Scaffold Design and Optimization for
Osteochondral Interface Tissue Engineering

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ABSTRACT

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A thin layer of calcified cartilage at the native cartilage-to-bone junction facilitates integration between deep zone articular cartilage and subchondral bone, while maintaining the integrity of the two distinct tissue regions. Regeneration of this interface remains a significant clinical challenge for long-term and functional cartilage repair. The strategy for osteochondral interface formation discussed in this thesis focuses on the design and optimization of a biomimetic scaffold for stable calcified cartilage formation. The ideal interface scaffold supports chondrocyte biosynthesis and the formation of calcified cartilage with physiologically-relevant mechanical properties. Furthermore, the interface scaffold allows for osteointegration and the maintenance of the calcified cartilage matrix. It is hypothesized that ceramic presence and zonal chondrocyte interactions regulate cell biosynthesis and mineralization, and these cell-matrix and cell-cell interactions are essential for calcified cartilage formation and maintenance.

Biomimetic design parameters for an interface scaffold were determined by characterizing the native interface in terms of mineral and matrix distribution. A composite hydrogel-hydroxyapatite scaffold was then designed to support formation of a functional calcified cartilage matrix. The hydrogel phase maintains the chondrocyte phenotype and allows for incorporation of ceramic particles, while the biomimetic ceramic phase is osteointegrative and decreases the need for cell-mediated mineralization. This scaffold was optimized in vitro based on hydrogel type, chondrocyte population, and ceramic particle size. The collective findings from these cell-ceramic interaction studies determined that hypertrophic chondrocytes, cultured in the presence of micron-sized hydroxyapatite particles, exhibit enhanced hypertrophy and matrix deposition. Scaffold ceramic dose and seeding density were also optimized for promoting calcified cartilage formation in vitro.

In order to implement the scaffold for integrative cartilage repair, a scaffold was designed to regenerate both uncalcified and calcified cartilage on a bilayered hydrogel scaffold. Furthermore, a polymer-ceramic nanofiber component was added to augment the original design for in vivo
implementation. The hydrogel-nanofiber composite scaffold was evaluated in vivo and found to support mineralization and osteointegration within the bone region while preventing endochondral ossification within the repair tissue. Finally, inspired by the stratified organization of zonal chondrocyte populations above the calcified cartilage interface, the layer hydrogel model was used to determine the role of zonal chondrocyte organization on calcified cartilage stability. This thesis collectively explores the cell-ceramic and cell-cell interactions, and their ramifications for calcified cartilage formation and maintenance. Specifically, ceramic presence promotes the deposition of a calcified cartilage matrix by hypertrophic chondrocytes in a dose-dependent manner, and furthermore, communication between surface zone and deep zone chondrocyte populations suppresses mineralization within articular cartilage above the calcified cartilage interface. It is anticipated that the scaffold design strategy developed in this thesis can also be applied to the regeneration of other complex interfaces where there are transitions from soft-to-hard tissue.
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LIST OF ABBREVIATIONS

ALP: Alkaline phosphatase  
CPC: Cetylpyridinium chloride  
DMMB: Dimethylmethylen blue  
DW: Dry weight  
DZ: Deep zone  
DZC: Deep zone chondrocytes  
EDAX: Electron dispersive X-ray  
EDTA: Ethylenediaminetetraacetic acid  
FTC: Full thickness chondrocytes  
FTIR-I: Fourier transform infrared imaging  
GAG: Glycosaminoglycan  
HA: Hydroxyapatite  
Ihh: Indian hedgehog  
IR: Infrared  
Micro-CT: Micro-computed tomography  
MZ: Middle zone  
MZC: Middle zone chondrocytes  
MMP: Metalloproteinase  
MSC: Mesenchymal stem cells  
OHP: Hydroxyproline  
PCR: Polymerase chain reaction  
PLGA: Polylactic-co-glycolic acid  
PMMA: Polymethylmethacrylate  
pNP: p-nitrophenol  
PTHrP: Parathyroid hormone-related protein  
SEM: Scanning electron microscopy  
SZ: Surface zone  
SZC: Surface zone chondrocytes  
SZP: Surface zone protein  
T3: Thyroid hormone  
TGA: Thermogravimetric analysis  
WW: Wet weight  
XRD: X-ray diffraction
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DEDICATION

This thesis is dedicated to Andrew, Michael, my mom, and the original Dr. Khanarian,
without whom another Dr. Khanarian could never exist.
CHAPTER 1: INTRODUCTION
1.1 Specific Aims

Osteoarthritis is the leading cause of disability among Americans and is responsible for over 600,000 joint replacements each year[1]. It is characterized by degeneration of articular cartilage, which results in painful joint motion. Due to the limited capacity of cartilage for self-repair, surgical intervention, such as lavage, subchondral drilling, microfracture, and cartilage grafts, is often required. Unfortunately, long-term success of these techniques is hindered by unwanted fibrocartilage formation, donor-site morbidity, and poor graft-to-bone integration.

The working hypothesis of this thesis is that the establishment of a stable osteochondral interface facilitates functional and integrative cartilage repair. The osteochondral interface is located between articular cartilage and bone. It consists of a calcified cartilage layer, within which hypertrophic chondrocytes are embedded in a mineralized matrix. This calcified cartilage layer allows for functional integration between cartilage and subchondral bone, while maintaining the integrity of the two distinct tissue regions. It also serves as a barrier to vascular invasion and enables pressurization of cartilage during physiologic loading. Without a stable osteochondral interface, there is osseous upgrowth into the cartilage compartment[2] and increased risk of mechanical failure[3]. Therefore, the goal of this thesis is to apply interface tissue engineering principles for the establishment of a stable osteochondral interface and cartilage-to-bone integration. In this effort, the first challenge is to regenerate calcified cartilage and the second, to maintain the integrity of both uncalcified and calcified cartilage over time.

The strategy for osteochondral interface formation discussed in this thesis centers on the design of a biomimetic scaffold for calcified cartilage regeneration. Specifically, the objectives of this thesis are to 1) design and optimize a scaffold for calcified cartilage formation and 2) promote stability and maintenance of the neo-calcified cartilage. The ideal interface scaffold should support chondrocyte biosynthesis and the formation of calcified cartilage with physiologically-relevant mechanical properties. Furthermore, the interface scaffold should allow for the maintenance of the calcified cartilage matrix, while prohibiting ectopic mineralization. Lastly, the interface scaffold must be osteointegrative. It is hypothesized that ceramic presence and zonal chondrocyte interactions regulate cell biosynthesis and mineralization, and these cell-matrix and cell-cell interactions are essential for calcified cartilage formation and homeostasis. To test this hypothesis, the specific aims of this thesis are as follows:
**Aim 1:** Identify design parameters for a biomimetic scaffold for cartilage-to-bone interface formation.

**Objective:** It is anticipated that characterization of the native osteochondral interface will provide biomimetic design parameters for an interface scaffold that supports the formation of calcified cartilage.

**Aim 2:** Design, characterize, and optimize a scaffold for the formation of a calcified cartilage matrix.

**Hypothesis:** By modulating cell type, hypertrophy and cell seeding density, as well as hydrogel type, ceramic particle size and dose, the interface scaffold will be optimized to support chondrocyte hypertrophy and matrix deposition.

**Aim 3:** Evaluate the efficacy of the interface scaffold for osteochondral interface regeneration.

**Hypothesis:** The scaffold system will support calcified cartilage regeneration *in vivo*.

**Aim 4:** Evaluate the maintenance of the calcified cartilage matrix formed.

**Hypothesis:** Zonal chondrocyte interactions will regulate chondrocyte biosynthesis and hypertrophy at the uncalcified-to-calcified matrix interface.

To obtain design parameters for a biomimetic interface scaffold, the native osteochondral interface is characterized in **Aim 1**. Specifically, matrix and ceramic distribution throughout the cartilage-to-bone transition are analyzed with Fourier Transform Infrared Spectroscopy Imaging (FTIR-I). Given that the native interface consists of a calcified cartilage layer, it is envisioned that a polymer-ceramic composite can be used as a biomimetic interface scaffold. **Aim 2** focuses on designing the interface scaffold for calcified cartilage formation. Specifically, studies examine the effects of cell type and seeding density, as well as ceramic particle size and dose. Although many current cartilage tissue engineering approaches use full thickness chondrocytes, obtained from all three zones of articular cartilage, the
responses of interface-relevant deep zone chondrocytes and hypertrophic chondrocytes are also evaluated. It is hypothesized that the interface scaffold regulates chondrocyte matrix deposition and hypertrophy and specifically, supports the deposition of a proteoglycan- and collagen-containing calcified matrix. Building upon the interface scaffold design and optimization performed in this thesis, a scaffold for use in combination with hydrogel-based cartilage grafts is developed and tested in vitro and in vivo (Aim 3). While the previous aims focus on the formation of a calcified cartilage matrix, Aim 4 addresses the maintenance of the newly formed interface. Given the stratified organization of articular cartilage above the calcified cartilage interface[4] and the reported role of zonal chondrocyte interactions in regulating chondrocyte mineralization[5], it is anticipated that the zonal organization of chondrocytes is necessary to maintain the neo-interface.

In this thesis, to achieve stable osteochondral interface repair, 1) biomimetic scaffold design and 2) optimization of chondrocyte-ceramic and zonal cellular interactions are explored. The approach of this thesis is unique as it aims to investigate the regeneration of the osteochondral interface, as well as the maintenance of the neo-interface. Furthermore, the scaffold design strategy developed in this thesis can also be applied to the regeneration of other soft-to-hard tissue transitions.

1.2 Significance

1.2.1 Clinical Problem

Osteoarthritis is the most common joint disease, especially among individuals over the age of 65, and is characterized by degeneration of articular cartilage[1]. Since patients often suffer from chronic pain and limited motion, osteoarthritis is the leading cause of work-related disability and therefore constitutes a substantial social and economic burden[6]. This debilitating condition is characterized by both macroscopic changes in joint architecture and microscopic changes within the tissue. Articular cartilage fibrillation and erosion are accompanied by subchondral bone thickening and osteophyte formation[7]. The proteoglycan loss and disruption of the collagen network associated with osteoarthritis[8-10] has been attributed to increased matrix metalloproteinase (MMP) and aggrecanase activity, as well as changes in chondrocyte phenotype[11]. Changes in matrix composition and
organization subsequently lead to compromised structural integrity[12] and lower mechanical properties[13].

The spontaneous repair response of articular cartilage is limited, due to restricted access to the vascular supply, lack of cell migration from surrounding tissue, and poor cell adherence to the cartilage matrix[14]. On the other hand, a robust repair response is seen in full-thickness defects that reach subchondral bone because there is access to progenitor cells in the bone marrow space and reparative factors from the humoral supply[15]. While techniques such as lavage are used for the clinical reduction of pain, other therapeutic strategies aim to elicit this spontaneous healing response[16]. A variety of shaving and drilling techniques expose articular cartilage to subchondral bone in order to allow access to progenitor cells and growth factors[17,18]. The repaired tissue is fibrocartilaginous in nature, consisting of a vascularized, scar-like tissue which is prone to ectopic mineralization and does not exhibit organization or composition that resembles that of healthy hyaline cartilage[19]. This fibrocartilage has inferior mechanical properties, does not integrate well with native tissue, and often degenerates over time[20,21]. In a study by Frisbie et al., equine full thickness femoral condyle defects that were subject to complete removal of the calcified cartilage interface and bone plate microfracture were compared to untreated controls[22]. Histological evaluation of osteochondral sections revealed the formation of fibrocartilage tissue above subchondral bone, with no distinct interface region. It was also found that any calcified cartilage present at the defect site was a result of incomplete removal during surgery and was not indicative of interface regeneration. These observations suggest that osteochondral interface formation is not achieved with current cartilage repair approaches.

1.2.2 Cartilage Tissue Engineering and the Need for an Interface

Tissue engineering techniques have been employed to regenerate cartilage, including the use of hydrogel-based cartilage grafts[23-33]. Paige et al. first introduced a cell-seeded scaffold to resurface lapine knees[28]. More recently, Malmonge et al. implanted polymethyl methacrylate-based hydrogel scaffolds into rat cartilage defects, and although an articulating surface was restored, osseous upgrowth was detected within the scaffold[23]. A space-filling chitosan solution has also been used to fill rabbit cartilage defects, and formation of a granular tissue was formed at the host-to-scaffold interface[24].
Rahfoth et al. treated full thickness defects with cell-seeded and acellular agarose implants[29], although no integration with native calcified cartilage was observed. Chondrocytes seeded in agarose and implanted into chondral defects have demonstrated healing in the dog model[30]. Chondrocyte-seeded alginate has also been shown to regenerate hyaline-like cartilage in vivo[31-33]. Despite the promising results of many of these techniques, the lack of a stable osteochondral interface during the healing process makes it unlikely that fibrocartilage tissue formation and ectopic mineralization will not compromise the long-term maintenance of the neo-cartilage[34,35].

Hunziker et al. elegantly demonstrated the importance of a barrier between cartilage and bone during healing of in vivo full thickness defects treated with a fibrinogen matrix[2]. It was found that a structural barrier placed between the cartilage and bone compartments, in place of the calcified cartilage layer, was necessary to maintain the integrity of the newly formed cartilage by preventing vasculature upgrowth and subsequent ectopic mineralization. In the past ten years, the focus has centered on developing osteochondral grafts instead, seeding cartilage-forming cells directly above a substrate that integrates with bone[36-38], developing bilayered scaffolds with cartilage and bone regions[39-47], fabricating continuous osteochondral constructs[48,49], or designing scaffolds with either a compositional or functional gradient[50-56]. As such, consistent formation of a calcified cartilage interface in these scaffolds has not been demonstrated.

1.3 Articular Cartilage and the Osteochondral Interface

1.3.1 Articular Cartilage

Articular cartilage is an avascular connective tissue located at the articulating ends of bones in the synovial joint and functions to transmit loads and lubricate joint motion[57]. Although the tissue has relatively low cellularity, it consists of a dense proteoglycan-collagen matrix. The matrix is comprised of fibrous components, predominantly collagen type II, with contributions from collagens III, VI, IX, X, XI, and XIV[58-61]. The non-fibrous components consist of polysaccharides, primarily glycosaminoglycans (GAG), and are often linked with non-collagenous proteins and other polysaccharides to form extensive proteoglycan aggregates[62]. The collagen network contributes to the tensile stiffness and strength of cartilage, whereas proteoglycans swell and resist compressive forces[57,63,64].
Both matrix composition and organization vary throughout the depth of the cartilage (Fig. 1.1); the tissue is subdivided into four different zones, which each differ in composition, cell morphology, and mechanical properties[4,65]. The surface zone occupies 5-10% of the total tissue. It has the highest water content, the lowest proteoglycan content, and is composed of thin collagen fibers (60-90 nm) arranged tangentially to the surface[66]. Surface zone chondrocytes (SZC) are characterized by a discoidal shape, arranged parallel to the surface[67]. These chondrocytes are also unique in that they express surface zone protein (SZP), also known as lubricin[68]. The next 40-50% is the middle zone, which is characterized by round chondrocytes distributed throughout a matrix with higher proteoglycan content relative to the surface zone and randomly oriented collagen fibers[66]. The third zone, or deep zone, comprises approximately 30% of the tissue. It is characterized by the highest proteoglycan content and larger collagen fibers that are organized perpendicular to the articular surface[66]. The cellularity of the deep zone is the lowest as compared to the surface and middle zones. The cells derived from this region, deep zone chondrocytes (DZC), are the largest in size[67] and are arranged in columnar groups. These chondrocytes, which are located directly above the calcified interface, also exhibit alkaline phosphatase (ALP) activity[69] and retain the ability to mineralize both in vitro[70] and in vivo[71].

**Figure 1.1. Cartilage-to-bone junction.** Cartilage covers the articulating surfaces of joints and is characterized by a stratified zonal organization of surface, middle, and deep zone chondrocytes layered above the calcified cartilage interface.
The zonal organization of cartilage serves an important role in regulating chondrocyte biosynthesis and mineralization. In a study by Jiang et al., zonal chondrocyte interactions were evaluated in a monolayer co-culture model[5]. It was found that SZC suppress DZC hypertrophy and mineralization. Moreover, the regulation of chondrocyte mineralization is mediated by local paracrine factors such as parathyroid hormone-related protein (PTHrP) and Indian hedgehog (Ihh), most likely through the PTHrP-Ihh negative feedback loop reminiscent of endochondral ossification[72].

1.3.2 Osteochondral Interface

During long bone development, secondary ossification centers form postnatally in each chondroepiphysis as the primary growth front approaches. The cartilage region between the primary and secondary ossification fronts is the growth plate which expands through a process of endochondral ossification until skeletal maturity[73]. In contrast to the growth plate which is resorbed in most species, biological and mechanical regulation of mineralization allows for a layer of calcified cartilage to persist at the cartilage-to-bone junction[74]. It has been shown that the articular cartilage-calcified cartilage unit functions as an analogous growth plate, with high regenerative potential towards the articular surface and hypertrophy in the calcified cartilage zone; as calcified cartilage is replaced with bone, new cartilage forms above[75].

This calcified cartilage layer is 80 to 240 µm thick in humans[76,77], where hypertrophic chondrocytes are embedded in a mineralized matrix. Although the calcification front was first identified using histological techniques in 1953[78], electron microscopy was later used to characterize the interface[79]. Bullough et al. directly visualized the calcification front by electron microscopy after removing the overlying non-calcified organic matrix[80]. While the calcified cartilage layer is highly interdigitated with subchondral bone[81], there is a distinct separation between cartilage and bone in terms of both vascular supply and matrix content. The collagen fibers that insert into the calcified cartilage layer from the deep zone abruptly end at the osteochondral junction[82,83], and blood vessels rarely breach the interface in healthy tissue[84,85]. The mineral phase of calcified cartilage is a carbonate-substituted form of hydroxyapatite (HA) and is very similar to the mineral found in bone in terms of chemistry and crystal size[74,86,87]. Even though a wide range of values has been reported for
the calcium content of the interface (1-28%)\cite{87,88}, a correlation between mineralization and mechanical properties has been demonstrated using nanoindentation\cite{89}. The underlying matrix is composed of collagen types II, IX, and X, and proteoglycans\cite{90,91}, where mineral deposits occur along collagen fibers\cite{83}. Matrix vesicles\cite{80} and alkaline phosphatase\cite{92,93}, which participate in cell-mediated mineral deposition by hypertrophic chondrocytes, are also found at the osteochondral interface.

This interdigitated calcified cartilage layer anchors non-calcified articular cartilage to subchondral bone and serves two important functions. Specifically, the calcified cartilage layer is a barrier which limits diffusion and is relatively impermeable to nutrients. The diffusion coefficient of small molecules in calcified cartilage has been measured for murine and equine specimens and ranges between (0.26-0.9 µm²/s)\cite{94,95}; this represents as much as a 100-fold decrease in transport as compared to the cartilage surface. It also prevents vascular invasion from subchondral bone into the cartilage compartment\cite{85} and allows for pressurization during loading\cite{96}. Secondly, the interface enables the transmission of forces across the joint and reduces stress concentrations at the interface\cite{82,97,98}. Studies have shown that the stiffness of the calcified cartilage layer is approximately 0.3 GPa and is intermediate between uncalcified cartilage and bone, thereby relieving the force gradient between these two tissues\cite{99}.

1.3.3 Age- and Disease-Related Changes

Age-dependent changes at the osteochondral interface have also been reported. Published studies have shown that the mature calcified cartilage region is avascular and relatively impermeable, whereas substantial vasculature facilitates molecular transport across calcified cartilage in immature rabbits\cite{100}. Advancement of the mineralization front is observed with age\cite{76,97,98} and has also been associated with osteoarthritis\cite{101-104}. Even after skeletal maturity, mineralization at the interface is balanced by cartilage growth, so that the calcified cartilage barrier is actively maintained\cite{76}. It has been postulated that cellular activity and mineralization is responsible for adapting to mechanical or disease-related changes \cite{105}.
1.3.4 Current Efforts in Osteochondral Interface Tissue Engineering

Published approaches to regenerate the osteochondral interface itself have largely been cell-based, with chondrocytes cultured in a mineralizing media and/or seeded directly on a calcium phosphate substrate. Kandel et al. first seeded deep zone chondrocytes on filter inserts pre-coated with collagen II and cultured these constructs in a mineralizing media containing 10 mM beta-glycerophosphate[106]. It was found that mineralized cartilage formed in the region directly adjacent to the insert. More recently, Allan et al. seeded deep zone chondrocytes at high density on porous calcium polyphosphate scaffolds, cultured in media with 10 mM beta-glycerophosphate[107]. The mineral formed in vitro was shown to be similar to that found in native tissue in terms of crystal structure, and improved interfacial mechanical properties were also measured. The next step is to address the functional requirements of cartilage-to-bone integration by combining cells with scaffolds for osteochondral interface tissue engineering. Recently, Jiang et al. evaluated 3-D osteoblast-chondrocyte co-culture on a biomimetic, continuous multi-phased osteochondral construct consisting of a hydrogel-based cartilage region, a polymer-ceramic composite microsphere bone region, and an interfacial region consisting of a hybrid of the hydrogel and polymer-ceramic composite[47]. It was found that osteoblast and chondrocyte co-culture on this scaffold system supported the formation of distinct yet continuous cartilaginous and osseous matrices, with pre-designed integration between these regions achieving a mineralized interfacial region within which direct osteoblast-chondrocyte interactions are encouraged. Collectively, these studies demonstrate the importance of cell-ceramic and cell-cell interactions on osteochondral interface formation.

1.4. Summary

Complex biological interfaces allow for functional integration between soft and hard tissues in the musculoskeletal system. Cartilage covers the articulating ends of tibiofemoral joints and facilitates smooth and painless motion. The distinct changes in mineral and matrix distribution from cartilage to bone, in addition to the structural organization of the tissue transition, are necessary for healthy joint functionality. The calcified cartilage layer allows for integration between articular cartilage and subchondral bone, while maintaining the integrity of the two distinct tissue regions. Despite advances in surgical techniques and the promise of tissue engineering for cartilage regeneration, there remains a
significant clinical need for functional and integrative cartilage repair methods. In order to address this challenge, this thesis focuses on the design and optimization of an innovative biomimetic composite scaffold system for calcified cartilage formation. The approach to the clinical challenge of biological fixation described in this thesis centers on functional interface tissue engineering.

Specifically, the native interface will be characterized with high-resolution imaging techniques in order to identify criteria for biomimetic scaffold design for interface regeneration (Chapter 2). Based on this characterization, a scaffold system will be designed and optimized through a series of in vitro studies (Chapters 3, 4 and 5). First, deep zone chondrocyte response to ceramic presence will be compared in a degradable (alginate, Chapter 3) and non-degradable hydrogel (agarose, Chapter 4). Then, non-hypertrophic and hypertrophic chondrocyte response will be evaluated, and the optimal chondrocyte phenotype will then be used to test the effect of ceramic size and dose. Cell response will be evaluated in terms of proliferation, matrix deposition, hypertrophy, and mineralization; scaffold mechanical properties will also be tested in compression and shear. The results from these scaffold characterization and cell-ceramic interaction studies will be utilized to develop an optimized biomimetic, composite scaffold for in vitro interface regeneration (Chapter 5).

In the second portion of the thesis (Chapters 6, 7, and 8), the interface scaffold will be evaluated for integrative cartilage repair. A layered hydrogel scaffold will be used to include a ceramic-free cartilage graft above the ceramic-containing calcified cartilage graft. Secondly, in order to augment scaffold integration with host tissue, a nanofiber component will be incorporated into the scaffold design. To examine the functional and integrative potential of the scaffold system, a rabbit osteochondral defect model will be used to evaluate interface formation and osteointegration. Lastly, Chapters 9 and 10 will focus on the role of zonal chondrocyte populations in maintaining the stability of uncalcified and calcified cartilage regions on the layered scaffold. This thesis will conclude with a summary and recommendations for future directions (Chapter 11).

While there have been tremendous advancements in the field of cartilage tissue engineering, including the use of stem cells, bioactive factors, novel biomaterials, and bioreactors, interface tissue engineering remains unexplored; tissue-to-tissue integration at these interfaces is a critical component of the next generation of multi-tissue and organ solutions. In summary, the approach outlined in this thesis
for improving cartilage repair is to develop a biomimetic composite scaffold system that is capable of regenerating the osteochondral interface and improving repair by hydrogel-based cartilage grafts. Furthermore, the methodologies developed throughout this work have relevance for other soft-to-hard, unmineralized-to-mineralized interfaces which are also critical for tissue functionality.
CHAPTER 2: MATRIX AND MINERAL DISTRIBUTION AT THE OSTEOCHONDRAL INTERFACE
2.1 Introduction

This thesis begins with a study that focuses on elucidating region-dependent and age-dependent changes in matrix and mineral across the cartilage-to-bone junction. Tissue interfaces are particularly challenging to study, since these tissue transitions are characterized by complex changes in content and organization over short distances. High resolution imaging techniques are well-suited to address the limitations of traditional qualitative characterization techniques. The findings from this study will provide the design parameters and benchmark assessment criteria for the development and testing of a biomimetic interface scaffold.

2.1.1 Background and Motivation

Although the osteochondral junction has been extensively characterized using histological and microscopy techniques[65,76,77,79-83,98,103,108-111], there is a critical need for quantitative assessment which is not subject to qualitative interpretation. Two such methods are Raman and Infrared (IR) spectroscopy which have been used to characterize biological tissue, whereby peak integration and the calculation of peak height or peak area ratios are used to generate high-resolution maps of the distribution, orientation, and composition of matrix components. Specifically, IR has been used to analyze a variety of connective tissues, including bone[112,113] and articular cartilage[114-117], and has been also useful for studying complex tissue transitions such as the ligament-to-bone interface[118]. Raman spectroscopy has also been used to analyze bone[119] and cartilage[120,121]. Studies have directly compared results from these two methods and have found their information to be comparable and complementary[122]. However, it is also important to recognize their differences. For Raman measurements, a polished surface of non-specific thickness can be used, whereas IR requires tissue processing of thin sections. Water interferes with the IR spectra, whereas wet specimens can be analyzed by Raman. On the other hand, IR spectroscopy collects a larger amount of data in a shorter period of time, which is especially advantageous for characterizing inhomogeneities at tissue interfaces.
2.1.2 Objectives

Given the regional variation in matrix content and organization of interface tissues, this study will use FTIR-I to characterize the osteochondral junction. It is anticipated that this technique will provide quantitative analysis of the molecular structure and spatial distribution of matrix components from uncalcified cartilage to bone. This study will first investigate region-dependent variations in collagen, proteoglycan, and mineral distribution, as well as collagen orientation across the interface regions of calf osteochondral specimens, which represent a well-characterized large animal model[114,123-126]. Given that the calcified cartilage tissue is a transitional region, it is anticipated that extracellular matrix composition are intermediate between articular cartilage and bone.

The second objective of this study is to evaluate age-dependent changes in matrix and mineral distribution, by comparing immature and mature specimens. This comparison provides information about critical tissue changes that occur during development. Given the functional differences between immature and mature cartilage, age-related structural changes are also expected. This is the first study to quantitatively analyze age-dependent changes in matrix distribution and organization of the bovine osteochondral interface, and findings of this study are anticipated to yield baseline benchmark parameters for evaluating the success of interface regeneration efforts.

2.2 Materials and Methods

2.2.1 Sample Isolation and Preparation

Osteochondral plugs were isolated from the tibiofemoral joints of three different immature and mature animals obtained from a local abattoir (Green Village Packing Company, Green Village, NJ). A sterile biopsy punch was used to extract 6 mm osteochondral plugs from the tibial plateau, containing regions of articular cartilage, calcified cartilage, and bone. Each specimen was divided into two halves to evaluate corresponding decalcified and non-decalcified sections were evaluated. To prepare the decalcified samples, osteochondral plugs were immediately fixed with 80% ethanol, supplemented with 1% cetylpyridinium chloride (CPC, Sigma, St. Louis, MO) to preserve proteoglycan[127], for 24 hours. Post-fixation, samples were rinsed in distilled water to remove the CPC before being demineralized in tris-hydroxymethylaminomethane (Tris, Sigma, St. Louis, MO) buffer containing 10%
ethylenediaminetetraacetic acid (EDTA, Sigma). Samples were then dehydrated using an ethanol series, cleared with xylene, and embedded in paraffin (Fisher Scientific, Pittsburgh, PA). The samples were microtomed (Reichert-Jung RM 2030 Microtome, Leica, Bannockburn, IL), and sections (7 µm) were placed immediately onto barium fluoride optical windows (Spectral Systems, Hopewell Junction, NY). The sections were deparaffinized in xylene, rehydrated with an ethanol series, and then dried overnight under vacuum. A second barium fluoride window was placed over the sample prior to infrared analysis.

The non-decalcified samples were fixed post isolation with 90% ethanol for 24 hours. Following fixation, samples were embedded in poly(methylmethacrylate) (PMMA) by modifying the methods of Erben et al.[128]. The samples were then sectioned (2 µm) with a Leica sliding microtome (SM2500S, Leica Microsystems Inc., Deerfield, IL) fitted with a tungsten carbide blade (Delaware Diamond Knives Inc., Wilmington, DE). Individual sections were dried and also placed between barium fluoride windows for analysis.

2.2.2 Fourier Transform Infrared Imaging

Fourier transform infrared imaging (FTIR-I) analysis was performed using an FTIR spectrometer (Spectrum 100, Perkin Elmer, Waltham, MA) coupled to an FTIR microscope imaging system (Spotlight 300, Perkin Elmer). The spectra were acquired with a spectral resolution of 8 cm\(^{-1}\) and a spatial resolution of 6.25 µm. The distribution of collagen and proteoglycan were mapped using the amide and carbohydrate peaks, respectively, while mineral distribution was mapped using the phosphate band. Since the carbohydrate and phosphate peaks overlap, decalcified samples were used for matrix analysis, whereas corresponding calcified samples were used for mineral analysis. For each sample, regions spanning across the entire interface progressing from the articular surface to bone were scanned and analyzed.

2.2.3 Spectra Analyses

The IR spectra were analyzed and spectroscopic images generated using ISYS 3.1.1 chemical imaging software (Spectral Dimensions Inc., Olney, MD) and MATLAB 7.0 R14 (The MathWorks Inc., Natick, MA). Prior to analysis, spectra were corrected by baseline subtraction using the ISYS software.
Relative collagen was estimated by integrating the peak area under the amide I band (1720-1590 cm$^{-1}$), and proteoglycan was estimated by integrating under a carbohydrate band associated with C-O-C and C-OH vibrations (1140-985 cm$^{-1}$), according to previous studies that have linearly correlated collagen and proteoglycan content to these respective band areas[114].

Additionally, collagen alignment was determined by scanning the demineralized samples with a gold-wire polarizer grid (Perkin Elmer) inserted in the path of the IR light, with the polarizer aligned at 0° and 90° with respect to the interface between cartilage and bone. As amide I and amide II bond vibrations are approximately orthogonal, previous studies have shown that the ratio of their band areas is an indicator of collagen fibril orientation when spectra are collected with polarized light[116]. Therefore in this study, spectra obtained with the polarizer were integrated under the amide I (1720-1590 cm$^{-1}$) and amide II bands (1590-1492 cm$^{-1}$), and numerical indices for collagen orientation were obtained by calculating the amide I-to-amide II band area ratio. Collagen orientation was categorized according to the parameters previously reported for cartilage analysis, namely amide I-to-amide II ratio values ≥ 2.7 indicated fibril orientation parallel to the interface and values ≤ 1.7 indicated perpendicular orientation. A ratio ranging from 1.7 - 2.7 indicated mixed fibril orientation.

The relative mineral-to-matrix ratio was calculated by integrating under the v1, v3 phosphate band contour (1200-900 cm$^{-1}$) and dividing by the amide I band area[129]. Prior to analysis of mineral distribution, the collected spectra were corrected for contributions from the PMMA embedding material. Specifically, spectra of pure PMMA were acquired, baseline corrected, and normalized by the peak at 1728 cm$^{-1}$, the highest peak in the PMMA spectrum. Sample spectra were likewise baseline corrected and normalized, and a pure PMMA spectrum was subtracted from the sample spectra to eliminate the PMMA background. Additionally, the normalized carbonate content in the apatite structure was estimated from the carbonate-to-matrix ratio, determined by integrating under the carbonate v2 band (890-850 cm$^{-1}$) and dividing by the area of the amide I band. Normalization with the amide I band accounts for any variations in sample thickness of the 1 to 2 um sections.
2.2.4 Data and Statistical Analyses

Line profiles of collagen, proteoglycan and mineral distribution from the articular surface to bone were generated. Relative values for matrix and mineral were also calculated for each tissue region, namely cartilage, calcified cartilage, and bone. Results are presented in the form of mean ± standard deviation, with n equal to the number of animals analyzed per group. Two-way analysis of variance (ANOVA), followed by a Tukey-HSD post-hoc test was performed to determine region- and age-dependent differences in relative matrix and mineral content (p<0.05). Statistical analysis was performed using the JMP IN software (version 4.0, SAS Institute, Cary, NC).

2.3 Results

2.3.1 Method Validation

The methodology used to analyze IR spectra is able to determine relative amounts and is not indicative of raw matrix or mineral content. It is also for this reason that although proteoglycan and collagen have overlapping spectra, the amide I and carbohydrate peaks are individually proportional to collagen and proteoglycan content in a linear manner[114]. Comparison with histological staining and biochemical analysis of fresh surface, middle, and deep zone explants was used to confirm the validity of IR analysis (data not shown).

Ethanol fixation and PMMA embedding have been shown to minimize interference that would otherwise be obtained in the acquired spectra from other fixation and embedding methods[129] and is therefore used in this study to determine the mineral profile across the interface. While the use of 90% ethanol is optimal for retaining mineral content of the specimen, this method of fixation quickly dehydrates the soft tissue above calcified cartilage and bone. As compared to the 80% ethanol fixation used for paraffin-embedded samples, the articular cartilage thickness decreased by approximately 15%, which was taken into account when comparing uncalcified and calcified tissue line scans. It is also expected that the degree of PMMA infiltration varies throughout the sample, especially in mineralized and non-mineralized regions. The technique for removal of the PMMA spectra used in this study allows for non-uniform subtraction of the embedding media, even if infiltration varies throughout the sample.
The decalcification of samples with EDTA was confirmed by negative histological staining of paraffin sections with von Kossa and Alizarin red. The spectra of all three tissue regions were also compared pre- and post-decalcification in order to assess the effect of the decalcification procedure. It was confirmed that the amide I band remains unchanged by decalcification. It was assumed that any matrix loss associated with decalcification or fixation was uniform throughout the sample and therefore has no significant effect on relative matrix quantities. The addition of CPC was used to improve retention of proteoglycan during fixation and decalcification, although proteoglycan was still present to some degree in the calcified specimens. Given the overlapping phosphate and carbohydrate peaks, it is therefore likely that residual proteoglycan content contributes to relative mineral content, although this contribution is negligible since the phosphate peak is approximately ten times higher than the carbohydrate peak.

2.3.2 Cartilage-to-Bone Transition

The cartilage-to-bone transition was characterized by distinct tissue regions which vary in matrix and mineral distribution (Fig. 2.1A). Representative spectra from articular cartilage, calcified cartilage, and subchondral bone confirmed the region-dependent changes in matrix and mineral (Fig. 2.1B). Whereas the amide I and II peaks were present in all three regions, the phosphate band was present only in calcified samples in the calcified cartilage and bone regions. Each sample contained approximately 60% cartilage and 30% bone, and the calcified cartilage interface was approximately 10%, or 150 µm in width. Articular cartilage was then further subdivided into the surface (10%), middle (25%), and deep zones (25%).

2.3.3 Region-Dependent Collagen Distribution and Orientation

Three distinct regions of collagen were evident from the quantitative mapping (Fig. 2.2). The zone closest to the articular surface had the lowest amount of collagen (p<0.05, Fig. 2.6). The remaining middle and deep zones had relatively higher collagen than the surface zone, although there was no significant difference in collagen distribution between middle/deep zone articular cartilage, calcified
cartilage, and bone regions (Fig. 2.6). Furthermore, collagen fibrils of immature samples were oriented only in the middle zone of articular cartilage, organized parallel to the osteochondral interface (Fig. 2.2).

