL of methacrylic anhydride (Sigma-Aldrich, Oakville, Canada) per 0.2 g of CS was added dropwise under continuous stirring (700 rpm). The pH was adjusted to 10 with 3 M sodium hydroxide. The reaction was then allowed to proceed for 1 h under continuous stirring (700 rpm), with the pH maintained at 10. The resulting product was precipitated in absolute ethanol for 15 min at 4 °C. The supernatant was then decanted and the precipitate was dissolved in dH\textsubscript{2}O. The solution was transferred into dialysis tubing with
a molecular weight cut-off of 3.5 kDa (SpectraPor™, ThermoFisher Scientific Inc.) and
dialyzed against 4 L of dH₂O for 72 h, with dH₂O changes every ~8 h. Purified MCS
solution was then neutralized with 3 M sodium hydroxide, snap frozen in liquid nitrogen
and lyophilized for 72 h. MCS was stored at -20°C under nitrogen and protected from
light until further use.

The target degree of methacrylation was 17%\textsuperscript{104}. In order to confirm the degree
of methacrylation, 10 mg of MCS was dissolved in 1 mL of deuterium oxide (Sigma-
Aldrich) and assessed by \textsuperscript{1}H NMR spectroscopy on an Inova 600 NMR spectrometer
(Varian, United States) following established methods\textsuperscript{139}.

### 2.3.2 Hydrogel crosslinking

Single-phase (no ECM) and composite MCS hydrogels incorporating the DNP or COL
particles were synthesized via previously-established photo-polymerization methods\textsuperscript{140}.
Briefly, MCS was added to PBS (1x) at a concentration of 20% (w/v). The sieved cryo-
milled DNP or COL particles were then added to the MCS pre-polymer solution at a
concentration of 5% (w/v). The MCS pre-polymer solution was left to dissolve overnight
under agitation (100 rpm) at 37°C. Immediately prior to fabrication, Irgacure 2959 photo-
initiator was dissolved in PBS (5 mg/mL) for 2.5 h, sterile filtered, and incorporated into
the MCS pre-polymer solution at a final concentration of 0.05% (w/v). The samples were
then transferred into 1 mL syringe moulds (ThermoFisher Scientific Inc.), and photo-
crosslinked for 2 minutes on each side (4 min total) through exposure to long-wavelength
ultraviolet light (365 nm, intensity of 12 mW/cm\textsuperscript{2}). The photo-crosslinked hydrogels
were then extruded from the syringe mould and cut into 50 µL sized scaffolds.

### 2.4 DNP characterization

#### 2.4.1 Histological characterization

Minced native NP and DNP samples were fixed in 10% formalin overnight, rinsed in
PBS, transferred to 70% ethanol, and processed for histology at the Robarts Molecular
Pathology Laboratory (London, ON). Samples were paraffin-embedded and sectioned (7
µm sections) using a Leica RM2235 microtome (Leica Biosystems, Concord, Canada).
Tissue sections were deparaffinized in xylene and rehydrated using an ethanol series (100%, 100%, 95%, 80%, 70%) followed by a dH₂O rinse. Sections were stained using standard methods for Toluidine Blue to visualize glycosaminoglycan (GAG) content, PicroSirius Red to visualize collagen content, and 4’,6-diamidino-2-phenylindole (DAPI) to visualize cell nuclei\textsuperscript{107}. Toluidine blue and PicroSirius Red stained tissue sections were visualized using an EVOS XL Core microscope (ThermoFisher Scientific Inc., Burlington, Canada) and a Nikon Optiphot polarizing microscope (Nikon Instruments Inc., Melville, United States), respectively. Fluorescent DAPI stained images were acquired using an EVOS FL fluorescence microscope (ThermoFisher Scientific Inc.).

2.4.2 Biochemical characterization
Cryo-milled DNP and cryo-milled native NP controls were digested in 125 µg/mL of papain enzyme in buffer solution (100 nM Na₂HPO₄, 5 mM EDTA, 5 mM L-Cysteine, pH 6.5) at 60 °C for ~18 hours for subsequent biochemical analyses, described in detail below.

2.4.2.1 Quantification of dsDNA
The double-stranded DNA (dsDNA) content in the DNP and native control samples was quantified using the Quant-iT™ PicoGreen® dsDNA kit (Molecular Probes, Invitrogen, Burlington, Canada) according to the manufacturer’s instructions. Briefly, an 8-point standard curve was generated by serial dilution of λ-DNA provided with the kit (100 µg/mL of TE buffer). Samples and standards were plated in technical triplicates (100 µL/well) at a 1:1 ratio with Quant-iT™ reagent (diluted 1:200 in TE buffer) and the fluorescence was measured using a CLARIOstar® spectrophotometer (BMG LABTECH Inc., Ortenberg, Germany; excitation 480 nm, emission 520 nm). dsDNA concentrations were calculated based on the standard curve and the values were normalized based on dry weight (expressed as ng dsDNA / mg dry tissue weight).

2.4.2.2 Glycosaminoglycan content
Sulphated glycosaminoglycan (sGAG) content was measured using the dimethyl methylene blue (DMMB) assay, as previously reported\textsuperscript{109}. Papain-digested samples were
diluted 1:30 with 1% bovine serum albumin (BSA) in PBS. An 8-point standard curve with a starting concentration of 250 µg/mL in 1% BSA was prepared through serial dilution of chondroitin sulphate sodium salt (Sigma C-6737) (10 mg/mL in 1% BSA). DMDB dye solution was then prepared by dissolving 1.6% DMDB dye in dH₂O supplemented with 1% ethanol and 0.2% formic acid (pH 5.3). Next, 10 µL of standards and samples were pipetted into 96-well plates in technical triplicates, and combined with 200 µL of DMDB dye solution. Absorbance was measured at 525 nm using a CLARIOstar® spectrophotometer (BMG LABTECH Inc.). sGAG concentrations were calculated based on the standard curve and the values were normalized to the dry tissue weight (expressed as µg of sGAG / mg dry tissue weight).

2.4.2.3 Hydroxyproline content

The hydroxyproline (OHP) assay was used as a measure of total collagen content, as previously described\textsuperscript{109}. Briefly, papain-digested samples were first hydrolyzed at a 1:1 ratio in 12 N hydrochloric acid at 110°C for 24 h, and neutralized with 6 N sodium hydroxide. An 8-point standard curve with a starting concentration of 16 µg / mL in dH₂O was prepared through the serial dilution of the hydroxyproline stock (100 µg/mL in dH₂O). Hydrolyzed samples (1:80 dilution in dH₂O), and standards were pipetted at a volume of 50 µL/well into a 96-well plate in technical triplicates. Samples and standards then underwent the sequential addition of: 50 µL of 0.05 M chloramine-T/20% 2-methoxyethanol (20 min incubation), 50 µL of 3.15 N perchloric acid (5 min incubation), and finally 50 µL of Ehrlich’s reagent (200 mg/mL of 4-dimethylaminobenzaldehyde in 2-methoxyethanol) (20 min incubation at 60°C). Prior to measurement, the plate was incubated at 4 °C for 5 min and left to stabilize at room temperature for 5 min. The absorbance at 560 nm was measured using a CLARIOstar® spectrophotometer (BMG LABTECH Inc.). Hydroxyproline concentrations were calculated based on the standard curve and values were normalized to dry tissue weight (expressed as µg OHP / mg dry tissue weight).
2.4.3 Immunohistochemical characterization

Minced native NP and DNP samples were embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, United States), and snap frozen in liquid nitrogen prior to cryo-sectioning (7-µm transverse sections). Tissue sections were fixed in acetone for 5 min and blocked in 10% goat serum in Tris-buffered saline supplemented with 0.1% Tween (TBST) for 1 h at room temperature. Tissue sections were then incubated for 1 h at 4 °C with primary antibodies diluted in TBST with 2% BSA against: collagen type I (1:100, ab34710, Abcam, Toronto, Canada), collagen type II (1:200, ab34712, Abcam), collagen type V (1:300, ab7046, Abcam), collagen type VI (1:300, ab6588, Abcam), fibronectin (1:150, ab23750, Abcam), laminin (1:200, ab11575, Abcam), or keratan sulphate (1:200, sc73518, Santa Cruz, Biotechnology). Tissue sections were then rinsed 3 times with TBST for 2 min at room temperature and incubated in either goat anti-rabbit IgG secondary conjugated to Alexa Fluor® 594 (1:200, ab150080, Abcam) or goat anti-mouse IgG secondary conjugated to Alexa Fluor® 680 (1:200, ab175775, Abcam), in 2% BSA in TBST for 1 h at room temperature. Slides were rinsed 3 times for 3 min in TBST and mounted with fluoroshield mounting medium (Abcam). Positive controls for antigen detection were performed on bovine dermis for all experiments (Appendix A: Supplementary Figure 1), and no primary antibody controls were also included in all trials. Images were acquired using a EVOS FL fluorescence microscope (Thermo Fisher Scientific Inc.).

2.4.4 Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) was performed on cryo-milled DNP and native NP tissue samples to visualize the ECM ultrastructure, using established protocols. Cryo-milled particles were coated in 5 nm osmium and visualized using a LEO1530 scanning electron microscope at an accelerating voltage of 1 kV and a working distance of 4.1 mm (Nanofabrication Facility, Western University).