Mature samples contained more collagen as compared to immature samples in the surface and deep zones, as well as the calcified cartilage and bone regions (p<0.05, Fig. 2.6). Similar to those of the immature specimens, the line scans of mature samples indicated increasing collagen from the articular surface to bone (Fig. 2.2). There was significantly higher collagen in the deep zone, calcified cartilage, and bone as compared to the surface zone. There was also higher collagen in the calcified cartilage and bone as compared to the middle zone (p<0.05). The relative amounts of collagen in deep zone cartilage, calcified cartilage, and bone were not significantly different from each other in mature samples.

Secondly, the collagen fibrils of mature samples were highly orientated, parallel to the articular surface in the surface zone and perpendicular to the cartilage-to-bone junction in the deep zone (Fig. 2.2). In the calcified cartilage layer between cartilage and bone, there was loss of collagen orientation.

2.3.4 Region-Dependent Proteoglycan Distribution

Similar to collagen distribution, relative amounts of proteoglycan also varied across the immature cartilage-to-bone junction (Fig. 2.3). The surface zone of immature specimens contained less proteoglycan than the middle and deep zones of articular cartilage, as well as calcified cartilage (p<0.05, Fig. 2.6). There were no significant differences between the middle zone, deep zone, and calcified cartilage region. Lastly, there was less proteoglycan in subchondral bone as compared to the deep zone and calcified cartilage. The carbohydrate peak was also normalized by the amide I peak to determine the relative proteoglycan distribution (Fig. 2.3). Normalized proteoglycan was highest in the surface and deep zones, and reflected the low relative amounts of collagen in the surface zone and high proteoglycan in the deep zone.

Mature osteochondral samples measured higher proteoglycan in the deep zone as compared to immature specimens, although there were no age-dependent differences in any other region (p<0.05, Fig. 2.6). Similar to immature samples, low relative proteoglycan at the surface zone and bone region and high relative proteoglycan in the deep zone and calcified cartilage region were also measured. There was significantly higher proteoglycan in the deep zone as compared to the surface zone, while no
differences in proteoglycan distribution were found across the middle zone, deep zone, calcified cartilage, and bone regions. Furthermore, normalized proteoglycan was uniformly distributed throughout the cartilage-to-bone transition (Fig. 2.3).

2.3.5 Region-Dependent Mineral Distribution

As expected, the uncalcified cartilage region of immature samples was mineral-free, whereas there was high relative mineral in subchondral bone (Fig. 2.4). Regions of high relative carbonate were found only in bone and not in the calcified cartilage region. The 150 µm interface region measured less normalized mineral than bone (p<0.05), with no significant variability among samples from different animals (Fig. 2.5). Furthermore, the line scans of normalized mineral distribution demonstrate that there was an exponential transition from uncalcified cartilage to bone (R²=0.98, 0.96, 0.99), with an average exponent of 17.7 ± 1.1.

Similarly, the articular cartilage region of mature samples was mineral-free, with high relative mineral in subchondral bone (Fig. 2.4). Although the normalized mineral content of the calcified cartilage region was not significantly different between mature and immature samples, mature samples measured lower relative mineral in the bone region (p<0.05, Fig. 2.6). It was also evident that calcified tissue of mature samples was less porous as compared to immature samples. Similar to immature samples, the calcified cartilage region of mature specimens had significantly less normalized mineral than bone and minimal variability among samples from different animals (Fig. 2.5). The mineral increase throughout the interface region was similarly modeled to fit an exponential curve for all three samples (R²=0.98, 0.97, 0.97), with an average exponent of 17.2 ± 4.6.

2.4 Discussion

The osteochondral interface is a region of calcified cartilage which persists at the epiphysis after skeletal maturity. The objective of this study was to characterize the relative amount, distribution, and organization of key matrix components across this critical junction in both immature and mature specimens. Region- and age-dependent changes in mineral, proteoglycan, and collagen, as well as collagen orientation, were detected and quantified across the multi-tissue regions of the interface.
The calcified cartilage region of both immature and mature bovine samples is approximately 150 µm in thickness and is consistent with previously reported values[65,77]. Throughout the entire calcified cartilage region, mineral content increases exponentially for both immature and mature specimens, from basal values in articular cartilage to high mineral content in bone. This phenomenon may be partially attributed to the fact that the cartilage-to-bone junction is interdigitated, meaning that there are mineral-poor regions adjacent to mineral-rich regions within the calcified cartilage transition zone. In order to determine whether the results from our study are an artifact of averaging hundreds of scans across the undulating interface, single line scans at various points along the specimen were graphed and also followed exponential increases (data not shown). Furthermore, the gradient in normalized mineral cannot be solely attributed to changes in the underlying matrix since unnormalized mineral distributions were also examined with similar results (data not shown). These findings indicate that, independent of uncalcified-calcified tissue interdigitation and location-dependent changes in collagen, an exponential gradient of mineral from articular cartilage to bone persists. These findings differ slightly from those presented in a study by Gupta et al., where quantitative backscattered electron imaging was used to determine the mineral distribution across human osteochondral samples; a gradient in mineral was measured only within a 30 µm region near the tidemark, with relatively uniform mineral distribution throughout the calcified cartilage and bone regions in human tissue[88]. Given the differences measured in cartilage thickness between different species[130], it is not unexpected that there are species-to-species variations in the osteochondral transition[108].

Although the normalized mineral content of calcified cartilage is not age-dependent, it is lower in the bone region of mature specimens as compared to immature specimens. This difference is attributed to the higher collagen content of mature specimens, since no significant difference in raw mineral values is detected (data not shown). Regardless of age, mineral content of the calcified cartilage region is less than that of subchondral bone. The calcified cartilage of the growth plate also contains less mineral than bone[131]. Reminiscent of other soft tissue-to-bone interfaces such as the transition found at the anterior cruciate ligament insertion site which has an exponential gradient in mineral from ligament, to mineralized fibrocartilage, and finally bone[118], it is not surprising that there is also a mineral gradient from articular cartilage to bone.
While the exponential gradient in mineral content is confined to the interface region, the distribution of both collagen and proteoglycan varies throughout the entire depth of the cartilage-to-bone transition. For immature samples, there is a clearly delineated portion of tissue close to the articular surface which is matrix-poor, followed by the middle and deep zones which are higher in both proteoglycan and collagen. Mature samples also exhibit increasing matrix content from the articular surface to the deep zone. Overall, these results are in agreement with published studies[4,132], which demonstrate a depth-dependent increase in matrix for both immature and mature bovine cartilage and chondrocytes isolated from each zone. A recent study by Kobrina et al. has further analyzed IR data using a “clustering” technique to clearly differentiate the different zones of mature lapine and bovine tissue with similar results[133]. On the other side of the osteochondral interface lies subchondral bone which, in agreement with previous studies, is a mineral- and collagen-rich matrix which is low in proteoglycan content[134].

While the depth-dependent increase in matrix content across articular cartilage is not age-dependent, there are a few distinct differences that occur during development. Mature samples are characterized by higher matrix content, specifically higher collagen in the surface and deep zone, as well as higher proteoglycan in the deep zone. Similar increases in matrix content have also been measured between fetal and newborn articular cartilage[132]. Secondly, the step-wise changes between cartilage regions observed in immature samples are replaced by a gradual transition in matrix content through the surface, middle, and deep zones for mature samples. This change in organization with age is also reflected in the orientation of collagen fibers. Whereas immature cartilage has oriented collagen fibers only in the middle zone, mature cartilage has collagen orientation parallel to the articular surface in the surface zone, mixed orientation in the middle zone, and perpendicular orientation in the deep zone. It has previously been shown that collagen is not well organized in fetal tissue and fiber orientation in fact increases with age[135,136]. Hunziker et al. has shown the transformation from fetal to mature cartilage tissue involves a process of resorption, similar to that of the growth plate, instead of internal tissue modeling[75]; all zones except the surface zone are resorbed and, instead of being replaced with bone, they are replaced by mature articular cartilage.
The critical cartilage-to-bone transition, evident in the line-scans across the osteochondral samples, is the focus of this study. The matrix of the calcified cartilage zone resembles that of deep zone cartilage for both immature and mature specimens. It is not surprising that the matrix content of the calcified cartilage zone is similar to the layer of cartilage directly above the interface. Chondrocytes at the mineralization front which have deposited a proteoglycan- and collagen-rich matrix become hypertrophic and mineralize. Interestingly, there is no collagen orientation in the calcified cartilage layer. This may be attributed to the high collagen X content which has a polygonal structure\cite{137}, as well as the distinct change in collagen orientation at the tidemark previously visualized by electron microscopy\cite{79}. While the relative collagen increases with age, no change in the amount of proteoglycan is evident. As the soft tissue-bone interface is crucial for musculoskeletal functionality, our long-term goal is to engineer a functional interface between cartilage and bone in order to achieve integrative repair. Results from this study provide the benchmark criteria for successful regeneration of the osteochondral interface, as well as new insights into the remodeling process which is critical for the healing response and long-term success of tissue engineering solutions. Future studies should focus on elucidating critical disease-related changes.

2.5 Conclusions

The depth-dependent changes in mechanical properties from cartilage to bone can be explained by changes in mineralization, as well as matrix content and organization, across this tissue transition. The results of this study show that from cartilage to bone, collagen content increases continuously and proteoglycan content peaks in the deep zone. Furthermore, mineral content increases exponentially throughout the calcified cartilage layer. Aside from the lack of fiber organization, the matrix of the calcified cartilage region resembles that of deep zone cartilage. With age, only the relative collagen content across the interface increases.

The results of this study collectively provide design criteria for the osteochondral interface scaffold. The exponential increase from basal to high mineral content, as opposed to a linear gradient, suggests that a tissue engineering solution can recapitulate this transition by juxtaposing mineral-free and mineral-containing regions. Furthermore, the calcified cartilage matrix is collagen- and proteoglycan-rich,
indicating that its matrix resembles that of the nonmineralized cartilage directly above it. Therefore, tissue engineering strategies aimed at regeneration of the osteochondral interface should promote the formation of a mineralized deep zone cartilage-like matrix.
Figure 2.1. Osteochondral characterization. Immature and mature osteochondral samples (n=3) show region-dependent (AC-articular cartilage, CC-calcified cartilage) changes in (A) histological staining (Picrosirius Red-collagen, polarized Picrosirius Red-collagen orientation, Alcian Blue-proteoglycan, von Kossa-mineral, 5x, scale bar=200 µm) for matrix and mineral, as well as (B) IR spectra (Am I-Amide I, Am II-Amide II).
Figure 2.2. Collagen distribution and alignment. Peak integration maps (n=3) of the amide I shows collagen distribution, and peak integration maps of the amide I, normalized by amide II, shows collagen orientation. The arrow indicates the position of the calcified cartilage region. Line scans (n=3) across the cartilage-to-bone junction reveal a step-wise increase in collagen for immature specimens and a gradual increase for mature specimens.
Figure 2.3. Proteoglycan distribution. Peak integration maps (n=3) of the carbohydrate band and normalized carbohydrate band show proteoglycan distribution. The arrow indicates the position of the calcified cartilage region. Line scans (n=3) across the cartilage-to-bone junction reveal a peak in proteoglycan content in deep zone cartilage for both immature and mature specimens.
Figure 2.4. **Mineral distribution and characterization.** Peak integration maps (n=3) of the phosphate band, normalized by amide I, shows mineral distribution. Peak integration maps (n=3) of the carbonate band, normalized by amide I, shows carbonate distribution. The arrow indicates the position of the calcified cartilage region. Line scans (n=3) across the cartilage-to-bone junction reveal a distinct transition from mineral-free to mineral-rich tissue regions for both immature and mature specimens.
Figure 2.5. Calcified cartilage transition. Results from three different samples (red, blue, and green) are graphed. The normalized phosphate content of the transition region is intermediate between uncalcified cartilage and bone (n=3, *p<0.05: difference with uncalcified cartilage, **p<0.05: difference with both uncalcified cartilage and calcified cartilage). The transition is modeled to an exponential fit (n=3) for both immature and mature specimens.
Figure 2.6. Age- and depth-dependent tissue transition. Both matrix and mineral content vary across the cartilage-to-bone junction for both immature and mature samples (n=3, *p<0.05: differences between zones/regions for immature samples, **p<0.05: differences between zones/regions for mature samples, ^p<0.05: differences between corresponding immature and mature zones/regions).
CHAPTER 3: A HYDROGEL-CERAMIC SCAFFOLD FOR OSTEOCHONDRAL INTERFACE FORMATION
3.1 Introduction

Based on previous tissue characterization studies and the results from Chapter 2, the ideal scaffold for osteochondral interface scaffold should support chondrocyte viability and the interface-relevant hypertrophic phenotype. Secondly, the scaffold should support proteoglycan and collagen deposition comparable to native deep zone cartilage, as well as interface-relevant mechanical properties. Furthermore, the scaffold should support formation of calcified cartilage while also prohibiting ectopic mineralization. Finally, the interface scaffold should be osteointegrative.

Guided by these design parameters, the scaffold design will consist of a biomimetic hydrogel-ceramic composite. Hydrogel scaffolds resemble the hydrated matrix of native cartilage and are commonly used for chondrocyte culture and cartilage tissue engineering applications; these scaffolds have been shown to maintain the chondrocyte phenotype and biosynthetic activity in vitro and in vivo. The ceramic phase within the scaffold consists of hydroxyapatite particles which have similar chemistry to mineral found in calcified cartilage. In comparison to scaffold-free osteochondral interface engineering approaches in which chondrocytes are seeded above ceramic substrates, relatively fewer chondrocytes are needed to seed the proposed hydrogel-ceramic scaffold. Secondly, ceramic content can be directly incorporated into the scaffold which decreases the need for cell-mediated mineralization.

3.1.1 Background and Motivation

The focus of this study, guided by the aforementioned design criteria, is to evaluate the potential of a hydrogel-ceramic composite scaffold to promote chondrocyte-mediated formation of a calcified cartilage-like matrix in vitro. The cartilage phase of the scaffold will be based on alginate, a hydrogel comprised of linear chains of covalently linked block-co-polymers of (1,4)-linked β-D-mannuronic acid and α-L-guluronic acid[138], that has been utilized extensively for chondrocyte culture[139-141] and cartilage tissue engineering[28,125,142]. Chondrocytes have been shown to maintain their native morphology and deposit a matrix rich in both proteoglycan and collagen when cultured in alginate, which also has the advantage of being biocompatible, non-immunogenic, and biodegradable. Moreover, the ambient gelation conditions associated with alginate have been reported to preserve the bioactivity of growth factors[143]. Although hydrogel-ceramic scaffolds have not been explored for calcified cartilage
regeneration, this type of composite scaffold has been tested for bone applications and shown to promote calcification in vitro[144,145].

3.1.2 Objectives

The first objective of this study is to determine the response of chondrocytes in the composite alginate-HA scaffold, focusing on changes in cell growth, biosynthesis, and mineralization, as well as scaffold compressive and shear mechanical properties. The second objective of this study is to identify a chondrocyte population that is optimal for calcified cartilage formation. Currently, the majority of cartilage and tissue engineering scaffolds are seeded with full thickness chondrocytes derived from all three zones of articular cartilage[37,146-148]. Given the promising results of the aforementioned cell-based approaches to interface tissue engineering, this study will compare the response of deep zone chondrocytes, the cells residing in the deep zone of cartilage and directly above the calcified cartilage interface[149], with that of commonly used full thickness chondrocytes. It is hypothesized that the presence of the hydroxyapatite phase within the alginate hydrogel scaffold will promote the formation of a calcified cartilage matrix, and differences in biosynthesis due to cell population are also expected.

3.2 Materials and Methods

3.2.1 Cells and Cell Culture

Primary articular chondrocytes were isolated from five neonatal calf knees (Green Village, NJ) following published protocols, and pooled together for seeding of this study[5]. Specifically, cells digested from the bottom third of articular cartilage was designated as deep zone chondrocytes (DZC). For comparison, chondrocytes from the full thickness of cartilage (FTC) were also isolated. Briefly, the cartilage pieces were digested for 16 hours with 0.1 w/v% collagenase (Sigma, St. Louis, MO) in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, GA), 2% antibiotics (10,000 U/ml penicillin, 10 mg/ml streptomycin), and 0.1% antifungal (amphotericin B). The cell suspension was then filtered (30 µm, Spectrum, Rancho Dominguez, CA) before plating. The isolated chondrocytes were maintained in high-density culture (4x10^5 cells/cm^2) in fully-supplemented DMEM with 10% FBS, 1% non-essential amino acids, 1%
antibiotics, and 0.1% antifungal for 48 hours before seeding. All media supplements were purchased from Cellgro-Mediatech unless otherwise specified.

### 3.2.2 Alginate Scaffold Fabrication, Characterization, and Culture

Medium viscosity sodium salt alginic acid (Sigma) was used to prepare a 2% alginate solution in phosphate buffered saline (PBS). A custom mold was used to crosslink the alginate scaffolds (10 mm diameter x 1.6 mm height) with 50 mM CaCl$_2$ (Sigma) and 150 mM NaCl (Sigma) for 30 minutes$^{[139]}$. Acellular and cellular alginate scaffolds with 1.5% hydroxyapatite (HA, 20 µm, Sigma) and corresponding samples without HA were fabricated. The experimental group consisted of cells in alginate scaffolds with hydroxyapatite (Alg+HA), whereas the control groups include cells in alginate (Alg), as well as corresponding acellular controls for alginate and alginate+HA groups. To form the cell-seeded scaffolds, chondrocytes were mixed into the alginate solution (1x10$^7$ cells/ml) prior to crosslinking. All samples were cultured under humidified conditions at 37°C and 5% CO$_2$, and maintained in ITS media composed of DMEM supplemented with 1% ITS+ Premix (BD Biosciences, San Jose, CA), 1% antibiotics, 0.1% antifungal, and 40 µg/ml proline (Sigma). The media was changed every other day and freshly supplemented with 50 µg/mL ascorbic acid (Sigma). In this study, chondrocyte response in the alginate+HA scaffolds was evaluated over four weeks of culture. Specifically, cell viability, proliferation, collagen and glycosaminoglycan (GAG) deposition, scaffold mechanical properties, mineralization, and hypertrophy were determined and compared between groups as well as over time.

Distribution of HA (n=2) in the as-fabricated acellular and cellular alginate+HA scaffolds were visualized by environmental scanning electron microscopy (ESEM, 15 kV, JEOL 5600LV, Tokyo, Japan), and elemental composition was ascertained using energy dispersive x-ray analysis (EDAX, 15kV, FEI Quanta 600, FEI Co., Hillsboro, OR). In addition, samples were weighed and desiccated for 24 hours (CentriVap Concentrator, Labconco Co., Kansas City, MO), after which the scaffold swelling ratio (n=4, wet weight/dry weight) and water content (n=4, water weight/wet weight) were calculated.

### 3.2.3 Cell Proliferation and Viability

Cell viability (n=2) was visualized using Live/Dead staining (Molecular Probes, Eugene, OR),
following the manufacturer’s suggested protocol. After washing in PBS, samples were imaged under confocal microscopy (Olympus Fluoview IX70, Center Valley, PA) at excitation and emission wavelengths of 488 nm and 515 nm, respectively. Cell proliferation (n=5) was determined using the PicoGreen® total DNA assay (Molecular Probes, Eugene, OR). Briefly, the samples were first rinsed with PBS and 300 μl of 0.1% Triton-X solution (Sigma) was used to lyse the cells. An aliquot of the sample (25 μl) was then added to 175 μl of the PicoGreen® working solution. Fluorescence was measured with a microplate reader (Tecan, Research Triangle Park, NC), at the excitation and emission wavelengths of 485 and 535 nm, respectively. Total cell number was obtained by converting the amount of DNA per sample to cell number using the conversion factor of 7.7 pg DNA/cell[150].

Cell size was determined by labeling the cells with the membrane dye calcein AM and imaging the samples under confocal microscopy (Olympus Fluoview IX70, Center Valley, PA). Three representative images were taken for each experimental group, with seven cells analyzed per image using ImageJ (National Institute of Health), and both the surface area (n=21) and aspect ratio (n=21) of each cell were calculated from the long and short axes of the 2D image projections.

3.2.4 Matrix Deposition

Collagen deposition (n=5) was quantified using the Sirius assay (Biocolor, Belfast, UK) according to the manufacturer’s suggested protocol, which detects type I-V collagen[151]. Bovine collagen I solution (Biocolor, UK) was used as a standard. Briefly, the samples were first desiccated for 24 hours and then digested for 16 hours at 60°C with papain (600 µg protein/ml, Sigma) in 0.1M sodium acetate (Sigma), 10 mM cysteine HCl (Sigma), and 50 mM ethylenediaminetetraacetate (Sigma). Absorbance was measured at 555 nm using a microplate reader (Tecan). Additionally, collagen distribution (n=2) was visualized by Picrosirius red staining. Briefly, the samples were first fixed in 10% neutral buffered formalin with 1% cetylpyridinium chloride (Sigma) for 24 hours, followed by dehydration with an ethanol series. The dehydrated samples were embedded in paraffin (Type 9, Richard-Allan Scientific, Kalamazoo, MI), sectioned from the center of the scaffold (7 μm) and mounted on microscope slides prior to staining and imaging.

Overall matrix deposition and cellularity was determined with H&E staining (n=2). Deposition of
types I, II, and X collagen (n=2) in the alginate and alginate+HA scaffolds was evaluated using immunohistochemistry. Monoclonal antibodies for type I collagen (1:20 dilution) and type II collagen (1:100) were obtained from Abcam (Cambridge, MA), and type X collagen antibody (1:1) was obtained from the Developmental Studies Hybridoma Bank (University of Iowa). After fixation, samples were treated with 1% hyaluronidase for 30 min at 37°C and incubated with primary antibody overnight. A FITC-conjugated secondary antibody (LSAB2 Abcam) was added and sections were imaged under confocal microscopy at excitation and emission wavelengths of 488 nm and 568 nm, respectively.

Sample glycosaminoglycan content (GAG, n=5) was determined with a modified 1, 9-dimethylmethylene blue (DMMB) binding assay[152-154], with chondroitin-6-sulfate (Sigma) as the standard. To account for the anionic nature of the carboxyl groups on the alginate hydrogel, the pH of the DMMB dye solution was adjusted to 1.5 with concentrated formic acid (Sigma) so that only the sulfated GAG-DMMB complexes were detectable. Additionally, the absorbance difference between 540nm and 595nm was used to improve the sensitivity in signal detection. Proteoglycan distribution (n=2) was visualized histologically with Alcian blue staining of paraffin embedded sections[150].

3.2.5 Scaffold Mechanical Properties

Scaffold mechanical properties (n=3) were determined following published protocols[155]. Briefly, scaffold diameter (d) was measured with a stereomicroscope (Bausch and Lomb, Rochester, NY) and testing was performed on a shear-strain controlled rheometer (TA instruments, New Castle, DE). Each sample was placed between two flat porous platens and immersed in DMEM to prevent dehydration. The equilibrium compressive Young’s modulus (E\text{eq}) of the sample was calculated at 15% compressive strain (ε) as follows:

$$E_{\text{eq}} = \frac{\sigma}{\varepsilon} \text{ where } \sigma = \frac{\Delta F}{\pi d^2 / 4},$$

where \(\Delta F\) is the change in equilibrium normal force due to the axial compression. The 15% compressive strain chosen here is within the physiological range for articular cartilage[156]. Finally, a dynamic shear test was performed (0.01 Hz to 10 Hz) with a logarithmic frequency sweep with the specified shear strain amplitude of 0.01 radian. The complex shear modulus was calculated as follows:
\[ G^* = \frac{Td}{2I_\gamma^*}, \]  

(2)

where, \( \gamma \) is the sinusoidal shear strain and \( T \) is the torque response. In general, \( G^* \) is a complex number and can be expressed as \( G^* = G' + iG'' \). The magnitude of the complex shear modulus (\( |G^*| \)) is therefore given by \( |G^*| = \sqrt{(G')^2 + (G'')^2} \), and the phase shift angle (\( \delta \)) between the applied strain and the torque response could be calculated from \( \delta = \tan^{-1}(G''/G') \).

3.2.6 Mineralization and Chondrocyte Hypertrophy

Mineralization potential was determined by measuring ALP activity (n=5) using an colorimetric assay based on the hydrolysis of p-nitrophenyl phosphate (pNP-PO_4) to p-nitrophenol (pNP)\[157\]. Briefly, the samples were lysed in 0.1% Triton-X solution, then added to pNP-PO_4 solution (Sigma) and allowed to react for 30 min at 37°C. The reaction was terminated with 0.1 N NaOH (Sigma), and sample absorbance was measured at 415nm using a microplate reader (Tecan). In addition, mineral distribution (n=2) was evaluated by von Kossa staining with 5% silver nitrate, followed by 30 minutes of UV exposure\[158\]. Additionally, media calcium concentrations (n=5) were quantified using the Arsenazo III dye (Pointe Scientific, Lincoln Park, MI), with absorbance measured at 620 nm using a microplate reader\[159\].

The expression of hypertrophic markers, such as MMP13 and type X collagen (n=3), were measured using reverse transcription followed by polymerase chain reaction (RT-PCR). The oligonucleotide primer sequences were: \( \beta \)-actin: CTGCCGCACTCAGAAAAT (sense), ACCGTGGTGCTAGAGGCC (antisense); MMP13: ACATCCCAAACGCCAGACAA (sense), GATGCAGCCGCAAGAAAT (antisense); Type X Collagen: TGGATCAAAGGCGGATGTG (sense), GCCCAGTAGGTCCAATGAC (antisense). Total RNA was isolated using the TRizol (Invitrogen) extraction method, with the isolated RNA reverse-transcribed into cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen). The cDNA product was then amplified for 35 cycles with recombinant Platinum Taq DNA polymerase (Invitrogen). Expression band intensities of relevant genes were analyzed semi-quantitatively and normalized to the housekeeping gene \( \beta \)-actin using ImageJ.
3.2.7 Statistical Analysis

Results are presented in the form of mean ± standard deviation, with \( n \) equal to the number of samples analyzed. A two-way analysis of variance (ANOVA) was performed to determine the effects of mineral presence and culturing time on cell response (proliferation, matrix deposition, ALP activity, solution calcium concentration, gene expression, or cell size), as well as scaffold parameters (weight, thickness, swelling ratio, or mechanical properties). The Tukey–Kramer post-hoc test was used for all pair-wise comparisons, and significance was attained at \( p<0.05 \). All statistical analyses were performed using JMP IN software (SAS Institute, Cary, NC).

Additionally, linear regression analysis of the correlation between mechanical properties and matrix deposition were performed. Specifically, mechanical properties and matrix content (GAG and collagen) from the cell-seeded alginate and alginate+HA groups (\( n=6 \)/group) were correlated at both day 14 and day 28 (\( R^2 \), slope, \( p \)-value). It is well established that the mechanical properties of cartilage are related to collagen and GAG content[160,161]. Therefore, the correlation between mechanical properties (\( E_{eq} \) and \( |G'| \)) and scaffold collagen and GAG content were determined both individually and in combination, using linear regression models.

3.3 Results

3.3.1 Acellular Scaffold Characterization

Acellular alginate and alginate+HA scaffolds were visualized with environmental electron microscopy (Fig. 3.1A). The alginate scaffold was homogeneous and uniform in appearance. While HA was distributed throughout the alginate+HA scaffold, aggregation of HA particles was observed at the bottom of the scaffold. Energy-dispersive X-ray analysis confirmed the presence of sodium (Na), chlorine (Cl), and calcium (Ca) in both the alginate and alginate+HA scaffolds as a result of the sodium-alginic salt and divalent Ca\(^{2+} \) cross-linking reaction (Fig. 3.1A). The presence of the phosphorus (P) peak was only detected in the alginate+HA scaffolds, accompanied by elevated peak intensity for Ca (Fig. 3.1A).

Both acellular alginate and alginate+HA scaffolds exhibited changes in swelling ratio over time (Fig. 3.1B), with a significant increase detected during the first 24 hours of culture. Thereafter, swelling of the alginate group remained unchanged until a significant decrease was observed after three weeks of
culture, which is most likely related to scaffold degradation. In contrast, the swelling ratio of the alginate+HA group increased significantly by day 28. It is noted that at all time points examined, the swelling ratio of the alginate+HA group remained significantly lower than that of the alginate group.

3.3.2 Cellular Scaffold Characterization

The cell-seeded alginate and alginate+HA scaffolds measured significantly higher wet weight as compared to their corresponding acellular controls at day 14 and day 28 (Fig. 3.1C). While there was no change in wet weight of the acellular scaffolds from day 1 to day 14, a significant increase was found over the first two weeks of culture in the cell-laden scaffolds. No significant difference in wet weight was detected between the cellular alginate and alginate+HA groups. The cell-seeded scaffolds measured a significantly larger diameter as compared to their corresponding acellular controls at day 28 (Fig. 3.1C), and again, no difference was found between chondrocyte-laden alginate and alginate+HA groups. Both types of scaffolds were significantly thicker as compared to their corresponding acellular controls at day 14. Furthermore, a greater scaffold thickness was measured for the alginate+HA group as compared to the alginate group at day 28 (p<0.05).

3.3.3 Cell Growth and Extracellular Matrix Deposition

Chondrocytes remained viable over time (Fig. 3.2A) and were spherical in shape in the alginate scaffolds. Similar to published studies of chondrocytes cultured in alginate[155], cell number decreased initially and stabilized thereafter (Fig. 3.2B). Interestingly, a significantly higher number of cells was found on the alginate+HA scaffolds as compared to the alginate control at day 7, 14, and 28.

Matrix deposition was uniformly distributed throughout the depth of the scaffolds (Fig. 3.2A). Collagen production increased in both types of scaffolds over time, with the alginate+HA group measuring significantly higher collagen content than the alginate control at day 28 (Fig. 3.3A). In order to account for differences in cell number between alginate and alginate+HA scaffolds, collagen content was also normalized to scaffold DNA content. It was found that collagen deposition per cell was 30% higher in alginate+HA scaffolds. These results were confirmed by histological staining (Fig. 3.3C). No positive staining was seen for type I collagen in either scaffold type, whereas type II collagen staining was present
in alginate scaffolds and strongly positive for cells cultured in the alginate+HA group. Proteoglycan deposition increased over time for both scaffold groups, and no significant difference between scaffold types was observed (Fig. 3.3B). At day 14, GAG deposition per cell was significantly lower in the HA-containing scaffolds only at day 14, but this difference was not observed at day 28. Histological analysis revealed that GAG deposition was evident throughout all scaffolds, with localization of matrix deposition clustered around individual chondrocytes (Fig. 3.3C).

3.3.4 Structure-Function Relationship

The mechanical properties of both acellular and cell-seeded alginate and alginate+HA scaffolds were assessed under unconfined compression and dynamic shear. The cell-seeded groups consistently exhibited higher $|G^*|$ and phase shift angle as compared to their respective acellular controls after two weeks of culture (Fig. 3.4, p<0.05). In general, a high phase shift angle (i.e., $\delta \to 90^\circ$) represents a highly viscous material, while a low value ($\delta \to 0^\circ$) indicates minimal internal energy damping in a material, with $\delta = 0^\circ$ defining an elastic material (i.e., no energy dissipation). Furthermore, the cell-seeded alginate+HA scaffolds measured significantly higher $|G^*|$ and phase shift angle than the alginate scaffolds at day 28 (Fig. 3.4).

To investigate the relationship between matrix deposition and scaffold mechanical properties, normalized matrix content (GAG, collagen content/wet weight) was systematically correlated with mechanical properties. For the alginate group, mechanical properties did not correlate with either GAG or collagen. Similarly for the alginate+HA group, no significant correlation was found between matrix components and either compressive modulus or phase shift angle. However for the alginate+HA group, when matrix content (Col, GAG) were individually correlated with $|G^*|$, a positive linear correlation with either GAG ($R^2=0.79$, Fig. 3.5A) or collagen content ($R^2=0.84$, Fig. 3.5B) was detected. Furthermore, when the matrix components were both correlated with mechanical properties (GAG+Col), the coefficient of determination between total matrix content and $|G^*|$ increased to 0.89 (p<0.05, Fig. 3.5C).
3.3.5 Mineralization and Hypertrophy

Chondrocyte ALP activity was evident in both alginate and alginate+HA scaffolds, albeit with a significant temporal decrease in activity observed in both groups. At day 7, ALP activity was significantly lower in the alginate+HA group as compared to the alginate group, although by day 28, cells in the alginate+HA group measured a higher ALP activity (Fig. 3.6A, p<0.05). Media calcium was significantly lower for the alginate+HA group as compared to the alginate controls and plain media at day 14 (Fig. 3.6B). In contrast, media for the acellular alginate+HA and alginate groups did not differ from plain media. Mineral deposition visualized by von Kossa confirmed the distribution of HA particles in the alginate+HA scaffold observed by ESEM (Fig. 3.6C). By day 28, positive von Kossa staining was detected in the matrix surrounding chondrocytes.

In terms of cell size, the average chondrocyte cross-sectional area was found to be $1164 \pm 541 \mu m^2$ in the alginate control at day 28 (Fig. 3.7A). In contrast, cells cultured in alginate+HA scaffolds measured a cross-sectional area of $2927 \pm 823 \mu m^2$ at day 28, representing a 250% increase as compared to the alginate control (p<0.05). Expression of MMP13 was detected in both scaffold groups, with a significant downregulation observed from day 14 to day 28 in the alginate+HA group (Fig. 3.7B). At day 14, MMP13 expression was significantly higher in the alginate+HA group as compared to the alginate group. Runx 2 expression decreased over time, with no difference observed between groups (data not shown). While minimal type X collagen expression and deposition was observed in the alginate group, it was upregulated for the alginate+HA group over time (Fig. 3.7C, p<0.05). In addition, type X collagen expression in the alginate+HA group was significantly higher than that of the alginate control and corresponded with strongly positive type X collagen staining.

3.3.6 Effect of Chondrocyte Population

Mineralization potential of and matrix deposition by deep zone chondrocytes (DZC) in alginate and alginate+HA scaffold were also compared to those of full thickness chondrocytes (FTC) collected from all three layers of articular cartilage (surface, middle, and deep). In terms of biosynthesis, no significant difference in total collagen content was observed between DZC and FTC when cultured in the alginate control. However, in the alginate+HA scaffold, significantly higher collagen deposition was
evident for the DZC group at day 28 as compared to the FTC group (Fig. 3.8A). Proteoglycan deposition in alginate scaffolds was significantly higher for the DZC versus the FTC group (Fig. 3.8B, p<0.05). In the alginate+HA scaffolds, higher proteoglycan deposition was also measured with DZC (p<0.05).

In terms of mineralization potential, DZC exhibited significantly higher ALP activity than FTC when cultured in alginate scaffolds for all time points tested. While DZC cultured in alginate+HA measured significantly higher ALP activity as compared to FTC at day 1, no significant difference between these groups was detected thereafter (Fig. 3.8C). By day 28, type X collagen expression was at basal levels for both DZC and FTC in alginate scaffolds. However, in the alginate+HA scaffolds, significantly higher type X collagen expression was evident for the DZC group as compared to the FTC group (Fig. 3.8D).

3.4 Discussion

The objective of this study is to evaluate the potential of a composite alginate+HA scaffold for osteochondral interface regeneration or more specifically, for the formation of a calcified cartilage matrix. To this end, the response of deep zone chondrocytes in alginate scaffolds with and without hydroxyapatite was determined over time and compared to those of full thickness chondrocytes. It was found that chondrocytes deposited a well-distributed proteoglycan- and collagen-rich matrix in alginate scaffolds. Interestingly, deep zone chondrocytes cultured in alginate+HA scaffolds exhibited elevated collagen deposition, resulting in higher compressive and shear mechanical properties than the ceramic-free alginate controls. Despite lower ALP activity, deep zone chondrocytes in the composite scaffold were found to be hypertrophic, with significantly greater cell size, as well as upregulated expression of MMP13 and type X collagen deposition. Moreover, these observed effects were chondrocyte population-dependent, as enhanced collagen deposition and hypertrophy was only observed for deep zone chondrocytes and not for full thickness chondrocytes which contains cells from all three cartilage zones. Collectively, the results of this study demonstrate that a composite alginate+HA hydrogel scaffold seeded with deep zone chondrocytes supports the formation of a calcified cartilage-like matrix.