2.4.5 Particle size distribution

Cryo-milled and sieved DNP (pooled from 4 bovine tails) and COL samples were analyzed using a Malvern Mastersizer 2000 (Malvern Instruments Ltd., Worcestershire,
United Kingdom) to determine their particle size distributions. In brief, ~300 mg samples of the particles were rehydrated in deionized water and analysed according to manufacturer’s instructions.

2.5 Hydrogel characterization

2.5.1 Gel content analysis

Gel content analysis was performed to quantify the amount of the MCS pre-polymer that was incorporated into the hydrogel network and confirm that the ECM particles were not interfering with the crosslinking efficiency. Following photo-crosslinking, MCS ± DNP/COL hydrogels were snap-frozen in liquid nitrogen and lyophilized for 24 h. The initial dry mass \( m_1 \) of each sample was recorded. Pre-polymer that was not crosslinked into the network was extracted by washing the samples 3 times in 10 mL of dH\(_2\)O for 3 h. Next, the hydrogels were snap-frozen in liquid nitrogen and lyophilized for 24 h. The final dry mass \( m_2 \) was recorded and the gel content (%) was calculated using the following equation:

\[
\text{Gel Content (\%)} = \left( \frac{m_2}{m_1} \right) \times 100
\]

2.5.2 Unconfined compression testing

Compression testing was performed to determine whether the incorporation of the ECM particles altered the mechanical properties of the hydrogels. To generate consistent scaffolds sized for mechanical testing, 110 µL cell-free single-phase (MCS only) and composite MCS+DNP/COL hydrogels were synthesized and photo-crosslinked individually within the syringe moulds. Following photo-crosslinking, the hydrogels were incubated in PBS overnight at 37 °C. Directly prior to mechanical testing, the height and diameter of the hydrogels were measured using digital calipers, to confirm a height to diameter ratio of ~1.5. Unconfined compression testing was carried out using the UniVert system (CellScale Biomaterials Testing, Waterloo, Canada) fitted with a 0.5 N load cell and a PBS bath maintained at 37 °C. Samples were subjected to 2 pre-conditioning cycles.
to a maximum of 10% strain at a rate of 0.05%/s. Hydrogel scaffolds then underwent 4 cycles of cyclic compression testing with a 0.01 N preload applied at the beginning of each cycle, and a maximum strain of 10% at a rate of 0.05%/s per cycle. Nominal stress was then calculated by dividing the applied force by the initial cross-sectional area of the scaffold. Young’s moduli were calculated from the slope of the linear region of the nominal stress-strain curve, with the stress values at 7% and 10% strain being used as the boundary conditions.

2.6 2-D and 3-D cell culture studies

2.6.1 Primary bovine NP cell isolation and culture

Primary NP cells were isolated using a protocol adopted from Séguin, et al. 2004. Briefly, NP tissue was dissected from bovine tails acquired from the local abattoir within 2 h post-mortem. Following dissection, NP tissues were placed in Ham’s F12 medium supplemented with 1% penicillin/streptomycin (P/S) and minced to ~2 mm³ using a scalpel. Minced tissues were transferred into fresh Ham’s F12 supplemented with 1% P/S and 0.5% protease XIV (Sigma, St. Louis, United States), and incubated for 1 hour at 37 °C. Next, the tissues were rinsed in PBS (1x) and then transferred into Ham’s F12 supplemented with 1% P/S and 0.1% Collagenase A (Roche, Laval, Canada), and incubated overnight (37°C, 5% CO₂). The resultant cell suspension was filtered through a 100 µm filter prior to seeding.

Cells were seeded in monolayer onto TCP at 25,000 cells/cm² and maintained in NP media (DMEM supplemented with 10% fetal bovine serum (FBS) and 1% P/S) at 37°C and 5% CO₂. Cells seeded onto TCP directly following isolation were designated as P0. Culture media was changed every 2 days and cells were passaged at 85-90% confluency through enzymatic dissociation with 0.25% trypsin (Gibco). At confluence, P0 cells were dissociated, centrifuged (1100 rpm for 5 min), resuspended in cryopreservation media (FBS supplemented with 10%v/v dimethyl sulfoxide (DMSO)), frozen at -80 °C overnight, rapidly thawed, and re-seeded onto TCP to generate the P0+1 passage level.
2.6.2 TCP NP cell passaging studies

Initial cell culture studies were performed to assess the effects of serial passaging on TCP and cryopreservation on the bovine NP cell morphology and gene expression patterns. Phase-contrast microscopy was used to assess the morphology across passage P0 to P5, as well as following cryopreservation in P0+1 samples. At each passage, once the NP cells reached ~80-90% confluency, they were rinsed in PBS (1x) and immediately imaged using a Leica DMI6000B inverted microscope equipped with a Leica DFC360FX camera. For the gene expression studies, P0 – P5 and cryopreserved P0+1 samples at 80% confluence were collected and processed for RNA extraction as described in detail below.

2.6.3 Cell seeding/passaging on the DNP/COL coatings

Culture studies were performed using the DNP and COL control coatings to assess whether culturing on the tissue-specific ECM could help to maintain the phenotype of NP cells serially passaged in monolayer culture. For these studies, NP cells (P0) were seeded at a density of 25,000 cells/cm² on the DNP coatings, COL coatings, or uncoated TCP controls, and cultured until TCP cells reached ~85% confluence (37°C, 5% CO₂). The NP cells cultured on the ECM coatings were passaging by digesting the DNP or COL with 0.25% collagenase A in serum-free media (DMEM supplemented with 1% P/S) for 45 min at 37 °C with agitation every 15 min. The TCP control cells were passaged using trypsin as described in Section 2.6.1. Cells were then centrifuged (1100 rpm for 5 min), rinsed in NP media to remove any remaining collagenase, centrifuged (1100 rpm for 5 min) and resuspended in NP media. Cells were re-seeded on their respective coatings at 25,000 cells/cm², and the process was repeated to generate cells from P0 to P3. At each passage, viability staining was performed as described in Section 2.7, and samples were collected for gene expression analysis as described in Section 2.8.

Additional studies were performed to assess the ability of the DNP coatings to rescue the phenotype of dedifferentiated NP cells. For these studies, samples of the bovine NP cells were serially passaged on TCP to passage 3 (P3) to generate the dedifferentiated cells. Un-passaged (P0) or dedifferentiated (P3) NP cells were seeded onto the DNP coatings,
COL coatings, or uncoated TCP at a density of 25,000 cells/cm², cultured for 48 h or 4 days in vitro (37°C, 5% CO₂), and processed for analysis of gene expression as described in Section 2.8.

### 2.6.4 Cell encapsulation within the composite MCS ± DNP/COL hydrogels

Culture studies were performed to assess the effects of incorporating tissue-specific ECM particles on the viability and phenotype of bovine NP cells encapsulated within the MCS hydrogels as a 3-D culture platform. Prior to cell culture, the MCS was decontaminated under UV light for 30 min and DNP/COL particles were decontaminated in 70% ethanol overnight followed by 3 successive 1 h rinses in PBS. For the studies, P0 or P3 cells were trypsinized (0.25%), centrifuged (1100 rpm for 5 min), and resuspended in NP media (DMEM + 10% FBS + 1% P/S) to obtain a concentration of 2 x 10⁷ cells/mL. The NP cell suspension (20% v/v) was combined with the MCS pre-polymer solution (80% v/v), the samples were well mixed with an 18G needle, and crosslinking was performed following the methods described in Section 2.3.2. Following crosslinking, 50 µL sized gels containing ~1x10⁶ NP cells were cultured individually within low-adherence 12-well tissue culture plates (Sarstedt, Nümbrecht, Germany) in NP media at 37 °C under 5% CO₂. NP cell viability in the MCS±DNP/COL hydrogels was qualitatively assessed through confocal microscopy at 24 h, 72 h, 7 day, and 14 day post-encapsulation, following the methods in the next section. Samples were also collected at each timepoint for gene expression analysis as described in Section 2.8.

### 2.7 LIVE/DEAD® cell viability characterization

Confocal imaging using a LIVE/DEAD® Viability/Cytotoxicity Assay (Invitrogen) kit was performed to assess the viability and distribution of cells on the 2-D and 3-D culture platforms. For the 2-D coating studies, the samples were rinsed with PBS and incubated at 37 °C in 2 μM Calcein-AM in PBS for 45 minutes to label live cells. For the 3-D hydrogel studies, the samples were rinsed with PBS and incubated in 4 μM EthD-1 and 2 μM Calcein AM in PBS for 30 minutes at 37 °C. Imaging was performed using a Zeiss LSM800 Confocal Microscope with Airyscan. Varying regions of the coatings and TCP
control samples were imaged at 10x magnification. The hydrogels were imaged followed published protocols\textsuperscript{104}. In brief, images were acquired at 5x magnification using the tiling function and z-stack features to visualize the entire cross-section of each hydrogel at 3-4 depths, separated by 50 µm, starting at the surface of the gel.

### 2.8 RT-qPCR gene expression analysis

For the 2-D coating studies, the cells were collected in 1 mL PureZOL (Bio-Rad Laboratories, Mississaug, Canada) with the use of a cell scraper. For the DNP or COL coatings, the samples were sonicated 3 times using a Model 100 Sonic Dismembrator (ThermoFisher Scientific Inc.) in 3 second bursts. For the cells cultured within the MCS±DNP/COL hydrogels, four 50 µL hydrogels were pooled and placed into 2 mL of PureZOL (Bio-Rad). A microtube pellet pestle was used to mechanical disrupt the hydrogels, followed by sonication ten times in 1 second bursts with the Model 100 Sonic Dismembrator.

Total RNA was extracted with the Aurum Total RNA Fatty and Fibrous Tissue kit (Bio-Rad) according to the manufacturer’s instructions. RNA concentration was determined using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific Inc.). Complimentary DNA was synthesized from 300 ng of RNA for the coating experiments, and 500 ng of RNA for both the TCP serial passaging and 3-D hydrogel experiments, using the iScript\textsuperscript{TM} cDNA synthesis kit (Bio-Rad). Gene expression was analyzed by SYBR-based real time qPCR using a Bio-Rad CFX-384 thermocycler. PCR reactions were run in triplicate, using 312 nM of forward and reverse primers, with 2x SsoFast EvaGreen Supermix (Bio-Rad). The PCR program consisted of the following: initial 2 minutes enzyme activation at 95°C, 10 seconds denaturation at 95°C, 30 seconds annealing/elongation at 60°C, for total of 45 cycles. Gene expression was calculated using the $\Delta\Delta^{Ct}$ method, normalized to expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase ($GAPDH$). All PCR primers were validated for efficiency and specificity, and sequences are displayed in Table 2.1. No template controls were included for all reactions.
Table 2.1 RT-qPCR List of genes and primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen Type 2 (<em>COL2A1</em>)</td>
<td>FWD: CCTCTGCGACGACATAATCT</td>
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<td>REV: GGTTCCTCTTTTCTGTCCTTTT</td>
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<tr>
<td>Collagen Type 1 (<em>COL1A1</em>)</td>
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<tr>
<td></td>
<td>REV: ACCAGGTTACCCGCTGTTT</td>
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<tr>
<td>SRY-box 9 (<em>SOX9</em>)</td>
<td>FWD: GCAAGCTCTGGAGACTGCTG</td>
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<tr>
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<td>REV: CGTTTCCTCAGCGACTTCCCTCC</td>
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<tr>
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<td>REV: CTATGTGCTCCGGGCAGT</td>
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<td>REV: AGACGGGCATTGTCGATCTG</td>
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<td>Forkhead Box A1 (<em>FOX1A1</em>)</td>
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<td>REV: AGAGGATAGGCGGTCCCTGTC</td>
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<td>Brachyury (<em>TBXT</em>)</td>
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<td>REV: TACCTTTGCAGCGGTCTGTG</td>
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<td><em>CD24</em></td>
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<td></td>
<td>REV: TTGTCCTTGACACCTCAGT</td>
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<tr>
<td>α-Smooth Muscle Actin 2 (<em>ACTA2</em>)</td>
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<td></td>
<td>REV: GCTTGAATTTGTGACCACCTCCT</td>
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<tr>
<td>Glyceraldehyde 3-phosphate dehydrogenase (<em>GAPDH</em>)</td>
<td>FWD: AAGGTCGGAGTGAAACGGATTTC</td>
</tr>
<tr>
<td></td>
<td>REV: ATTGATGGCGACGTAGTCAA</td>
</tr>
</tbody>
</table>
2.9 Statistical analysis

All numerical data are represented as mean ± standard deviation (SD). All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, San Diego, United States). A Kolmogorov-Smirnov test was done to compare the particle size distribution of the cryo-milled DNP and COL particles. The biochemical characterization data was analysed using a two-tailed unpaired Student’s t-test. The effects of serial passaging on TCP on NP gene expression was analysed by one-way ANOVA with a Tukey’s post-hoc comparison of the means. Gene expression levels of NP cells seeded on 2-D coatings or encapsulated within the 3-D hydrogels were analyzed by two-way ANOVA with a Tukey’s post-hoc comparison of means. Differences with p<0.05 were considered to be statistically significant.
Chapter 3

3 Results

3.1 Characterization of decellularized nucleus pulposus

The first aim of this study was to validate a detergent-free protocol for nucleus pulposus (NP) decellularization, with the overall goal of enhancing cell extraction and the retention of key extracellular matrix (ECM) components, including glycosaminoglycans (GAGs) and collagens. Briefly, NP tissue was dissected from bovine intervertebral discs (IVDs). The minced NP tissue was then subjected to a two-step protocol involving a single freeze-thaw cycle in deionized water (dH2O) to lyse cells, followed by a 5 h enzymatic digestion in deoxyribonuclease (DNase) and ribonuclease (RNase) to generate the decellularized NP (DNP).

Cellular extraction was qualitatively assessed using 4’,6-diamidino-2-phenylindole (DAPI) fluorescent staining for nuclei (Figure 3.1A). Compared to the native NP, DNP tissue contained fewer cells, although some residual nuclei were detected. Quantitative analysis of dsDNA content revealed an average dsDNA content of 11.1 ± 5.9 ng/mg tissue dry weight in the DNP as compared to 73.0 ± 2.6 ng/mg in the native NP, which represented a significant reduction in dsDNA content by ~85% following decellularization (Figure 3.1B). These findings support that the NP decellularization protocol was effective at extracting cells and cellular debris.

The GAG content of the NP tissues following decellularization was qualitatively assessed through toluidine blue staining of histological sections (Figure 3.2A). The staining intensity was decreased in the DNP compared to the native NP tissue, suggesting that there was extraction of GAGs during the tissue processing. These findings were corroborated through the quantification of sulphated GAGs (sGAGs) using the dimethyl methylene blue (DMMB) assay (Figure 3.2B). More specifically, the DNP contained significantly lower sGAG content (149.1 ± 15.9 µg/mg tissue dry weight) compared to the native NP (271.8 ± 38.7 µg/mg), indicating that ~45% of the native sGAGs were retained following processing.
Figure 3.1. Decreased cellular content following nucleus pulposus decellularization

(A) Representative 4',6-diamidino-2-phenylindole (DAPI) stained sections of native nucleus pulposus tissue (NP) and decellularized NP (DNP) showed a reduction in cell nuclei (pseudo-coloured in white) following decellularization. Scale bars = 200 µm. (B) Biochemical analysis of double-stranded DNA (dsDNA) content in the native NP and DNP measured using the PicoGreen Assay showed a significant reduction in dsDNA content in DNP relative to the native NP. Data reported as mean ± SD (N=4). Student’s two-tailed t-test; *** p<0.0001.
Figure 3.2 Sulphated glycosaminoglycan (sGAG) content was reduced following nucleus pulposus decellularization. (A) Representative histological sections of native nucleus pulposus (NP) and decellularized NP (DNP) stained with Toluidine Blue to detect GAGs (purple). GAG staining was detected throughout both DNP and native NP tissue sections, although staining intensity appeared to be reduced within DNP relative to NP. Scale bars = 200 µm. (B) Biochemical analysis of sGAG content using the dimethyl methylene blue (DMMB) assay showed a retention of ~45% of sGAGs within the DNP relative to the native NP. Data reported as mean ± SD (N=4). Student’s two-tailed t-test; **p<0.001.

The spatial distribution of collagens was visualized in DNP and native NP using picrosirius red staining of histological sections (Figure 3.3A). Polarized light microscopy revealed a similar spatial distribution of dense collagen networks in both the DNP and native NP tissues. These findings were confirmed by quantification of the tissue hydroxyproline content, which was not significantly different between the DNP (64.8 ±
4.5 µg/mg tissue dry weight) and native NP tissue (56.6 ± 5.4 µg/mg tissue dry weight) (Figure 3.3B).

**Figure 3.3. Collagen content was unaffected by the decellularization of nucleus pulposus tissue.** (A) Representative PicroSirus Red staining of native nucleus pulposus (NP) and decellularized NP (DNP) imaged by polarized light. Staining revealed a complex network of collagen fibers of varying sizes, with similar patterns detected in the DNP and native NP tissues. Scale bars = 200 µm. (B) Biochemical analysis of hydroxyproline (OHP) content as a measure of total collagen content using the hydroxyproline assay showed no significant difference between DNP and native NP tissues. Data reported as mean ± SD (N=4). Students two-tailed t-test; p<0.05.
Lastly, cryo-milled DNP and NP particles were visualized through scanning electron microscopy (SEM). DNP and native NP samples both included particles of a varying range of sizes and were found to have similar ultrastructures (Figure 3.4), suggesting that the decellularization process did not substantially alter the ECM architecture following milling.