It was observed here that while the addition of hydroxyapatite to alginate had no effect on total proteoglycan deposition, it resulted in hypertrophy and a significant increase in collagen synthesis by
deep zone chondrocytes. On the other hand, the presence of HA tempers GAG production while simultaneously enhancing collagen deposition by individual chondrocytes. This is not unexpected as decreased proteoglycan content, and specifically loss of large proteoglycan aggregates, has been reported during cartilage mineralization[162]. Both chondrocyte hypertrophy and elevated collagen deposition are also suggestive of calcified cartilage matrix formation, since it has been postulated that collagen allows for aggregation of mineral crystals as well as organized crystal growth and mineral deposition by hypertrophic chondrocytes[91]. In this study, both matrix elaboration and scaffold degradation contributed to changes in mechanical properties over time. As expected for an ionotropic gel such as alginate, degradation occurs as a result of decrosslinking, as evidenced by the lower wet weight found in the acellular scaffolds by day 28[163]. However, in the cell-laden alginate and alginate+HA scaffolds, matrix deposition compensated for hydrogel degradation and resulted in significantly higher shear mechanical properties[139].

More specifically, higher matrix synthesis by deep zone chondrocytes cultured in alginate+HA scaffolds resulted in significantly greater magnitude of dynamic shear modulus and phase shift angle by day 28. While no structure-function relationship was evident in the correlation analysis for the alginate scaffolds, the magnitude of dynamic shear modulus was found to correlate positively with matrix deposition in the alginate+HA scaffolds. Furthermore, there was an increase in the magnitude of the correlation coefficient when both proteoglycan and collagen were related to the magnitude of dynamic shear modulus, suggesting the formation of a functional cartilage matrix[160,161]. It is noted that while the resulting scaffold mechanical properties in this study were only a fraction of those of the native calcified cartilage interface[89], the positive correlations between cell-mediated matrix deposition and scaffold mechanical properties demonstrate the potential regeneration of both biomimetic and functional tissue in the hydrogel-ceramic composite scaffold. While the mechanical properties of the Alginate+HA scaffold do not yet meet those of calcified cartilage[99], we plan to optimize the scaffold (e.g. HA content) and culture conditions (e.g. mechanical loading) to potentially increase its mechanical properties in future studies.

In this study, it was observed that deep zone chondrocytes seeded in alginate+HA scaffolds became hypertrophic, as evidenced by the increase in cell size and the upregulation of both type X
collagen and MMP13 over time. This may be analogous to endochondral ossification, during which proliferating chondrocytes mature into hypertrophic chondrocytes that express type X collagen and MMP13, exhibit alkaline phosphatase activity and eventually mineralize[137,164-167]. However, unlike bone there was no evidence of type I collagen deposition and the scaffold remains rich in type II collagen, thereby more closely resembling cartilage. Interestingly, the presence of hydroxyapatite in the alginate hydrogel promoted chondrocyte hypertrophy without an accompanying significant increase in mineralization potential. It is possible that the pre-incorporated ceramic phase of the composite scaffold is biomimetic, and its availability diminishes the need for further cell-mediated mineralization.

As full thickness chondrocytes have been widely used for cartilage tissue engineering, another objective of this study was to determine the optimal chondrocyte population for osteochondral interface regeneration. To this end, the response of full thickness chondrocytes was compared to that of deep zone chondrocytes in alginate and alginate+HA scaffolds. It was found that deep zone chondrocytes produced more collagen and proteoglycan, and exhibited higher ALP activity than full thickness chondrocytes. The higher mineralization potential and proteoglycan biosynthesis observed here for deep zone chondrocytes in the alginate controls are in agreement with published studies[5,168]. Interestingly, mineralization potential and hypertrophic marker expression were only upregulated when deep zone chondrocytes were cultured in alginate+HA scaffolds. Furthermore, our previous studies of surface zone and middle zone chondrocyte seeded in hydrogel scaffolds measured basal ALP activity over time, with no response to HA presence[169]. These results suggest that deep zone chondrocytes seeded in the alginate+HA scaffold represents an optimal combination for osteochondral interface tissue engineering.

When comparing zonal populations of cells it is also critical to recognize the presence of progenitor cells that reside in cartilage[170], especially as the enzymatic digest will yield a mix population of cells. However, given the serum-free conditions, lack of growth factor stimulation, and relatively low cell seeding density in this study, it is not anticipated that progenitor cells will contribute significantly to the observed cell response.
3.5 Conclusions

In summary, it is observed that the alginate+HA composite scaffold promotes deep zone chondrocyte-mediated formation of a calcified cartilage-like matrix, with higher mechanical properties than ceramic-free alginate scaffolds. While this study demonstrates that deep zone chondrocytes are responsive to hydroxyapatite presence, the mechanical properties of this degradable hydrogel system in serum-free media are not sufficient to meet the demands of the osteochondral interface. Moreover, it is challenging to control hydroxyapatite particle distribution in alginate. Therefore, a non-degradable hydrogel will be explored to optimize deep zone chondrocyte-mediated calcified cartilage formation.
Figure 3.1. Scaffold characterization. (A) Hydrogel scaffolds were visualized with environmental electron microscopy (15 kV, 100x, Day 1, n=2). Energy-dispersive X-ray analysis confirmed the presence of phosphorus (P) in the alginate+HA scaffolds. (B) At all time points examined, the swelling ratio of the alginate+HA group was lower than that of the alginate group (*p<0.05, n=4). (C) Cell culture resulted in higher wet weight, larger scaffold diameter and thickness as compared to corresponding acellular controls (^p<0.05, n=4). Alginate+HA scaffolds were significantly thicker than the alginate scaffolds by day 28 (*p<0.05), with no difference in wet weight or diameter observed between the cell-seeded groups.
Figure 3.2. Cell viability, morphology, and growth. (A) Chondrocytes remained viable over time in the hydrogel scaffolds (15kV, 10x, n=2), with uniform matrix deposition throughout the depth of the scaffold (H&E, 5x, n=2). (B) Cell number decreased from day 1 to day 7, and remained unchanged thereafter (#p<0.05, n=5). A higher number of cells was consistently found on the alginate+HA scaffolds as compared to the alginate control (*p<0.05).
Figure 3.3. Extracellular matrix deposition. (A) Collagen production increased in both types of scaffolds over time (#p<0.05, n=5), with the alginate+HA group measuring significantly higher collagen content than the alginate control by day 28 (*p<0.05). (B) While proteoglycan deposition increased over time for both scaffold groups (#p<0.05, n=5), no significant difference between groups was observed. (C) While there was minimal staining for type I collagen in both groups, there was strong positive type II collagen staining for the alginate+HA group at day 28 (10x, 20x, n=2).
**Figure 3.4. Mechanical properties.** The compressive modulus (A), magnitude of the complex shear modulus (B), and phase shift angle (C) of both acellular and DZC-seeded scaffolds were determined. The cell-seeded groups consistently exhibited higher \( |G^*| \) and phase shift angle as compared to their respective acellular controls \(^p<0.05, n=3\). Moreover, by day 28, a higher \( |G^*| \) and phase shift angle were found in the alginate+HA scaffolds as compared to the alginate control \(^*p<0.05\).
Figure 3.5. Structure-function correlation between matrix content and scaffold mechanical properties. A positive correlation between magnitude of dynamic shear modulus and (A) glycosaminoglycan (GAG), as well as (B) collagen (Col) content, was identified for the alginate+HA group only (n=6, *p<0.05). (C) A positive correlation between $|G^*|$ and glycosaminoglycan (GAG), as well as collagen (Col) content, was identified for the alginate+HA group only (*p<0.05). Furthermore, the coefficient of determination ($R^2$) between matrix content and $|G^*|$ increased when both GAG and collagen are correlated with mechanical properties (GAG+Col).
Figure 3.6. Mineralization potential and mineral distribution. (A) Cell ALP activity decreased over time in both scaffold groups (#p<0.05, n=5). At day 7, a significantly lower ALP activity was found in the alginate+HA group, although by day 28, the alginate+HA group measured a higher ALP activity than the alginate group (*p<0.05). (B) Alginate+HA scaffolds had lower media [Ca] at Day 14 as compared to alginate controls (*p<0.05, n=5). (C) In addition to pre-incorporated HA, a mineralized matrix was only observed in the alginate+HA group (10x, n=2).
Figure 3.7. Chondrocyte hypertrophy. (A) Cells in the alginate+HA scaffolds measured a larger cross-sectional area than those in the alginate control (*p<0.05, n=20). Expression of hypertrophic markers such as (B) MMP13 and (C) type X collagen were significantly higher in the alginate+HA group at day 14 (*p<0.05, n=3), and the upregulation of type X collagen persisted at day 28 with strong positive staining. Type X collagen staining was positive only for the alginate+HA group at day 28 (n=2).
Figure 3.8. Effect of chondrocyte population. (A) By day 28, higher collagen content was measured for the alginate+HA scaffolds seeded with deep zone chondrocytes (DZC) versus full thickness chondrocytes (FTC) (*p<0.05, n=5). (B) Higher proteoglycan deposition was detected in the DZC-seeded alginate and alginate+HA groups as compared to those seeded with FTC (*p<0.05, n=5). (C) Higher ALP activity was measured for DZC as compared to FTC at day 1 (*p<0.05, n=5). (D) At day 28, higher type X collagen expression was measured for the alginate+HA scaffolds seeded with DZC versus FTC (*p<0.05, n=5).
CHAPTER 4: OPTIMIZATION OF FUNCTIONAL HYDROGEL-CERAMIC SCAFFOLD FOR CARTILAGE-TO-BONE INTERFACE FORMATION
4.1 Introduction

In Chapter 3, hydroxyapatite (HA) particles incorporated in alginate hydrogels were shown to support chondrocyte viability, collagen deposition, and hypertrophy. While these results are promising and demonstrate the feasibility of hydrogel-ceramic composites for osteochondral interface regeneration, the composite alginate scaffold has several limitations. First, the relatively long gelation time of alginate, which is controlled by the inward diffusion of divalent cations, allows HA particles to aggregate at the bottom of the gel. It is therefore difficult to control HA distribution in alginate. Secondly, given the ionotropic nature of alginate, whereby cross-linking is achieved most commonly with calcium ions, it would be more optimal to have a scaffold system whose calcium content is modulated only with the pre-incorporation of hydroxyapatite particles. Another limitation of the alginate-ceramic scaffold is that its mechanical properties are substantially lower than that of cartilage or the native osteochondral interface. To address these concerns, we will evaluate the response of deep zone chondrocytes in agarose hydrogels.

4.1.1 Background and Motivation

This study focuses on the design and optimization of an agarose-ceramic composite scaffold for osteochondral interface tissue engineering. By pre-incorporating a ceramic phase into the hydrogel, scaffold mechanical properties and osteointegration are expected to be enhanced. Agarose is a well-characterized hydrogel that has been used extensively for chondrocyte culture and cartilage tissue engineering[47,171-173]. Moreover, physiologically-relevant mechanical properties which approach those of the native cartilage have been reported with chondrocyte-laden agarose constructs[30,171].

4.1.2 Objectives

In this study, the hydrogel-composite scaffold will be optimized based on cell type, HA size, and dose for formation of a calcified cartilage-like matrix in vitro. The first objective of this study is to compare the response of interface-relevant cells in HA-free and HA-containing agarose scaffolds. While deep zone chondrocytes (DZC) have been utilized for regeneration of calcified cartilage[106,107,174,175], hypertrophic chondrocytes are the resident cell population identified at the native osteochondral
interface[90]. In this study, to induce DZC hypertrophy, thyroid hormone (T3) will be used to increase cell alkaline phosphatase activity and upregulate collagen X expression[137,176]. It is hypothesized that compared to unstimulated DZC, the induced hypertrophic chondrocytes (DZC+T3) will better support the formation of a calcified cartilage-like matrix when cultured in HA-containing agarose scaffolds.

The second objective of the study is to optimize the hydrogel-ceramic composite scaffold in terms of HA particle size and dose. At the native osteochondral interface, chondrocytes are embedded within a calcified matrix, which is comprised of HA crystals on the order of 15-20 nm, as well as micron-sized HA aggregates[74,87]. The effects of incorporating nano- versus micron-sized HA particles in agarose scaffolds will therefore be investigated, with a size-dependent response anticipated for hypertrophic chondrocytes. In addition to particle size, the dose-dependent effects of HA particles will be investigated. The calcium content of native calcified cartilage has been estimated to range from 1 to 28%[88]. Therefore a dose-dependent effect on cell response is anticipated, and given that mineral content correlates positively with tissue mechanical properties[89], it is expected that scaffold mechanical properties will also be modulated by HA dose. Findings from these studies will help to identify the optimal parameters (cell phenotype, HA size, dose) for biomimetic interface scaffold design and the formation of calcified cartilage in vitro.

4.2 Materials and Methods

4.2.1 Cells and Cell Culture

Primary chondrocytes were isolated from the deep zone cartilage of five immature bovine knee joints (Green Village, NJ) by segregating the bottom third of articular cartilage and removing any remaining calcified cartilage[5]. Briefly, the isolated cartilage pieces were pooled and digested for 16 hours with collagenase type II (3.5 activity units/mg, Worthington, Lakewood, NJ) in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, GA), 2% antibiotics (10,000 U/ml penicillin, 10 mg/ml streptomycin), and 0.2% antifungal (amphotericin B). The cell suspension was then filtered before plating (30 µm, Spectrum, Rancho Dominguez, CA). Isolated chondrocytes were maintained in fully-supplemented media for 48 hours before seeding[174]. All media supplements were purchased from Cellgro-Mediatech unless otherwise specified.
4.2.2 Agarose and Agarose-Hydroxyapatite Scaffold Fabrication

Sterile 4% agarose solution (Type VII, Sigma) was made in phosphate buffered saline (PBS) and combined 1:1 (by volume) with cell suspension (20 x 10^6 cells/mL). The cell-agarose mixture was cast between two glass platens and allowed to gel at room temperature for 15 minutes, resulting in a final seeding density of 10 x 10^6 cells/ml in 2% agarose. To fabricate HA-containing scaffolds, ceramic particles were first mixed with the cell suspension, then combined 1:1 with a 4% agarose solution to form 2% agarose scaffolds containing 1.5 w/v% HA. Individual disks (5 mm diameter x 2.4 mm height) were cored with a sterile biopsy punch (Sklar Instruments, West Chester, PA). Both acellular and cellular scaffolds were cultured under humidified conditions at 37°C and 5% CO₂, maintained in ITS media consisting of DMEM supplemented with 1% ITS+ Premix (BD Biosciences, San Jose, CA), 1% antibiotics, 0.1% antifungal, and 40 µg/ml proline (Sigma). The media was changed every other day and supplemented each time with fresh 50 µg/ml ascorbic acid (Sigma).

In order to induce hypertrophy, DZC were stimulated with triiodothyronine (T3, Sigma, St. Louis, MO) during the first three days of culture[176]. The optimal dose was determined by assessing chondrocyte ALP activity after stimulation with 0, 10, 25, 200, 1000, or 2000 nM of T3 (Fig. 4.1). By day 7, a significantly higher alkaline phosphatase (ALP) activity was detected for DZC cultures stimulated with 10 nM of T3 as compared to HA-free control, with the highest ALP activity observed in the 25 nM T3 group. However, no significant difference in ALP activity was measured beyond 25 nM of T3. Based on these observations, chondrocyte hypertrophy was induced by stimulation with 25 nM of T3 in this study.

The responses of DZC and DZC stimulated by T3 (DZC+T3) were compared in HA-free and HA-containing agarose scaffolds over a two-week culturing period. The effect of HA size on DZC and DZC+T3 was evaluated by comparing cell response in agarose scaffolds with micron-sized (18-28 µm, Sigma) and nano-sized (140-240 nm, Nanocerox, Ann Arbor, MI) HA particles. Next, utilizing the optimal chondrocyte population (DZC vs. DZC+T3) and HA size (micron vs. nano), the effect of ceramic dose was investigated by comparing scaffolds with 0%, 1.5%, 3.0%, or 6.0 w/v% HA. In this study, a scaffold HA content range of 0-6% by wet weight is equivalent to a range of 0-70% by dry weight. These values were selected in order to be able to test HA doses which are lower, comparable or higher than those reported for the native interface.
4.2.3 Scaffold Characterization

As-fabricated scaffolds were characterized in terms of ceramic distribution, content, and chemistry. Gross morphology of the scaffolds was assessed with a stereoscope in both cross-sectional and top views. Distribution of HA particles (n=2) was visualized using environmental scanning electron microscopy (ESEM, 15 kV, JEOL 5600LV, Tokyo, Japan). Scaffold water content (wet weight-dry weight/wet weight, n=3) was determined following desiccation for 24 hours (CentriVap Concentrator, Labconco Co., Kansas City, MO), and ceramic content (ash weight/dry weight, n=3) was measured by thermogravimetric analysis (TA Q-50, TA Instruments, New Castle, DE). The sample was heated to 100°C, followed by a 20°C/minute ramp to 700°C, and the residual weight was then determined. Elemental composition (n=2) of the scaffolds was ascertained by energy dispersive x-ray analysis (EDAX, 15kV, FEI Quanta 600, FEI Co., Hillsboro, OR).

The size, chemistry, structure of micro-HA and nano-HA particles used in the fabrication of the composite scaffolds were also compared. The diameter (n=18) of the micron-sized particles was measured directly using ImageJ. A zeta-sizer was used to determine the size of nano-HA (Zetasizer Nano-ZS Test Measurement System, Malvern, Worcestershire, UK). Briefly, a particle solution of 10% dextran solution and 1% HA was sonicated prior to the measurement to prevent particle aggregation. Dynamic light scattering was then used to directly measure the diffusion of particles and convert to size via the Stokes-Einstein relationship. Ceramic chemistry was examined using Fourier transform infrared spectroscopy (FTIR, FTS 3000MX Excalibur Series, Digilab, Randolph, MA), whereby the samples were air-dried in a chemical hood, and FTIR spectra were collected in absorbance mode (400 scans, resolution at 4 cm⁻¹). Finally, crystallographic structure of HA was confirmed with X-ray diffraction (XRD, X-ray Diffractometer, Inel, Artenay, France). The samples were evaluated over a range of 0 to 120 degrees, with a step size of 0.029 degrees.

4.2.4 Cell Proliferation and Matrix Deposition

Cell proliferation (n=5) was determined using the PicoGreen® total DNA assay (Molecular Probes, Eugene, OR). Briefly, the samples were lysed with 500 μl of 0.1% Triton-X solution (Sigma), and an aliquot of the sample (25 μl) was added to 175 μl of PicoGreen® working solution. Fluorescence was
measured with a microplate reader (Tecan, Research Triangle Park, NC), at the excitation and emission wavelengths of 485 and 535 nm, respectively. Total cell number was obtained by converting the amount of DNA per sample to cell number using the conversion factor of 7.7 pg DNA/cell[150].

Collagen deposition (n=5) was quantified with a modified hydroxyproline assay[177]. Briefly, the samples were first desiccated for 24 hours and then digested for 16 hours at 60°C with papain (600 µg protein/ml) in 0.1M sodium acetate (Sigma), 10 mM cysteine HCl (Sigma), and 50 mM ethylenediaminetetraacetate (Sigma). A 40 µl aliquot of the digest was hydrolyzed with 10 µl of 10 M sodium hydroxide and autoclaved for 25 minutes. The hydrolyzate was then oxidized by a buffered chloramine-T reagent for 25 minutes before the addition of Ehrlich’s reagent. Sample absorbance was measured at 550nm (Tecan), and the collagen content was obtained by interpolation along a standard curve of bovine collagen I (Sigma). Additionally, collagen distribution (n=2) was visualized by Picrosirius red staining. Briefly, the samples were first fixed in 10% neutral buffered formalin with 1% cetylpyridinium chloride (Sigma) for 24 hours, followed by dehydration with an ethanol series. Prior to staining and imaging, the dehydrated samples were embedded in paraffin (Type 9, Richard-Allan Scientific, Kalamazoo, MI), sectioned from the center of the scaffold (7 µm sections), and mounted on microscope slides.

Deposition of collagen I, II, and X (n=2) were evaluated using immunohistochemistry. Monoclonal antibodies for collagen I (1:20 dilution) and collagen II (1:100 units dilution) were purchased from Abcam (Cambridge, MA), while collagen X antibody (1:1 dilution) was obtained from the Developmental Studies Hybridoma Bank (University of Iowa). Paraffin sections were treated with 1% hyaluronidase for 30 min at 37°C to remove proteoglycan and incubated with primary antibody overnight. A FITC-conjugated secondary antibody (1:200 dilution, LSAB2 Abcam) was used and sections were imaged via confocal microscopy at excitation and emission wavelengths of 488 nm and 568 nm, respectively.

Sample glycosaminoglycan content (GAG, n=5) was determined with a modified 1,9-dimethylmethylene blue (DMMB) binding assay[152-154], with chondrotin-6-sulfate (Sigma) as the standard. The absorbance difference between 540nm and 595nm was used to improve the sensitivity in
signal detection. Distribution of GAG (n=2) was visualized histologically by Alcian blue staining of paraffin-embedded sections[150].

4.2.5 Scaffold Mechanical Properties

Scaffold mechanical properties (n=3) were determined using a shear-strain controlled rheometer (TA instruments, New Castle, DE) following published protocols[155]. Briefly, each sample was placed between two flat porous platens and immersed in DMEM to prevent dehydration. First, the equilibrium compressive Young’s modulus (E_{eq}) was calculated at 15% compressive strain, which is within the physiological range for articular cartilage[156]. Secondly, a dynamic shear test was performed (0.01 Hz to 10 Hz) with a logarithmic frequency sweep at a shear strain amplitude of 0.01 radian. Both the magnitude of the complex shear modulus (|G^*|) and phase shift angle (δ) between the applied strain and the resulting torque were determined.

4.2.6 Mineralization and Chondrocyte Hypertrophy

Alkaline phosphatase (ALP) activity (n=5) was quantified using an enzymatic assay based on the hydrolysis of p-nitrophenyl phosphate (pNP-PO_4) to p-nitrophenol (pNP)[157]. Briefly, the samples were lysed in 0.1% Triton-X solution, then added to pNP-PO_4 solution (Sigma) and allowed to react for 30 min at 37°C. Sample absorbance was measured at 415 nm. In addition, mineral distribution (n=2) was evaluated with Alizarin red staining and by von Kossa staining with 5% silver nitrate, followed by 30 minutes of UV exposure[158]. Additionally, media calcium concentrations (n=5) were quantified using the Arsenazo III dye (Pointe Scientific, Lincoln Park, MI), with absorbance measured at 620 nm using a microplate reader[159].

The expression of collagen X, matrix metalloproteinase-13 (MMP13), Indian Hedgehog (Ihh), and parathyroid hormone-related protein (PTHrP) were measured at day 14 using reverse transcription followed by polymerase chain reaction (RT-PCR, n=3), with custom-designed primers. The oligonucleotide primer sequences were: GAPDH: GCTGGTGCTGAGTATGTGGT (sense), CAGAAGGTGCAGAGATGATGA (antisense); Collagen X: TGGATCCAAAGGCGATGTG (sense), GCCCAGTAGGTCCATTAAGGC (antisense); MMP13: ACATCCCAAACGCCCAGACAA (sense),
GATGCAGCCGAGAAGAAT (antisense); Ihh: ATCTCGGTGATGAACCAGTG (sense),
CCTTCGTAATGCAGCGACT (antisense); PTHrP: ACCTCGGAGGTGTCCCCTAA (sense),
GCCCTCAGCATCATACAGACCCCAA (antisense). Total RNA was isolated via TRIzol (Invitrogen) extraction,
and then reverse-transcribed into cDNA using the SuperScript III First-Strand Synthesis System
(Invitrogen). The cDNA product was amplified with recombinant Platinum Taq DNA polymerase
(Invitrogen). Expression band intensities of relevant genes were analyzed semi-quantitatively and
normalized to the housekeeping gene glyceraldehydes 3-phosphate dehydrogenase (GAPDH). Finally,
cell size (n=12) was determined by computing cell surface area from the long-axis and short-axis of
individual cells (32x) at day 14.

4.2.7 Statistical Analysis

Results are presented in the form of mean ± standard deviation, with n equal to the number of
samples analyzed. Multi-way analysis of variance (ANOVA) was performed to determine the effects of T3
stimulation, HA presence and size, as well as dose and culturing time. The Tukey–Kramer post-hoc test
was used for all pair-wise comparisons, and significance was attained at p<0.05. All statistical analyses
were performed using the JMP IN software (SAS Institute, Cary, NC). In addition, to evaluate structure-
function relationships, correlations between mechanical properties (E<sub>eq</sub>, |G*|, δ) and scaffold collagen and
GAG content were determined either individually (GAG or collagen) or in combination (GAG+collagen)
using linear regression models[174]. Specifically, mechanical properties and matrix content from the HA-
free, micro-HA, and nano-HA groups at were correlated (R<sup>2</sup>, slope, p-value).

4.3 Results

4.3.1 Scaffold Characterization

Acellular HA-free agarose scaffolds were compared to those with either micro-HA or nano-HA in
terms of particle distribution, scaffold chemistry, and ceramic content. The HA-free and HA-containing
scaffolds were visualized with environmental scanning electron microscopy (ESEM). While the agarose
scaffold was homogeneous and uniform in appearance, ceramic particles were uniformly distributed
throughout the micro-HA and nano-HA scaffolds (Fig. 4.2A). Energy-dispersive X-ray analysis detected the presence of calcium (Ca) and phosphorus (P) peaks only in the HA-containing scaffolds (Fig. 4.2A).

Micro-HA and nano-HA powders were characterized in terms of ceramic chemistry and structure. Analysis by FTIR confirmed the presence of carbonate (1400-1500 cm$^{-1}$) peaks in both micro-HA and nano-HA, as well as peaks corresponding to three phosphate vibration modes (962 cm$^{-1}$, 1040-1092 cm$^{-1}$, 561-601 cm$^{-1}$, Fig. 4.2B). Furthermore, the 002, 211, 112, 300, 202, 301, 102, 210, and 212 crystal planes were identified in the XRD diffraction patterns of both micro-HA and nano-HA[178]. These findings confirm the similarity between the micro and nano particles in terms of chemistry and crystallinity.

As expected, scaffolds containing HA measured higher wet weight and dry weight, and therefore, lower water content as compared to HA-free controls (p<0.05, Fig.4.2C). While there was no difference in wet weight between the 1.5% HA and 3% HA scaffolds, a significantly higher wet weight was found with the addition of 6% HA. A dose-dependent increase in dry weight also corresponded with a dose-dependent decrease in water content (p<0.05). In terms of ceramic content, no significant difference was found between the micro-HA and nano-HA scaffolds, and the 3% and 6% HA groups measured 43.8±1.2% (mg HA/mg DW) and 66.3±0.8% (mg HA/mg DW), respectively. The HA-free scaffolds measured a compressive modulus of 2.9±0.2 kPa and $|G^*|$ of 4.8±0.6 kPa (Fig. 4.2C). While the addition of 1.5% HA and 3% HA did not significantly alter mechanical properties, the presence of 6% micro-HA resulted in a significant increase in both compressive modulus (4.3±0.2 kPa) and $|G^*|$ (8.7±1.1 kPa). No significant difference in phase shift angle was found among any of the groups tested. In addition, no significant difference in scaffold dimensions and mechanical properties were found between the acellular and cellular scaffolds (data not shown).

4.3.2 Effect of HA Presence and Size on Deep Zone Chondrocytes

To assess the effect of ceramic presence and particle size on cell response, DZC were seeded in HA-free and HA-containing agarose scaffolds. Histology staining revealed that both cells and matrix deposition were uniform throughout the depth of the scaffolds for all groups examined. Moreover, cell number remained relatively constant over time for the HA-free and nano-HA groups (Fig. 4.3). In contrast, a significant increase in cell number was measured for the micro-HA group during the first week
of culture. By day 14, a higher cell number was found in the micro-HA group as compared to the HA-free group (p<0.05), with no difference detected between the nano-HA and micro-HA groups. While no significant difference in matrix content was detected between the scaffold groups examined, a significant increase in both GAG and collagen deposition was found from day 1 to day 7 for all scaffold groups, with no further increase detected thereafter. As expected, mineralization potential of DZC decreased over time for all groups (p<0.05), with no significant difference seen between HA-free and HA-containing scaffolds. While the expression of genes relevant to chondrocyte maturation (collagen X, MMP13, Ihh, PTHrP), were detected in all DZC-seeded scaffolds at day 14, no significant difference was found between groups.

4.3.3 Effect of HA Presence and Size on Hypertrophic Chondrocytes

For hypertrophic chondrocytes (DZC+T3), a significant increase in cell number was measured between day 7 and day 14 for the HA-free and nano-HA groups (Fig. 4.4). No change in cell number was found over time for the micro-HA group. At both day 7 and day 14, significantly higher cell number was measured in the HA-free and nano-HA scaffolds as compared to micro-HA scaffolds. Proteoglycan deposition increased over the first week of culture for all groups, with no change thereafter (p<0.05, Fig. 4.4). By day 14, significantly higher GAG content was measured in the micro-HA scaffold as compared to both nano-HA and HA-free scaffolds. While no change in collagen content was measured during the first week of culture, significant increases were observed for all groups between day 7 and day 14. The highest collagen content was found in the micro-HA group as compared to either the HA-free or nano-HA groups (p<0.05). Histological staining at day 14 confirms the deposition of GAG and collagen for all groups, with strongly positive staining evident in the micro-HA scaffold. While no positive staining for collagen I was evident for any of the groups tested, collagen II staining was observed in HA-free, micro-HA, and nano-HA scaffolds after two weeks of culture.

No significant change in ALP activity was detected for hypertrophic chondrocytes in HA-free scaffolds during the two weeks of culture (Fig. 4.5). In contrast, a decrease in ALP activity was first measured from day 1 to day 7 when these cells were cultured in HA-containing scaffolds (p<0.05), followed by a significant increase in ALP activity during the second week of culture. By day 14,
significantly higher ALP activity was measured in HA-containing scaffolds as compared to the HA-free group, with no difference detected between the micro-HA and nano-HA groups.

Von Kossa staining at day 14 confirms the uniform distribution of HA inside the hydrogel scaffold, as well as evidence of mineralization throughout the hydrogel (Fig. 4.5). A significant decrease in media calcium concentration was measured at day 14 for both acellular and cell-laden micro-HA groups as compared to plain media control. In contrast, no such change in calcium concentration was seen for the nano-HA group. Furthermore, no significant difference in media calcium was measured between corresponding acellular and cell-laden scaffolds for both the HA-free and HA-containing groups.

Chondrocyte hypertrophy was also assessed by measuring cell size, the expression of hypertrophic markers, and deposition of collagen X. In terms of cell size, while there was no change in chondrocyte aspect ratio, the hypertrophic DZC cultured in HA-containing scaffolds measured a larger surface area by day 14 (p<0.05), with no significant difference detected between the micro-HA and nano-HA groups. Collagen X expression was upregulated at day 14 for hypertrophic chondrocytes cultured in HA-containing scaffolds as compared to HA-free scaffolds. Immunohistochemistry confirmed the deposition of collagen X in HA-containing groups, with no difference found between micro-HA and nano-HA groups.

While there was no difference in MMP13 and PTHrP expression between groups, \textit{Ihh} was upregulated in the HA-containing scaffolds as compared to the HA-free scaffold (p<0.05). Again, no difference in \textit{Ihh} expression was measured between micro-HA and nano-HA groups.

\textit{4.3.4 Effect of HA Presence and Size on Scaffold Mechanical Properties}

For the hypertrophic chondrocyte-seeded groups, mechanical properties of the HA-containing scaffolds were compared to those of HA-free scaffolds at day 14. As expected, all cell-seeded scaffolds measured significantly higher compressive modulus, magnitude of dynamic shear modulus, and phase shift angle over their corresponding acellular controls (Fig. 4.6). After two weeks of culture, the micro-HA group measured higher compressive and shear moduli as compared to the HA-free control (p<0.05), with no statistical difference detected between the nano-HA and HA-free scaffolds. No difference in phase shift angle between the micro-HA scaffold and HA-free control was measured, although a significantly lower angle was found for the nano-HA group as compared to all other groups.
In order to determine the structure-function relationship of the hydrogel-ceramic composites, linear correlation analysis was performed for hypertrophic chondrocytes seeded in HA-free, micro-HA and nano-HA scaffolds (Fig. 4.6). Not surprisingly, a positive correlation between GAG and compressive modulus was observed for all scaffolds. Interestingly, higher correlation coefficients were found between GAG and compressive modulus for the micro-HA ($R^2=0.93$) and nano-HA groups ($R^2=0.83$) as compared to the HA-free group ($R^2=0.74$). There was also a positive correlation between GAG and $|G^*|$ for all groups, which again was higher for the micro-HA ($R^2=0.93$) and nano-HA scaffolds ($R^2=0.91$) as compared to the HA-free scaffolds ($R^2=0.75$). With respect to the phase shift angle, a correlation with GAG was detected for the HA-free group ($R^2=0.67$) and micro-HA group ($R^2=0.86$), with no positive correlation evident in the nano-HA group.

Collagen content was also correlated to scaffold mechanical properties. Positive correlation was found between collagen and compressive modulus for HA-free ($R^2=0.74$) and micro-HA groups ($R^2=0.71$), with the highest correlation seen in the nano-HA group ($R^2=0.96$). Collagen content also correlated with $|G^*|$ for the HA-free ($R^2=0.77$), micro-HA ($R^2=0.90$), and nano-HA ($R^2=0.83$) groups. Similar to GAG, collagen correlated with phase shift angle only for the HA-free ($R^2=0.68$) and micro-HA ($R^2=0.78$) groups.

Correlations were also performed with both GAG and collagen to determine their synergistic effects. No significant correlation between combined matrix content and any of the mechanical properties investigated was seen in the HA-free group. In contrast, for the micro-HA scaffolds, significant correlations were measured with combined matrix content and compressive modulus ($R^2=0.93$), $|G^*|$ ($R^2=0.96$), and phase shift angle ($R^2=0.89$). For the nano-HA group, combined matrix content also correlated with compressive modulus ($R^2=0.97$) and $|G^*|$ ($R^2=0.91$).

4.3.5 Effect of Micro-HA Dose on Hypertrophic Chondrocytes

Given the significantly higher ALP activity, matrix content, and mechanical properties measured with hypertrophic chondrocytes (DZC+T3) cultured in agarose scaffolds with micro-HA, scaffold design is further optimized by testing the response of DZC+T3 as a function of HA dose in (0%, 1.5%, 3%, and 6w/v% micro-HA). Significant increases in cell number were measured over time at all doses of HA, with no apparent difference found as a function of HA content (Fig. 4.7A). While the deposition of GAG
increased over the 14-day culturing period for all groups tested (p<0.05), the 1.5% and 3% HA groups measured significantly higher GAG content at day 14 as compared to the 0% HA and 6% HA groups. Similarly, collagen deposition increased between day 1 and day 14 in all scaffolds (p<0.05), and significantly higher collagen content was measured in 1.5% and 3% HA scaffolds as compared to the 0% HA scaffold after two weeks of culture. No difference in collagen content was found between the 6% HA and the other groups examined. Histological staining confirmed positive and uniform deposition of GAG and collagen in all scaffolds by day 14 (Fig. 4.7A).

The ALP activity of hypertrophic chondrocytes cultured in HA-containing scaffolds increased significantly over time (Fig. 4.8). After two weeks of culture, chondrocytes in the 1.5%, 3%, and 6% HA scaffolds measured significantly higher ALP activity as compared to the 0% HA group, with the highest ALP activity found in the 3% HA group. As expected, staining intensity for both calcium (Alizarin red) and phosphate (von Kossa) increased with increasing HA dose. Interestingly, there was evidence of mineral deposition between pre-incorporated HA particles (Fig. 4.8). In terms of hypertrophic markers, collagen X was significantly upregulated in the 3% HA scaffold as compared to the 0% HA group, with an additional four-fold increase seen in the 6% HA group by day 14. Upregulation of MMP13 was detectable only in the 6% HA scaffold (p<0.05). In addition, by day 14, Ihh and PTHrP expression were significantly downregulated for the 3% HA and 6% HA groups as compared to the 0% HA and 1.5% HA groups.

4.3.6 Effect of Micro-HA Dose on Scaffold Mechanical Properties

The mechanical properties of acellular 1.5% and 3% HA scaffolds were not significantly different from those of the acellular 0% HA scaffolds by day 14, although both higher compressive and shear moduli were measured for the acellular 6% HA group (p<0.05, Fig.4.7B). When the scaffolds were seeded with hypertrophic chondrocytes, the 3% HA and 6% HA groups measured a higher compressive modulus than the 0% HA group (p<0.05), with no significant difference seen between the 3% HA and 6% HA groups. No difference in compressive modulus was found for the 1.5% HA group as compared to the 0% HA group. In terms of magnitude of dynamic shear modulus, a dose-dependent increase was observed in the 1.5% HA and 3% HA groups (p<0.05). The |G*| of the 6% HA scaffolds was higher than that of the 0% HA or 1.5% HA scaffolds (p<0.05), although it was not significantly different as compared
to the 3% HA scaffold. No difference in phase shift angle was detected between the 0% HA, 1.5% HA, and 6% HA groups, although a significantly higher angle was measured for the 3% HA group at day 14 with respect to the 0% HA group.