Figure 3.4. Scanning electron microscopy (SEM) of cryo-milled native nucleus pulposus (NP) and decellularized nucleus pulposus (DNP) showing a similar extracellular matrix ultrastructure. Images captured at magnifications of 2,500x and 10,000x. 2,500x magnification scale bar = 5 µm. 10,000x magnification scale bar = 1 µm. (N=1).
3.2 Characterization of the extracellular matrix in decellularized nucleus pulposus

Immunohistochemical staining was performed to characterize the effects of the detergent-free decellularization protocol on the ECM composition of the NP (Figure 3.5). Analysis of collagen type I (COLI), collagen type II (COLII), collagen type V (COLV), collagen type VI (COLVI), laminin (LN), and fibronectin (FN) revealed similar staining intensities and spatial distribution patterns in the DNP compared to the native NP. Although the spatial distribution of keratan sulphate (KS) was similar in the DNP and native NP, a decrease in staining intensity was noted in the DNP compared to the native NP tissue. (Positive tissue controls and no primary antibody controls are included in Appendix A: Supplementary Figure 1.)
Figure 3.5. Immunofluorescence analysis confirmed the maintenance of key NP ECM constituents following decellularization. Representative images showing the presence and distribution of collagen type I (COL I), collagen type II (COL II), collagen type V (COL V), collagen type VI (COL VI), laminin (LN), and fibronectin (FN), with similar distribution patterns and staining intensities in the decellularized NP (DNP) and the native NP. Keratan sulphate (KS) showed a similar spatial distribution pattern with qualitatively decreased staining intensity within the DNP relative to the native NP. Scale bars = 200 µm. (N=3).

3.3 Phenotype characterization of primary bovine NP cells in monolayer culture

Primary bovine NP cells were plated in monolayer on tissue culture plastic (passage P0), and consecutively passaged up to five times (P5) to assess the stability of cell phenotype following in vitro cell expansion. Cells at P0 were also cryopreserved, thawed, seeded onto tissue culture plastic (TCP), and grown to confluence (P0+1) to assess the effects of cryopreservation on cell phenotype. Phase-contrast microscopy showed there to be two cell types present at each passage level: small round cells and elongated spindle-shaped cells. Additionally, while no qualitative differences were seen in spindle-shaped morphology, rounded cells appeared to decrease in number within later passages as compared to early passages. No differences were noted in the cryopreserved group (Figure 3.6).

RT-qPCR gene expression analysis was performed to assess the expression of ECM genes (COL2A1, COL1A1) and nucleus pulposus-associated genes (SOX9, KRT8, KRT18, KRT19, FOXA1, PAX1) at each passage. Serial passaging of primary NP cells in monolayer culture was associated with a general loss of cell type-specific gene expression. NP cells expanded beyond P3 showed a significant decrease in COL2A1 expression relative to un-passaged cells (P0), while no significant difference in COL1A1 gene expression was detected (Figure 3.7A). NP cells showed a similar loss of cell-type specific markers over serial passages, with a significant decrease in the expression of KRT8, KRT18, and KRT19 detected in cells by P1 relative to P0 (Figure 3.7B). Cryopreserved and re-seeded cells also showed a significant decrease in KRT8, KRT18, and
KRT19 expression relative to P0 cells. Although the expression of SOX9 and PAX1 in primary NP cells were not significantly altered by passage in vitro, the expression of FOXA1 was significantly decreased in NP cells at P5 relative to P0. Together, these findings suggest that the NP phenotype is largely affected following the P1 passage as seen by a decrease in NP-specific marker expression, with increased dedifferentiation seen by P3, as marked by decreased COL2A1 expression.

Figure 3.6. Morphology of primary bovine NP cell monolayer cultures following serial passage. Primary bovine NP cells were dissociated from tissues and plated in monolayer culture (passage P0). Cells were expanded in vitro and consecutively passaged at ~85 % confluence up to passage 5. At confluence, some P0 cells were trypsinized, cryopreserved, and subsequently thawed into monolayer culture (P0+1). Cell morphology appeared consistent with increased passages in monolayer culture. Images are representative of N=3 individual cell preparations. Scale bar = 200 µm.
Figure 3.7. RT-qPCR gene expression analysis of serially passaged primary bovine NP cells grown in monolayer on tissue culture plastic. Primary bovine NP cells were dissociated from tissues and plated in monolayer culture (passage P0). Cells were expanded in vitro and consecutively passaged up to passage 5. At confluence, P0 cells were trypsinized, cryopreserved, and subsequently thawed into monolayer culture (P0+1). Gene expression analyses were performed on NP cells at each passage to quantify (A) NP ECM genes (COL2A1, COL1A1), and (B) NP phenotype markers (SOX9, KRT8, KRT18, KRT19, SOX9, FOXA1, PAX1). Relative gene expression was determined using the ΔΔCt method normalized to expression of the housekeeping gene GAPDH, and expressed relative to unpassaged (P0) cells. Data is presented as mean ± SD, N=3. One-way ANOVA with Tukey’s post-hoc correction was performed. *p<0.05, **p<0.005, ***p<0.0005, ****p<0.0001.
3.4 Assessment of primary bovine NP cells cultured on ECM coatings

Primary bovine NP cells were imaged using calcein-AM staining to assess their viability when grown on TCP, DNP coatings, or type I collagen (COL) coatings generated from commercially-sourced bovine tendon collagen (Figure 3.8). A high density of live cells was observed on all culture substrates, across all passages. Of note, NP cells cultured on TCP consistently reached confluence approximately 3 days post-seeding, as compared to NP cells cultured on DNP or COL coatings, which were seen to be less confluent at the same timepoint. These results confirm the capacity of the DNP coatings to support the attachment and viability of primary bovine NP cells across different passage levels in vitro.
Figure 3.8. A high density of viable primary bovine NP cells were visualized across passages P0 to P3 on tissue culture plastic and ECM-coated surfaces. Representative confocal microscopy images showing calcein-AM stained live cells (green) following monolayer culture (P0) and serial passaging (P1-P3) on uncoated tissue culture plastic (TCP), decellularized NP (DNP) coatings, or type I collagen (COL) coatings. Images are representative of N=3 individual cell preparations. Scale bar = 200 μm.

To assess the effects of culture on DNP coatings on the maintenance of the NP cell phenotype across passages in monolayer cultures, RT-qPCR analysis was used to quantify the expression of ECM genes (*COL2A1, COL1A1*), NP-associated markers (*KRT8, KRT19, SOX9, FOXA1, PAX1*), notochord-associated markers (*CD24, SHH, TBXT*), and fibroblast-associated markers (*ACTA2, FSP1*). In contrast to NP cells cultured on TCP, which showed a significant decrease in *COL2A1* expression following passage, NP cells cultured on DNP or COL coatings showed no significant change in *COL2A1* and *COL1A1* gene expression with passaging (Figure 3.9A). Compared to primary (P0) NP cells cultured on TCP, primary (P0) NP cells cultured on DNP coatings showed a significant decrease in the expression of NP-associated markers, *KRT8, KRT19*, and *SOX9* - a change not induced by culture on COL coatings (Figure 3.9B). Similar to NP cells serially passaged on TCP, primary NP cells cultured on COL coatings showed a significant decrease in the expression of the NP-associated markers *KRT8, KRT19, SOX9*, and *FOXA1* following the first passage. Although serial passage on ECM coatings did not significantly alter the expression of the notochord-associated markers *CD24* and *TBXT*, the expression of *SHH* was significantly decreased in NP cells following passage on TCP and COL (Figure 3.9C). Compared to primary (P0) NP cells cultured on TCP, primary (P0) NP cells cultured on DNP coatings showed a significant decrease in the expression of *SHH* while primary (P0) NP cells cultured on COL coatings showing a significant increase in *SHH* (Figure 3.9C). Lastly, the expression of *ACTA2*, a myofibroblast marker, was significantly decreased in primary NP cells grown on DNP and COL coatings relative to cells cultured on TCP at P0 (Figure 3.9D). On both TCP and COL coatings, expression of *ACTA2* was significantly decreased in passaged NP cells. Taken together these findings suggest that *in vitro* culture of primary bovine NP cells on DNP coatings was unable to maintain the NP phenotype across serial passages.
Figure 3.9. RT-qPCR gene expression analysis of serially passaged primary bovine NP cells grown in monolayer on tissue culture plastic or ECM coatings. Primary bovine NP cells (P0) were cultured in monolayer on uncoated tissue culture plastic (TCP), decellularized NP (DNP) coatings, or type I collagen (COL) coatings and serially passaged (P1-P3) on the same culture substrate. Gene expression analysis was performed to quantify expression of (A) NP ECM genes (COL2A1, COL1A1), (B) NP-associated markers (SOX9, KRT8, KRT19, FOXA1, PAX1), (C) notochord-associated markers (CD24, TBXT, SHH), and (D) fibroblast markers (ACTA2, FSP1). Relative gene expression was determined using the ΔΔCt method normalized to expression of the housekeeping gene GAPDH, and expressed relative to P0 cells on TCP. Data is presented as mean ± SD, N=3. Two-way ANOVA with Tukey’s post-hoc correction was performed. *p<0.05, **p<0.005, ***p<0.0005, ****p<0.0001.

In order to determine if ECM coatings could restore the NP cell phenotype in 2-D culture, dedifferentiated late passage NP cells (passaged 3 times on TCP) or primary un-passaged (P0) NP cells were seeded onto TCP, DNP coatings or COL coatings, and gene expression was assessed after 2 days or 4 days. Following 2 days, culture of P0 NP cells on COL coatings induced a significant increase in COL2A1 expression compared to cells cultured on either TCP or DNP (Figure 3.10A). However, there was no significant difference in COL2A1 expression induced by ECM coatings following 4 days of culture relative to P0 cells on TCP at 2 days. Both, P0 cells on DNP coatings and P3 cells on COL induced a significant increase in COL1A1 expression at 4 days compared to P0 cells on TCP at 2 days. In general, culture of dedifferentiated NP cells on ECM coatings did not reverse the loss of expression of NP and notochord-associated markers. In fact, expression of KRT8, KRT19, and SHH were significantly reduced in P0 NP cells cultured on DNP coatings at both 48 h and 4 days compared to cells cultured on TCP or COL coatings at the same timepoints (with the exception of KRT19 expression in P0 cells at 4 day; Figure 3.10B,C). KRT8 and KRT19 expression were significantly decreased in P3 cells at both time points compared to P0 cells, unaffected by cultured on ECM coatings. No significant differences were detected in SOX9 expression at each timepoint, showing no effect associated with cell passage or culture substrate (Figure 3.10B). Although no significant differences were detected in the expression of the notochordal markers CD24 or TBXT between P0 and P3 cells on various coatings, SHH expression was significantly
decreased in P3 cells compared to P0 cells and was not altered by culture on DNP or COL coatings (Figure 3.10C). Lastly, the expression of fibroblast-associated markers was largely unchanged in NP cells by passage or culture conditions (Figure 3.10D). Taken together, these findings suggest that culture of dedifferentiated P3 NP cells on DNP coatings alone was unable to rescue the NP specific phenotype.
Figure 3.10. RT-qPCR gene expression analysis of primary bovine NP cells grown on uncoated tissue culture plastic or ECM coatings, and cultured in vitro for 48 h and 4 days. Un-passaged (P0) and late passage (P3) primary bovine NP cells were seeded onto uncoated tissue culture plastic (TCP), decellularized NP (DNP) coatings, or type I collagen (COL) coatings. Cells were cultured in vitro for 2 days (2d) or 4 days (4d). Gene expression analysis was performed to quantify expression of (A) NP ECM genes (COL2A1, COL1A1), (B) NP-associated markers (SOX9, KRT8, KRT19), (C) notochord-associated markers (CD24, TBXT, SHH), and (D) fibroblast markers (ACTA2, FSP1). Relative gene expression was determined using the \( \Delta \Delta C_t \) method normalized to expression of the housekeeping gene GAPDH, and expressed relative to P0 cells on TCP at 2 days. Data is presented as mean ± SD, N=3. Two-way ANOVA with Tukey’s post-hoc correction was performed. *p<0.05, **p<0.005, ***p<0.0005.

3.5 Characterization of decellularized nucleus pulposus and bovine tendon collagen cryo-milled particles

Previous studies within our lab investigating human adipose-derived stromal cells (ASCs) encapsulated within methacrylated chondroitin sulphate (MCS) hydrogels in combination with cryomilled decellularized adipose tissue (DAT) particles determined that ECM particle size can modulate the differentiation of the encapsulated cells\(^{139}\). Based on the previous findings that showed favorable bioactivity with a smaller particle size, lyophilized DNP and COL particles were sieved through a 125 µm stainless steel mesh following cryo-milling. The particles that passed through the filter were collected and their size was analyzed using a Malvern Mastersizer instrument. Volume fraction analysis confirmed that the majority of the particles in both the DNP and COL groups were less than 125 µm (Figure 3.11A). While the particles ranged from 3 µm to 550 µm for the DNP samples and 2 µm to 280 µm for COL samples, there was no significant difference in the particle size distribution between the two groups (Figure 3.11B). These findings confirm that particle size was not likely to be a confounding factor influencing the response of the cells encapsulated within the MCS+DNP versus MCS+COL hydrogels.
Figure 3.11. Particle size distribution analysis confirmed that cryo-milled DNP and COL particles were of a similar size range. (A) Volume fraction and (B) particle size distribution of cryo-milled DNP and COL particles that passed through the 125 µm stainless steel mesh. The majority of the particles were less than 125 µm and there was no significant difference in the particle size distributions between the DNP and COL groups, as determined by a Kolmogorov–Smirnov test (p<0.05) (N=3).

3.6 Physical characterization of composite hydrogels

To generate the hydrogel platform for the 3-D cell culture studies, chondroitin sulphate (CS), a prominent GAG within the native NP, was functionalized with methacrylate groups through established protocols. The purity and degree of methacrylation of the MCS was confirmed by 1H NMR spectroscopy (Appendix A: Supplementary Figure 2). Hydrogel constructs of MCS ± DNP/COL (20% w/v MCS, 5% w/v ECM), were synthesized via UV-crosslinking. Following crosslinking and swelling, the hydrogels appeared macroscopically similar, with slight variation in colour; MCS hydrogels appeared clear and colourless, while MCS+DNP and MCS+COL hydrogels were opaque with a slight off-white colour. (Figure 3.12).
Figure 3.12. Macroscopic images of MCS±DNP/COL hydrogels following UV-crosslinking and swelling. MCS hydrogels were transparent, while the MCS+DNP or MCS+COL were opaque with an off-white colour.

Gel content analysis was performed to assess the crosslinking efficacy by measuring the amount of MCS polymer that was successfully incorporated into the hydrogel network following UV-crosslinking. Gel content did not differ significantly between the hydrogel groups, with average values of 80.4 ± 2.5% for MCS, 81.5 ± 0.5% for MCS+DNP, and 82.8 ± 2.1% for MCS+COL (Figure 3.13A). These results confirm that the incorporation of 5% w/v DNP or COL particles into MCS hydrogels did not interfere with the crosslinking process.

To assess the stiffness of the hydrogels, unconfined bulk compression testing was performed. Consistent with the gel content analysis, no significant differences were detected in the stiffness of the varying hydrogel groups, with Young’s moduli values of 160.9 ± 41.5 kPa for MCS, 202.1 ± 45.5 kPa for MCS+DNP, and 202.3 ± 23.2 kPa for MCS+COL (Figure 3.13B).
Figure 3.13. Gel content and mechanical analysis of composite MCS ± DNP/COL hydrogels. (A) There was no significant difference in the gel content between any of the hydrogel groups, confirming that the incorporation of the ECM particles did not interfere with crosslinking (n=5 individual hydrogels/trial, N=3 independent trials). (B) Similarly, there was no significant difference in Young’s moduli between the hydrogel groups, as determined through unconfined bulk compression testing (n=2-3 individual hydrogels/trial, N=4-5 independent trials). One-way ANOVA with Tukey’s post hoc comparison of means (p<0.05).

3.7 In vitro assessment of primary bovine NP cells encapsulated within MCS ± DNP/COL composite hydrogels

3.7.1 LIVE/DEAD® analysis of encapsulated NP cells

Primary bovine NP cells were encapsulated within MCS, MCS+DNP, or MCS+COL composite hydrogels to assess the capacity of the three-dimensional (3-D) culture platforms to support NP cell viability in culture. Both un-passaged (P0) (Figure 3.14) and late passage (P3) (Figure 3.15) cells were encapsulated, to assess the impact of ECM incorporation on the viability of both primary and dedifferentiated NP cells following 24 h, 72 h, 7 days, or 14 days of in vitro culture.

Following encapsulation, P0 NP cells showed a uniform distribution throughout all hydrogels (Figure 3.14). In general, the MCS, MCS+DNP, and MCS+COL groups showed high cell viability across all timepoints assessed. Qualitative observations
suggested that fewer viable cells were present in the MCS group following 7 and 14 days of culture as compared to the MCS+DNP or MCS+COL groups. Notably, NP cell clustering was observed after 7 and 14 days in culture within the MCS+DNP and MCS+COL hydrogels.

Following encapsulation, P3 NP cells also showed a uniform cell distribution across all hydrogel groups (Figure 3.15). Cell viability was similar in all hydrogels up to 7 days in culture, although the viability of NP cells in MCS appeared reduced following 14 days in culture compared to MCS+DNP or MCS+COL groups. NP cell clustering was apparent following 14 days of culture within both the MCS+DNP and MCS+COL groups.

These findings support that the MCS+DNP hydrogels are capable of maintaining high cellular viability of both P0 and P3 primary bovine NP cells up to 14 days in culture. Additionally, the incorporation of ECM particles might allow for higher cellular viability as compared to MCS only hydrogels.
Figure 3.14. Primary bovine NP cells remained viable following encapsulation and in vitro culture within MCS ± DNP/COL up to 14 days. Unpassaged P0 primary bovine NP cells were encapsulated within MCS, MCS+DNP or MCS+COL hydrogels, and cultured in vitro for 24 h, 72 h, 7 days, or 14 days. Representative confocal microscopy images were captured at each time point showing live cells stained with calcein-AM (green), dead cells with ethidium homodimer-1 (red), and auto-fluorescence of DNP and COL particles (blue). Images were captured at depths of either 75 µm or 150 µm. Top panels: Tiled confocal image showing full hydrogel cross-sectional area. Scale bars = 1 mm. Bottom panels: High magnification hydrogel image. Scale bars = 200 µm. Images are representative of N=3 individual cell preparations.
Figure 3.15. P3 bovine NP cells remained viable following encapsulation and in vitro culture within MCS ± DNP/COL up to 14 days. Late passage P3 primary bovine NP cells were encapsulated within MCS, MCS+DNP or MCS+COL hydrogels, and cultured in vitro for 24 h, 72 h, 7 days, or 14 days. Representative confocal microscopy images were captured at each time point showing live cells stained with calcein-AM (green), dead cells with ethidium homodimer-1 (red), and auto-fluorescence of DNP and COL particles (blue). Images were captured at depths of either 75 µm or 150 µm. Top panels: Tiled confocal image showing full hydrogel cross-sectional area. Scale bars = 1 mm. Bottom panels: High magnification hydrogel image. Scale bars = 200 µm. Images are representative of N=3 individual cell preparations.