4.4 Discussion

The goal of this study is to design and optimize a hydrogel-hydroxyapatite (HA) scaffold for calcified cartilage formation. To this end, the response of deep zone chondrocytes and hypertrophic chondrocytes were compared in agarose scaffolds with and without HA, and the ceramic phase was also optimized in terms of HA particle size and dose in order to promote hypertrophy and calcified matrix deposition. While ceramic presence had minimal effect on biosynthesis by deep zone chondrocytes, it significantly modulated both matrix deposition and mineralization by hypertrophic chondrocytes. Moreover, while hypertrophy was upregulated in HA-containing hydrogels independent of ceramic size, enhanced matrix deposition was observed only in scaffolds with micron-sized HA. When the response of hypertrophic chondrocytes was further assessed as a function of micro-HA content, a dose-dependent effect on biosynthesis and the expression of hypertrophic markers was found. It was observed that chondrocyte matrix deposition, alkaline phosphatase (ALP) activity, as well as scaffold compressive and shear moduli were the highest in scaffolds containing 3% micron-sized HA particles, resulting in a significant structure-function correlation between matrix content and mechanical properties of the hydrogel-ceramic scaffold.

The results of this study suggest that hypertrophic chondrocytes are optimal for calcified cartilage regeneration in the agarose-HA scaffold. While no change in deep zone chondrocyte biosynthesis and hypertrophy were detected here in the presence of HA, hypertrophic chondrocytes exhibited enhanced mineralization potential with the addition of HA. To ascertain whether the observed effects of micro-HA are in response to ceramic presence or are merely the result of the inclusion of a particulate phase within the hydrogel matrix, the hypertrophic chondrocytes were also cultured in agarose scaffolds with inert, micron-sized glass beads. It was found that bead presence had no effect on chondrocyte biosynthesis or mineralization potential, demonstrating that the effects observed were due to HA (Fig. 4.9). Published studies have shown that cartilage mineralization is proceeded by accumulation of critical ions such as
calcium and phosphate[179,180], it is possible that the composite hydrogel-ceramic scaffold can serve as a reservoir of these ions which regulate chondrocyte mineralization. This is confirmed by the precipitation of calcium from the media seen in both acellular and cellular HA-containing groups. In addition to hypertrophy and ALP activity, hypertrophic chondrocytes measured higher GAG and collagen deposition in agarose scaffolds containing micro-HA. As mineral deposits in calcified cartilage are often associated with collagen fibers[91], the elevated collagen deposition may facilitate cell-mediated mineralization. Characterization studies of growth plate cartilage has reported a localized increase in proteoglycan deposition by hypertrophic chondrocytes surrounding mineral clusters[181], whereby the proteoglycans have been postulated to serve as nucleation sites for further mineralization[182]. These results collectively suggest that cell-mediated mineralization, coupled with GAG and collagen synthesis, occurred in agarose scaffolds containing micro-HA. Not only was the total matrix content higher in scaffolds with micro-HA, both GAG and collagen synthesis per cell were significantly greater than those of the HA-free scaffolds.

It is interesting to note that while chondrocyte hypertrophy is not particle size-dependent, enhanced matrix deposition is found only when the hypertrophic chondrocytes are cultured in a hydrogel matrix containing micro-HA. These effects may be attributed to the fact that cell-ceramic interactions are significantly different for particles which are of similar size to individual cells versus those which are an order of magnitude smaller, as is the case for the nano-HA group. Furthermore, the mineral present in native calcified cartilage is reported to consist of large aggregates, which more closely approximate the micro-HA particles in size. Interestingly, higher matrix content in the micro-HA group also translated into greater mechanical properties, which is beneficial for functional cartilage repair since native calcified cartilage is critical for load transfer from cartilage to bone. More importantly, the strongly positive correlation found here between total matrix content and the magnitude of dynamic shear modulus in the micro-HA group is indicative of the deposition of a functional cartilage matrix, mimicking the interaction between proteoglycans and collagen which have been reported to strengthen cartilage in shear[161].

It is observed here that the response of hypertrophic chondrocytes to micro-HA is also dose-dependent. Chondrocyte ALP activity is found to be the highest in the 3% micro-HA scaffolds, accompanied by upregulated collagen X. Interestingly, PTHrP, which has been shown to suppress
chondrocyte hypertrophy[183], is expressed at basal levels with 3% HA. It is likely that in the HA-containing agarose system, hypertrophy is being induced by multiple factors, including thyroid hormone stimulation, presence of HA, and their synergistic interactions. Moreover, the addition of 1.5% and 3% micro-HA to the hydrogel also enhances matrix deposition by hypertrophic chondrocytes relative to the HA-free control. For example, collagen deposition per cell is significantly higher for the 1.5% and 3% groups as compared to the control. Decreased biosynthesis with 6% HA may be explained by the lack of space for extensive matrix elaboration given the large number of HA particles pre-incorporated into the hydrogel. While no significant difference in matrix content between scaffolds with 1.5% and 3% HA were found at day 14, scaffold mechanical properties increased with ceramic content. It is likely that the proteoglycan-collagen network is more effectively reinforced by the greater number of ceramic particles in the 3% HA than in the 1.5% HA scaffolds.

In this study, HA was uniformly distributed throughout the agarose hydrogel. This is likely due to the fact that upon cooling, agarose solutions set into a gel in which chain segments are stabilized by hydrogen bonding[184], unlike the cross-linking of alginate which is limited by the rate of ion diffusion[174]. Consequently, agarose crosslinks uniformly and rapidly close to its gelation temperature (30°C), which prevents sedimentation and results in uniform ceramic distribution within the scaffold. Furthermore, the lack of background staining for calcium or phosphorus in the agarose control confirms that this scaffold design allows for a controlled investigation of ceramic presence on chondrocytes biosynthesis. Moreover, while the as-fabricated alginate and agarose scaffolds measured comparable compressive and shear moduli, the cell-laden agarose scaffolds exhibited higher mechanical properties than the chondrocyte-seeded alginate scaffolds after two weeks of culture. These effects were enhanced with the addition of HA to the hydrogel, with a nearly 10-fold increase in mechanical properties for micro-HA-containing agarose scaffolds as compared to alginate-micro HA scaffolds.

It is also interesting to note that the response of deep zone chondrocytes varied in alginate and agarose composite scaffolds. Whereas chondrocyte hypertrophy and collagen deposition were upregulated in HA-containing alginate scaffolds (Chapter 3), no such response was seen for deep zone chondrocytes in HA-containing agarose. In contrast, deep zone chondrocyte response in alginate closely resembles these responses of hypertrophic chondrocytes in this study, which exhibited hypertrophy and
elevated matrix synthesis in scaffolds containing HA. One possible explanation for the differences is that, unlike in agarose, the relatively high concentration of calcium in alginate hydrogels post cross-linking may regulate chondrocyte maturation. Wang et al. reported that activity of calcium-sensing receptors by extracellular calcium coincides with an increase in collagen X expression and ALP activity in CFK2 cells[185]. The addition of calcium chloride has also been shown to increase collagen X synthesis by hypertrophic chondrocytes[186]. Therefore, while the agarose system which has significant functional advantages over alginate, the addition of a bioactive ceramic which releases mineral inductive ions may further enhance the responses observed in this study, and their effects will be investigated in future studies.

Calcified cartilage formation is in general the result of chondrocyte hypertrophy and mineralization within a proteoglycan- and collagen-rich matrix. In this study, an optimal HA content and size were identified, as the hypertrophic chondrocytes produced a matrix rich in proteoglycan and collagen within the composite scaffold containing 3.0 w/v% of micron-sized HA particles. These response are likely due to the fact that the mineral content of the 3.0% HA group most closely approximates that of native tissue[87], and micron-size aggregates are abundant at the osteochondral interface[74]. The addition of the biomimetic mineral phase within the scaffold not only modulates chondrocyte biosynthesis and hypertrophy, it also minimizes the need for extensive cell-mediated mineral deposition. Furthermore, the hydrogel-ceramic scaffold recapitulates biomimetic synergy between proteoglycan and collagen, resulting in physiologically relevant structure-function relationships which are likely beneficial for clinical implementation.

Clinically, it is envisioned that the hydrogel-ceramic scaffold may be utilized in combination with hydrogel-based cartilage tissue engineering grafts, whereby the ceramic phase is used to promote calcified cartilage formation and osteointegration, and the hydrogel phase of the composite would facilitate integration with a hydrogel-based cartilage graft. Future studies will focus on optimizing the composite hydrogel scaffold system in terms of cell seeding density and mineral chemistry, with the long-term goal of achieving functional and integrative cartilage repair.
4.5 Conclusions

This study compares the response of deep zone chondrocytes and hypertrophic chondrocytes cultured in HA-free and HA-containing agarose scaffolds. The ceramic phase of scaffold was optimized by testing both HA particle size and dose. Although deep zone chondrocyte response is not affected by either ceramic presence or size, hypertrophic chondrocytes exhibit higher ALP activity in the presence of HA and deposit more proteoglycan and collagen in the agarose+HA scaffolds with micro-HA versus nano-HA particles. In terms of ceramic dose, the 3% micro-HA group is optimal for calcified cartilage formation, measuring the highest matrix content, mineralization, and mechanical properties. In addition to allowing uniform distribution of ceramic particles throughout the scaffold, the mechanical properties of agarose+HA scaffold are significantly higher than those of alginate+HA scaffolds. These results demonstrate that the optimized agarose+HA composite supports the formation of a calcified cartilage-like matrix and is a promising scaffold for osteochondral interface tissue engineering.
Figure 4.1. T3 optimization to induce chondrocyte hypertrophy. Exogenous addition of 10 nM T3 significantly increased DZC ALP activity at day 14 (*p<0.05), with higher ALP activity measured with the addition of 25 nM T3. No further increases in ALP activity were detected with increasing T3 dose. The addition of 25 nM T3 significantly increased ALP activity at both day 7 and day 14 (*p<0.05), and no differences in GAG or collagen deposition were detected. (*p<0.05: difference between groups; #p<0.05: change over time).
Figure 4.2. Scaffold characterization. (A) Light microscopy images of HA-free and HA-containing scaffolds (top-view and side-view). There is no change in scaffold dimensions (n=5) with the addition of HA particles. Environmental Scanning Electron Microscopy (ESEM, 100x) reveals uniform particle distribution and Energy Dispersive X-ray Analysis (EDAX) confirms the presence of calcium (Ca) and phosphorus (P) in the HA-containing scaffolds. (B) Fourier Infrared Spectroscopy (FTIR) and X-ray Diffraction confirms that there are no differences in chemistry and crystallinity between micro-HA and nano-HA. (C) The addition of HA resulted in a dose-dependent decrease in scaffold water content (*p<0.05: relative to HA-free, **p<0.05: relative to HA-free and 1.5% HA groups, ***relative to HA-free, 1.5%, and 3% HA groups). Significant increases in compressive modulus (*p<0.05, n=3) and magnitude of dynamic shear modulus (**p<0.05, n=3) were found for the 6% HA group.

<table>
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<th>Day 1, Acell</th>
<th>WW (mg)</th>
<th>% Water</th>
<th>DW (mg)</th>
<th>% Ceramic</th>
<th>HA Size (µm)</th>
<th>E (kPa)</th>
<th></th>
<th>(kPa)</th>
<th>δ(degrees)</th>
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<td>0.7 ± 0.3</td>
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<td>N/A</td>
<td>2.9 ± 0.2</td>
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<td>0.9 ± 0.7</td>
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<tr>
<td>1.5% Nano HA</td>
<td>42.7 ± 0.9*</td>
<td>96.7 ± 0.4*</td>
<td>1.4 ± 0.2*</td>
<td>27.8 ± 5.6</td>
<td>0.19 ± 0.05</td>
<td>3.5 ± 0.9</td>
<td>5.3 ± 0.8</td>
<td>2.1 ± 1.5</td>
<td></td>
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<tr>
<td>1.5% Micro HA</td>
<td>42.6 ± 2.0*</td>
<td>97.0 ± 0.3*</td>
<td>1.3 ± 0.1*</td>
<td>26.9 ± 1.7</td>
<td>23.54 ± 5.62</td>
<td>3.1 ± 0.2</td>
<td>4.7 ± 0.8</td>
<td>1.5 ± 0.3</td>
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<tr>
<td>3% Micro HA</td>
<td>46.0 ± 1.2**</td>
<td>95.1 ± 0.5**</td>
<td>2.2 ± 0.3**</td>
<td>43.8 ± 1.2***</td>
<td>23.54 ± 5.62</td>
<td>3.6 ± 0.5</td>
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<tr>
<td>6% Micro HA</td>
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<td>91.6 ± 1.1***</td>
<td>3.9 ± 0.4***</td>
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Figure 4.3. Effect of HA presence and size on deep zone chondrocytes. A higher cell number was measured at day 14 for the micro-HA group as compared to HA-free control (*p<0.05, n=5). Both cells and matrix are uniformly distributed throughout the scaffolds (H&E, 10x, bar= 500 μm, Day 14, n=2), and GAG as well as collagen content increased for all groups over the first week of culture (#p<0.05, n=5). Cell ALP activity decreased over time for all groups (#p<0.05, n=5), with no change detected in the expression of hypertrophic markers due to either the presence or size of HA particles (n=3).
Figure 4.4. Effect of HA presence and size on hypertrophic chondrocytes: biosynthesis. While a significant increase in cell number was measured over time for the HA-free and nano-HA groups (#p<0.05, n=5), no change was measured in micro-HA scaffolds. Both GAG and collagen content were significantly higher at day 14 for the micro-HA group (*p<0.05, n=5). Corresponding histology reveals strongly positive matrix staining for the micro-HA group (Alcian Blue for GAG and Picrosirius Red for collagen, 10x, bar=200 μm, Day 14, n=2). More specifically, strongly positive collagen II staining and minimal collagen I staining, was observed for all groups (Immunohistochemistry, 10x, bar=200 μm, Day 14, n=2).
Figure 4.5. Effect of HA presence and size on hypertrophic chondrocyte: mineralization. The presence of HA significantly elevated ALP activity in hypertrophic chondrocytes by day 14 (*p<0.05, n=5), with a corresponding increase in cell size in the HA-containing scaffolds (*p<0.05, bar=25 μm, 32x, n=12). Both type X collagen and Ihh expression are upregulated by day 14 (*p<0.05, n=3), with positive staining for type X collagen found at day 14 in the HA-containing scaffolds independent of particle size (10x, bar=200 μm, n=2). A significant decrease in media calcium was only found for the acellular and cell-laden micro-HA groups as compared to plain media control (*p<0.05, n=5), with no difference seen due to HA size. Von Kossa and Alizarin red staining confirm the uniform distribution of HA particles in the HA-containing scaffolds, with strongly positive mineral staining seen in the micro-HA group (10x, bar=200 μm, Day 14, n=2).
Figure 4.6. Effect of HA presence and size on hypertrophic chondrocytes: scaffold mechanical properties. (A) The micro-HA containing scaffold measured a higher compressive modulus and magnitude of dynamic shear modulus as compared to the HA-free control (*p<0.05, Day 14), and the addition of nano-HA resulted in a lower phase shift angle (*p<0.05). Note that mechanical properties of all cell-laden scaffolds were significantly higher than those of the corresponding acellular controls (*p<0.05, Day 14, n=3). (B) For the micro-HA group, linear regression analysis revealed a strong positive correlation between scaffold mechanical properties (E, |G*|, and δ) and matrix content (GAG, Col) as well as matrix interactions (GAG+Col) (*p<0.05, Day 14, n=6).
Figure 4.7. Effect of HA dose on hypertrophic chondrocytes: growth, biosynthesis and scaffold mechanical properties. (A) While there is no dose-dependent effect on cell growth, the addition of 1.5% and 3% micro-HA resulted in higher GAG and collagen deposition by day 14 (*p<0.05, n=5), as confirmed by histology (Alcian Blue for GAG and Picosirius Red for collagen 10x, bar=200 μm, Day 14, n=2). (B) Cell-laden scaffolds measured significantly higher compressive modulus, magnitude of dynamic shear modulus, and phase shift angle as compared to corresponding acellular controls at day 14 (n=3). A dose-dependent increase in scaffold compressive and shear moduli was found, with those of the 3% and 6% HA groups being significantly higher than that of the 0% HA group (*p<0.05, n=3). In addition, cell-laden scaffolds with 3% HA measured a higher phase shift angle as compared to the 0% HA group (*p<0.05, n=3).
Figure 4.8. Effect of HA dose on hypertrophic chondrocytes: mineralization. A dose-dependent effect in ALP activity was found, with the highest enzyme activity detected in the 3% HA groups (*p<0.05, n=5). Cell ALP activity also increased over time for the both the 3% and 6% HA groups (#p<0.05, n=5). Similarly, a dose-dependent increase in type X collagen expression was found, with the highest collagen X and MMP13 expression detected in the 6% HA group by day 14. In contrast, significant suppression of PTHrP and Ihh expression was found at the higher HA doses (3% and 6% HA, *p<0.05, n=5). For mineralization, both von Kossa and Alizarin red staining revealed the presence of pre-incorporated HA particles, as well as cell-mediated mineral deposition (10x, bar=200 μm, Day 14, n=2).
Figure 4.9. Effect of inert particle presence on hypertrophic chondrocytes. No change in cell number was detected over time of between groups. While ALP activity, GAG and collagen deposition increased over time (#p<0.05), no significant differences were measured between groups.
CHAPTER 5: EFFECT OF CELL DENSITY ON CALCIFIED CARTILAGE FORMATION
5.1 Introduction

In Chapter 4, chondrocyte response was evaluated on hydrogel scaffolds containing hydroxyapatite. The results demonstrate that ceramic presence, size, and dose regulate matrix deposition and mineralization by hypertrophic chondrocytes. Furthermore, structure-function relationships were determined by demonstrating positive correlations between matrix content and mechanical properties. This chapter investigates the effects of scaffold cell seeding density on the formation of calcified cartilage matrix.

5.1.1 Background and Motivation

Although previous studies optimizing the composite hydrogel-ceramic scaffold used a seeding density of 10 million cells/mL, to approximate the cellularity of the mature lower radial zone in human cartilage[65], low scaffold mechanical properties remain a significant challenge. Cell density is an important parameter which modulates tissue formation; in native cartilage, the transition from tissue development to tissue homeostasis with age is accompanied by a decrease in chondrocyte density[187]. Inspired by these findings, studies have used higher seeding densities for tissue regeneration in vitro and have achieved greater matrix deposition and scaffold mechanical properties[148,155,188].

5.1.2 Objectives

The objective of this study is to vary cell seeding density in the hydrogel-ceramic composite scaffold. It is hypothesized that increasing initial cell seeding density will result in higher matrix deposition and subsequently, enhanced scaffold mechanical properties. The seeding density resulting in tissue with ceramic content, matrix content, and mechanical properties which most closely resemble those of native calcified cartilage will be identified as the optimal density for calcified cartilage regeneration in vitro.

5.2 Materials and Methods

5.2.1 Cells and Cell Culture

Primary cells were isolated from the bottom 30% of articular cartilage, designated as the deep zone, of five immature bovine knee joints (Green Village, NJ) following published protocols[5]. Briefly, the
isolated cartilage pieces were digested for 16 hours with collagenase type II (3.5 activity units/mg, Worthington, Lakewood, NJ) in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, GA), 2% antibiotics (10,000 U/ml penicillin, 10 mg/ml streptomycin), and 0.1% antifungal (amphotericin B). The cell suspension was then filtered before plating (30 µm, Spectrum, Rancho Dominguez, CA). Isolated chondrocytes were maintained in fully-supplemented media[174]. All media supplements were purchased from Cellgro-Mediatech unless otherwise specified.

5.2.2 Agarose Scaffold Fabrication

Cells from each of the five calves were pooled together prior to scaffold seeding, and composite agarose+HA scaffolds were fabricated as previously described (Chapter 4). Briefly, sterile 4% agarose solution (Type VII, Sigma) was made in phosphate buffered saline (PBS) and combined 1:1 (by volume) with the cell-media suspension. Composite agarose+HA scaffolds were fabricated by pre-incorporating 3 w/v% HA (20-25 µm, Sigma) in the cell suspension prior to casting. The effects of cell seeding density on chondrocyte biosynthesis and scaffold mechanical properties were investigated by comparing scaffolds with 10 million/mL, 20 million/mL and 30 million/mL over a six week culturing period. All samples were cultured under humidified conditions at 37°C and 5% CO₂ and maintained in ITS media, consisting of DMEM supplemented with 1% ITS+ Premix (BD Biosciences, San Jose, CA), 1% antibiotics, 0.1% antifungal, and 40 µg/ml proline (Sigma). The media was changed every other day and supplemented with fresh 50 µg/mL ascorbic acid (Sigma). Deep zone chondrocytes (DZC) were stimulated with 25 nM triiodothyronine (DZC+T3, Sigma, St. Louis, MO) for the first three days of culture to induce the hypertrophic phenotype[5,176].

5.2.3 Scaffold Characterization

To determine scaffold height and diameter (n=5), stereoscope scaffold images were analyzed with ImageJ (National Institute of Health). Water content (water weight/scaffold wet weight, n=5) was measured following desiccation for 24 hours (CentriVap Concentrator, Labconco Co., Kansas City, MO), and ash weight (n=5) was determined by heating the sample to 700°C for 1 hour in an oven (Thermolyne,
Finally, scaffold chemistry was examined using Fourier transform infrared spectroscopy (FTIR), whereby samples before and after heating were air-dried and spectra were collected in attenuated total reflectance mode (ATR, 16 scans, Frontier FT-IR, Perkin Elmer, Waltham, MA).

5.2.4 Cell Proliferation and Matrix Deposition

Cell proliferation (n=5) was determined using the PicoGreen® total DNA assay (Molecular Probes, Eugene, OR), following the manufacturer's suggested protocol. Briefly, the samples were first rinsed with phosphate buffered saline (PBS, Sigma) and the cells were lysed in 300 μl of 0.1% Triton X solution (Sigma). An aliquot of the sample (25 μl) was then added to 175 μl of the PicoGreen® working solution. Fluorescence was measured with a microplate reader (Tecan, Research Triangle Park, NC), at the excitation and emission wavelengths of 485 and 535 nm, respectively. Total cell number in the sample was obtained by converting the amount of DNA per sample to cell number using the conversion factor of 7.7 pg DNA/cell [150].

As previously described (Chapter 4), collagen deposition (n=5) and glycosaminoglycan (GAG) deposition (n=5) were quantified using a simplified hydroxyproline assay [177] and a modified 1,9-dimethylmethylen blue (DMMB) binding assay [152], respectively. Additionally, matrix distribution (n=2) was visualized by Picrosirius red staining for collagen and Alcian blue staining for GAG. Briefly, the samples were first fixed in 10% neutral buffered formalin with 1% cetylpyridinium chloride (Sigma) for 24 hours, followed by dehydration with an ethanol series. Prior to staining and imaging, the dehydrated samples were embedded in paraffin (Type 9, Richard-Allan Scientific, Kalamazoo, MI), sectioned (7 μm), and mounted on microscope slides.

5.2.5 Hypertrophy and Mineralization

The expression of collagen X, MMP13, Ihh, and PTHrP (n=5) were measured at day 14 using reverse transcription followed by real-time polymerase chain reaction (RT-PCR). Total RNA was isolated using the TRIzol (Invitrogen) extraction method. The isolated RNA was reverse-transcribed into cDNA
using the SuperScript III First-Strand Synthesis System (Invitrogen). PCR reactions (25 ul) were carried out using a SYBR GreenER qPCR SuperMix (Invitrogen) on an iCycler instrument (Bio-Rad, Hercules, CA). Relative expression data was quantified using $2^{-\Delta\Delta Ct_{\text{sample}}}$ where $Ct$ is the cycle threshold. Deposition of collagen X collagen ($n=2$) was evaluated using immunohistochemistry. Monoclonal antibody for collagen X (1:1 dilution) was obtained from the Developmental Studies Hybridoma Bank (University of Iowa). After fixation, samples were treated with 1% hyaluronidase for 30 min at 37°C and incubated with primary antibody overnight. A FITC-conjugated secondary antibody (LSAB2 Abcam) was added and sections were imaged under confocal microscopy at excitation and emission wavelengths of 488 nm and 568 nm, respectively.

Alkaline phosphatase (ALP) activity ($n=5$) was quantified using an enzymatic assay based on the hydrolysis of $p$-nitrophenyl phosphate ($p$NP-PO$_4$) to $p$-nitrophenol ($p$NP)[157]. Briefly, the samples were lysed in 0.1% Triton-X solution, then added to $p$NP-PO$_4$ solution (Sigma) and allowed to react for 30 min at 37°C. Sample absorbance was measured at 415nm using a microplate reader (Tecan). Scaffold mineral distribution was visualized with both von Kossa and Alizarin red staining ($n=2$). Microcomputerized tomography ($\mu$CT) was also performed to reconstruct 3D images of mineral distribution. Samples were aligned along their axial direction and stabilized in a 1.5mL centrifuge tube that was clamped in the specimen holder of a vivaCT 40 system (SCANCO Medical AG, Basserdorf, Switzerland). The length of the scaffold was scanned at 15 µm isotropic resolution. The standard $\mu$CT evaluation protocol was performed for the $\mu$CT images[189]. The tissue was processed by Gaussian filtering to decrease the noise influence and then thresholded to extract the mineralized phase.

5.2.6 Mechanical Properties

Scaffold mechanical properties ($n=4$) were determined following published protocols[174]. The diameter ($d$) of each sample was measured with a stereomicroscope (model PP&E 56939; Bausch and Lomb, Rochester, NY) and mechanical tests were performed on a shear-strain controlled rheometer (ARES-LS1, TA instruments, New Castle, DE). Briefly, each sample was placed between two flat porous platens and immersed in PBS to prevent dehydration. The equilibrium compressive Young’s modulus ($E_{eq}$) of the sample was calculated at 15% strain. A dynamic shear test was then performed (0.01 Hz to
10 Hz) with a logarithmic frequency sweep with the specified shear strain amplitude of 0.01 radian, from which the magnitude of the complex shear modulus (|G^*|) and phase shift angle (δ) between the applied strain and the torque response were calculated at 1Hz[155].

5.2.7 Statistical Analysis

Results are presented in the form of mean ± standard deviation, with n equal to the number of samples analyzed. A two-way analysis of variance (ANOVA) was performed to determine the effects of cell seeding density and culturing time. The Tukey–Kramer post-hoc test was used for all pair-wise comparisons, and significance was attained at p<0.05. All statistical analyses were performed using the JMP IN software (SAS Institute, Cary, NC). In addition, to evaluate structure-function relationships, correlations between mechanical properties (E_eq, |G^*|, δ), as well as scaffold matrix, ceramic, and water content were determined using linear regression models[174].

5.3 Results

5.3.1 Effect of Seeding Density on Matrix Deposition

Agarose scaffolds with 3% HA content were seeded at three different cell densities, 10 million/mL, 20 million/mL, and 30 million/mL. No change in cell number was found from day 1 to day 7 for all groups (Fig. 5.1A). However, by day 14, a significant increase in cell number was seen for the 10 million/mL group, and by day 28, a significant decrease in cell number was found for the 30 million/mL group. No change in cell number was detected during the six-week culturing period for the 20 million/mL group, and after four weeks of culture, there was no difference in cell number/wet weight between groups. Total cell number did not change over time for the 10 million/mL and 30 million/mL groups, although significant increases were measured for the 20 million/mL group between day 7 and day 28. After six weeks of culture, the highest cell number was found in the 30 million/mL scaffold (p<0.05), although no difference was detected between the 10 million/mL and 20 million/mL scaffolds.

Over the six weeks of culture, significant increases in weight % collagen were measured for all groups (Fig. 5.1B). Significantly higher collagen content was found in the 30 million/mL scaffold as compared to 10 million/mL scaffold at day 14, and by day 28, higher collagen content was measured in
the 20 million/mL and 30 million/mL scaffolds (p<0.05). Interestingly, after six weeks of culture, the lowest collagen content was measured in the 30 million/mL scaffold. Similar trends were observed when total collagen content was normalized by cell number, although further increases in collagen production per cell were found at day 42 only for the 10 million/mL and 20 million/mL groups (p<0.05). After six weeks of culture, collagen per cell was again lowest in the 30 million/mL scaffold. In terms of total matrix content, total collagen content was higher for the 20 million/mL scaffold as compared to the 10 million/mL scaffold by day 42 (p<0.05), with no significant difference between the 30 million/mL group and any other group (Fig. 5.3).

Proteoglycan content, when normalized by weight, significantly increased over the first week of culture for all groups (Fig. 5.1C). Further increases in GAG were measured for the 20 million/mL and 30 million/mL groups between day 14 and day 28, as well as the 10 million/mL and 20 million/mL groups between day 28 and day 42 (p<0.05). By day 7, scaffolds with 20 million/mL and 30 million/mL cells measured significantly higher proteoglycan content as compared to the 10 million/mL group. By day 28, the highest GAG content found in the 30 million/mL scaffold, intermediate content in the 20 million/mL scaffold, and lowest in the 10 million/mL scaffold. After six weeks of culture, there was higher proteoglycan content in the 20 million/mL and 30 million/mL scaffolds as compared to the 10 million/mL scaffold. When proteoglycan content was normalized by cell number, significant increases over time were again measured for all groups over the first week of culture. Further increases were similarly measured for the 20 million/mL and 30 million/mL groups by day 28 and for the 10 million/mL and 20 million/mL groups by day 42 (p<0.05). Although the highest GAG deposition per cell was again found in the 30 million/mL group at day 28, the 20 million/mL group had the highest GAG by day 42 (p<0.05). In terms of total matrix content, significantly higher GAG was measured in the 20 million/mL and 30 million/mL scaffolds as compared to the 10 million/mL scaffold after six weeks of culture (Fig. 5.3).

Histological staining confirmed strong positive staining for collagen and proteoglycan in all scaffold groups at day 28. There was uniform positive staining for matrix throughout the scaffolds, with no edge effects or depth-dependent variations (Fig. 5.1 and 5.3).
5.3.2 Effect of Seeding Density on Mineralization Potential and Mineral Deposition

Chondrocytes seeded in all groups exhibited increasing ALP activity from day 7 to day 14 (Fig. 5.2A, p<0.05). The ALP activity of chondrocytes in the 10 million/mL and 30 million/mL groups decreased by day 28, with the highest enzymatic activity measured for the 20 million/mL group after four weeks of culture (p<0.05). By day 42, a significant decrease in ALP activity was measured for the 10 million/mL and 20 million/mL groups. In addition to the scaffold-incorporated HA content, the pattern of von Kossa staining changes over time for all groups (Fig. 5.2B). At day 1, distinct HA particles were visible throughout the scaffold, while relatively diffuse positive staining was visible after four weeks of culture.

Collagen X, MMP13, Ihh, and PTHrP were expressed at day 14 by all groups (Fig. 5.2B). Significantly higher MMP13 was detected for the 20 million/mL and 30 million/mL groups as compared to the 10 million/mL group, and Ihh was upregulated for the 30 million/mL group (p<0.05). No differences in PTHrP and collagen X expression were found at day 14 between groups. Corresponding collagen X immunohistochemistry at day 28 showed positive staining for all scaffold groups, with the most positive staining evident in the 20 million/mL scaffold (Fig. 5.2C). After six weeks of culture, a step-wise increase in ash weight was measured between the 10 million/mL, 20 million/mL and 30 million/mL (Fig. 5.3, p<0.05).

In order to further assess mineral distribution for the 20 million/mL group over time, µCT analysis was performed for both acellular and cell-seeded scaffolds for comparison (Fig. 5.2D). It was evident that cell-seeded scaffolds contained higher mineral content as compared to acellular scaffolds at both day 14 and day 28. In terms of scaffold chemistry, the ceramic phase within the scaffold was characterized by phosphate peak (900-1200 cm\(^{-1}\)), whereas scaffold spectra contained both phosphate peaks and the amide I (1590-1720 cm\(^{-1}\)) and amide II peaks (1492-1590 cm\(^{-1}\)).

5.3.3 Structure-Function Relationships

Scaffolds seeded with 20 million/mL chondrocytes were larger in terms of scaffold diameter, thickness, wet weight, and dry weight by day 42 (Fig. 5.3, p<0.05). Interestingly, the 30 million/mL group measured significantly higher wet weight as compared to both the 10 million/mL and 20 million/mL
groups. Despite differences in wet weight, no difference in normalized water content was measured between groups. Interestingly, a non-uniform scaffold shape was evident for the 20 million/mL and 30 million/mL groups, although this was not seen for the 10 million/mL group.

Scaffolds seeded at 20 million/mL measured a compressive modulus of 68 kPa and magnitude of the complex shear modulus of 186 kPa at day 42 and were significantly higher than the mechanical properties of the 10 million/mL scaffolds. Scaffolds seeded at 30 million/mL also measured a higher compressive modulus and magnitude of the complex shear modulus as compared to the 10 million/mL group (p<0.05). There was no difference in phase shift angle between scaffold groups.

Normalized GAG and collagen content at day 42 were plotted against both compressive modulus and the magnitude of the complex shear modulus to determine the presence of linear correlations (Fig. 5.4). No significant correlations between GAG and scaffold mechanical properties were found. On the other hand, collagen content demonstrated negative linear correlations with both compressive modulus ($R^2=0.54$) and magnitude of the complex shear modulus ($R^2=0.72$). Compressive modulus and magnitude of the complex shear modulus were also found to positively correlate with the ratio of GAG-to-collagen ($R^2=0.63$, $R^2=0.71$), water content ($R^2=0.54$, $R^2=0.82$), and ash weight ($R^2=0.29$, $R^2=0.53$).

### 5.4 Discussion

The objective of this study is to optimize calcified cartilage formation using a composite hydrogel-hydroxyapatite (HA) scaffold. To this end, the response of induced hypertrophic chondrocytes in agarose scaffolds with HA was determined as a function of cell seeding density. Chondrocytes maintained their hypertrophic phenotype and deposited a uniform proteoglycan- and collagen-rich matrix in all scaffolds groups over the 42-day culturing period. Seeding density was shown to regulate chondrocyte proliferation and mineralization, as well as the balance between proteoglycan and collagen within the matrix.

Cell density for all scaffold groups reaches an equilibrium value of approximately 5000 cells/mg after four weeks of culture, with cell proliferation only evident for the lowest seeding density group. Enhanced proliferation of chondrocytes at lower seeding densities is in agreement with previously published studies. Porcine chondrocytes seeded on alginate sponges demonstrated higher proliferative
capacity at lower seeding densities[190]. In a study by Kelly et al., full thickness chondrocytes were seeded on agarose scaffolds at 10 million/mL and 60 million/mL and, similar to this study, higher cell proliferation was measured for the group with the lower seeding density in serum-containing media[191]. It is also important to note that in this study, total cell number per scaffold significantly increases over the 6-week culturing period for all groups; the decrease over time measured for the 30 million/mL group reflects increasing scaffold wet weight and not cell loss.

Despite the fact that all scaffolds have similar normalized cell number after four weeks of culture, there are significant differences in matrix content at that time between groups. Although proteoglycan deposition is similar between groups after two weeks of culture, scaffolds with higher initial cell seeding density reach higher proteoglycan values at earlier timepoints. These results differ from previous studies with full thickness chondrocytes which have measured enhanced proteoglycan deposition only at seeding densities higher than 20 million/mL[191]. One possible explanation is that HA enhancement of GAG deposition is not uniform for all seeding densities. Collagen deposition is significantly lower and also lags behind proteoglycan synthesis. Interestingly, the group with the highest proteoglycan content no longer has the highest collagen content by six weeks of culture. It is possible that collagen deposition in this group is hindered by insufficient space or high proteoglycan content[192]. By calculating the GAG-to-collagen ratio, it is possible to determine overall changes in matrix composition. Over time, the ratio decreases for the 10 million/mL group, whereas there is no change for the 20 million/mL group. Interestingly, the GAG-to-collagen ratio peaks at day 28 for the 30 million/mL group, decreasing thereafter. At both day 28 and day 42, the highest seeding group measures the highest ratio as compared to both the 10 million/mL and 20 million/mL groups, and may represent a fundamental shift in chondrocyte biosynthesis and tissue properties.