3.7.2 Gene expression analysis of encapsulated NP cells

The effects of incorporating DNP within the MCS hydrogels on gene expression of the NP ECM genes, NP-associated markers, notochord-associated markers and fibroblast-associated markers were assessed in encapsulated primary un-passaged (P0) bovine NP cells following 24 h, 72 h, 7 days, and 14 days of culture to determine whether the incorporation of the ECM within a 3-D culture platform could maintain the NP cell phenotype (Figure 3.16). Overall, the incorporation of the DNP or COL particles did not significantly alter the expression of any of the genes relative to the MCS alone hydrogels at all timepoints investigated. P0 NP cells showed a decrease in COL2A1 expression over time in the MCS+DNP and MCS+COL hydrogel groups (Figure 3.16A), while COL1A1 expression was not significantly altered over time in culture for any of the groups. Of the NP-associated markers, KRT19 expression significantly decreased over time in the MCS and MCS+DNP hydrogels and SOX9 expression significantly decreased over time in all groups (Figure 3.16B). Interestingly, PAX1 expression was significantly increased in the NP cells encapsulated within the MCS+DNP hydrogels at 14 days and the MCS+COL hydrogels at 7 days relative to their expression levels at 24 h. Of the notochord-associated markers, CD24 expression was significantly reduced over time in the MCS+DNP hydrogel group (Figure 3.16C). No differences were observed in the expression of the fibroblast-associated markers over time for any of the groups (Figure 3.16D). Taken together, these findings suggest that the incorporation of the DNP particles was not
sufficient to maintain the phenotype of the primary bovine NP cells encapsulated and cultured within the MCS hydrogels.

To assess the potential for encapsulation within the 3-D MCS+DNP hydrogels to rescue the phenotype of dedifferentiated late passage (P3) NP cells, primary NP cells were first serially passaged on TCP to P3, and then encapsulated within the MCS, MCS+DNP, or MCS+COL hydrogels and cultured for up to 14 days. Similar to the findings with the P0 cells, the incorporation of the DNP or COL particles did not significantly alter the expression of any of the genes relative to the MCS alone hydrogels at all timepoints investigated. The expression of the ECM genes $COL2A1$ and $COL1A1$ did not significantly change over time for any of the groups (Figure 3.17A). Of the NP-associated markers, $KRT8$ expression levels significantly decreased over time for all groups (Figure 3.17B). Similarly, expression of the notochord-associated marker CD24 significantly decreased over time in the MCS+DNP group (Figure 3.17C). Similar to the P0 encapsulated cells, expression of the fibroblast-associated markers $ACTA2$ and $FSP1$ was not altered over time in any of the hydrogel groups (Figure 3.17D). The notochord-associated marker $SHH$ was not detected in any of the samples. Taken together, these results show that culturing within the 3-D MCS hydrogels either with or without the incorporation of the ECM was insufficient to restore the NP phenotype of encapsulated P3 dedifferentiated cells.
Figure 3.16. RT-qPCR gene expression analysis of P0 primary bovine NP cells encapsulated within MCS ± DNP/COL scaffolds and cultured in vitro up to 14 days. Unpassaged (P0) primary bovine NP cells were encapsulated within MCS, MCS+DNP, or MCS+COL hydrogels, and cultured in vitro for 24 h, 72 h, 7 days, or 14 days. Gene expression analysis was performed to quantify expression of (A) NP ECM-associated genes (*COL2A1, COL1A1*), (B) genes associated with the NP phenotype (*SOX9, KRT8, KRT19, PAX1, FOXA1*), (C) notochord-associated markers (*CD24, TBXT, SHH*), and (D) fibroblast cell markers (*ACTA2, FSP1*). Relative gene expression was determined using the ΔΔCt method normalized to expression of the housekeeping gene *GAPDH*, and expressed relative to cells encapsulated within MCS at 24 h. Data is presented as mean ± SD, N=3. Two-way ANOVA with Tukey’s post-hoc correction was performed. * indicates significant difference in gene expression at 24 h within specific hydrogel groups. * p<0.05.
Figure 3.17. RT-qPCR gene expression analysis of P3 primary bovine NP cells encapsulated within MCS ± DNP/COL scaffolds and cultured in vitro up to 14 days. Late passage (P3) primary bovine NP cells were encapsulated within MCS, MCS+DNP, or MCS+COL hydrogels, and cultured in vitro for 24 h, 72 h, 7 days, or 14 days. Gene expression analysis was performed to quantify expression of (A) NP ECM-associated genes (COL2A1, COL1A1), (B) genes associated with the NP phenotype (SOX9, KRT8, KRT19, PAX1, FOXA1), (C) notochord-associated markers (CD24, TBXT), and (D) fibroblast cell markers (ACTA2, FSP1). Relative gene expression was determined using the ΔΔC_t method normalized to expression of the housekeeping gene GAPDH, and expressed relative to cells encapsulated within MCS at 24 h. Data is presented as mean ± SD, N=3. Two-way ANOVA with Tukey’s post-hoc correction was performed. *, †, ‡ indicates significant difference in gene expression at 24 h within specific hydrogel groups. * p<0.05, † p<0.005, ‡ p<0.0005.
Chapter 4

4 Discussion

4.1 Summary of findings

In the first aim of the current study, a novel detergent-free decellularization protocol for nucleus pulposus (NP) was developed and validated. Characterization of the resultant decellularized NP (DNP) demonstrated that the process was effective at extracting cells (~85% removal), while retaining key extracellular matrix (ECM) constituents including various collagens, fibronectin, and laminin. The process resulted in a reduction in sulphated glycosaminoglycan (GAG) content (~45% retention), which was found to be equivalent to⁹⁶ or an improvement⁹⁸ over current detergent-based NP decellularization protocols in the literature.

In the second aim, coatings were successfully made from α-amylase digested DNP or purified collagen (COL) that were found to be stable in culture and could support the attachment and growth of bovine NP cells in monolayer culture. However, serial passaging of primary bovine NP cells on DNP coatings was unable to maintain the NP phenotype across serial passages based on analysis of gene expression. Further, RT-qPCR analysis suggested that culture of dedifferentiated P3 NP cells on DNP coatings was unable to rescue the NP-specific phenotype.

In the final aim, MCS hydrogels incorporating cryo-milled DNP or COL particles were generated, which could be used to encapsulate bovine NP cells and were found to support their viability in culture over 14 days. MCS ± DNP/COL hydrogels were then applied as 3-D culture platforms to investigate the effects of DNP on primary bovine NP cell phenotype. The incorporation of the DNP particles was not sufficient to maintain the phenotype of the P0 primary bovine NP cells encapsulated and cultured within the MCS hydrogels. ECM incorporation within the MCS hydrogels was also insufficient to restore the NP phenotype of encapsulated P3 dedifferentiated cells based on gene expression analysis.
4.2 General discussion

The ECM has been shown to play a key role in modulating cell fate and function\textsuperscript{106}, as such, there is growing interest in developing ECM-derived bioscaffolds capable of harnessing these bio-modulatory effects. ECM-derived scaffolds have previously been shown to modulate cell viability, proliferation, and lineage specific differentiation\textsuperscript{106}. This presents a promising solution for treatments targeting intervertebral disc degeneration, which aims to promote nucleus pulposus (NP) regeneration and/or repair.

One area of research is NP decellularization, from which ECM-based scaffolds mimicking the native NP biochemical composition and mechanical properties can be developed. To date, various NP decellularization protocols have been developed, demonstrating the ability of DNP to provide cell-instructive cues\textsuperscript{77,80,103}. To date, published NP decellularization protocols utilize a wide variety of decellularization techniques including freeze-thaw cycles and enzymatic treatments, and all include detergents, such as sodium dodecyl sulphate (SDS), Triton X-100, deoxycholic acid, or a combination thereof\textsuperscript{80,96–98,101–103}. The use of detergents is effective at extracting cells, however it can cause unwanted ECM protein denaturation, proteoglycan loss, structural alterations, and growth factor elimination, limiting the bioactivity of the resultant decellularized ECM\textsuperscript{87}. Additionally, if not adequately cleared, residual detergent may elicit cytotoxic effects\textsuperscript{87}.