It is also interesting to note that overall matrix deposition, as well as matrix deposition per cell doubles for the 20 million/mL and 30 million/mL groups at day 28, which corresponds with the ALP activity peak and the onset of mineralization. Just as pre-inciporporated HA content results in higher matrix deposition[193], it is likely that cell-mediated mineralization also upregulates chondrocyte biosynthesis even further. The initial increase in mineralization potential over the first two weeks of culture for all groups is attributed to the thyroid hormone stimulation, since unstimulated cultures measure decreasing
ALP activity from day 1 (data not shown). Interestingly, there is a prolonged peak in mineralization potential with the intermediate seeding density of 20 million/mL, and corresponds to strongly positive collagen X deposition. Given that the pre-incorporated scaffold ceramic content is the same for each group, these findings indicate that the cell-to-ceramic ratio is critical for regulation of hypertrophy and mineralization. After six weeks of culture, cell-mediated mineralization is evident in both histological staining and density-dependent increases in scaffold ash weight. Interestingly, those scaffolds with higher mineral content also measure higher collagen content. Given the biomimetic interaction which exists in vivo between collagen fibers and mineral deposits[91], it not unexpected that mineralization and collagen deposition are interrelated within the in vitro scaffold culture.

While it is initially unexpected that collagen does not positively correlate with scaffold mechanical properties, it is important to consider changes in matrix distribution and organization which may occur for the 20 million/mL and 30 million/mL groups. In a non-degradable system such as agarose, increased matrix deposition and water content due to swelling crowds the polymer matrix once all available space is accommodated. This results in the non-uniform scaffold shape observed in this study, which is also reminiscent of cartilage curling and may similarly signal the presence of residual stress within the scaffold[194]. The high degree of water uptake is a result of the higher GAG content within the scaffolds. Furthermore, the relationship between GAG and collagen is critical for understanding scaffold mechanical properties, since hydrated proteoglycans have been shown to pre-stress the collagen network and increase the shear modulus or articular cartilage[160]. Lastly, it is important to consider the effects of mineralization of scaffold mechanical properties. Previous studies using a similar agarose-HA system have demonstrated that pre-incorporated HA content significantly increases scaffold mechanical properties at 3% and 6%, which approximate the range of mineral content of scaffold in this study[193].

The results of this study collectively demonstrate that initial cell seeding density modulates chondrocyte biosynthesis, mineralization, and scaffold mechanical properties, all of which are important for osteochondral interface tissue engineering. Specifically for calcified cartilage formation in composite agarose-hydroxyapatite scaffolds, a seeding density of 20 million/mL is most optimal. Based on the functional requirements of the calcified cartilage interface, chondrocytes within this scaffold group exhibited the highest biosynthetic activity and mineralization potential. Furthermore, increasing the
seeding density from 10 million/mL to 20 million/mL resulted in higher compressive and shear mechanical properties. Further increases in seeding density hindered matrix biosynthesis. Despite the promising results demonstrated in this study, collagen production remained significantly lower than proteoglycan production. Future studies will focus on implementing this interface scaffold for in vivo tissue formation.

5.5 Conclusions

Cell seeding density is a key tissue engineering parameter which regulates chondrocyte biosynthesis and scaffold properties. Mechanical properties of the 20 million/mL and 30 million/mL scaffolds are higher than those of the 10 million/mL group. However, since ALP activity and matrix content are highest for the 20 million/mL group, the intermediate seeding density is chosen as optimal for calcified cartilage formation in hydrogel-ceramic composite scaffolds.
Figure 5.1. Effect of seeding density on matrix deposition. (A) By day 14, there was a significant increase in cell number only for the 10 million/mL group, and by day 28, there was a significant decrease in cell number only for the 30 million/mL group (#p<0.05, n=5). Histological staining confirms positive and uniform staining for proteoglycan and collagen in all scaffold groups by day 28 (n=2, Day 28, 10x, scale bar=200 um). (B) After four weeks of culture, the 20 million/mL and 30 million/mL groups had significantly higher collagen content as compared to the 10 million/mL group (*p<0.05, n=5). (C) By day 28, there was a step-wise increase in GAG content with increasing cell seeding density (*p<0.05, n=5).
Figure 5.2. Effect of seeding density on hypertrophy and mineralization. (A) The ALP activity of the 20 million/mL group was highest at day 28 (*p<0.05, n=5). (B) Upregulation of MMP13 was measured for the 20 million/mL and 30 million/mL groups, whereas higher Ihh was measured only for the 30 million/mL group (*p<0.05, n=5, Day 14). (C) Von Kossa staining of the scaffolds revealed diffuse staining, and collagen X deposition was most positive for the 20 million/mL group (10x, n=2, Day 28). (D) FTIR characterization of the scaffold shows both HA and protein presence and µCT shows higher mineral content in cell-seeded scaffolds as compared to acellular controls at Day 14 and Day 28 (20 million/mL).
Figure 5.3. Scaffold properties. The 20 million/mL group measured higher scaffold diameter, thickness, wet weight, dry weight, compressive modulus, and magnitude of dynamic shear modulus at day 42 as compared to the 10 million/mL group (*p<0.05, n=5), and there was a further increase in wet weight for the 30 million/mL group as compared to the 20 million/mL group (**p<0.05, n=5). Higher %GAG was found for the 20 million/mL group as compared to the 10 million/mL group (*p<0.05, n=5), although significantly lower % collagen was measured for the 30 million/mL group (*p<0.05, n=5). Staining for matrix and mineral was uniform throughout the scaffolds for all groups (n=2, Day 42, scale bar=3 mm).

<table>
<thead>
<tr>
<th>Day 42</th>
<th>10 M/mL</th>
<th>20 M/mL</th>
<th>30 M/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter (mm)</td>
<td>6.1 ± 0.3</td>
<td>7.1 ± 0.3*</td>
<td>7.3 ± 0.1*</td>
</tr>
<tr>
<td>Thickness (mm)</td>
<td>2.8 ± 0.3</td>
<td>3.3 ± 0.3*</td>
<td>3.4 ± 0.1*</td>
</tr>
<tr>
<td>Wet Weight (mg)</td>
<td>61.5 ± 5.9</td>
<td>100.9 ± 3.7*</td>
<td>118.9 ± 5.6**</td>
</tr>
<tr>
<td>Dry Weight (mg)</td>
<td>9.0 ± 0.8</td>
<td>15.0 ± 0.5*</td>
<td>16.4 ± 1.5*</td>
</tr>
<tr>
<td>Water (%)</td>
<td>85.4 ± 0.0</td>
<td>85.2 ± 0.0</td>
<td>86.2 ± 1.3</td>
</tr>
<tr>
<td>Ash Weight (mg)</td>
<td>3.7 ± 0.8</td>
<td>5.6 ± 0.6*</td>
<td>6.9 ± 0.6**</td>
</tr>
<tr>
<td>GAG (%)</td>
<td>1.26 ± 0.25</td>
<td>1.74 ± 0.27*</td>
<td>1.82 ± 0.30*</td>
</tr>
<tr>
<td>GAG (mg)</td>
<td>0.85 ± 0.15</td>
<td>1.87 ± 0.30*</td>
<td>2.12 ± 0.39*</td>
</tr>
<tr>
<td>Collagen (%)</td>
<td>0.94 ± 0.10</td>
<td>0.89 ± 0.12</td>
<td>0.71 ± 0.10*</td>
</tr>
<tr>
<td>Collagen (mg)</td>
<td>0.66 ± 0.15</td>
<td>0.96 ± 0.16*</td>
<td>0.82 ± 0.13</td>
</tr>
<tr>
<td>E (kPa)</td>
<td>54 ± 9</td>
<td>68 ± 10*</td>
<td>75 ± 4*</td>
</tr>
<tr>
<td></td>
<td>105 ± 14</td>
<td>186 ± 41*</td>
<td>235 ± 20*</td>
</tr>
<tr>
<td>δ (deg)</td>
<td>14.4 ± 2.5</td>
<td>14.9 ± 1.3</td>
<td>13.1 ± 0.9</td>
</tr>
</tbody>
</table>
Figure 5.4. Structure-function relationships. While there was no correlation between GAG content and scaffold mechanical properties, negative correlations were detected between collagen content and both compressive modulus and $|G^*|$. Ash weight correlated only with $|G^*|$ ($R^2=0.53$), whereas water content correlated with both compressive modulus ($R^2=0.54$) and $|G^*|$ ($R^2=0.82$). Finally, the ratio between GAG and collagen also demonstrated a positive correlation between compressive modulus ($R^2=0.63$) and $|G^*|$ ($R^2=0.71$).
CHAPTER 6: UNCALCIFIED-TO-CALCIFIED CARTILAGE SCAFFOLD
6.1 Introduction

In order to implement the interface scaffold for integrative cartilage repair, the hydrogel-ceramic composite will be used in conjunction with a hydrogel-based cartilage graft to form articular cartilage above the calcified cartilage interface. It is critical that the integrity of each tissue region is maintained over time. Specifically, mineral presence and mineralization must be confined to the calcified region, and co-culture of the uncalcified and calcified scaffolds must not impede matrix deposition in either region.

6.1.1 Background and Motivation

In healthy joints, the matrix-rich region directly above the calcified cartilage interface remains mineral-free. Similarly, in order to implement an interface scaffold for clinical repair, an unmineralized proteoglycan-collagen matrix must be maintained above the hydrogel-ceramic interface scaffold. Returning to the original cell-ceramic interaction model developed in Chapter 4, where hydroxyapatite (HA) was directly incorporated into agarose scaffolds for calcified cartilage formation, analogous models were used to assess the maintenance of uncalcified cartilage above the interface. In order to determine the behavior of cells seeded above interface scaffold (FTC or more specifically, DZC), an equivalent amount of micro-HA (1.5w/v%-1 mg/well) was pre-coated on the bottom of cell culture plates prior to culture of the cell-seeded agarose scaffolds (10 million cells/mL, Fig. 6.1). It was found that the ALP activity of both FTC and DZC decreased over time for both groups. While there was no effect on mineralization potential of FTC or DZC, FTC experienced significantly higher GAG deposition and lower collagen deposition in the presence of HA. No effect on DZC GAG deposition was detected, although higher collagen content was found in the group cultured with HA (p<0.05). Within a full thickness defect where the calcified cartilage interface is compromised, chondrocytes within the scaffold are most likely exposed to systemic factors such as thyroid hormone (T3). It was found that while DZC+T3 measured higher ALP activity at day 7 in the presence of HA, there were no significant differences in scaffold matrix content (Fig. 6.2). These findings demonstrate that in addition to monitoring chondrocyte hypertrophy and localization of mineralization to the calcified cartilage interface, matrix deposition must also be monitored on bilayered uncalcified-calcified scaffolds.
While the previously described models are useful for determining chondrocyte response to HA outside the scaffold, it is also necessary to incorporate the ceramic particles directly into the calcified cartilage layer of the bilayered hydrogel scaffold. Therefore, a region of DZC seeded in agarose was layered above an acellular region of HA particles in agarose (1.5 w/v%, Fig. 6.2). Since it has been previously speculated that ions such as calcium participate in modulating chondrocyte mineralization and biosynthesis within the hydrogel-ceramic composite scaffolds, it is not unexpected the encapsulation of HA within agarose affects chondrocyte response. This was confirmed by preliminary data which shows that DZC+T3 measured enhanced ALP activity and matrix content in the bilayered scaffold. This bilayered scaffold will be expanded upon in this chapter to incorporate the optimized cell-seeded interface scaffold from Chapter 4.

6.1.2 Objectives

This study assesses the integrity of uncalcified and calcified matrix regions on a single scaffold. Bilayered scaffolds with ceramic-free and ceramic-containing layers correspond to uncalcified and calcified tissue regions. Although previous studies have developed layered hydrogel designs for cartilage tissue engineering applications [195-197], this is the first study to layer ceramic-free and ceramic-containing agarose. Full thickness chondrocytes seeded in agarose hydrogel has previously been used to regenerate cartilage in vitro[148] and will therefore be used as the uncalcified cartilage graft in this study. In order to assess the integrity of each layer, ‘uncalcified’ and ‘calcified’ regions within the composite scaffolds are compared to corresponding ‘uncalcified-alone’ and ‘calcified-alone’ controls.

To this end, the first objective of the study is to demonstrate the feasibility of fabricating layered hydrogel scaffolds which maintain their integrity in culture. Secondly, the effect of uncalcified-calcified co-culture on each respective region will be assessed, in terms of cell proliferation, matrix deposition, and hypertrophy. Since ceramic presence outside the hydrogel scaffold was shown to upregulate deep zone chondrocyte alkaline phosphatase activity, it is hypothesized that co-culture with the interface scaffold will also modulate chondrocyte mineralization.
6.2 Materials and Methods

6.2.1 Cells and Cell Culture

Primary articular chondrocytes were isolated from five immature bovine knee joints (Green Village Packaging, Green Village, NJ). Deep zone chondrocytes (DZC) were isolated from the bottom 30% of articular cartilage[5,175,198]. Chondrocytes isolated from the entirety of articular cartilage were designated as full thickness chondrocytes (FTC). Briefly, the separated cartilage pieces were incubated for 16 hours with 0.1 w/v% collagenase (Sigma, St. Louis, MO) in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, GA), 2% antibiotics (10,000 U/ml penicillin, 10 mg/ml streptomycin), and 0.1% antifungal (amphotericin B). The cell suspension was then filtered before plating (30 µm, Spectrum, Rancho Dominguez, CA). The isolated chondrocytes were maintained in fully-supplemented DMEM with 10% FBS, 1% non-essential amino acids, 1% antibiotics, and 0.1% antifungal for 48 hours before seeding. All media supplements were purchased from Cellgro-Mediatech unless otherwise specified.

6.2.2 Layered Scaffold Fabrication and Culture

Cell-seeded hydrogel scaffolds were fabricated by mixing the cell suspension at a volume ratio of 1:1 with sterile 4% agarose (Type VII, Sigma) solution in phosphate buffered saline (PBS) at a seeding density of $10^7$ cells/ml. Hydroxyapatite (HA, Sigma) was pre-mixed with cell suspension prior to the addition of agarose to fabricate agarose+HA scaffolds (Chapter 4). The cell-agarose suspension of each layer was cast and allowed to gel at room temperature for 5 minutes prior to casting the subsequent layer. Individual disks (5 mm diameter, 0.75 mm thickness/layer) were cored with a sterile biopsy punch (Sklar Instruments, West Chester, PA). Samples were cultured under humidified conditions at 37°C and 5% CO$_2$ and maintained in ITS media, consisting of DMEM supplemented with 1% ITS+ Premix (BD Biosciences, San Jose, CA), 1% antibiotics, 0.1% antifungal, and 40 µg/ml proline (Sigma). The media was changed every other day and supplemented with fresh 50 µg/mL ascorbic acid (Sigma). In order to induce DZC hypertrophy, cultures were stimulated with 25 nM triiodothyronine (T3, Sigma, St. Louis, MO) for the first three days of culture[5,176].

Co-culture scaffolds comprised of FTC-seeded agarose were gelled above DZC-seeded agarose
with 3w/v% HA (DZC+HA, Fig. 6.3). Cell response was measured in each region by first separating scaffolds into HA-free and HA-containing layers. Chondrocyte biosynthesis and mineralization potential were quantified at days 1, 14, and 28 and subsequently compared to single-culture controls, namely uncalcified-alone and calcified-alone scaffolds.

6.2.3 Chondrocyte Biosynthesis and Hypertrophy

Cell proliferation (n=5) was determined using the PicoGreen® total DNA assay (Molecular Probes, Eugene, OR). Briefly, the samples were lysed with 500 μl of 0.1% Triton-X solution (Sigma), and an aliquot of the sample (25 μl) was then added to 175 μl of the PicoGreen® working solution. Fluorescence was measured with a microplate reader (Tecan, Research Triangle Park, NC), at the excitation and emission wavelengths of 485 and 535 nm, respectively. Total cell number was obtained by converting the amount of DNA per sample to cell number using the conversion factor of 7.7 pg DNA/cell[150].

Collagen deposition (n=5) was quantified using a simplified hydroxyproline assay[177]. Briefly, the samples were first desiccated for 24 hours and then digested for 16 hours at 60°C with papain (600 μg protein/ml) in 0.1M sodium acetate (Sigma), 10 mM cysteine HCl (Sigma), and 50 mM ethylenediaminetetraacetate (Sigma). A 40 μl aliquot of the digest was hydrolyzed with 10 μl 10 M sodium hydroxide and autoclaved for 25 minutes. The hydrolyzate was then oxidized by a buffered chloramine-T reagent for 25 minutes before the addition of Ehrlich’s reagent. Sample absorbance was measured at 550nm (Tecan), and the collagen content was obtained by interpolation along a standard curve of bovine type I collagen (Sigma). Additionally, collagen distribution (n=2) was visualized by Picosirius red staining. Briefly, the samples were first fixed in 10% neutral buffered formalin with 1% cetylpyridinium chloride (Sigma) for 24 hours, followed by dehydration with an ethanol series. Prior to staining and imaging, the dehydrated samples were embedded in paraffin (Type 9, Richard-Allan Scientific, Kalamazoo, MI), sectioned (7 μm), and mounted on microscope slides.

Sample glycosaminoglycan content (GAG, n=5) was determined with a modified 1,9-dimethylmethylene blue (DMMB) binding assay[152-154], with chondroitin-6-sulfate (Sigma) as the standard. The absorbance difference between 540nm and 595nm was used to improve the sensitivity in
signal detection. Distribution of GAG (n=2) was visualized histologically by Alcian blue staining of paraffin embedded sections[150].

Quantitative alkaline phosphatase (ALP) activity (n=5) was determined using an enzymatic assay based on the hydrolysis of p-nitrophenyl phosphate (pNP-PO_4) to p-nitrophenol (pNP)[157]. Briefly, the samples were lysed in 0.1% Triton-X solution, then added to pNP-PO_4 solution (Sigma) and allowed to react for 30 min at 37°C. Sample absorbance was measured at 415nm using a microplate reader (Tecan). The expression of collagen X, matrix metalloproteinase-13 (MMP13), and Indian Hedgehog (Ihh) were measured using reverse transcription followed by polymerase chain reaction (RT-PCR, n=3), with custom-designed primers (Chapter 4). Total RNA was isolated via TRIzol (Invitrogen) extraction, and then reverse-transcribed into cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen). The cDNA product was amplified with recombinant Platinum Taq DNA polymerase (Invitrogen). Expression band intensities of relevant genes were analyzed semi-quantitatively and normalized to the housekeeping gene glyceraldehydes 3-phosphate dehydrogenase (GAPDH).

6.2.4 Statistical Analysis

Results are presented in the form of mean ± standard deviation, with n equal to the number of samples analyzed. A two-way analysis of variance (ANOVA) was performed to determine the effects of co-culture, as well as culture time. The Tukey–Kramer post-hoc test was used for all pair-wise comparisons, and significance was attained at p<0.05. All statistical analyses were performed using the JMP IN software (SAS Institute, Cary, NC).

6.3 Results

6.3.1. Characterization of Multi-Layered Scaffolds

For the duration of the culturing period, the layered scaffold remained intact, and no layer-to-layer separation was observed for any samples. The uncalcified and calcified portions of the scaffold were readily distinguishable; while the FTC layer was relatively translucent, the HA-containing layer was more opaque (Fig. 6.3). A clear distinction between the two regions was also evident in brightfield images (Fig. 6.3).
6.3.2. Effect of Uncalcified-Calcified Co-Culture on Cell Proliferation and Hypertrophy

Uniform cell density was measured for all scaffold layers and scaffold groups at day 1 (Fig. 6.3). There was no change in FTC cell number over the 28-day culturing period for either group. Similarly, there was no change in DZC cell number over time, although higher cellularity was measured for the DZC+HA co-cultured group at day 28.

The ALP activity of chondrocytes within the calcified region was significantly higher than those from the uncalcified region (Fig. 6.3). Decreasing ALP activity was measured for FTC groups over time (p<0.05), and no significant difference in mineralization potential was detected with co-culture in the uncalcified zone. The ALP activity of DZC in the single-culture group was maintained during the first two weeks of culture and decreased significantly thereafter. On the other hand, a significant increase in ALP activity was measured in the calcified region of the co-cultured scaffold between day 1 and day 14, although it decreased to basal levels after four week of culture (p<0.05). The expression of hypertrophic markers, namely collagen X, MMP13, and Ihh, were also assessed at day 14 in the calcified region. It was found that co-culture with FTC significantly upregulated DZC+HA collagen X expression.

6.3.3. Effect of Uncalcified-Calcified Co-Culture on Matrix Deposition

The layered scaffold resulted in two distinct regions of matrix deposition, which was confirmed by histological staining for both GAG and collagen (Fig. 6.4). Minimal GAG deposition was measured over time by FTC in single-culture (Fig. 6.4). On the other hand, there was a significant increase in GAG between day 1 and day 14 for FTC in co-culture, measuring higher GAG content than the single-culture control (p<0.05). However, by day 28, no difference between FTC groups was detected. Significant GAG deposition was also measured in the calcified region during the first two weeks of culture, with no further change in GAG content thereafter, and no difference between single-culture and co-cultured groups was detected.

Significant collagen deposition was measured in the uncalcified region during the first two weeks of culture, with no further change in collagen content thereafter (Fig. 6.4). No difference in FTC collagen deposition between single-culture and co-cultured groups was detected. There was increasing collagen
content for the duration of the culturing period for DZC+HA, with significantly higher collagen content at day 28 for the co-cultured group.

6.4 Discussion

In this study, the clinical application of the interface scaffold for cartilage repair was tested by layering an agarose cartilage graft above the hydrogel-ceramic scaffold. This bilayered scaffold was used to assess the integrity of the ceramic-free and ceramic-containing regions which mimic the uncalcified and calcified cartilage tissues at the native cartilage-to-bone junction. Findings from this study demonstrate that two distinct, yet continuous matrix regions are maintained in the ‘uncalcified’ and ‘calcified’ layers of the scaffold. While the ‘calcified’ region does not promote mineralization within the uncalcified region, chondrocyte hypertrophy is maintained within the agarose+HA matrix.

The ‘calcified’ region measures higher overall matrix content as compared to the ‘uncalcified’ region. This difference in matrix deposition may be attributed to multiple factors. First, there are different chondrocyte populations within each region (SZC+MZC+DZC vs. DZC), with DZC measuring the highest overall matrix synthesis and SZC measuring the lowest in culture; given the same number of DZC and SZC+MZC+DZC, lower matrix deposition is expected by FTC. Unpublished data from our laboratory has also shown that zonal chondrocyte interactions suppress matrix deposition in tri-culture. Furthermore, ceramic presence enhances matrix deposition by deep zone chondrocytes, whereas full thickness chondrocyte biosynthesis remains unaffected (Chapter 3). Therefore, higher matrix deposition within the ‘calcified’ region is attributed to a combination of ceramic-enhanced matrix synthesis and the mixed zonal population within the ‘uncalcified’ region. Although DZC matrix deposition is enhanced in co-culture at day 28, matrix content per cell is not significantly different and is therefore not a result of the increased cellularity on the bilayered scaffold.

Thyroid hormone stimulation serves multiple purposes in this study. First, it induces hypertrophy in DZC, and further enhances mineralization potential in the presence of HA (Chapter 4). Although FTC are also exposed to T3, their ALP activity remains at basal levels[5]. It is therefore not unexpected that higher mineralization potential is measured by DZC within the ‘calcified’ region. Secondly, thyroid hormone simulates the diseased model where chondrocytes are exposed to systemic factors from the
bleeding defect; the concentration of T3 in the bovine blood supply is approximately 2 nM[199]. Although unpublished data from our laboratory has shown that ceramic presence, regardless of proximity to the scaffold, upregulates DZC ALP activity (data not shown), zonal chondrocyte interactions in this study are sufficient to suppress any increase in hypertrophy within the ‘uncalcified’ region[5]. Interestingly, hypertrophy within the ‘calcified’ region is enhanced when layered below FTC. Although it is unlikely that the presence of SZC and MZC upregulates DZC hypertrophy, autocrine communication among DZC has been shown to enhance mineralization[5].

Previous scaffold designs for osteochondral tissue engineering have also formed mineral-free and mineral-containing regions on a single scaffold. One modality involves seeding cartilage-forming cells directly above a calcium-phosphate substrate[37]. Other scaffolds have incorporated a hydrogel-based cartilage layer above devitalized bone[46]. The presence of bone, even in co-culture, was shown to decrease matrix deposition in the cartilage region and inhibited functional cartilage formation in vitro. One possible explanation for the significant decrease in chondrocyte biosynthesis is the high HA content within a 4 mm bone core in contrast to the approximately 1 mg contained within the ‘calcified’ layer of this study. Future in vivo studies are required to determine whether presence of native bone at the defect site will also have detrimental effects on articular cartilage and calcified cartilage matrix formation.

6.5 Conclusions

The results of this study collectively demonstrate that the hydrogel-ceramic interface scaffold can be implemented in a layered scaffold design to regenerate both articular cartilage and calcified cartilage. Chondrocyte response within the ‘uncalcified’ region of the scaffold is not affected by the presence of the hydroxyapatite-containing interface scaffold, and two distinct matrix regions are maintained in culture.
Figure 6.1. Effect of HA presence on cartilage matrix formation. Full thickness chondrocytes (FTC) and deep zone chondrocytes (DZC) were seeded in agarose and cultured in wells containing either plain media or well pre-coated with HA. Both FTC and DZC measured decreasing ALP activity (n=5) over time (#p<0.05). Higher GAG content (n=5) was found in FTC scaffolds in the presence of HA (*p<0.05), although no difference was found in DZC scaffolds. While there was lower collagen content (n=5) in FTC scaffolds in the presence of HA (*p<0.05, higher collagen deposition was measured in DZC scaffolds in the presence of HA.
Figure 6.2. Effect of HA encapsulation on cartilage matrix formation. Deep zone chondrocytes stimulated with thyroid hormone (DZC+T3) were seeded in agarose and cultured in wells pre-coated or in bilayered scaffolds containing a region of acellular agarose+HA. Although HA particle presence outside the scaffold resulted in higher ALP activity (n=5) at Day 7 (*p<0.05), there was no effect on matrix deposition (n=5). On the other hand, when HA particles were incorporated within agarose, higher ALP activity, GAG content, and collagen content were measured (*p<0.05).
Figure 6.3. Layered cartilage graft with osteochondral interface. Two distinct regions were evident within the hydrogel scaffold, representing the matrix-poor agarose region and matrix-rich agarose+HA region (Day 14, 10x, scale bar=500 um). Higher DZC cell number (n=5) was measured at day 28 in co-culture (*p<0.05). FTC measured decreasing ALP activity (n=5) over time for both groups (#p<0.05), whereas there was a peak for DZC in co-culture at day 14 (*p<0.05).
Figure 6.4. Proteoglycan and collagen deposition. Two distinct regions were evident in the histological staining of the hydrogel scaffold (n=2, Day 14, 10x, scale bar=200 um). Significant FTC GAG deposition (n=5) was measured only in the co-culture group at day 14 (*p<0.05). Co-culture had no effect on DZC GAG deposition. Significant collagen deposition (n=5) was measured for all groups during the first two weeks of culture (#p<0.05), and higher DZC collagen was detected in co-culture by day 28 (*p<0.05).
CHAPTER 7: HYDROGEL-NANOFIBER SCAFFOLD SYSTEM FOR INTERFACE REPAIR
7.1 Introduction

In Chapter 6, it was shown that the interface scaffold can be used in conjunction with a ceramic-free graft to regenerate both articular cartilage and calcified cartilage in vitro. The next step is to augment the design of the interface scaffold to facilitate its clinical implementation.

7.1.1 Background and Motivation

The dual roles of the biomimetic ceramic phase within the scaffold are to promote formation of a calcified cartilage matrix and facilitate integration with bone. Given its high water content and porosity, it is anticipated that a hydrogel-based scaffold will not be sufficient to promote dense calcified tissue formation and osteointegration when first implanted. Therefore, the goal of this study is to augment the hydrogel interface scaffold for in vivo implementation. To accomplish this task, the use of nanofiber scaffolds will be explored. Nanofibers are particularly advantageous for tissue engineering, as their properties can be modulated during fabrication[200,201] to meet the scaffold design criteria. They have been investigated with promising results for bone[202], meniscus[203], intervertebral disk[204], cartilage[205], ligament[206], and tendon[207] repair applications.

7.1.2 Objectives

To this end, a composite nanofiber-hydrogel scaffold is developed for osteochondral repair. A "cup" design, whereby a nanofiber mesh is formed into the shape of a cylindrical defect and contains the hydrogel scaffold, is envisioned. This design allows for ease of handling and manipulation of the hydrogel. It is also anticipated that the biomimetic interface between implant and tissue may facilitate healing. The first objective of this study is to compare chondrocyte response with and without the polymer nanofiber component. It is anticipated that the presence of the nanofiber will not decrease matrix deposition or downregulate hypertrophy of chondrocytes seeded within the hydrogel interface scaffold. Secondly, a ceramic phase will be incorporated within the nanofiber at the base of the scaffold. In addition to facilitating osteointegration with the underlying native bone, this design also allows for a relatively dense layer of ceramic to be present at the osteochondral interface. Similar to chondrocyte
response in composite hydrogel scaffolds, it is anticipated that ceramic presence within the nanofiber will also promote matrix deposition and hypertrophy.

7.2 Materials and Methods

7.2.1 Cells and Cell Culture

Primary chondrocytes were isolated from the deep zone cartilage of five immature bovine knee joints (Green Village, NJ) following published protocols\[5\]. Briefly, the isolated cartilage pieces were digested for 16 hours with collagenase type II (3.5 activity units/mg, Worthington, Lakewood, NJ) in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, GA), 2% antibiotics (10,000 U/ml penicillin, 10 mg/ml streptomycin), and 0.1% antifungal (amphotericin B). The cell suspension was then filtered (30 µm, Spectrum, Rancho Dominguez, CA) and plated in fully-supplemented media[174]. All media supplements were purchased from Cellgro-Mediatech unless otherwise specified.

7.2.2 Scaffold Fabrication, Culture, and Characterization

Nanofiber scaffolds made from poly(D,L-lactide-co-glycolide) 85:15 co-polymer (PLGA, $M_w = 123.6$ kDa; Lakeshore Biomaterials, Birmingham, AL) were produced using electrospinning[207]. Briefly, a 35% (v/v) solution of PLGA was mixed with 55% N,N-dimethylformamide (Sigma-Aldrich, St. Louis, MO) and 10% ethyl alcohol. The polymer solution was loaded into a 5-mL syringe with a 18.5-gauge stainless steel blunt-tip needle and was dispensed using a syringe pump (Harvard Apparatus, Holliston, MA; 1 mL/h) and electrospun at 8 to 10 kV onto a collecting surface. Nanofiber scaffolds were approximately 100 µm thick, with a fiber diameter of approximately 600 nm and pore diameter of 5 µm[207]. Composite PLGA+hydroxyapatite (PLGA+HA, 140-240 nm, Nanocerox, Ann Arbor, MI) nanofiber mesh was also fabricated by pre-incorporating 15% HA nano-particles within the electrospinning solution via sonication. Nanofiber scaffolds were then fabricated by sintering PLGA mesh into cylindrical cups in a custom-designed device (6 mm diameter, 5 mm height) at 60°C for 30 minutes. Nanofiber scaffolds with PLGA+HA bases were fabricated by removing mesh from the bottom of the PLGA scaffolds and re-sintering PLGA+HA mesh.
Sterile 4% agarose solution (Type VII, Sigma) was made in phosphate buffered saline (PBS) and combined 1:1 (volume ratio) with the cell suspension (20 x10^6 cells/ml) for a final concentration of 10^7 cells/ml seeded in 2% agarose. Composite agarose+HA was also fabricated by pre-incorporating HA (20-25 µm, 3w/v%, Sigma) in the cell suspension. The cell-agarose suspension was then added into each nanofiber scaffold (250 µl) and allowed to gel at room temperature for 15 minutes. Both PLGA scaffolds (PLGA walls and PLGA base) and PLGA+HA scaffolds (PLGA walls and PLGA+HA base) were filled with either agarose or agarose+HA. Nanofiber-free agarose and agarose+HA controls with 3w/v% HA, seeded with 10^7 cells/ml, were also fabricated as previously described (Chapter 4).

Samples were cultured under humidified conditions at 37°C and 5% CO₂ and maintained in ITS media, consisting of DMEM with 1% ITS+ Premix (BD Biosciences, San Jose, CA), 1% antibiotics, 0.1% antifungal, and 40 µg/ml proline (Sigma). The media was changed every other day and supplemented with fresh 50 µg/ml ascorbic acid (Sigma). In order to induce hypertrophy, DZC were stimulated with 25 nM triiodothyronine (T3, Sigma, St. Louis, MO) for the first three days of culture[176] (Chapter 4). A corresponding set of samples served as unstimulated controls.

As-fabricated scaffolds were visualized (n=2) under environmental scanning electron microscopy (ESEM, 20 kV, JEOL 5600LV, Tokyo, Japan), and elemental composition (n=2) was ascertained by energy dispersive x-ray analysis (EDAX, 20 kV, FEI Quanta 600, FEI Co., Hillsboro, OR).

7.2.3 Chondrocyte Biosynthesis and Hypertrophy

At days 1, 14, and 28, the nanofiber mesh was removed from the hydrogel and each portion was analyzed separately. Cell proliferation (n=5) was determined using the PicoGreen® total DNA assay (Molecular Probes, Eugene, OR). Briefly, the samples were lysed with 500 µl of 0.1% Triton-X solution (Sigma), and an aliquot of the sample (25 µl) was added to 175 µl of the PicoGreen® working solution. Fluorescence was measured with a microplate reader (Tecan, Research Triangle Park, NC), at the excitation and emission wavelengths of 485 and 535 nm, respectively. Total cell number was obtained by converting the amount of DNA per sample to cell number using the conversion factor of 7.7 pg DNA/cell[150].
Collagen deposition (n=5) was quantified with a simplified hydroxyproline assay[177], and collagen content was obtained by interpolation along a standard curve of bovine type I collagen (Sigma). Sample glycosaminoglycan content (GAG, n=5) was determined with a modified 1,9-dimethylmethylene blue (DMMB) binding assay[152-154], with chondroitin-6-sulfate (Sigma) as the standard. The absorbance difference between 540nm and 595nm was used to improve the sensitivity in signal detection. Additionally, collagen and GAG distribution (n=2) were visualized by Picrosirius red and Alcian blue staining, respectively[174]. Matrix penetration into the nanofiber scaffold was also visualized by staining of frozen sections (n=2). Briefly, one corresponding set of nanofiber-hydrogel samples which was not separated, were similarly fixed in 10% neutral buffered formalin. These were then embedded in 5% polyvinyl alcohol (PVA, Sigma-Aldrich) and 7 µm thick sections were obtained using a cryostat (Hacker-Bright OTF model, Hacker Instruments and Industries, Winnsboro, SC).

Deposition of collagen I, II, and X (n = 2) were evaluated using immunohistochemistry. Monoclonal antibodies for collagen I (1:20 dilution) and collagen II (1:100 dilution) were purchased from Abcam (Cambridge, MA), and collagen X antibody (1:1 dilution) was obtained from the Developmental Studies Hybridoma Bank (University of Iowa). After fixation, samples were treated with 1% hyaluronidase for 30 min at 37°C to remove proteoglycan and incubated with primary antibody overnight. A FITC-conjugated secondary antibody (1:200 dilution, LSAB2 Abcam) was used and sections were imaged via confocal microscopy at excitation and emission wavelengths of 488 nm and 568 nm, respectively. Quantitative alkaline phosphatase (ALP) activity (n=5) was determined using an enzymatic assay based on the hydrolysis of p-nitrophenyl phosphate (pNP-PO₄) to p-nitrophenol (pNP)[157]. In addition, mineral distribution (n=2) was evaluated by Alizarin red staining.

7.2.4 Statistical Analysis

Results are presented in the form of mean ± standard deviation, with n equal to the number of samples analyzed. A two-way analysis of variance (ANOVA) was performed to determine the effects of nanofiber and ceramic presence, as well as culturing time. The Tukey–Kramer post-hoc test was used for all pair-wise comparisons, and significance was attained at p<0.05. All statistical analyses were performed using the JMP IN software (SAS Institute, Cary, NC).
7.3 Results

7.3.1. Composite Nanofiber-Hydrogel Scaffold

The individual fibers that comprise the nanofiber component of the scaffold were visible under ESEM post-sintering (Fig. 7.1A). Incorporation of HA was assessed with EDAX, confirming the presence of phosphorus (P) and calcium (Ca) peaks for the PLGA+HA mesh (scaffold base); these peaks were not present in the spectra of PLGA mesh (scaffold side). Analysis of the nanofiber mesh (PLGA and PLGA+HA) removed from the agarose (Ag) and agarose+HA (Ag+HA) hydrogel also revealed increasing cellularity and matrix content over time (data not shown). By day 28, significantly higher cell number, GAG, and collagen content were measured on the PLGA+HA mesh cultured adjacent to the agarose+HA hydrogel (Fig. 7.1B). Histology sections at the gel-nanofiber interface also confirmed that there was continuous matrix deposition and evidence of tissue ingrowth. In terms of ALP activity, a peak in mineralization potential at day 14 was measured only for cells on PLGA+HA mesh adjacent to the agarose hydrogel (p<0.05).

7.3.2. Agarose Hydrogel Culture in Nanofiber Cups

Agarose hydrogels cultured in nanofiber cups were compared to nanofiber-free controls; the nanofiber cups were comprised either entirely of PLGA (Ag in PLGA NF) or PLGA walls with PLGA+HA base (Ag in HA NF) (Fig. 7.2). There was no change in cell number over the culturing period for any of the scaffold groups. Whereas the ALP activity of cells within the nanofiber-free hydrogel did not change over time, a peak at day 14 was measured for the PLGA NF group (p<0.05). Similarly, ALP activity significantly increased between day 1 and day 14 for the HA NF group, although it was also maintained during the last two weeks of culture. By day 28, the nanofiber groups measured higher ALP activity as compared to the nanofiber-free hydrogel, with the highest values detected for the nanofiber group with the PLGA+HA base (p<0.05).

Proteoglycan deposition was measured in all scaffolds for the duration of the experiment (Fig. 7.2). While there was no difference between groups at day 14, significantly higher GAG was found in the nanofiber-containing scaffolds at day 28. Similarly, significant collagen deposition was evident in both nanofiber-free hydrogel and nanofiber-containing scaffolds during the first two weeks of culture, with no
differences between groups. Further increases in collagen were measured for only the nanofiber-containing scaffolds at day 28, with significantly higher collagen as compared to nanofiber-free hydrogel.

7.3.3. Agarose+Hydroxyapatite Culture in Nanofiber Cups

Agarose+HA hydrogels cultured in nanofiber cups were compared to nanofiber-free controls; the nanofiber cups were again comprised either entirely of PLGA (Ag in PLGA NF) or PLGA walls with PLGA+HA base (Ag in HA NF) (Fig. 7.3). There was no change in cell number over the culturing period for any of the scaffold groups. The ALP activity of nanofiber-free hydrogel was maintained through day 14 and significantly decreased thereafter. On the other hand, significant increases in ALP activity were detected for PLGA NF and HA NF groups during the first two weeks of culture and were higher than the nanofiber-free hydrogel at day 28 (p>0.05). Both proteoglycan and collagen deposition were measured in all scaffolds for the duration of the experiment, with no differences detected between groups (Fig. 7.3).

7.3.4. Response of Non-Hypertrophic Chondrocytes in Nanofiber Cups

The response of non-hypertrophic chondrocytes was assessed in nanofiber cups (PLGA wall and PLGA+HA base), and compared to corresponding T3-stimulated cultures (Fig. 7.4). For those chondrocytes seeded in agarose hydrogel, no change in cell number was measured in the absence of T3. On the other hand, a significant increase in cell number was found between day 14 and day 28 for the T3-stimulated group. The ALP activity of T3-free chondrocytes decreased over time and reached basal levels by day 14 (p<0.05), whereas significantly higher ALP activity was measured for the T3-stimulated group at both day 14 and day 28. Proteoglycan deposition was detected during the first two weeks of culture for both groups, with a further increase by day 28 for the T3-stimulated group (p<0.05). Although there was no significant collagen deposition between day 1 and day 14, higher collagen content was measured in both scaffolds by day 28 (p<0.05). Significantly higher GAG and collagen content were measured for the T3-stimulated group by four weeks of culture.

For those chondrocytes seeded in agarose+HA hydrogel, an increase in cell number was found between day 1 and day 14 in the absence of T3, whereas a delayed increase between day 14 and day 28 was detected with T3 stimulation (p<0.05). After four weeks of culture, the T3-stimulated groups
measured significantly higher cell number as compared to the T3-free group. The ALP activity of T3-free chondrocytes again significantly decreased over time, whereas significantly higher ALP activity was measured for the T3-stimulated group at both day 14 and day 28. Proteoglycan deposition was detected during the first two weeks of culture for both groups, with a further increase by day 28 for the T3-stimulated group (p<0.05). There was significant collagen deposition throughout the culturing period for both groups. Although there was no difference in GAG content between groups at day 28, significantly higher collagen content was measured for the T3-stimulated group.

Ceramic presence had no effect on the ALP activity or matrix deposition of T3-free cultures. On the other hand, T3 stimulation in combination with HA presence, significantly enhanced cell number at day 28. The combined effect of HA presence and T3 stimulation also resulted in significantly higher ALP activity at both day 14 and day 28. Lastly, the combined effect of HA presence and T3-stimulation enhanced collagen deposition after four weeks of culture (p<0.05).

7.4 Discussion

In this study, a novel nanofiber-hydrogel tissue engineered cup scaffold is developed for calcified cartilage regeneration, promoting deposition of a collagen II- and proteoglycan-rich matrix in ceramic-containing scaffolds (Fig. 7.5). Furthermore, the presence of PLGA+HA nanofiber enhances chondrocyte hypertrophy in both agarose and agarose+HA hydrogels, although mineralization and collagen X deposition is evident only in agarose+HA hydrogels. There is also evidence of matrix outgrowth at the edges of the hydrogel onto the nanofiber, with higher cellularity, matrix, and mineralization potential, on PLGA+HA nanofiber. These results collectively demonstrate that the nanofiber cup scaffold with PLGA walls and PLGA+HA base is well-suited for both tissue infiltration and osteointegration.

Previous studies have shown that induced hypertrophic chondrocytes seeded on agarose hydrogels (Chapter 4) and nanofiber mesh[208] measure higher matrix deposition and mineralization potential in the presence of HA. It is interesting to note that ceramic presence in the nanofiber, as compared to the ceramic-free nanofiber, had no effect on matrix deposition in this study. Since additional HA content within the nanofiber cup does not modulate matrix deposition of chondrocytes seeded within hydrogel scaffolds which already contain ceramic, it is postulated that proteoglycan and collagen
synthesis are dominated by ceramic presence within the hydrogel. Similarly, ceramic presence within the nanofiber had no effect on agarose+HA scaffolds, although additional HA within the composite cup further enhanced ALP activity of agarose scaffolds.

In order to study the combination of micro-HA particles within the agarose (inside the scaffold) and nano-HA particles within the nanofiber (outside the scaffold), the ceramic ‘outside’ model from Chapter 6 was again used. Analogous to the agarose+HA scaffold seeded in the PLGA+HA nanofiber cup, DZC+T3 were seeded in agarose scaffolds with micro-HA, cultured in wells pre-coated with nano-HA (Fig. 7.6). Control groups consisted of either micro-HA or nano-HA both inside and outside the scaffold in order to keep total ceramic content constant, as well as ceramic-free agarose. Interestingly, the combination of micro-inside and nano-outside resulted in the highest ALP activity, and may explain the enhanced mineralization found for the agarose+HA cultured in the HA-containing cup. Since collagen content was only regulated by the presence of micro-HA inside the scaffold and there was no significant difference in proteoglycan, enhanced matrix deposition within the cup cultures must be attributed to other factors, such as soluble PLGA degradation products[209].

The findings of this study also demonstrate that non-hypertrophic chondrocytes such as T3-free deep zone chondrocytes or full thickness chondrocytes can form articular cartilage within the nanofiber scaffold, since they have not shown a propensity towards hypertrophy or mineralization. It is envisioned that this scaffold will serve four important functions in vivo. First and foremost, ceramic within the hydrogel promotes calcified cartilage formation. Secondly, ceramic within the nanofiber mesh is expected to further promote integration with surrounding bone. Unpublished data from our laboratory has already demonstrated the osteointegrative potential of PLGA+HA mesh in vivo using a bone core model. Next, it is envisioned that the entire nanofiber cup will promote continuous tissue integration from host-to-implant. In a study by Li et al., chondrogenesis of mesenchymal stem cells, similar to those expected at the defect site, was reported on poly(e-caprolactone) nanofiber scaffolds[205]. A biodegradable cell-seeded nanofibrous hydrogel has also been used to promote functional cartilage-to-cartilage integration[210]. These findings indicate that cells from within the scaffold, as well as those from the host tissue, will be able to attach and deposit matrix on the nanofiber. Finally, during early tissue formation, it is anticipated
that the nanofiber will serve as a temporary barrier against vascular invasion and ectopic mineralization, although this remains to be tested in an intra-articular model.

7.5 Conclusions

Nanofiber scaffolds are biomimetic and have the potential to improve scaffold-to-tissue integration, in terms of cell-mediated matrix deposition and osteointegration. A novel hydrogel-nanofiber cup was developed in this study, composed of PLGA nanofiber walls and PLGA+HA nanofiber base. Chondrocyte biosynthesis, hypertrophy, and consequently, calcified cartilage formation, are enhanced within this nanofiber scaffold as compared to nanofiber-free controls. Furthermore, it is feasible to form articular cartilage in the cup scaffold since non-hypertrophic chondrocytes seeded in agarose hydrogel deposit an uncalcified cartilage matrix.
Figure 7.1. Composite nanofiber-hydrogel scaffold. Characterization (n=2, 20 kV, 1000x, scale bar=50 μm) of the composite nanofiber (NF) scaffold (scale bar=3 mm) confirmed the presence of HA-free (walls) and HA-containing regions (base). Analysis of the nanofiber surrounding the hydrogel scaffolds measures cell and matrix infiltration, and this was confirmed by histology of the nanofiber-hydrogel interface (n=2, Day 28, 10x, scale bar=200 μm). There was a peak in ALP activity at Day 14 for chondrocytes on the PLGA+HA nanofiber surrounding the agarose hydrogel (n=5, #p<0.05), which is higher than all other groups (*p<0.05).
Figure 7.2. Agarose hydrogel in nanofiber cups. While the presence of the nanofiber (NF) mesh did not affect DZC+T3 cell number (n=5), both PLGA and PLGA+HA mesh measured higher proteoglycan deposition (n=5), collagen deposition (n=5), and ALP activity (n=5) by day 28 as compared to Ag (*p<0.05: difference between groups; #p<0.05: change over time).
Figure 7.3. Agarose+hydroxyapatite hydrogel in nanofiber cups. While the presence of the nanofiber (NF) mesh did not affect DZC+T3 cell number (n=5) or matrix deposition (n=5), both PLGA and PLGA+HA mesh resulted in higher ALP activity (n=5) at day 28 as compared to Ag+HA (*p<0.05: difference between groups; #p<0.05: change over time).
Figure 7.4. Non-hypertrophic chondrocyte response in nanofiber cups. Higher cell number (n=5) was measured for Ag+HA scaffolds with the addition of T3 at Day 28 (^p<0.05), as well as with HA presence (*p<0.05). Higher ALP activity (n=5) was also measured for T3-stimulated groups (^p<0.05). Higher GAG and collagen deposition (n=5) were measured for Ag scaffolds with T3 stimulation (^p<0.05), although only collagen was enhanced for Ag+HA scaffolds with T3 stimulation (*p<0.05). The combination of HA presence and T3 stimulation further increased ALP activity and collagen deposition (*p<0.05) at both day 14 and day 28. (^p<0.05: effect of T3; *p<0.05: effect of HA; #p<0.05: change over time).
**Figure 7.5. Matrix characterization.** The matrix within the composite scaffold stained positive for proteoglycan (Alcian Blue) and collagen (Picosirius Red), with minimal collagen I staining and strongly positive collagen II staining (Day 28, 10x, bar=200 um). There was positive mineral staining (Alizarin red) only for the HA-containing groups, and collagen X staining was present only in T3-stimulated scaffolds containing HA.
Figure 7.6. Combined effect of HA inside and outside hydrogel scaffold on DZC+T3. Lower cell number (n=5) was measured for groups with micro-HA inside the scaffolds (M/M and M/N, *p<0.05) as compared to the HA-free control at Day 14. All ceramic-containing groups exhibited elevated ALP activity (n=5) as compared to the HA-free control, with the highest found for the M/N group (*p<0.05). While there was continuous GAG deposition (n=5) over time for all groups (#p<0.05), ceramic presence had no effect at any timepoint. After two weeks of culture, groups with micro-HA inside the scaffold (M/M and M/N) measured higher collagen content (n=5, *p<0.05) as compared to the HA-free control. (*p<0.05: effect of HA; #p<0.05: change over time).
CHAPTER 8: IN VIVO INTERFACE REPAIR
8.1 Introduction

The previous chapters of this thesis described the characterization of the osteochondral interface and have demonstrated the feasibility of forming calcified cartilage in vitro. Two models for clinical implementation were developed, namely a bilayered uncalcified-calcified hydrogel scaffold (Chapter 6) and nanofiber cup-hydrogel composite (Chapter 7). The interface scaffold can be implanted in three different modalities to achieve integrative cartilage repair. One option is to pre-culture a cell-seeded scaffold in vitro prior to implantation into the patient. The second option is to directly implant a cell-seeded scaffold; in this scenario, tissue formation results from the scaffold cells as well as infiltration of repair cells from the native defect. The third option, and the one used in the following pilot animal study, is to implant an acellular scaffold to direct the response of host cells. While the results from Chapter 5 demonstrate the feasibility of culturing a calcified cartilage matrix in vitro, an animal model will be used in Chapter 8 to assess calcified cartilage formation, integration, and mineralization in vivo of an acellular scaffold.

8.1.1 Background and Motivation

This study is required because scaffold systems to enhance soft tissue-to-bone integration must be tested using relevant in vivo models to account for the presence of vasculature, the full complement of in vivo cytokines, hormones, growth factors, cell types, and physiologic loading which are essential for graft-to-host interaction. In vitro models alone are not adequate to evaluate the biologic response due to the complexity of the processes being studied, and there is insufficient data available for the design of non-living models. This in vivo evaluation will provide invaluable feedback for future scaffold optimization and implant evaluation in larger animal models.

8.1.2 Objectives

The goal of this study is to test the bilayered and nanofiber cup designs for osteochondral interface repair in vivo by assessing calcified cartilage formation, integration, and ectopic mineralization. It is hypothesized that the hydrogel-ceramic phase of the scaffold will support calcified cartilage formation and osteointegration, while the nanofiber-ceramic phase will further enhance host-to-scaffold integration.
and protect the repair tissue from ectopic mineralization. To this end, the cartilage-to-bone interface will be surgically reconstructed utilizing the interface scaffold, hydrogel-ceramic composites either with or without a nanofiber component. These experimental groups will be compared to empty defects, as well as the native osteochondral interface.

The rabbit is chosen as it is a well-established model for evaluating cartilage healing[14,15,20,29,39,211-217]. Although large animal models such as goat, sheep, and horse more closely resemble human anatomy, smaller animal alternatives such as rabbit are often used for pilot studies. Specifically, the New Zealand White rabbit will be used in this study and is the lowest species in which the response can be evaluated because the rabbit knee joint is of the minimally adequate size for a 3 to 4 mm critical-sized defect. Although there are differences between the rabbit and human cartilage thickness and matrix content, the interface morphology are relatively similar and of primary importance in this study[75,98].

8.2 Materials and Methods

8.2.1 In Vivo Model

In this Institutional Animal Care and Use Committee–approved pilot study, four female New Zealand White rabbits weighing between 3.0 and 3.5 kg had bilateral surgery to create one osteochondral defect in the medial femoral condyle and two osteochondral defects in the femoral groove. Animals were anesthetized with ketamine (35 mg/kg) and xylazine (5 mg/kg), and anesthesia maintenance was administered via isoflurane (1-4%) inhalation. With use of a standard aseptic technique, each knee was approached through a medial parapatellar incision with the patella dislocated laterally. Critical-sized cartilage defects (3.5 mm diameter, 4 mm depth) were created with a surgical drill (Stryker, Mahwah, NJ). Acellular hydrogel and nanofiber-hydrogel 4 mm scaffolds were subsequently press-fit into the defects so that they were flush with the articular surface. Experimental groups consisted of PLGA+HA nanofiber scaffolds with agarose (n=4) and agarose+HA (n=4). PLGA nanofiber scaffolds with agarose served as scaffold controls (n=4), and empty defects were used as negative controls (n=4, Fig. 8.1A). Once the scaffolds were implanted, the patella was reduced, the joint capsule was closed with interrupted sutures, and the wound was closed in anatomical layers. Postoperatively the animals were allowed to move freely
within their cages. At four weeks, rabbits were euthanized and samples and their knee joints were isolated to harvest the tissue samples.

### 8.2.2 Scaffold Fabrication

Nanofiber scaffolds composed of poly(D,L-lactide-co-glycolide) 85:15 co-polymer (PLGA, $M_w = 123.6$ kDa; Lakeshore Biomaterials, Birmingham, AL) were produced using electrospinning[207]. Briefly, a 35% (v/v) solution of PLGA was mixed with 55% N,N-dimethylformamide (Sigma-Aldrich, St. Louis, MO) and 10% ethyl alcohol. The polymer solution was loaded into a 5-mL syringe with a 18.5-gauge stainless steel blunt-tip needle and was dispensed using a syringe pump (Harvard Apparatus, Holliston, MA; 1 mL/h) and electrospun at 8 to 10 kV onto a collecting surface. Composite PLGA+hydroxyapatite (PLGA+HA, 140-240 nm, Nanocerox, Ann Arbor, MI) nanofiber mesh was also fabricated by pre-incorporating 15% HA nano-particles within the electrospinning solution via sonication. Nanofiber scaffolds were then fabricated by sintering PLGA and PLGA+HA mesh in a custom-designed device (4 mm diameter, 4 mm height) at 60°C for 30 minutes. Post-assembly, scaffolds were UV-sterilized.

Agarose scaffolds (2%, Type VII, Sigma) and composite agarose+HA scaffolds (20-25 µm, 3w/v%, Sigma) were fabricated as previously described (Chapters 4 and 6). Nanofiber-free scaffolds agarose scaffolds were 4 mm in diameter, whereas the hydrogel scaffolds with nanofiber were 3.5 mm in diameter. Agarose+HA scaffolds were bilayered and composed of 1 mm agarose layered above 3 mm agarose+HA in order to match the uncalcified-to-calcified tissue transition within the defect. Composite hydrogel-nanofiber scaffolds were assembled by press-fitting at the time of surgery.

### 8.2.3 Tissue Imaging

Micro-computerized tomography (micro-CT) was used to visualize mineralized tissue formation ex vivo. Samples were aligned along their axial direction, stabilized in a 50mL centrifuge tube that was clamped in the specimen holder, and scanned by a vivaCT 40 system (SCANCO Medical AG, Basserdorf, Switzerland). The sample was scanned at 15 µm isotropic resolution and standardized micro-CT evaluation protocol was performed for all images[189]. The tissue was processed by Gaussian filtering to decrease the influence of noise and then thresholded to extract the mineralized phase using the standard
protocol of Scanco software for µCT analysis. Specifically, two different threshold values were used to assess the presence of mineralized tissue within each defect site (Fig. 8.2). The high threshold of 300 was determined to be optimal for native bone, whereas the low threshold of 200 was determined by using the mineral-free agarose scaffold as a blank control. Images in cross-section were taken from the middle 1 mm of the defect.

A high-resolution imaging system developed for small animal research (Vevo 770, VisualSonics Inc., Toronto, CA) was used in this study, with a high-frequency ultrasound probe (RMV-707B, 30 MHz) at a focal depth of 12.7 mm from the surface of the transducer. B-mode full-view images were acquired at a frame rate of 100 Hz (Fig. 8.2); the field of view was 12 mm × 12 mm. The ultrasound probe was placed above the joint, perpendicular to the articular surface.

8.2.4 Histology

The femoral head was dissected from each rabbit, and corresponding decalcified and calcified samples were randomly chosen for histological examination. Calcified samples were fixed in 10% formaldehyde supplemented with 1% CaCl₂ to preserve mineral, and decalcified samples were fixed in 10% neutral-buffered formalin supplemented with 1% CPC for 48 hours to preserve matrix. Calcified samples were subsequently embedded in PMMA according to an established protocol[128]. Decalcified samples were decalcified in EDTA. Following serial dehydration, samples were then embedded in paraffin. Both PMMA and paraffin blocks were sectioned (7 µm) and stained with Safranin O and von Kossa to visualize proteoglycan and mineral, and Goldner’s trichrome was used to visualize osteoid and mature bone (Fig. 8.3).

8.3 Results

8.3.1. Empty Defect

The empty defect served as a negative control to all the scaffold groups. As expected, the integrity of all three tissue regions, namely articular cartilage, calcified cartilage, and bone were disrupted. Four weeks after surgery the surface of the defect was covered with white, opaque tissue; the surface of the defect was not flush with the articular surface and sloped towards a dimple in the center of the defect.
The micro-CT images, both at the low and high threshold, revealed mineralization along the sides of the defect and a mineral-free portion in the center of the defect (Fig. 8.2); the boundaries of the original defect were not readily apparent. Von Kossa staining confirmed the presence of mineral along the sides of the defect, while the central region of the defect remained unfilled by repair tissue (Fig. 8.3).

There was a distinct demarcation between repair tissue and native tissue (Fig. 8.4, cartilage-repair interface), where a change in tissue quality was observed (repair-bulk). Whereas native cartilage contained very few cells, the repair tissue was highly cellular, fibrous and disorganized (top panel-region of interest). Within this repair tissue, isolated areas of bone-forming cells were also evident (bottom panel-region of interest). Fibrous tissue was also evident in the area lining the empty defect (top panel-scaffold-tissue interface). A mineralized front was present on the sides and bottom of the defect, with a region of interdigitated calcified cartilage and uncalcified cartilage (top panel-repair-bone interface). At this mineralization front, the cells resembled hypertrophic chondrocytes and there was positive staining for osteoid (bottom panel-repair-bone interface).

8.3.2. Agarose Implant

The agarose served as a hydrogel control to hydrogel-ceramic group and nanofiber-free agarose group. Similar to the empty defect, the cartilage surface was almost completely filled with white, opaque tissue after four weeks of implantation (Fig. 8.1); unlike the empty defect, the surface of the repair tissue was flush with the articular surface. Micro-CT shows that the majority of the defect not occupied by agarose was filled with mineralized tissue (Fig. 8.2). Although the agarose implant was initially flush with the cartilage, mineralized tissue formed above the scaffold. Von Kossa staining confirmed the presence of dense mineral above the agarose scaffold, which was continuous with the adjacent bone (Fig. 8.3).

The repair tissue was interdigitated with the adjacent native tissue (Fig. 8.5, cartilage-repair interface), although a region of acellular tissue was located at this junction. The repair tissue more closely resembled healthy cartilage, with rounded cells as compared to the granular appearance of the empty defect (repair-bulk). The hydrogel region was relatively acellular and matrix-free, although there were isolated regions of bone marrow invasion (bottom panel-scaffold). The boundary between the agarose scaffold and native tissue was characterized by clearly delineated osteoid (bottom panel-
scaffold-tissue interface). Agarose presence was also characterized by a robust immune response, namely presence of macrophages and giant cells at the periphery of the scaffold (top panel-region of interest). Whereas endochondral ossification engulfed the empty defect from all sides, a mineralization front was shown to move upward towards the articular surface from above the agarose implant; a region of interdigitated calcified cartilage and uncalcified cartilage (repair-bone interface) also stained positive for osteoid (bottom panel-repair-bone interface).

8.3.3. Agarose-Ceramic Implant

After four weeks of implantation, the surface of the defect was covered with white repair tissue (Fig. 8.1), although it was more disorganized relative to the empty and agarose groups and not flush with the articular surface. Micro-CT detected only partial filling of the defect with mineralized tissue along the sides and bottom of the scaffold (Fig. 8.2); the outline of the original defect was clearly visible. Von Kossa staining confirmed that there was no mineralization within the center of the defect where the implant was located (Fig. 8.3). On closer inspection (Fig. 8.6), HA particles were evident at the edges of the hydrogel scaffold, as well outside the scaffold in the surrounding tissues (top panel-scaffold-tissue interface).

The repair tissue was well-integrated with the adjacent native tissue (repair-surface interface), although the articulating surface at the juncture was highly irregular. The repair tissue was more cellularized and fibrous as compared to the agarose-only group (repair-bulk). Similar tissue, albeit less dense, was also evident in the area lining the implant (top panel-scaffold-tissue interface). The scaffold itself was invaded by bone marrow (scaffold). The mineralization fronts between repair tissue and bone (bottom panel-repair-bone interface) and scaffold and bone (bottom panel-scaffold-tissue interface) were relatively disorganized as compared to other groups. Isolated regions of mineralized tissue were also scattered throughout the defect (top panel-region of interest).

8.3.4. Agarose Implant in Nanofiber Scaffold

The agarose-PLGA composite implant served as a control to all nanofiber groups. Unlike the other groups, the majority of the defect was not covered with white, opaque tissue (Fig. 8.1). The original
defect boundary was clearly visible using micro-CT, and the majority of the defect was void of mineralized tissue (Fig. 8.2); in the low threshold images, only a narrow line of mineralization was present across the center of the defect. Von Kossa staining confirmed the presence of discontinuous mineralization along the sides of the implant, and there were isolated regions of mineral scattered throughout the repair tissue as well (Fig. 8.7).

A region of highly cellular tissue, approximately 25 µm in thickness, was located between the native tissue and repair tissue (cartilage-repair interface); the repair tissue itself was also highly cellular and disorganized. Although the majority of the hydrogel scaffold was matrix-free, there was invasion from the bone marrow in isolated regions (scaffold-bulk). The presence of the nanofiber, approximately 70 µm thick, was also evident at the scaffold-to-tissue interface (bottom panel-scaffold-tissue interface), although it was not readily visible at all points along the hydrogel-tissue interface. While there was no calcified tissue regeneration in the majority of the scaffold, a mineralized front was evident near the wound edges of the osteochondral interface (repair-bone interface). It is interesting to note that mineralization was less organized as compared to other groups, with fragmented mineralization (top panel-region of interest). Large aggregates of macrophages and bone-forming cells were also scattered within the repair tissue (bottom panel-region of interest).

8.3.5. Agarose Implant in Nanofiber-Ceramic Scaffold

Four weeks post-implantation, the surface of the tissue was only partially filled with white tissue (Fig. 8.1), and except for sporadic mineralization across the top of the scaffold, micro-CT images indicated that the majority of the defect was void of mineralized tissue (Fig. 8.2). Von Kossa staining confirmed the presence of mineralization only surrounding the scaffold (Fig. 8.3). On closer examination, individual mineral deposits were also evident in the region adjacent to the nanofiber (Fig. 8.8, top panel-region of interest).

The repair tissue was integrated with the adjacent native tissue (cartilage-repair interface) and resembled the tissue formed in the empty defect (repair-bulk). The scaffold itself was acellular and void of matrix deposition or tissue invasion (scaffold), although it was lined with a layer of cells (bottom panel-region of interest). Fibrous tissue was also found lining the defect, and mineralization was observed
within the nanofiber itself (top panel-scaffold-tissue interface), with a region of interdigitated calcified cartilage and uncalcified cartilage (top panel-repair-bone interface). Unlike the nanofiber-free groups, there was limited endochondral ossification advancing from the osteochondral interface at the edges of the wound.

8.3.6. Agarose-Ceramic Implant in Nanofiber-Ceramic Scaffold

After four weeks of implantation, the surface of the tissue was again only partially restored (Fig. 8.1). The micro-CT image indicated the presence of scattered mineral deposits along the top of the defect (Fig. 8.2), and von Kossa confirmed positive staining for an organized mineral layer above the above the agarose-ceramic scaffold (Fig. 8.3). Von Kossa staining also confirmed the presence of a 100 µm layer of continuous mineralization surrounding the entire nanofiber scaffold.

The repair tissue was well-integrated with the adjacent native tissue (Fig. 8.9, cartilage-repair interface), with an acellular and interdigitated junction. Interestingly, it was more cartilage-like as compared to all other groups, with rounded cell morphology and columnar cell organization (repair-bulk). The hydrogel implant itself was acellular and matrix free (scaffold), and was almost completely surrounding by mineralized tissue which infiltrated the nanofiber mesh (top panel-scaffold-tissue interface). Individual HA particles were visible, although similar to the agarose+HA scaffold, HA particles were also found outside the scaffold (top panel-scaffold-tissue interface). There was limited interdigitation of uncalcified and calcified tissue at the junction between repair tissue and new mineralized tissue (top panel-repair-bone interface). Similar to the other experimental groups, giant cells were seen adjacent to the scaffold (bottom panel-repair-bone interface). It is interesting to note that the region directly above the hydrogel scaffold, was intermediate between mature bone and unmineralized fibrocartilage and contained isolated mineral deposits (top panel-region of interest).

8.4 Discussion

The long-term research goal of this thesis is to facilitate calcified cartilage regeneration, by engineering a functional interface that mimics the native structure and composition of the cartilage-to-bone junction. Specifically, this study assesses the efficacy of a biomimetic polymer-ceramic scaffold to
promote calcified cartilage formation and integration, while preventing ectopic mineralization. Osteochondral defects in a rabbit model are repaired with the tissue-engineered composite scaffold which consists of a hydrogel-ceramic composite contained within a nanofiber-ceramic cup. It was found that scaffold ceramic promotes mineral deposition, whereas the nanofiber component regulates the quality of the repair tissue.

The defect model disrupts articular cartilage, calcified cartilage, and bone, with limited damage to the surrounding tissue. Without the presence of a scaffold, the osteochondral defect is filled with mineralizing fibrocartilage. This repair response is very similar to that previously reported in other studies[19,214]. The presence of a fibrin blood clot introduces mesenchymal stem cells to the defect site, and these cells take on a flattened, elongated appearance with time. The repair tissue is fibrous and subject to endochondral ossification. Spontaneous repair is dramatically improved with the presence of a scaffold. It is proposed that the agarose scaffold acts to physically support and position the blood clot during repair. This was also found in a study by Yokoto et al., where an acellular, double-network hydrogel implanted at an optimal depth was shown to promote spontaneous hyaline cartilage repair[213]. It was postulated that by supporting the fibrin clot, the repair cells are exposed to mechanical loading which enhances cartilage-like matrix formation. Unfortunately, this tissue also undergoes vascular invasion and subsequent endochondral ossification.

Instead of promoting mineralization above the scaffold, as is the case with the ceramic-free hydrogel, hydroxyapatite presence within the hydrogel redirects mineralization to the periphery of the scaffold within bone instead of above the scaffold near the osteochondral interface. Two factors may be contributing to this phenomenon. First, HA particles are localized in this periphery region after four weeks of implantation. The displacement of particles may be due to mechanical compression of the agarose scaffold which is observed with all groups; although the scaffold was implanted flush with the articular surface, mechanical forces and formation of repair tissue are most likely confined the scaffold. Secondly, the formation of a mineralized capsule around the implant may be an immune response to quarantine the scaffold from native tissue. Given the ubiquitous presence of macrophages and giant cells, the role of this robust foreign body response should be investigated further to determine its effects on tissue formation and repair.
The presence of the nanofiber component also serves a secondary function, namely as a barrier to preventing endochondral ossification. Endochondral ossification emanates from both sides of the defect and the mineralization front continues to engulf the repair tissue until a physical or functional barrier is present. It is possible that while the hydrogel-ceramic scaffold promotes mineralization below the osteochondral interface, nanofiber-ceramic barrier prevents endochondral ossification and protects the neo-cartilage tissue, resulting in stable interface repair. Likewise, the presence of a stable osteochondral interface may further promote the integrity of the articular cartilage region. The presence of hyaline-like tissue at the articular surface is likely responsible for formation or regulation of the distinct osteochondral interface for this scaffold group and will be investigated further in Chapter 9.

The design of this scaffold also has a few limitations which should be addressed in subsequent studies. Since rabbit cartilage is very thin, the nanofiber cup was press-fit deep within the bone compartment, and the interface region of the scaffold was not located at the interface of native tissue. Secondly, there was limited tissue formation within the scaffolds since they were acellular. In order to investigate the role of chondrocyte biosynthesis, analogous constructs were cultured *in vitro*; full thickness chondrocytes (FTC) were seeded within the HA-free agarose portion and deep zone chondrocytes (DZC) were seeded within the HA-containing agarose portion (*Fig. 8.10*). For the FTC-only groups, cup presence had no effect on chondrocyte ALP activity, although both the PLGA and PLGA+HA cups significantly increased GAG and collagen deposition by day 28. On the other hand, the PLGA+HA cup suppressed FTC/DZC ALP activity, with no corresponding effect on matrix deposition. These results are in agreement with previous findings from *Chapter 7* which found that the nanofiber cups enhanced matrix deposition only in hydrogel groups without HA.

The imaging techniques explored in this study are also critical for assessing repair within the joint. In addition to being clinically relevant for tissue monitoring in patients, non-invasive imaging techniques are particularly advantageous because they do not require processing of histology samples which often complicate the interpretation of tissue morphology. One such imaging technique is ultrasound, shown alongside the micro-CT images in *Fig. 8.2*, which has been used for many *in vivo* applications such as assessing strain within vessel walls[218] and myocardial mechanics[219], as well as identification of breast cancer[220] and intravascular plaques[221]. Konofagou *et al.* has also demonstrated the feasibility
of imaging poroelastic tissue-like materials such as articular cartilage[222] and mapping their axial and lateral tissue strains.

A 50 MHz transducer has been previously used to image rat knee joints; the B-mode images indicated the presence of smooth articular surface (white), bone (black), and the cartilage-bone interface (irregular and echoic)[223]. These results are very similar to the images from our study, which depict the cartilage surface, as well as the scaffold-to-bone, repair tissue-to-bone, and empty-to-bone interfaces within the bone region. Future studies can be explored to use quantitative parameters derived from ultrasound to measure tissue quality and amount. In a study by Hattori et al., rabbit cartilage defects treated with PLGA scaffolds were imaged with ultrasound[224]. By transforming the echograms into wavelet maps, a linear correlation was demonstrated between the maximum magnitudes from the wavelet map with the histological grading score. A subsequent study by Brown et al. examined relative ultrasound reflection from normal and diseased tissue[225]. It was found the relationship between reflection from the cartilage surface and bone at the osteochondral junction provided information about cartilage degeneration. The ultrasound pulse-echo signal from cartilage and bone regions of osteochondral samples have been correlated with matrix and mineral content[226]. Future studies will investigate further the use of high-resolution ultrasound to monitor osteochondral interface formation.