The current study aimed to refine and validate a detergent-free NP decellularization protocol. Characterization of the resulting DNP demonstrated significant cell removal (\textasciitilde85\%) and preservation of key ECM components such as collagens and glycoproteins (fibronectin and laminin), and retention of \textasciitilde45\% of the native glycosaminoglycan (GAG) content. Previous detergent-based NP decellularization protocols have reported slightly higher (\textasciitilde96\%)\textsuperscript{77} or lower (\textasciitilde73\%)\textsuperscript{97} reductions in double stranded DNA (dsDNA) content, indicating that our detergent-free protocol is just as effective at clearing cellular content. The GAG loss we report is consistent with previously reported NP decellularization protocols\textsuperscript{96–99}, which might be attributed to the solubility of GAGs in an aqueous environment. Of note, some detergent-based NP decellularization protocols report low (16\%) GAG retention\textsuperscript{80,98}. In fact, a study by Elder \textit{et al.} reported that increased detergent
incubation time was associated with increased GAG loss\textsuperscript{142}. Our protocol circumvented the use of detergents through a combination of freeze-thaw cycles and enzymatic digestions. Freeze-thaw cycles cause intracellular ice crystal formation leading to cell membrane lysis, with minimal alteration to the ECM ultrastructure\textsuperscript{87,91}. Additionally, freeze-thaw cycles can increase tissue porosity, potentially allowing for increased efficacy of subsequent enzymatic treatments\textsuperscript{119}. Previous studies utilizing freeze-thaw cycles for the decellularization of other connective tissues such as cartilage, reported a reduction of cellular content, similar to our results\textsuperscript{143}. The second step of the decellularization protocol involved treatments with deoxyribonuclease (DNase) and ribonuclease (RNase), endonucleases which hydrolyze deoxyribonucleotides and ribonucleotides, respectively. Although enzymes are effective at clearing nuclear content, their bioactivity is often limited to the surface of the tissue\textsuperscript{87}. Most NP decellularization protocols maintain the intact native NP throughout the decellularization process\textsuperscript{80,96,99,101,102}, potentially limiting the efficacy of enzymatic treatments. As such, the native NP was minced prior to decellularization, increasing tissue surface area to improve decellularization efficacy.

ECM-derived bioscaffolds are compelling for the development of cell culture platforms that recapitulate the complex biochemical composition and biomechanical properties of the native tissue. The Flynn lab has previously done extensive work in the development and characterization of various ECM-derived bioscaffolds including foams, microcarriers, coatings, and hydrogels from various decellularized tissue sources (adipose, trabecular bone, cartilage)\textsuperscript{104,107,110,122,144}. While previous research in the Flynn lab has largely focused on bioscaffold platforms incorporating decellularized adipose tissue (DAT)\textsuperscript{107,110,122,144}, minimal research has been done investigating the potential uses of DNP in the context of NP cell bioscaffold platforms\textsuperscript{95}. Wachs \textit{et al}, previously developed a pepsin-digested porcine DNP scaffold which had the capacity to support cellular viability up to 21 days in culture and promote GAG production\textsuperscript{96}. Similarly, Yu \textit{et al.} digested DNP with pepsin to generate a DNP scaffold capable of gelation into a hydrogel, which showed increased type II collagen, aggrecan, and \textit{SOX9} gene expression\textsuperscript{103}. Zhou \textit{et al.} incorporated genipin, a cross-linking agent, to form DNP hydrogels\textsuperscript{80}. Finally, various groups have directly seeded regenerative cells onto DNP
tissue following decellularization\cite{97,98,102,145}. However, to our knowledge no study to date has developed DNP coatings for NP cell culture. Previous studies have used pepsin to digest the ECM in order to generate ECM-derived coatings\cite{120,121}. Pepsin, a proteolytic enzyme, however is non-specific which may impact the bioactivity of the resultant ECM. Previous studies done in the Flynn lab have developed an \( \alpha \)-amylase digestion protocol for coating synthesis, which allows for the preservation of collagen fibrils and prevents the fragmentation of ECM proteins when compared to pepsin-digested ECM\cite{110}. As such, to generate DNP coatings, an \( \alpha \)-amylase digestion protocol previously developed by Shridhar et al. was applied to DNP\cite{110}.

The majority of in vitro studies involve the expansion of cells on rigid 2-D tissue culture plastic (TCP). These platforms support cell attachment and proliferation, allowing for cell expansion\cite{82,138}. However, monolayer culture of bovine primary NP cells on TCP results in loss of the NP phenotype with serial passaging as measured by decreased NP-associated marker expression\cite{82}. Due to the limitations in accessing human NP cells, bovine NP cells were selected for the current study based on their ease of sourcing and similarity in cellular composition compared to the human NP\cite{6}. Consistent with previous studies, our characterization demonstrated a reduction in the mRNA expression of NP-associated markers and type-II collagen in primary bovine NP cells with serial passaging\cite{82}. Loss of the NP phenotype in monolayer culture can be attributed to many factors, including culture substrate stiffness, trypsinization during passaging, and/or oxygen tension\cite{146,147,148}. Primary bovine NP cells are known to adopt an elongated spindle-shape morphology in monolayer culture with some cells maintaining the round morphology\cite{82,138}. Similar to Ashraf et al.\cite{82}, we noted two distinct NP cell morphologies in primary bovine NP monolayer culture, with a decrease observed in rounded cell morphology with increased passaging. This morphological change might be associated with the dedifferentiation of NP cells, which was also noted by Ashraf et al. through gene expression analysis\cite{82}. Since the healthy native NP is avascular, NP cells reside in a largely hypoxic environment. A previous study Jaworski, et al. showed porcine NP cells to better maintain the NP phenotype when cultured under hypoxic conditions (1% O\(_2\)) as compared to normoxic conditions (21% O\(_2\))\cite{147}, Within our studies bovine NP cells were
cultured under normoxic conditions (21% O₂), which might have contributed to the dedifferentiation seen in monolayer culture.

Given the limitations with NP cell expansion on TCP, there is a need for cell culture substrates that more closely mimic the native NPs complex composition and mechanical properties. To integrate cell-ECM interactions, previous studies have coated TCP with purified ECM components such as collagen, laminin, and fibronectin. However, these culture platforms fail to recapitulate the complex biochemical composition of the native NP. ECM-derived coatings have previously been generated from a variety of tissues and have shown to guide cell attachment, proliferation, and lineage specific differentiation of stem cells in culture.

Our findings demonstrate that culture of primary bovine NP cells on DNP coatings maintained cell viability compared to TCP across multiple passages, suggesting the potential of DNP as a cell culture substrate. However, in contrast to studies showing α-amylase derived DAT bioscaffolds to have biomodulatory effects, our study concluded that DNP coatings did not have bioactive effects on primary bovine NP cells, as measured by the inability of DNP coatings to maintain the NP phenotype over serial passages in monolayer. Additionally, we showed that DNP coatings were unable to rescue the NP phenotype in dedifferentiated NP cells, as measured by gene expression analysis. Various factors have previously been identified to regulate cellular responses on ECM coatings, including: surface topography, ligand presence, and mechanical properties. Surface topography has previously been found to differ between tissues such as bladder, small intestine, and liver when generated into coatings. Specific processing methods (pepsin digest vs α-amylase digest) have also been found to impact surface topography within the same tissue source, generating distinct ultrastructures. Additionally, responses to coating ultrastructure might be dependent on cell type being examined. As such, variations in tissue source and cell type, might explain the cell-instructive bioactivity seen within Shridhar et al. α-amylase digested DAT coatings and not within our α-amylase digested DNP coatings. Matrix stiffness has previously been found to largely modulate NP cell phenotype, with stiffer matrices leading to increased dedifferentiation. Shridhar et al. found α-amylase digested DAT to have a
matrix stiffness of ~36 kPa, which is higher than that of the native bovine NP (~6-19 kPa\textsuperscript{152,153}), potentially contributing to the inability to maintain or rescue the NP phenotype.

Chondroitin sulphate (CS) is the most abundant GAG within the native NP\textsuperscript{7}, as such methacrylated chondroitin sulphate (MCS) was chosen for the development of a 3-D NP cell culture platform. MCS hydrogels have previously been studied as a platform for the incorporation of decellularized trabecular bone and decellularized adipose tissue, which were shown to modulate ASC lineage specific differentiation\textsuperscript{107}. Additionally, MCS hydrogels incorporating ECM particles are tunable in their cell-cell and cell-ECM properties, which play key roles in modulating cellular phenotype\textsuperscript{154}.

Recognizing the important role which physical properties play within bioscaffolds in modulating cellular behaviour, composite hydrogels underwent physical characterization. First, cryo-milled ECM particles were characterized, as previous studies within the Flynn lab determined that ECM particle size plays a key role in the regulation of cellular differentiation within MCS hydrogels, with smaller particles allowing for better hydrogel integration and bioactive effects\textsuperscript{137}. Results showed no significant differences between DNP and COL particle distributions, suggesting that particle size was not a confounding factor in the observed cellular responses. Our analysis of gel content demonstrated that the incorporation of ECM particles (DNP or COL) at 5 %w/v did not alter the crosslinking efficiency. Moreover, the crosslinking efficiency within our MCS hydrogels was consistent with previous studies\textsuperscript{107}. Matrix stiffness has been shown to modulate NP cell proliferation, and matrix deposition when encapsulated within hydrogels\textsuperscript{116}.

Unconfined bulk compression testing demonstrated that the incorporation of ECM particles did not alter the mechanical stiffness of the hydrogels, which were found to have a Young’s modulus value of ~190 kPa. This material stiffness might be a result of the incorporation of 20% MCS in comparison to previous studies investigating MCS at a concentration of 10% have resulted in Young’s moduli of ~125 kPa\textsuperscript{107}. Importantly, the stiffness of the native human NP has been reported to be ~5-25 kPa\textsuperscript{118,155}, while the native bovine NP has been reported to be ~6-19 kPa\textsuperscript{152,153}, indicating that further optimization of
hydrogel platforms will be required to generate materials that mimic the native tissue’s mechanical properties.