8.5 Conclusions

The results of this study collectively demonstrate that the combination of a layered ceramic-containing hydrogel within a ceramic-containing nanofiber cup is the optimal design for interface formation: the agarose scaffold 1) supports a fibrin clot for neo-cartilage formation and 2) localizes ceramic particles to promote mineralization at the periphery of the scaffold, while the nanofiber-ceramic mesh 3) prevents invasion of endochondral ossification and promotes osteointegration. The combined effect of promoting mineralization and preventing endochondral ossifications allows for the development of a stable osteochondral interface.
Figure 8.1. Osteochondral interface defect model. Each of the six groups was implanted within the femoral groove or medial condyle (Ag:Agarose, HA: Hydroxyapatite, NF: Nanofiber). The scaffolds were press-fit into the defects, and after four week of culture, tissue formation was observed (scale bar=3 mm).
Figure 8.2. Defect imaging. Mineralized tissue formation was visualized by micro-CT at two thresholds and ultrasound after four weeks of implantation.
**Figure 8.3. Defect histology.** Histology was used to visualize mineral, matrix, and cell morphology in plastic-embedded sections (Safranin O and von Kossa and Goldner’s Trichrome, bar=500 um and 1 mm).
Figure 8.4. Empty defect. The top panel shows Safranin O and von Kossa (SO+VK), and the bottom panel shows Goldner’s trichrome staining (Tri). Interface regions are imaged (32x, bar=100 um and 50 um) from regions indicated in the overview image (5x, bar=1 mm).
Figure 8.5. Agarose implant. The top panel shows Safranin O and von Kossa (SO+VK), and the bottom panel shows Goldner's trichrome staining (Tri). Interface regions are imaged (32x, bar=100 um and 50 um) from regions indicated in the overview image (5x, bar=1 mm).
Figure 8.6. Agarose-ceramic implant. The top panel shows Safranin O and von Kossa (SO+VK), and the bottom panel shows Goldner’s trichrome staining (Tri). Interface regions are imaged (32x, bar=100 um and 50 um) from regions indicated in the overview image (5x, bar=1 mm).
Figure 8.7. Agarose implant in nanofiber scaffold. The top panel shows Safranin O and von Kossa (SO+VK), and the bottom panel shows Goldner’s trichrome staining (Tri). Interface regions are imaged (32x, bar=100 um and 50 um) from regions indicated in the overview image (5x, bar=1 mm).
Figure 8.8. Agarose implant in composite nanofiber scaffold. The top panel shows Safranin O and von Kossa (SO+VK), and the bottom panel shows Goldner's trichrome staining (Tri). Interface regions are imaged (32x, bar=100 um and 50 um) from regions indicated in the overview image (5x, bar=1 mm).
Figure 8.9. Agarose-ceramic implant in composite nanofiber scaffold. The top panel shows Safranin O and von Kossa (SO+VK), and the bottom panel shows Goldner’s trichrome staining (Tri). Interface regions are imaged (32x, bar=100 um and 50 um) from regions in the overview image (5x, bar=1 mm).
**Figure 8.10. In vitro controls.** Full thickness chondrocytes (FTC) and deep zone chondrocytes (DZC) were cultured in agarose scaffolds and layered agarose/agarose+3% micro-HA scaffolds (10 million/mL). Hydrogel scaffolds in nanofiber cups were compared to nanofiber-free scaffolds. Nanofiber cups had no effect on FTC ALP activity, while the FTC/DZC group measured lower ALP in the PLGA+HA cup at Day 28 (*p<0.05). Increases in GAG and collagen deposition were measured for all groups during the first two weeks of culture (#p<0.05). Although the PLGA+HA nanofiber cup had no effect on FTC/DZC matrix deposition, higher GAG and collagen content were found for FTC cultured in PLGA and PLGA+HA nanofiber cups at Day 28 (*p<0.05).
CHAPTER 9: MAINTENANCE OF UNCALCIFIED-TO-CALCIFIED CARTILAGE JUNCTION
9.1 Introduction

In order to achieve long-term functional cartilage repair, the mineralization front between mineral-free articular cartilage and the mineral-containing interface scaffold must be maintained. Therefore, this chapter returns to the layered hydrogel model from Chapter 6 and adds a level of complexity, namely the zonal organization of chondrocytes within uncalcified cartilage.

9.1.1 Background and Motivation

The maintenance and integrity of both uncalcified and calcified cartilage regions is critical for normal joint function. This balance is disrupted in diseased tissue, and the result is matrix loss\cite{8-10} and advancement of the calcification front into articular cartilage\cite{71,103}. These changes represent a deviation from homeostasis at the cartilage-to-bone junction.

In a study by Jiang et al., interactions between chondrocytes isolated from each of three articular cartilage zones were evaluated, and it was found that zonal paracrine communication regulates chondrocyte hypertrophy and mineralization in a monolayer model\cite{5}. Microarray analysis of healthy and osteoarthritic cartilage has also indicated that the expression of nearly 200 genes, including matrix genes, bone-related genes, and proteinase genes, varies among the different cartilage zones\cite{227}; interestingly, similar expression patterns were observed in healthy and diseased tissue, indicating the presence of common regulatory mechanisms within cartilage. Inspired by these findings and the zonal organization of cartilage\cite{4}, physiologically-relevant layered hydrogel models have been previously developed to understand interactions between surface, middle, and deep zone chondrocytes\cite{169}. Similar to the results from the monolayer culture, surface zone chondrocytes prevent T3-induced increases in deep zone chondrocyte hypertrophy and ectopic mineralization in hydrogel culture as well. These findings collectively demonstrate the role of zonal chondrocyte interactions in preventing mineralization above the calcification front.

9.1.2 Objectives

Specifically, the goal of this study is to assess the integrity of uncalcified and calcified matrix regions on a single scaffold. In Chapter 6, uncalcified and calcified regions were formed \textit{in vitro} using a
bilayered scaffold of agarose and agarose+HA; the HA-containing layer was seeded with hypertrophic chondrocytes, and the HA-free layer was seeded with FTC. The objective of this study is to determine whether the organization of zonal chondrocyte populations within the uncalcified cartilage region regulates biosynthesis and mineralization at the scaffold interface. To this end, two layered models are tested; the first has an ‘uncalcified’ region comprised of a mixed population of chondrocytes from all three zones (data from Chapter 6) and the second has a biomimetic ‘uncalcified’ region of layered surface, middle, and deep zone chondrocytes.

9.2 Materials and Methods

9.2.1 Cells and Cell Culture

Primary articular chondrocytes were isolated from five immature bovine knee joints (Green Village Packing, Green Village, NJ). Due to differences in cartilage thickness between animals and isolation sites, a standardized protocol to isolate zonal chondrocyte populations was developed based on published reports[5,175,198]. Specifically, cells from the top 10% closest to the articular surface and bottom 20% closest to the calcified tissue were designated as surface zone (SZC) and deep zone chondrocytes (DZC), respectively. Chondrocytes isolated from the entirety of articular cartilage were designed as full thickness chondrocytes (FTC). Briefly, the separated cartilage pieces were incubated for 16 hours with 0.1 w/v% collagenase (Sigma, St. Louis, MO) in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, GA), 2% antibiotics (10,000 U/ml penicillin, 10 mg/ml streptomycin), and 0.1% antifungal (amphotericin B). The cell suspension was then filtered before plating (30 µm, Spectrum, Rancho Dominguez, CA). The isolated chondrocytes were maintained in fully-supplemented DMEM with 10% FBS, 1% non-essential amino acids, 1% antibiotics, and 0.1% antifungal for 48 hours before seeding. All media supplements were purchased from Cellgro-Mediatech unless otherwise specified.

9.2.2 Layered Scaffold Fabrication and Culture

Cell-seeded hydrogel samples were fabricated by mixing the cell suspension at a volume ratio of 1:1 with sterile 4% agarose (Type VII, Sigma) solution in phosphate buffered saline (PBS) at a seeding
density of $10^7$ cells/ml. Hydroxyapatite (HA, Sigma) was pre-mixed with cell suspension prior to the addition of agarose to fabricate agarose+HA scaffolds (Chapter 4 and 6). The cell-agarose suspension of each layer was cast and allowed to gel at room temperature for 5 minutes prior to casting the subsequent layer. Individual disks (5 mm diameter, 0.75 mm thickness/layer) were cored with a sterile biopsy punch (Sklar Instruments, West Chester, PA). Samples were cultured under humidified conditions at $37^\circ$C and 5% CO$_2$ and maintained in ITS media, consisting of DMEM supplemented with 1% ITS+ Premix (BD Biosciences, San Jose, CA), 1% antibiotics, 0.1% antifungal, and 40 µg/ml proline (Sigma). The media was changed every other day and supplemented with fresh 50 µg/mL ascorbic acid (Sigma). In order to induce DZC hypertrophy, cultures were stimulated with 25 nM triiodothyronine (T3, Sigma, St. Louis, MO) for the first three days of culture[5,176].

Co-culture scaffolds comprised of either FTC-seeded agarose or layered SZC/MZC/DZC-seeded agarose (SMD) were gelled above DZC-seeded agarose with 3% HA (DZC+HA, Fig. 9.1A). Cell response was measured in each region by first separating layered scaffolds into HA-free and HA-containing regions. Chondrocyte biosynthesis and mineralization potential was quantified at days 1, 14, and 28 and subsequently compared to single-culture controls, namely uncalcified-alone and calcified-alone scaffolds.

9.2.3 Cell Proliferation and Matrix Deposition

Cell proliferation (n=5) was determined using the PicoGreen® total DNA assay (Molecular Probes, Eugene, OR). Briefly, the samples were lysed with 500 µl of 0.1% Triton-X solution (Sigma), and an aliquot of the sample (25 µl) was then added to 175 µl of the PicoGreen® working solution. Fluorescence was measured with a microplate reader (Tecan, Research Triangle Park, NC), at the excitation and emission wavelengths of 485 and 535 nm, respectively. Total cell number was obtained by converting the amount of DNA per sample to cell number using the conversion factor of 7.7 pg DNA/cell[150].

Collagen deposition (n=5) was quantified using a simplified hydroxyproline assay[177]. Briefly, the samples were first desiccated for 24 hours and then digested for 16 hours at $60^\circ$C with papain (600 µg protein/ml) in 0.1M sodium acetate (Sigma), 10 mM cysteine HCl (Sigma), and 50 mM ethylenediaminetetraacetate (Sigma). A 40 µl aliquot of the digest was hydrolyzed with 10 µl 10 M
sodium hydroxide and autoclaved for 25 minutes. The hydrolyzate was then oxidized by a buffered chloramine-T reagent for 25 minutes before the addition of Ehrlich’s reagent. Sample absorbance was measured at 550nm (Tecan), and the collagen content was obtained by interpolation along a standard curve of bovine type I collagen (Sigma). Additionally, collagen distribution (n=2) was visualized by Picosirius red staining. Briefly, the samples were first fixed in 10% neutral buffered formalin with 1% cetylpyridinium chloride (Sigma) for 24 hours, followed by dehydration with an ethanol series. Prior to staining and imaging, the dehydrated samples were embedded in paraffin (Type 9, Richard-Allan Scientific, Kalamazoo, MI), sectioned (7 μm), and mounted on microscope slides.

Sample glycosaminoglycan content (GAG, n=5) was determined with a modified 1,9-dimethylmethylen blue (DMMB) binding assay[152-154], with chondroitin-6-sulfate (Sigma) as the standard. The absorbance difference between 540nm and 595nm was used to improve the sensitivity in signal detection. Distribution of GAG (n=2) was visualized histologically by Alcian blue staining of paraffin embedded sections[150].

9.2.4 Chondrocyte Hypertrophy and Mineralization

Quantitative alkaline phosphatase (ALP) activity (n=5) was determined using an enzymatic assay based on the hydrolysis of p-nitrophenyl phosphate (pNP-PO₄) to p-nitrophenol (pNP)[157]. Briefly, the samples were lysed in 0.1% Triton-X solution, then added to pNP-PO₄ solution (Sigma) and allowed to react for 30 min at 37°C. Sample absorbance was measured at 415nm using a microplate reader (Tecan). The expression of collagen X, matrix metalloproteinase-13 (MMP13), and Indian Hedgehog (Ihh) were measured using reverse transcription followed by polymerase chain reaction (RT-PCR, n=3), with custom-designed primers (Chapter 3). Total RNA was isolated via TRIzol (Invitrogen) extraction, and then reverse-transcribed into cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen). The cDNA product was amplified with recombinant Platinum Taq DNA polymerase (Invitrogen). Expression band intensities of were analyzed semi-quantitatively and normalized to the housekeeping gene glyceraldehydes 3-phosphate dehydrogenase (GAPDH). In addition, mineral distribution (n=2) was evaluated by Alizarin red staining.
9.2.5 Mechanical Testing

Depth-dependent mechanical testing of the layered osteochondral scaffolds was performed using a custom-fabricated device[126]. Digital image correlation was used to analyze the strain throughout the sample when 5% over strain was applied to the sample. Briefly, each scaffold was cut in half diametrically and one half was loaded onto a custom unconfined compression device mounted on the motorized stage of an inverted microscope. The initial uncompressed thickness ($h_0$) of the specimen was measured optically and an axial tare strain of 5% of the initial sample thickness was applied. The sample was allowed to equilibrate for 20 minutes, and images of the sample cross-section were then taken. An additional compression of 5% was then applied and a second set of images was acquired after allowing the sample to equilibrate again for 20 minutes. Image analyses were performed using an optimized digital image correlation technique producing accurate axial displacements (considered to be the z-axis) and axial strain fields.

9.2.6 Fourier Transform Infrared Imaging

Histology samples were sectioned (7 µm) and placed immediately onto barium fluoride optic windows (Spectral Systems, Hopewell Junction, NY). The sections were deparaffinized in xylene, rehydrated with an ethanol series, and then dried overnight under vacuum. A second barium fluoride window was placed over the sample prior to IR analysis. Fourier transform infrared imaging (FTIR-I) analysis was performed using an FTIR spectrometer (Spectrum 100, Perkin Elmer, Waltham, MA) coupled to an FTIR microscope imaging system (Spotlight 300, Perkin Elmer). The spectra were acquired with a spectral resolution of 8 cm$^{-1}$ and a spatial resolution of 6.25 µm. The content and distribution of collagen and proteoglycan were mapped for regions spanning mineral-free region of the sample.

The IR spectra were analyzed and spectroscopic images generated using ISYS 3.1.1 chemical imaging software (Spectral Dimensions Inc., Olney, MD) and MATLAB 7.0 R14 (The MathWorks Inc., Natick, MA). Prior to analysis, spectra were corrected by baseline subtraction using the ISYS software. The collected spectra were also corrected for contributions of the agarose scaffold material. Specifically, spectra of pure agarose were acquired, baseline corrected, and normalized by the peak at 1068 cm$^{-1}$, the
highest peak in the agarose spectrum. Sample spectra were likewise baseline corrected and normalized, and a pure agarose spectrum was subtracted from the sample spectra to eliminate the scaffold background. Collagen content was then estimated by integrating the peak area under the Amide I band (1720-1590 cm⁻¹), and proteoglycan content was estimated by integrating under a carbohydrate band associated with C-O-C and C-OH vibrations (1140-985 cm⁻¹). These analyses are based on methods developed by Camacho et al., and have shown that collagen and proteoglycan content correlates with C-O-C and C-OH band areas, respectively[114].

9.2.7 Statistical Analysis

Results are presented in the form of mean ± standard deviation, with n equal to the number of samples analyzed. A two-way analysis of variance (ANOVA) was performed to determine the effects of co-culture, as well as culture time. The Tukey–Kramer post-hoc test was used for all pair-wise comparisons, and significance was attained at p<0.05. All statistical analyses were performed using the JMP IN software (SAS Institute, Cary, NC).

9.3 Results

9.3.1. Effect of Uncalcified-Calcified Co-Culture on Chondrocyte Biosynthesis

Uniform cell density was measured for all scaffold layers and scaffold groups at day 1 (Fig. 9.1B). For the scaffolds with FTC layered above DZC+HA, no change in cell number was measured over time in both the uncalcified and calcified regions. Whereas co-culture had no effect on FTC cell number, significantly higher cell number was measured for the DZC+HA co-culture group at day 28. For the scaffolds with SMD layered above DZC+HA, a significant increase in cell number was measured between day 1 and day 14 in both the uncalcified and calcified regions. Co-culture had no effect on either SMD or DZC+HA cell number at any timepoint.

The highest level of GAG deposition was measured in the DZC+HA region, followed by SMD, and finally FTC with the lowest (p<0.05, Fig. 9.2A). For the scaffolds with FTC layered above DZC+HA, proteoglycan deposition was measured during the first two weeks of culture in both the uncalcified and calcified regions (p<0.05). Co-culture resulted in significantly higher GAG deposition by FTC as
compared to the single-culture control at day 14, although there was no difference by day 28. On the other hand, co-culture had no effect on GAG deposition within the DZC+HA layer. For scaffolds with SMD layered above DZC+HA, increases in scaffold GAG content were again measured from day 1 to day 14 in both the uncalcified and calcified regions (p<0.05). While there was no further change in GAG for the calcified region, GAG deposition significantly increased between day 14 and day 28 for the uncalcified region. Co-culture had no effect on either SMD or DZC+HA GAG deposition at any timepoint. It is evident from histological staining for GAG that proteoglycan deposition was more positive in the DZC region of the SMD uncalcified scaffold in both single-culture and co-culture, with comparable staining between DZC and DZC+HA layers (Fig. 9.2B).

Similar to GAG deposition, the highest level of collagen deposition was measured in the DZC+HA region, followed by SMD, and finally FTC with the lowest (p<0.05, Fig. 9.3A). For scaffolds with FTC layered above DZC+HA, significant collagen deposition was measured during the first two weeks of culture in both the uncalcified and calcified regions. Further increases in collagen content were measured during the last two weeks of culture in the DZC+HA region. Higher collagen deposition was deposited in the calcified region for the co-culture group by day 28 (p<0.05), with no corresponding difference in the FTC layer. For the scaffolds with SMD layered above DZC+HA, significant collagen deposition was measured throughout the duration of the experiment in both the uncalcified and calcified regions. While co-culture had no effect on collagen biosynthesis, higher matrix content was quantified in the calcified region in co-culture as compared to the single-culture control at day 14. It is again evident from histological staining that collagen deposition was more positive in the DZC region of the SMD uncalcified scaffold in both single-culture and co-culture, with comparable staining between DZC and DZC+HA layers (Fig. 9.3B).

9.3.2. Effect of Uncalcified-Calcified Co-Culture on Chondrocyte Hypertrophy

The ALP activity of chondrocytes within the calcified region was significantly higher than those from the uncalcified region (Fig. 9.4A). Decreasing ALP activity over time was measured for FTC layered above DZC+HA (p<0.05) and remained unaffected by co-culture. On the other hand, DZC ALP activity within the calcified region of this scaffold significantly increased during the first two weeks of culture,
decreasing thereafter to basal levels by day 28; higher values were measured in co-culture for the DZC+HA region at day 14 as compared to the single-culture control (p<0.05). For scaffolds with SMD layered above DZC+HA, ALP activity did not change over time in either the uncalcified or calcified regions. The ALP activity of SMD was unaffected by co-culture, although significantly higher values were measured in co-culture for the DZC+HA region at day 28.

The expression of hypertrophic markers, namely collagen X, MMP13, and Ihh, were also assessed at day 14 in the calcified region (Fig. 9.4B). It was found that while co-culture with SMD had no effect on chondrocyte hypertrophy, co-culture with FTC significantly upregulated collagen X expression. It is evident from histological staining for mineral that scaffold mineral was restricted to the calcified region, with no sign of cell-mediated mineralization within the uncalcified region (Fig. 9.4C).

9.3.3. Characterization of Multi-Layered Scaffolds

Each zone within the layered scaffold was readily distinguishable by day 28. The FTIR-I images of the uncalcified region confirmed the presence of a GAG- and collagen-rich poor region near the top of the scaffold and a matrix-rich region in the deep zone directly above the calcified layer (Fig. 9.5A). The top of the scaffold was relatively transparent, whereas the rest of the scaffold was translucent. Furthermore, depth-dependent changes in mechanical properties were measured, with maximum deformation in the surface zone, decreasing throughout the deep zone, and approaching zero strain in the calcified cartilage region (Fig. 9.5B).

9.4 Discussion

In this study, layered agarose scaffolds were used to mimic the native organization of zonal chondrocyte populations. While it has been previously demonstrated that surface zone chondrocytes regulate deep zone chondrocyte hypertrophy[5], results from this study suggest that deep zone chondrocytes in turn regulate the hypertrophy of calcified cartilage. Furthermore, despite the gradient from low to high matrix content through its depth, biomimetic layering of zonal chondrocytes results in overall higher matrix deposition in uncalcified cartilage as compared to a mixed population of full thickness chondrocytes.
Layered hydrogel scaffolds have been previously used to recapitulate the native organization of cartilage[173,196,197,228,229] and are a particularly advantageous model for determining zonal chondrocyte interactions. First and foremost, chondrocytes maintain their native morphology in hydrogel culture. Secondly, it facilitates the layering of chondrocyte populations in order to mimic the organization of native cartilage[4]. Lastly, it is possible to separate layers of the scaffold for endpoint analysis to discern the individual responses of each layer.

As previously discussed in Chapter 6, the ‘calcified’ region measures higher overall matrix content as compared to the ‘uncalcified’ region. This study supports the hypothesis that either mixed tri-culture of all three cartilage zones (FTC) suppresses matrix deposition within ‘uncalcified’ cartilage or conversely, matrix deposition is enhanced in the layered tri-culture model. In a study by Ng et al., a synergistic increase in GAG in a layered scaffold of SZC and MZC+DZC was measured[195], although there was no change in collagen deposition. Significantly higher matrix deposition was also achieved in a layered scaffold of SZC and DZC[229]. These findings indicate that zonal chondrocyte biosynthesis is affected by both chondrocyte interactions and organization.

Hypertrophy and mineralization potential are regulated on the bilayered scaffold. The findings of this study confirm that zonal chondrocyte interactions, regardless of zonal organization, are sufficient to suppress increases in hypertrophy within the ‘uncalcified’ region. Whereas chondrocyte hypertrophy within the ‘calcified’ region is upregulated in co-culture with FTC, no such enhancement is seen for the layered ‘uncalcified’ region. This suggests that the zonal organization of chondrocytes also regulates hypertrophy within calcified cartilage.

The collective changes in matrix composition in the biomimetic layered scaffolds, namely from a matrix-poor and HA-free region near the articular surface, to matrix-rich and HA-free region and finally, matrix-rich and HA-containing region in calcified cartilage, mirrors the depth-dependent scaffold properties of native tissue[126] and other biomimetic hydrogel scaffold designs[173]. Furthermore, there is no evidence of matrix discontinuities at the layer interfaces. These results demonstrate that the cell-cell and cell-ceramic interactions within the layered biomimetic scaffold regenerate stable uncalcified and calcified cartilage regions.
9.5 Conclusions

While the biomimetic layering of zonal chondrocyte populations above the interface scaffold is ideal for tissue regeneration and recapitulation of depth-dependent mechanical properties, this study demonstrates that full thickness chondrocytes are an acceptable alternative cell source for forming uncalcified cartilage matrix above the calcified cartilage matrix.
Figure 9.1. Cell proliferation in layered agarose model. (A) The cartilage portion of the model consists of either full thickness chondrocytes (FTC) or layered surface, middle, and deep zone chondrocytes (SZC, MZC, DZC, SMD), and the calcified cartilage portion consists of DZC embedded in an agarose+HA composite. (B) Higher cell number (n=5) was measured for DZC+HA when layered with FTC (*p<0.05: difference between groups; #p<0.05: change over time).
Figure 9.2. GAG deposition in layered agarose model. (A) Higher GAG deposition (n=5) was measured for FTC when layered with DZC+HA (*p<0.05: difference between groups; #p<0.05: change over time). (B) For the SMD scaffolds, GAG staining (n=2) was localized to the DZC region directly above the DZC+HA layer (Day 28, 10x, scale bar=200 um).
Figure 9.3. Collagen deposition in layered agarose model. (A) Higher collagen deposition (n=5) was measured for DZC+HA when layered with FTC or SMD (*p<0.05: difference between groups; #p<0.05: change over time). (B) For the SMD scaffolds, collagen staining (n=2) was localized to the DZC region directly above the DZC+HA layer (Day 28, 10x, scale bar=200 um).
Figure 9.4. Mineralization in layered agarose model. (A) An increase in ALP activity (n=5) at day 14 was measured for DZC+HA layered with FTC (*p<0.05: difference between groups; #p<0.05: change over time). (B) Co-culture with FTC upregulated DZC+HA collagen X expression (n=3, Day 14). (C) For the SMD scaffolds, mineral staining (n=2) was localized to the DZC+HA region (Day 28, 10x, scale bar=200 um).
Figure 9.5. Layered scaffold characterization. (A) FTIR-I characterization of the uncalcified region confirmed the transition from low to high matrix content seen in the scaffold histology. (B) The transition in matrix and mineral content was visualized by histological staining (Alizarin red-mineral, Alcian Blue-GAG, Picrosirius Red-collagen, 10x, scale bar=500 um) corresponded with depth-dependent changes in scaffold strain (Day 28, n=3).
CHAPTER 10: ZONAL CHONDROCYTE INTERACTIONS REGULATE CHONDROCYTE HYPERTROPHY AND CARTILAGE HOMEOSTASIS
10.1 Introduction

In Chapter 9, zonal chondrocyte organization within articular cartilage was shown to regulate chondrocyte mineralization. Therefore, cell-to-cell communication will be further explored in Chapter 10 to determine the role of each zonal chondrocyte population and their mode of interaction. These effects will also be studied in the bovine explant model, thereby linking the biomimetic hydrogel model and native tissue characterized in Chapter 2.

10.1.1 Background and Motivation

It has been speculated that communication between multiple cell types at tissue-to-tissue junctions participate in formation and regulation of biological interfaces. Specifically, there is evidence that osteoblast-fibroblast interactions regulate fibrocartilage formation at the ligament-to-bone interface[158], and interactions between chondrocytes and osteoblasts regulate mineralization at the cartilage-to-bone interface[150]. These cell-cell interactions also have ramifications for multi-phased scaffold designs which promote interface formation by controlling the spatial distribution of each tissue and interface region [47,230].

10.1.2 Objectives

The first objective is to develop an explant model to study the effect of zonal chondrocyte interactions on cartilage maintenance. Explants have been used extensively in the literature to study cartilage biology and mechanics[231-233]. Inspired by these findings and the zonal organization of cartilage[4], physiologically-relevant models will include single-culture controls to evaluate the effects of co-culture and tri-culture on matrix content and mineralization. Thyroid hormone will be used to stimulate chondrocyte hypertrophy and mineralization[5,176], similar to the diseased condition where chondrocytes are exposed to systemic factors from the vascular supply and the regulation of chondrocytes is disrupted. Given the native structure of articular cartilage, it is postulated that zonal interactions suppress chondrocyte mineralization and promote the maintenance of matrix content.

The second objective of this study is to use analogous layered hydrogel co-culture and tri-culture models to evaluate the biosynthetic activity of individual chondrocyte populations. These models will be
further developed to understand the mechanism by which chondrocyte mineralization is regulated. Since it has been shown that biological \([5,72,183]\) and mechanical \([102,234,235]\) cues regulate cartilage homeostasis, it is anticipated that both chondrocyte signaling and physiologic mechanical loading will mediate chondrocyte communication in the biomimetic hydrogel model.

10.2 Materials and Methods

10.2.1 Cells and Cell Culture

Primary articular chondrocytes were isolated from immature bovine knee joints (Green Village Packing, Green Village, NJ). Due to differences in cartilage thickness between animals and isolation sites, a standardized protocol to isolate zonal chondrocyte populations was developed based on published reports\([5,175,198]\). Specifically, cells from the top 10\% closest to the articular surface, middle 30\%, and bottom 20\% closest to the calcified tissue were designated as surface zone (SZC), middle zone (MZR), and deep zone chondrocytes (DZC), respectively. Briefly, the separated cartilage pieces were incubated for 16 hours with 0.1 w/v\% collagenase (Sigma, St. Louis, MO) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5\% fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, GA), 2\% antibiotics (10,000 U/ml penicillin, 10 mg/ml streptomycin), and 0.1\% antifungal (amphotericin B). The cell suspension was then filtered before plating (30 µm, Spectrum, Rancho Dominguez, CA). The isolated chondrocytes were maintained in fully-supplemented DMEM with 10\% FBS, 1\% non-essential amino acids, 1\% antibiotics, and 0.1\% antifungal for 48 hours before seeding. All media supplements were purchased from Cellgro-Mediatech unless otherwise specified.

10.2.2 Explant Model

Analogous co-culture and tri-culture explants models were developed to assess chondrocyte hypertrophy and mineralization. Cartilage explants were isolated with a 6 mm biopsy punch (Sklar Instruments, West Chester, PA) from immature bovine knees and again separated into the surface zone (SZ), middle zone (MZ), and deep zone (DZ). The experimental groups consisted of SZ-, MZ-, and DZ-alone (single-culture), SZ+DZ (co-culture), and SZ+MZ+DZ (segregated tri-culture). Chondrocyte response in these groups was compared with those in full-thickness explants (FTC, unsegregated tri-
culture). Chondrocyte response in single-, co-, and tri-culture were evaluated over three weeks. Each layer was analyzed separately to assess the individual response of each zonal population, namely SZ, MZ, and DZ.

10.2.3 3D Hydrogel Model

Agarose Type VII (4%, Sigma) was used in this study to fabricate single- and multi-layered scaffolds, similar to previously published protocols[195], by combining agarose in a 1:1 ratio with cells for a final seeding density of 10 million chondrocytes/mL. The experimental groups consisted of layered tri-culture (SZC/MZC/DZC), layered co-culture (SZC/MZC), (MZC/DZC), (SZC/DZC) and mixed co-culture (SZC+DZC), whereas the control groups consisted of SZC-alone, MZC-alone, and DZC-alone single-cultures. Samples were cultured under humidified conditions at 37°C and 5% CO₂ and maintained in ITS media, consisting of DMEM supplemented with 1% ITS+ Premix (BD Biosciences, San Jose, CA), 1% antibiotics, 0.1% antifungal, and 40 µg/ml proline (Sigma). The media was changed regularly and supplemented with fresh 50 µg/mL ascorbic acid (Sigma) and 3 mM β-glycerophosphate (Sigma). All groups were stimulated with 50 nM triiodothyronine (T3, Sigma) for the first three days of culture to induce chondrocyte hypertrophy[5,176].

In order to characterize the zonal model, SZC, MZC, and DZC were analyzed in terms of cell proliferation, matrix deposition, and hypertrophy. Chondrocyte phenotype, hypertrophy, and mineralization were then evaluated in single-culture, co-culture, and tri-culture groups over a three week culturing period. The layered scaffolds were separated prior to analysis in order to determine the individual response of each chondrocyte population.

10.2.4 Role of Dynamic Loading

To assess the effects of dynamic loading on the layered co-culture model, scaffolds were loaded in unconfined compression according to previously published protocols[172]. Briefly, a nominal ±5% compressive sinusoidal strain at 1 Hz frequency was applied, superimposed above a 10% tare strain, in unconfined compression with impermeable loading platens. Dynamic compressive loading was carried out at 37°C and 5% CO₂ in a humidified incubator for 3 hours/day for 7 days. Free-swelling, unloaded
controls were positioned adjacent to the loading device. The effects of dynamic loading on ALP activity and hypertrophy in the layered co-culture model were analyzed over 7 days of culture.

10.2.5 Role of PTHrP

Exogenous 100 nM parathyroid hormone-related protein (PTHrP, 1-40, Sigma) was added to DZC-alone cultures to mimic the effect of co-culture. To further evaluate the role of PTHrP in mediating the interaction between zonal chondrocyte populations, the actions of PTHrP were blocked using a PTHrP antagonist, parathyroid hormone (PTH, 7-34, Bachem, San Carlos, CA)[5,236]. Exogenous 1 µM PTH was added to SZC+DZC co-cultures for the duration of the experiment under serum-free conditions. The effects of the exogenous PTHrP and PTH antagonist on ALP activity and hypertrophy were analyzed over one week of culture.

10.2.6 Explant Content and Chondrocyte Response

As previously described[174], cell proliferation (n=4,5) was determined using the PicoGreen® total DNA assay (Molecular Probes, Eugene, OR), following the manufacturer’s suggested protocol. Fluorescence was measured with a microplate reader (Tecan, Research Triangle Park, NC), at the excitation and emission wavelengths of 485 and 535 nm, respectively. Total cell number in the sample was obtained by converting the amount of DNA per sample to cell number using the conversion factor of 7.7 pg DNA/cell[150].

Following papain digestion, collagen deposition (n=4,5) was quantified using a modified hydroxyproline assay[177]. Sample absorbance was measured at 550nm (Tecan), and the collagen content was obtained by interpolation along a standard curve of bovine type I collagen (Sigma). Total GAG deposition (n=4,5) was measured using a modified 1,9-dimethylmethylene blue (DMMB) dye-binding assay with chondroitin-6-sulfate (Sigma) as a standard. Absorbance was measured at both 540 nm and 595 nm for improved signal detection. Collagen and GAG distribution (n=2) were evaluated by Picrosirius red and Alcian blue staining, respectively.

Cell mineralization was determined by measuring ALP activity and mineral deposition. Quantitative ALP activity (n=4,5) was measured using an enzymatic assay based on the hydrolysis of $p$-
nitrophenyl phosphate (pNP-PO₄) to p-nitrophenol (pNP)[157], and sample absorbance was measured at 415 nm using a microplate reader (Tecan). Mineral distribution (n=2) was evaluated by von Kossa staining with 5% silver nitrate and 30 minutes of UV exposure[158]. Additionally, media calcium concentrations (n=4,5) were quantified using Arsenazo III dye (Pointe Scientific, Lincoln Park, MI), and absorbance was measured at 620 nm using a microplate reader (Tecan).

The expression of chondrocyte-relevant markers (n=3) were measured at day 7 using reverse transcription followed by polymerase chain reaction (RT-PCR) with custom-designed primers as follows:

<table>
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<th>Primer</th>
<th>Sense</th>
<th>Antisense</th>
</tr>
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<tbody>
<tr>
<td>B-actin</td>
<td>CTGCAGGCACTCCAGAAACTA</td>
<td>ACCGTGTGGGCGTGAGGTC</td>
</tr>
<tr>
<td>SZP</td>
<td>TGCAACGCTAGGCAGTA</td>
<td>CATCCAGAAATAATGACCTCAAT</td>
</tr>
<tr>
<td>SOX9</td>
<td>TACTGGTCGGCCAGCTTC</td>
<td>AACGGCTCGAGCAAGAAT</td>
</tr>
<tr>
<td>Type X Collagen</td>
<td>TGGATCAAAGGCGATGTG</td>
<td>GCCCCAGTAGGTCCATTAAGGC</td>
</tr>
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<td>MMP13</td>
<td>ACATCCCAAACGCCAGCAAA</td>
<td>GATGCAGCGCCAGAAGAAT</td>
</tr>
<tr>
<td>Ihh</td>
<td>ATCTCGGTGATGAACGACT</td>
<td>CCTTCGTAATGACGACT</td>
</tr>
<tr>
<td>PThrP</td>
<td>ACCTCGGAGGTGTCCTCAA</td>
<td>GCCCTCATCATCAGCCAA</td>
</tr>
<tr>
<td>PThrP-receptor</td>
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<td>TCCTTGACGAAGATGCTCAC</td>
</tr>
<tr>
<td>TGF- β1</td>
<td>ACACGCTTCAAGTGACATT</td>
<td>CATGAGGACGAGAAAGGG</td>
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<tr>
<td>TGF-β3</td>
<td>CTTCACGCTGTCCAGTGG</td>
<td>GGAAGGCCTCAATCCCTCTGC</td>
</tr>
</tbody>
</table>

Total RNA was isolated using the TRIzol reagent (Invitrogen) extraction method. The isolated RNA was reverse-transcribed into cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen) following the manufacturer’s suggested protocol, and the cDNA product was amplified with recombinant Platinum Taq DNA polymerase (Invitrogen). Expression band intensities of relevant genes were analyzed semiquantitatively and normalized against that of the housekeeping gene β-actin.

10.2.7 Statistical Analysis

Results are presented in the form of mean ± standard deviation, with n equal to the number of samples analyzed. A two-way analysis of variance (ANOVA) was performed on all quantitative data to determine the effects of co-culture and culturing time on zonal chondrocyte hypertrophy, mineralization,
and matrix content. The Tukey–Kramer post hoc test was used for all pair-wise comparisons, and significance was attained at p<0.05. All statistical analyses were performed using the JMP IN software (SAS Institute, Cary, NC).

10.3 Results

10.3.1. Zonal Explant Model

In order to establish baseline characterization of the explant model, each zone was characterized at day 1 in terms of cellularity, matrix content, and mineralization potential (Fig. 10.1). Chondrocytes within SZ, MZ, and DZ remain viable following zonal separation. In agreement with the 10:30:20 isolation protocol, SZ wet weight was the lowest, followed by DZC, and finally MZC with the highest tissue wet weight. Similarly, SZ dry weight was the lowest of all three zones, although there was no difference between MZ and DZ dry weights. Water content decreased throughout the three zones of cartilage, with a significant drop from 77.4% in SZ to 61.7% in DZ. Similarly, cellularity was highest in SZ and significantly lower in the MZ and DZ. In terms of matrix content, proteoglycan content was lowest in SZ and higher in MZ and DZ, whereas collagen content was lowest in DZ and higher in SZ and MZ (p<0.05). The ALP activity of cells in DZ was significantly higher than cells from both SZ and MZ.

Surface zone (SZ), middle zone (MZ), and deep zone (DZ) cartilage explants in single-culture, co-culture (SZ+DZ, SD), and tri-culture (SZ+MZ+DZ, SMD), were compared to corresponding zones in full thickness (FTC) explants to assess cartilage homeostasis (Fig. 10.1). In order to determine overall changes in matrix content, explant dry weight was measured over time for all explant groups. For SZ, dry weight increased over the first week of culture for all groups (p<0.05), with no further change thereafter. Although there was no difference in dry weight between SZ groups at day 7, significantly higher weight was measured for the SD group as compared to FTC. On the other hand, no change in MZ dry weight was found over time or between groups. Lastly, DZ-alone measured a significant increase in dry weight from day 1 to day 7; no change was found for any other group. There was no further change in DZ dry weight after three weeks of culture, at which time no statistical difference between groups was detected.