Final experiments focused on assessing the effects of DNP-containing hydrogels on primary (P0) or in vitro dedifferentiated (P3) bovine NP cells in 3D culture. Composite MCS hydrogels incorporating ECM particles (DNP or COL) appeared to contain more viable cells compared to MCS only hydrogels following extended culture. Previous work has shown hydrogels functionalized with ECM proteins, such as laminin, have increased cell retention potentially attributed to increased cell adhesion to ECM proteins. In the native NP, both notochord cells and mature bovine NP cells are localized within cell clusters. Of note, P0 and P3 NP cells encapsulated within ECM-containing hydrogels showed evidence of cell clustering following prolonged culture. Previous studies showed that porcine NP cell clustering was associated with increased ECM ligands present within the culture platform, suggesting that the incorporation of ECM had a bioactive effect promoting clustering and the adoption of a cell morphology more representative of NP cells as observed in situ. Together these studies suggest potential beneficial effects of ECM incorporation in 3D hydrogels over prolonged culture periods.

Importantly, culture in ECM-containing 3-D hydrogels did not maintain the expression of notochord- or NP-associated markers in primary (P0) cells, nor restore the NP phenotype in dedifferentiated (P3) bovine NP cells. Compared to culture on 2-D coatings, primary (P0) bovine NP cells encapsulated in 3-D hydrogels appeared to maintain the expression of a subset of NP- and notochord-associated markers; however, these effects were independent of the incorporation of ECM particles. Further studies are required to specifically compare marker level expression between 2D and 3D culture platforms relative to that of cells within the native NP. Our findings are consistent with previous studies that suggested culture of NP cells in 3-D is more effective at retaining the NP phenotype over 2-D cell culture. This might be a result of NP cells being capable of retaining their rounded morphology within 3-D culture, while 2-D culture results in a spindle-shaped morphology. Similar to the current work, previous studies investigating hydrogel platforms in the context of NP regeneration have shown high levels of NP cell survival; however, these studies generally lack phenotypic cell characterization thus
limiting the interpretation of their findings\textsuperscript{96,160,161}. In contrast to our study, a self-crosslinking scaffold developed from porcine DNP by Zhou, \textit{et al.} resulted in significant upregulation of \textit{COL2A1}, \textit{SOX9}, \textit{KRT19}, and \textit{PAX1} expression in human ASCs after 14 days of culture\textsuperscript{80}. The difference in cell type, processing methods and tissue source may contribute to the differences noted\textsuperscript{80}. Additionally, the self-crosslinking scaffolds produced had a Young’s moduli more closely resembling that of native NP (16 kPa)\textsuperscript{80}, further suggesting that hydrogel stiffness might be an important factor modulating cell behaviour in our hydrogel. Highlighting the important of mechanical properties on cell behavior, Xu \textit{et al.} demonstrated that encapsulation of rat NP cells within gelatin hydrogels at increasing stiffness resulted in increased cell dedifferentiation\textsuperscript{116}. Additionally, increased hydrogel stiffness decreases matrix porosity potentially limiting, cell survival, proliferation, migration, ECM production, and nutrient diffusion, which can impact cellular behaviour and phenotype\textsuperscript{162}. Together, these results suggest that high hydrogel stiffness may contribute to the loss of NP cell phenotype we observed in 3-D culture. Additionally, the hydrogel stiffness might be having a negative impact on NP cell phenotype, ultimately opposing the potential bioactive effects of the DNP. Future studies should focus on the development of hydrogels with mechanical properties more closely matching those of the native NP.

\section*{4.3 Limitations}

In the current thesis, an effective method for decellularizing bovine nucleus pulposus (NP) was developed. Additionally, the decellularized NP (DNP) was further processed to generate coatings and composite hydrogels, which were applied as 2-D and 3-D culture platforms for assessing the effects of the NP-specific ECM on the phenotype of cultured primary bovine NP cells through gene expression analysis. However, there are limitations in the study design including the limited number of biological replicates, potential for cell donor variability, and lack of native tissue controls, which should be considered and addressed in the future to further confirm the trends in the gene expression data.

Small sample size leads to a decrease in statistical power, increasing the chances of type-I and type-II errors. The gene expression studies in the current thesis included a sample
size of N=3. In the future, it would be worthwhile to repeat the studies with additional cell donors, to validate the findings and increase the statistical power of the analysis.

In the studies, the specific cattle breeds from which the tails were isolated were not known, and may have varied between trials. Further, the bovine primary NP cells were isolated from bovines ranging between 9-30 months of age. During this time frame, cellular changes can occur within the NP, as cells transition from notochord cells at birth to mature adult NP cells\(^5\). As such, differences in the age of the cattle could also have contributed to the observed variability in gene expression levels between the biological replicates. One alternative to consider in the future could be pooling cells isolated from multiple tails to be able to perform multiple replicates with the same batch of cells in order to better understand variability attributed to the cells versus the culture platforms.

Lastly, gene expression analysis was conducted using either P0 cells at varying time points as controls for the \(\Delta\Delta C_T\) gene expression analysis. However, it is difficult to assess the ability of the DNP to rescue or maintain the native NP phenotype when comparing to cells that have been extracted from the tissues and cultured. Future studies should isolate RNA directly from native NP to utilize as a native NP control to be able to more fully assess the capacity of the DNP culture platforms to maintain or rescue the native NP phenotype. Additionally, it would be beneficial to compare the phenotype of the cells cultured on the 2-D and 3-D culture platforms, to gain insight into which culture platform is better at maintaining/rescuing the NP phenotype.

### 4.4 Future directions

The body of work documented in this thesis demonstrates the capacity of DNP to be incorporated within novel 2-D and 3-D cell culture platforms. Future studies should focus on further characterizing the cellular response to 2-D and 3-D DNP-derived scaffolds. In our studies, the NP phenotype was assessed through gene expression analysis. Building from this work, it would be interesting to assess the phenotypic response at the protein level through techniques such as western blotting, enzyme-linked immunosorbent assay (ELISA) or immunohistochemistry. Additionally, the ability of DNP scaffolds to stimulate NP cells into producing NP-specific ECM is important for the translation from
in vitro to in vivo. As such future studies could assess the production of key NP ECM markers such as type II collagen or GAG production, through western blotting, histological staining (Toluidine blue, picrosirius red), biochemical assays or immunohistochemistry.

In the current study MCS hydrogels were found to have a stiffer matrix than that of the native NP. Due to the large role which biomechanical properties play in modulating cellular behaviour, future studies, should explore other natural polymers with stiffness values more closely mimicking the native NP. Hyaluronic acid (HA) is a GAG abundantly found within the NP. HA has previously been studied, and functionalized with methacrylate to generate photo-crosslinkable gels, similar to MCS. The mechanical properties of methacrylated HA (MHA) hydrogels can be tuned through modulation of the UV exposure time, degree of methacrylation, and concentration of MHA, to generate hydrogels with moduli (~40 kPa) that more closely resemble that of the native NP. Primary bovine NP cells encapsulated within MHA hydrogels maintained cell survival for 56 days in culture, and were shown to express aggrecan and type II collagen at the gene level. As such, MHA may be a promising alternative polymer for the incorporation of DNP and it would be interesting to compare the cellular response between the MCS and MHA platforms in future work.

Lastly, the exploration of more clinically relevant cell sources such as bone marrow derived-mesenchymal stromal cells (MSC) or induced pluripotent stem cells (iPSC) could be investigated within our DNP-derived bioscaffolds, as these cell types would allow for autologous cellular treatment increasing translatability. MSCs have previously been explored towards NP regeneration and have shown great potential in their ability to differentiate towards an NP-like phenotype. iPSCs have also been explored, and shown to differentiate into an NP-like phenotype although not studied as extensively as MSC.
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Supplementary Figure 1. Representative images of positive controls for immunohistochemical staining. Extracellular matrix specific markers (red) are counterstained with DAPI (blue) to visualize cell nuclei. Bovine skin was used as a tissue positive control for collagen type I (COL I), collagen type II (COL II), Collagen type V (COL V), collagen type VI (COL VI), laminin (LN), fibronectin (FN) and keratan sulphate (KS). Scale bar = 200 µm.
Supplementary Figure 2. Representative $^1$H-NMR spectra of methacrylated chondroitin sulphate (MCS). Key peaks have been identified and correlated to the chemical structure of the MCS polymer, comprised of repeating units of D-glucuronic acid and N-acetyl galactosamine. Peaks at ~6.1 ppm (i) and ~5.65 ppm (ii) correspond to the vinyl groups on the grafted methacrylate functional group. The peak at ~1.85 ppm (iv) corresponds to the methyl group protons on the methacrylate which was functionalized to the chondroitin sulphate. The peak at ~1.95 ppm (iii) corresponds to the proton on the methyl group of the native N-acetyl residues on the chondroitin sulphate. Integration of the protons confirmed a target degree of methacrylation of 17%. The absence of non-specific peaks in the spectra confirmed the absence of chemical contaminants in the purified MCS polymer. Spectra is representative of N=8 polymer batches.
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