In order to assess scaffold hydration, explant wet weight was also measured over time. For SZ, wet weight increased from over the first week of culture for all groups (p<0.05), with further increases by
day 21 for the SD and SMD groups. Although there was no difference in wet weight between SZ groups at day 7, significantly higher weight was measured for the SD and SMD groups as compared to SZ-alone. Higher SZ wet weight was also measured for the SD group as compared to FTC (p<0.05). No change in MZ wet weight was found during the first week of culture, although there was a significant increase for MZ-alone by day 21. The weight of MZ-alone was significantly higher that both the SMD and FTC groups. Lastly, DZ wet weight increased from day 1 to day 7 for all groups except FTC. There was a further increase in wet weight measured after three weeks of culture for the SD group, measuring a higher weight as compared to FTC. By day 21, the water content of MZ-alone was significantly higher than both SZ-alone and DZ-alone. In the FTC model, water content of DZ was lower than both SZ and MZ (p<0.05). Although SZ and DZ water content remained unchanged in co-culture and tri-culture, MZ-alone water content was significantly higher than in FTC. Given the variability in tissue swelling among groups and zones, dry weight was used to normalize all cell and matrix data.

10.3.2. Cartilage Homeostasis: Matrix

A significant decrease in cell number of all SZ groups was measured during the first week of culture, although higher number was found in the SMD and FTC groups as compared to the SZ-alone and SD groups at day 7 (p<0.05, Fig. 10.2). No further change in cell number was detected thereafter, although differences between groups were no longer detected by day 21. On the other hand, there was a decrease in MZ cell number over the first week of culture for only the single-culture group (p<0.05), and at day 7, MZ-alone contained significantly less cells than SMD and FTC groups. By day 21, no difference in MZ cellularity between groups was detected. Similarly, there was a decrease in DZ cell number over the first week for only the single-culture group (p<0.05), and at day 7, DZ-alone contained significantly less cells than SMD and FTC groups. There was a delayed decrease in cell number for SMD and FTC by day 21, resulting in no difference between groups after three weeks of culture.

There was no change in SZ proteoglycan content over the first week of culture for all groups, with a significant increase measured between day 7 and day 21 for the FTC group, and no difference between groups was detected at either timepoint (Fig. 10.2). For MZ GAG content, there was no difference over time or between groups. Finally, DZ GAG decreased over the first week of culture for the single-culture
and SD groups (p<0.05). There was a further decrease for DZ-alone by day 21, resulting in significantly lower GAG content as compared to all other groups. Staining for proteoglycan confirmed that SZ groups had less positive staining as compared to the MZ and DZ.

The collagen content of SZ significantly decreased uniformly over the first week of culture, followed by an increase for the tri-culture groups by day 21 (p<0.05, Fig. 10.2). The collagen content of MZ also significantly decreased between day 1 and day 7 for all groups, with a further decrease after three weeks of culture for only the FTC group. No differences in MZ collagen were detected between groups at either timepoint. Finally, there was a significant decrease in DZ collagen for the single-culture and SD groups, measuring lower collagen as compared to the tri-culture groups at day 7. A delayed decrease after three weeks of culture was measured for the tri-culture groups. Less positive collagen staining was evident for SZC single-culture and co-culture groups.

Unnormalized matrix content is also important to consider, given the significant changes in explant weight (data not shown). While there was no change in MZ GAG content over time, there was in fact a 5- to 9-fold increase in SZ GAG, and DZ experienced an 80% loss in GAG only in single-culture. Similarly, there was an increase in SZ collagen, although MZ and DZ exhibited a 20% to 70% decrease over time.

10.3.3. Cartilage Homeostasis: Mineralization

Mineralization potential of SZ and MZ remained at basal levels over time and for all groups (Fig. 10.3). The ALP activity of DZ increased over the first week of culture for all groups (p<0.05) and was significantly lower for SD and SMD groups as compared to single-culture at day 7. Between day 7 and day 21, DZ ALP activity significantly decreased for single-culture, SD, and FTC groups. After three weeks of culture, all co-culture and tri-culture groups measured lower DZ ALP activity as compared to DZ-alone (p<0.05).

Corresponding von Kossa staining at day 21 indicated negligible staining for all SZ and MZ groups (Fig. 10.3). Only DZ-alone stained positive for mineral, with negligible staining evident for all co-culture and tri-culture groups. Positive von Kossa staining for mineral was visible only in the DZ-alone group. In order to further explore the mineral presence in DZ explants, single-culture and FTC groups
were compared with T3-free controls, as well as with the addition of inorganic phosphate. It was found that without T3 stimulation, there was no positive mineral staining in either group. Furthermore, the addition of inorganic phosphate resulted in increased positive staining for the DZ-alone group and minimal positive staining for the FTC group.

Hypertrophic markers, namely collagen X, MMP-13, and Ihh were all expressed by DZ in all groups at day 7. Furthermore, collagen X expression was downregulated in all co-culture and tri-culture groups as compared to single-culture control, and Ihh expression was downregulated in only the tri-culture groups. Between groups, the expression of MMP-13 remained unchanged.

10.3.4. Agarose Tri-Culture Model

Each chondrocyte population, namely SZC, MZC, and DZC, was characterized in terms of cell proliferation, ALP activity, and matrix deposition in both single-culture scaffolds and layered tri-culture scaffolds (Fig. 10.4). There were significant decreases in SZC and MZC cell number by day 21 only in single-culture, as well as a decrease in SZC cell number between day 1 and day 7 in tri-culture. Of all three cell types, only DZC measured significant cell proliferation during the first week, followed by a decrease by day 21. Furthermore, DZC measured the highest cellularity of all cell types by day 21 in both groups (p<0.05). The ALP activity of SZC and MZC remained at basal levels in single-culture for the duration of the experiment, while a significant increase was measured between day 1 and day 7 in tri-culture. The ALP activity of DZC increased during the first week in both single-culture and tri-culture groups, and a subsequent decrease was found for only the tri-culture group by day 21 (p<0.05). Furthermore, DZC measured the highest ALP activity of all cell types at day 7 in both single-culture and tri-culture.

In terms of matrix synthesis, SZC GAG deposition remained at basal levels for the duration of the experiment. On the other hand, a significant increase was measured for MZC between day 1 and day 7 in tri-culture and day 7 and day 21 in single-culture. Proteoglycan deposition by DZC increased during the first week in both groups, and remained stable thereafter. By day 21, MZC measured the highest GAG activity, followed by DZC, and finally SZC in single-culture (p<0.05). On the other hand, there was no significant difference between MZC and DZC in tri-culture, both measuring higher GAG/cell as
compared to SZC. Minimal SZC collagen synthesis was measured over time in the single-culture group, whereas an increase was measured during the first week in tri-culture (p<0.05). Significant MZC collagen synthesis was measured in both groups between day 1 and day 7, with no change thereafter. Finally, DZC collagen deposition remained at basal levels for both groups.

10.3.5. Effect of Tri-Culture on Chondrocyte Response

The response of SZC, MZC, and DZC were compared between the single-culture and tri-culture groups (Fig. 10.4). Significantly lower SZC cellularity was found in tri-culture groups at both day 7 and day 21. On the other hand, no difference in MZC cell number was detected between groups at any timepoint. While DZC cell number was lower in the tri-culture group at day 7 (p<0.05), no difference was found between groups by day 21. No differences in ALP activity between single-culture and tri-culture groups were also found for SZC and MZC. However, DZC measured a significantly lower ALP activity in tri-culture as compared to single-culture control at day 7.

In terms of matrix, proteoglycan deposition by SZC remained at basal levels for the culturing period, with no significant difference between single-culture and tri-culture groups. Proteoglycan deposition by MZC was significantly higher in tri-culture at day 7, although no difference between groups was found by day 21. Similarly, GAG deposition by DZC was higher in tri-culture only at day 7 (p<0.05). In order to quantify scaffold content, GAG/wet weight is also reported. During the first week of culture, significant GAG accumulation was measured in MZC and DZC groups. While no difference was found between single-culture and tri-culture groups for MZC, higher DZC GAG/wet weight was found for tri-culture groups at both day 7 and day 21 (p<0.05). No significant difference in SZC collagen deposition between single-culture and tri-culture groups was detected. Collagen deposition by MZC was significantly higher in tri-culture at day 7, although no difference between groups was found by day 21. Similarly, deposition by DZC was higher in tri-culture only at day 7 (p<0.05). During the first week of culture, significant collagen accumulation was measured in SZC and MZC groups. While no difference was found between single-culture and tri-culture groups for MZC, higher SZC collagen/wet weight was found for tri-culture groups at both day 7 and day 21 (p<0.05). Corresponding histology showed strongly positive staining for GAG and collagen by DZC as compared to SZC and MZC.
10.3.6. Zonal Chondrocyte Interactions

In order to elucidate the specific zonal chondrocyte interactions present in the tri-culture model, chondrocyte response was further assessed in SZC+MZC, MZC+DZC, and SZC+DZC co-culture scaffold and again compared to single-culture controls (Fig. 10.5). In a layered scaffold comprised of SZC and MZC, there was significant accumulation of GAG by MZC in single-culture and co-culture between day 1 and day 7 (p<0.05). Both SZC and MZC GAG deposition were higher in co-culture at day 7, although no differences were detected by day 21. On the other hand, no accumulation of collagen was measured in the co-culture group for either SZC or MZC, and was significantly lower in the co-culture group as compared to the single-culture controls at day 7. By day 21, there were significant decreases in collagen for the single-culture controls, resulting in no significant difference between groups after three weeks of culture. Finally, ALP activity remained at basal levels for the duration of the experiment, with no difference between groups at any timepoint.

In a layered scaffold comprised of MZC and DZC, there was significant GAG deposition by both cell types from day to day 7, with no change thereafter. Furthermore, GAG deposition was higher in co-culture scaffolds at day 7 (p<0.05). Both MZC and DZC collagen content increased during the first week in co-culture, with no change thereafter. Collagen accumulation was lower in the co-culture group as compared to single-culture control at day 7, although differences were no longer present by day 21. Finally, MZC ALP activity remained at basal levels for the duration of the experiment. A peak in DZC ALP activity was measured at day 7 (p<0.05), with no difference between co-culture and single-culture groups at either day 7 or day 21.

In a layered scaffold comprised of SZC and DZC, there was significant GAG deposition by both cell types in co-culture from day to day 7, with a decrease in DZC GAG accumulation by day 21. Whereas there was no difference in DZC GAG content between groups, GAG deposition by SZC was higher at day 21. Both SZC and DZC collagen content increased during the first week in co-culture, with a significant decrease thereafter only for the single-culture controls. No differences in collagen accumulation were detected at day 7, although higher collagen was measured for DZC in co-culture after three weeks of culture (p<0.05). Finally, both SZC and DZC ALP activity increased between day 1 and
day 7 in co-culture (p<0.05). There was no difference between groups for SZC ALP activity, although a significantly lower value was measured for DZC in co-culture at day 7.

10.3.7. Co-Culture Model to Study Hypertrophy and Mineralization

The simplified co-culture model of SZC layered above DZC was used to further explore the regulation of hypertrophy (Fig. 10.6). The two layers were visible in the brightfield and histology images of the layered scaffold; the matrix-rich DZC region was opaque and the matrix-poor SZC region was relatively translucent. Chondrocytes within the SZC layer expressed SZP, which was significantly higher in the co-culture group. Basal levels of SZP were measured for DZC both in single-culture and co-culture at day 7. On the other hand, Sox9 was expressed by both SZC and DZC. In co-culture, there was no change in SZC Sox9 expression, although expression by DZC was higher (p<0.05).

Stimulation with T3 was used to promote chondrocyte mineralization (Fig. 10.6). In the absence of T3, there was no change in media calcium for SZC, DZC or co-culture groups as compared to plain media. Furthermore, there was minimal von Kossa staining at 21 days. With the addition of T3, DZC-alone cultures measured lower media calcium at day 4 as compared to plain media, SZC-alone and the co-culture group (p<0.05). By day 7, both DZC-alone and co-culture groups measured lower media calcium as compared to plain media and SZC-alone. Corresponding von Kossa staining for T3-stimulated groups as indicated no positive staining for SZC-alone group while there was strongly positive staining for the DZC-alone group. Furthermore, co-culture histology indicated staining for mineral in the DZC region not adjacent to the SZC layer.

As expected, SZC collagen X expression was at basal levels in single-culture and was significantly higher in co-culture (Fig. 10.7). The expression of SZC MMP13 and Ihh were also higher in the co-culture scaffold (p<0.05). On the other hand, DZC expressed high collagen X, with a significant decrease in co-culture at day 7. The expression of MMP-13 was at basal levels for DZC both in single-culture and co-culture, and a significant decrease in DZC Ihh was detected in the co-culture group. These changes in hypertrophic marker expression were also accompanied by significant changes in chondrocyte regulatory markers, namely PTHrP, TGF-β1, and TGF-β3. Higher SZC PTHrP and TGF-β1, as well as DZC TGF-β1 expression, were measured at day 7 (p<0.05).
10.3.8. Mechanism of Zonal Chondrocyte Communication

To assess the effect of SZC-to-DZC proximity, the layered co-culture model previously described was compared to a mixed co-culture of SZC+DZC. When the activity of both SZC and DZC were considered in combination, significantly lower ALP activity was measured in the mixed co-culture group as compared to the layered co-culture group at day 7 (Figure 10.8A). Layered co-culture scaffolds with and without the application of dynamic loading were also considered. The application of dynamic loading did not affect the integrity of the layered constructs and as expected, SZC ALP activity was at basal levels in both unloaded and loaded groups (data not shown). On the other hand, the ALP activity of DZC was significantly lower for the loaded group as compared to the unloaded control at day 7.

Exogenous addition of PTHrP to DZC single-cultures resulted in a significant decrease in ALP activity at day 7 (Fig. 10.8B). The blocking of PTHrP in layered co-culture, on the other hand, resulted in a significant increase in DZC ALP activity, with no change in SZC mineralization potential.

10.4 Discussion

The objectives of this study were to determine the role of zonal chondrocyte interactions in regulating cartilage homeostasis and formation. To this end, we established physiologically-relevant explant and hydrogel models. The results of this study collectively demonstrate that tri-culture of all three cartilage zones mitigates the loss of deep zone matrix content, while co-culture with the surface zone prevents ectopic mineralization. Similar to the explant model, deep zone chondrocyte mineralization is suppressed in the presence of surface zone chondrocytes. While chondrocyte proteoglycan deposition is enhanced by zonal interactions, collagen deposition is suppressed in all interaction models except in the co-culture of surface and deep zone chondrocytes. These findings collectively demonstrate the importance of understanding zonal chondrocyte interactions for cartilage tissue engineering applications.

Characterization of each cartilage zone confirms the three different cartilage matrices found in native tissue. To summarize, the surface zone of articular cartilage has the highest cellularity, with a matrix composed primarily of collagen. The middle zone and deep zone layers have lower cellularity with high GAG content. The deep zone layer has the lowest collagen content for all three zones, as well as significant mineralization potential. This characterization is in agreement with previously conducted
studies which have looked at the depth-dependent distribution of cells and matrix within immature cartilage explants[132]. Zonal chondrocyte populations mirror the biosynthetic activity in in vitro culture of the cartilage zone from which they were derived. Specifically, low GAG and high collagen deposition were measured by chondrocytes close to the articular surface, high matrix overall deposition by middle zone chondrocytes, and high proteoglycan and low collagen deposition by deep zone chondrocytes. Only those chondrocytes farthest from the articular surface measured significant alkaline phosphatase activity over time. These findings are also in agreement with previous studies which ascertained the biosynthetic activity of chondrocyte subpopulations in vitro[4,5,237-242].

Layering of zonal chondrocyte populations have been previously used to mimic the native organization of cartilage[173,196,228,229]. A layered hydrogel model is particularly advantageous model for determining zonal chondrocyte interactions. First and foremost, chondrocytes maintain their 3D morphology in hydrogel culture. Secondly, it is possible to separate layers of the scaffold for endpoint analysis to discern the individual responses of each cell type. In contrast to the 2D co-culture model where there is direct cell-cell contact[5], surface, middle, and deep zones chondrocytes were instead separated and therefore limited cellular communication to autocrine and paracrine factors. The findings of this study indicate that there is a synergistic increase in matrix with the co-culture of surface and deep zone chondrocytes. On the other hand, there is a shift from collagen to proteoglycan deposition in tri-culture of all three zones. In a study by Sharma et al, layered hydrogel constructs consisting of surface zone and deep zones chondrocytes were compared to single-cell controls[229]. It was found that despite decreased deep zone chondrocyte cellularity in co-culture, comparable GAG and collagen were measured as compared to single-culture controls. In another study by Ng et al, surface zone chondrocytes were co-cultured with a mixed combination of middle and deep zone chondrocytes in a bilayered agarose scaffold[195]. As compared to single-culture controls, no synergistic increase in matrix biosynthesis was observed. Differences between models may be attributed to variations in seeding density, the presence of serum, and thyroid hormone stimulation.

This study also uses an explant model to assess matrix homeostasis. Explant cartilage cultures provide a well-characterized, stable in vitro system that enables the study of chondrocytes within their native extracellular matrix[231,232,243-245]. Cartilage explants measured an overall decrease in
normalized matrix content in this study, although this loss was partially mitigated by tri-culture of all three zones. This is in agreement with Bian et al. which demonstrated that full thickness explants can be maintained in culture for six weeks in serum-free conditions[245]. However, no decrease in explant dry weight over time was detected and points to a net increase in unnormalized matrix content. Although both GAG and collagen were deposited within the surface zone over time, the ratio of GAG-to-collagen increased from 0.17 to nearly 0.80, while in the remaining zones, collagen loss was not accompanied by changes in proteoglycan. The changes within the surface zone are not dependent on zonal chondrocyte interactions. The uniform increases in GAG deposition within this zone in vitro indicates that it is suppressed within the native joint. On the other hand, zonal chondrocyte interactions mediate matrix loss in the zone closest to the other osteochondral interface. The presence of surface zone cartilage improves deep zone GAG retention, whereas the middle zone is necessary for collagen retention. Since no difference between the segregated (SZ+MZ+DZ) and non-segregated (FTC) was detected, matrix homeostasis is mostly likely regulated via paracrine communication between the cartilage layers.

In addition to modulating matrix content, mineralization and hypertrophy is regulated by zonal chondrocyte interactions. In this study, thyroid hormone was used to disrupt the self-regulation of deep zone chondrocytes by promoting hypertrophy and mineralization in deep zone explant and chondrocyte cultures. Interestingly, surface zone and middle zone chondrocytes were largely unaffected and maintained basal ALP activity and negative staining for mineral. This confirms that immature deep zone chondrocytes are a unique cell population that retains some of the characteristics of growth plate chondrocytes, namely the potential to become hypertrophic and mineralize in vitro[70]. Similar to the findings of the 2D direct co-culture model[5], the presence of surface zone chondrocytes, alone and in combination with middle zone chondrocytes, suppressed the induced increase in deep zone hypertrophy in both the agarose and explant models.

Since the different zonal populations in vivo and in the chondrocyte interaction models are not in direct contact, paracrine factor transport is necessary for the regulation of mineralization. Evidence for this is the greater suppression in the mixed co-culture model as compared to the layered; surface and deep zone chondrocytes are in closer proximity in the mixed model, and therefore the paracrine factors have a shorter distance to travel. Dynamic loading, by facilitating transport within the model, enhances
the effects of co-culture[246]. Not only is the application of dynamic loading a technique to determine the issue of transport, it is also physiologically-relevant and needed for the maintenance of healthy cartilage[234].

Similar to the 2D co-culture model, regulation of cartilage mineralization is partially regulated by PTHrP. Interestingly, it is only upregulated in the surface zone, whereas deep zone expression remains unchanged. Previous studies have demonstrated that in fact PTHrP expression is localized near the articular surface[247,248]. Blocking of PTHrP only partially reverses the effects of co-culture. One explanation for this is the incomplete blocking with PTH, given the competitive blocking of the PTHrP receptor[249]. Secondly, it is possible that other co-factors are also responsible, such as members of the TGF-β family which are upregulated in co-culture and have been shown to inhibit terminal chondrocyte differentiation[250]. In a study by Pateder et al., growth plate chondrocytes stimulated with TGF-β measured higher PTHrP in a dose-dependent manner, and furthermore, TGF-β1 and TGF-β3 were found to be more potent than TGF-β2[251].

Interestingly, the pattern of regulatory marker expression is different for the agarose and explant co-culture models (Fig. 10.9). While no difference in surface zone regulation was measured in co-culture, enhanced PTHrP and TGF-β1 were upregulated in full thickness explants. On the other hand, TGF-β1 and TGF-β3 were suppressed in the deep zone of all co-culture and tri-culture groups. In addition to the presence of cell-matrix interactions present in cartilage explants, it is also postulated that there are significant differences in the biology of chondrocytes suspended within agarose and those in their native environment which should be explored further. One significant difference is the expression of PTHrP receptor found only in cartilage explants and suggests variations in signaling efficiency and feedback. Despite their differences, both hydrogel and explant models are useful tools for studying cartilage maintenance and cell-to-cell interactions. Our results suggest that in immature animals, maintenance of the articular surface is directly responsible for regulation of mineralization at the interface. The method of communication most likely involves PTHrP signaling and is mediated through dynamic loading of tissue. Finally, the stratified organization of all three layers of articular cartilage is critical for maintaining cartilage matrix content in culture, although this scaffold may not represent the ideal scaffold for cartilage regeneration.
10.5 Conclusions

The results from this study collectively demonstrate that zonal chondrocyte interactions regulate chondrocyte biosynthesis, hypertrophy, and mineralization. These findings have important ramifications for designing the optimal scaffold which achieves cartilage formation and maintenance; while layered surface zone and deep zone scaffolds are optimal for high collagen deposition and suppression of mineralization, long-term culture of all three zones is necessary for retention of matrix content.
The role of zonal interactions was investigated in the explant model by isolating surface zone (SZ), middle zone (MZ), and deep zone (DZ) explants. Chondrocytes remained viable in the explant post-separation (Day 1, 20x, bar=100 μm). Water content and cellularity were significantly lower in the DZ as compared to the SZ (*p<0.05). GAG content was lowest in the SZ (*p<0.05), and collagen content was lowest in DZ (**p<0.05). High ALP activity was detected on the DZ (*^p<0.05: significant difference with respect to SZ; **^p<0.05: significant difference with respect to MZ). For single-culture explants, increases in SZ and DZ wet and dry weights were detected over the first week of culture (#p<0.05). For SZ+DZ co-culture (SD), increases in SZ and DZ wet weights were also detected for the duration of the experiment (#p<0.05). For SZ+MZ+DZ tri-culture (SMD) and full thickness explants (FTC), lower MZ wet weight was detected as compared to the single-culture control at Day 21 (*p<0.05).
Figure 10.2. Matrix homeostasis. HigherSZ, MZ, and DZ cell number were measured in the tri-culture groups as compared to single-culture controls at Day 7 (*p<0.05). Zonal chondrocyte interactions had no effect on SZ or MZ matrix deposition. On the other hand, higher DZ GAG content was found for all co-culture and tri-culture groups at Day 21, and higher DZ collagen content was measured in tri-culture at day 7 (*p<0.05). Corresponding GAG and collagen staining showed strongly positive staining in the deep zone of full thickness explants (Day 21, 10x, 250 um).
**Figure 10.3. Ectopic mineralization.** The ALP activity of chondrocytes within SZ and MZ remained at basal levels over time and for all groups. There was a significant suppression of DZ ALP activity in all co-culture and tri-culture groups at Day 21 as compared to the single-culture control (*p<0.05). Von Kossa staining (Day 21, 10x, bar=250 um) showed positive staining only in the DZ-alone scaffold. The expression was collagen X and Ihh were significantly downregulated in the tri-culture groups at Day 7 (*p<0.05).
Figure 10.4. Effect of tri-culture on chondrocyte biosynthesis. Chondrocytes isolated from the surface zone (SZC), middle zone (MZC), and deep zone (DZC) were cultured in single-culture and tri-layered scaffolds. There was lower SZC and DZC cell number at Day 7 in tri-culture (*p<0.05). DZC ALP activity was lower in tri-culture at Day 7 (*p<0.05). During the first week of culture, significant increases in MZC tri-culture, DZC single-culture, and DZC tri-culture GAG/cell, as well as SZC tri-culture, MZC single-culture, and SZC tri-culture collagen/cell were measured (#p<0.05). Corresponding histology for collagen and GAG show the most matrix deposition for the DZC groups (Day 7, 20x, bar=100 um).
Figure 10.5. Zonal chondrocyte interactions. Significantly higher GAG content and lower collagen content were measured at Day 7 in SZC+M Zac (SM) and MZC+DZC (MD) scaffolds (*p<0.05). Decreased DZC ALP activity was measured in SZC+DZC (SD) co-culture scaffolds at Day 7 (*p<0.05).
Figure 10.6. Co-culture model to study regulation of deep zone chondrocyte mineralization. Layered scaffolds of SZC+DZC result in corresponding matrix-rich and matrix-poor regions (Day 7, 5x, bar=400 um). Positive SZP expression was expressed only by SZC, whereas both SZC and DZC expressed Sox9 at Day 7. Without T3 stimulation, no calcium uptake of positive staining for mineral was detected. With T3 stimulation, decreased media calcium was detected for the DZC-alone and layered co-culture groups at Day 21 (*p<0.05). Positive von Kossa staining was also seed for the DZC-alone and DZC portion of the layered scaffold (Day 21, 10x, bar=100 um).
Figure 10.7. Mechanism of zonal chondrocyte interactions. The expression of collagen X and MMP13 were upregulated by SZC in co-culture, whereas there was significant down-regulation of DZC collagen X and Ihh at day 7 (*p<0.05). These changes corresponded to higher SZC PTHrP and TGF-β3 and DZC TGF-β1 expression (*p<0.05).
Figure 10.8. Role of PTHrP and dynamic loading. (A) The suppression of ALP activity was enhanced in mixed co-culture as compared to layered co-culture (*p<0.05). DZC ALP activity suppression was also enhanced with the application of dynamic loading (*p<0.05). (B) Exogenous addition of PTHrP to DZC-alone cultures decreased ALP activity to basal levels (*p<0.05). The addition of PTH to layered co-culture scaffolds reversed the effect of co-culture and resulted in higher DZC ALP activity at Day 7 (*p<0.05).
Figure 10.9. Zonal chondrocyte regulation in cartilage explants. Higher SZ PTHrP, PTHrP-r and TGF-β1 expression were measured in FTC tri-culture (*p<0.05). Lower MZ PTHrP, TGF-β1 and TGF-β3, as well as lower DZ TGF-β1 and TGF-β3, were measured in FTC tri-culture at Day 7 (*p<0.05).
CHAPTER 11: SUMMARY AND FUTURE DIRECTIONS
11.1 Summary

The objective of this thesis was to design an interface scaffold for calcified cartilage regeneration, given the long-term goal of functional and integrative cartilage repair. The ideal osteochondral interface scaffold should support chondrocyte biosynthesis and the formation of calcified cartilage with physiologically-relevant mechanical properties. Furthermore, the interface scaffold should allow for the maintenance of the calcified cartilage matrix, while prohibiting ectopic mineralization. Lastly, the interface scaffold should be osteointegrative. It was hypothesized that ceramic presence and zonal chondrocyte interactions regulate cell biosynthesis and mineralization, and these cell-ceramic and cell-cell interactions are essential for calcified cartilage formation and homeostasis. To evaluate these hypotheses, studies were designed to address four specific aims. **Aim 1** focused on characterizing the native osteochondral interface in terms of matrix and mineral distribution from the articular surface to subchondral bone. To this end, Fourier Transform Infrared Spectroscopy was used to evaluate region- and age-dependent changes in matrix and mineral content in bovine osteochondral explants (*Chapter 2*). Biomimetic design parameters were derived from results of this characterization study, as well as from previous studies which have studied the interface using microscopy and histological techniques.

A hydrogel-based scaffold was designed for the formation of calcified cartilage, since hydrogels are especially well-suited to maintain the chondrocyte phenotype and their biosynthetic activity. Furthermore, a hydrogel-based interface scaffold can be easily integrated with other hydrogel-based cartilage grafts. Within the hydrogel phase, biomimetic ceramic was incorporated during fabrication. The rationale behind the incorporation of hydroxyapatite was two-fold; the inclusion of biomimetic mineral content diminishes the need for cell-mediated mineralization and ceramic presence facilitates osteointegration with underlying bone. Ceramic presence was also shown to modulate chondrocyte hypertrophy and biosynthesis, and the optimization of these cell-ceramic interactions within the interface scaffold was the focus of **Aim 2**. An agarose-based hydrogel system was chosen over alginate in order to better control ceramic distribution and achieve higher mechanical properties. Next, it was demonstrated that chondrocyte response to hydroxyapatite is population- and phenotype-dependent (*Chapter 3 and 4*). In contrast to full thickness chondrocytes, hypertrophic chondrocytes, induced with thyroid hormone stimulation, measured higher matrix deposition and mineralization in the hydrogel-
ceramic scaffolds as compared to hydrogel-alone scaffolds. Furthermore, ceramic size and dose were shown to modulate the response of these hypertrophic chondrocytes (Chapter 4). Only micron-sized particles (as opposed to nano-particles) consistently promoted the formation of a calcified cartilage matrix, and additionally, an optimal ceramic dose was identified (3w/v%).

Based on the optimized scaffold design, in vitro calcified cartilage formation was evaluated at three different cell seeding densities (Chapter 5). Within this agarose+HA scaffold, induced hypertrophic chondrocytes deposited mineral and a proteoglycan-collagen matrix. These cell-mediated changes were also reflected in higher mechanical properties and biomimetic structure-function relationships. Building upon the results from the previous aim, the hydrogel-ceramic composite was implemented for integrative cartilage repair in Aim 3. To this end, the feasibility of layering of HA-free and HA-containing agarose scaffolds was evaluated in Chapter 6. The findings of this study indicated that articular cartilage and calcified cartilage could be formed on a single, bilayered scaffold. Next, a nanofiber cup design was developed in order to add a biomimetic interface between the implant and tissue, as well as a temporary osteochondral barrier until tissue development was achieved (Chapter 7). In addition, these designs were tested in an animal model (Chapter 8). Finally, in Aim 4, the maintenance of the osteochondral interface was explored, focusing on the role of the stratified organization of surface, middle, and deep zone chondrocytes above calcified cartilage and the role of zonal chondrocyte interactions. The major findings of each chapter are briefly highlighted below.

11.1.1 Osteochondral Interface Characterization and Rationale for Interface Scaffold Design

The first step towards biomimetic scaffold design is characterization of the native tissue. While articular cartilage has been extensively characterized, the thin calcified cartilage interface between articular cartilage and bone is particularly challenging to study since it is not readily separable from bone. Previously, microscopy and histological techniques have been used to characterize the osteochondral interface[67,79-81,90,93,97,103,109,110,252]. In Chapter 2, matrix and mineral distribution across the cartilage-to-bone transition were identified without separating the calcified cartilage layer from cartilage or bone, using a high-resolution imaging technique to quantify the relative amounts of proteoglycan, collagen, and mineral based on their unique spectral signatures[112,114,116,129]. It was determined
that the calcified cartilage layer resembles deep zone cartilage, containing comparable proteoglycan and collagen content, albeit with mineral content. Furthermore, the transition from basal mineral content within articular cartilage to higher mineral content in bone is exponential. The mineral content of the calcified cartilage transition region is significantly lower as compared to bone and also reflects the interdigitated nature of the interface. These trends are preserved with age, although only the immature model was used for tissue regeneration applications in this thesis.

These findings, along with the findings of previous studies, provide the design criteria for the interface scaffold. In addition to supporting chondrocyte viability and the hypertrophic phenotype, chondrocytes within this scaffold must deposit a collagen- and proteoglycan-rich matrix. The interface scaffold must support calcified cartilage formation and biomimetic structure-function relationships. Furthermore, mineralization must be confined to only the interface scaffold. Finally, a scaffold designed for interface tissue engineering should enable biological fixation with surrounding tissue.

11.1.2 Interface Scaffold Design and Optimization

Based on the interface characterization performed in Chapter 2, a hydrogel-ceramic composite scaffold was designed and developed for in vitro calcified cartilage formation. The scaffold consists of two phases: the hydrogel phase supports the chondrocyte phenotype and biosynthesis, and the ceramic phase is pre-incorporated to mimic the mineral found at the native interface[74,86,87]. Both alginate (Chapter 3) and agarose (Chapter 4) hydrogels were tested. These materials have been used extensively for chondrocyte culture and cartilage tissue engineering applications[29,123,139-141,171-173,188,191,196,253-256], although agarose was found to be more optimal for distributing ceramic particles and achieving higher mechanical properties in culture.

It was hypothesized that interface-relevant deep zone chondrocytes are better suited for calcified cartilage formation as compared to full thickness chondrocytes, since these cells are located directly above the interface in native tissue and have shown promise for calcified cartilage formation[70,106,107,175,257,258]. Hypertrophic chondrocyte response was also tested in Chapter 4 by stimulating cultures with thyroid hormone[5,176], and it was found that these cells are in fact more
appropriate since they exhibit enhanced matrix deposition and hypertrophy in ceramic-agarose composite scaffolds.

The ceramic phase of the interface scaffold was also optimized in terms of ceramic size and dose (Chapter 4). Micron-sized and nano-sized particles were used for comparison in order to determine the role of mineral aggregates within the native tissue as compared to individual crystals[74]. Ceramic dose was varied to encompass the wide range of values reported for the mineral content of calcified cartilage[87,88,131]. The results of these cell-ceramic optimization studies demonstrated that only micron-sized particles promote calcified cartilage formation. Furthermore, there is an optimal range of ceramic dose which promotes deposition of a functional calcified cartilage-like matrix; this range reflects the trade-off between ceramic-induced enhancement in cell-mediated matrix deposition and decreased available space at very high doses. At an intermediate seeding density of 20 million chondrocytes/mL, in a scaffold comprised of 3 w/v% ceramic, calcified cartilage formation was achieved after six weeks in vitro (Chapter 5).

11.1.3 Interface Scaffold Implementation for Repair

In order to implement this scaffold for cartilage repair applications, a cartilage graft is also required to regenerate articular cartilage. One of the added advantages of a hydrogel-based system is that layered scaffolds are easily fabricated[173]. The feasibility of using a bilayered scaffold, with full thickness chondrocytes in agarose layered above deep zone chondrocytes in agarose+ceramic, was demonstrated in Chapter 6. Ceramic presence within the calcified cartilage layer does not adversely affect chondrocytes within the uncalcified layer, and there were no indications of ectopic mineralization above the interface scaffold. Next, a ceramic-containing nanofiber cup was added (Chapter 7). The purpose of the nanofiber is to further promote osteointegration with bone. Secondly, the biomimetic nanofiber mesh is envisioned as a temporary osteochondral barrier until dense tissue development is achieved within the hydrogel. Finally, the cup design facilitates handling and implantation of the scaffold. The cup design itself was also shown to enhance calcified cartilage formation.

An osteochondral defect model in mature New Zealand White rabbits (Chapter 8) was used to test the efficacy of the interface scaffold for regenerating the osteochondral interface in vivo. As
compared to an empty defect which is filled with mineralized fibrocartilage, the presence of a hydrogel scaffold enhances the quality of the repair tissue, while ceramic incorporation within the hydrogel promotes mineralization surrounding the scaffold. The nanofiber-ceramic mesh serves two roles by preventing invasion of endochondral ossification and promoting osteointegration. The implant comprised of a polymer-ceramic nanofiber and a bilayered agarose/agarose-ceramic scaffold was shown to promote the formation of a stable uncalcified-to-calcified interface four weeks post-implantation.

11.1.4 Interface Scaffold Maintenance

Although calcified cartilage formation was demonstrated in vitro and in vivo in Aims 2 and 3, the maintenance of this interface is also critical to long-term tissue functionality. A variety of age- and disease-related changes disrupt interface homeostasis, namely advancement of the mineralization front into uncalcified cartilage and thickening of subchondral bone[71,102,103]. Within articular cartilage, zonal chondrocyte interactions have been shown to regulate mineralization[5]. Therefore, the role of the zonal organization of articular cartilage, consisting of stratified surface, middle, and deep zone chondrocytes[4], was investigated with respect to the calcified cartilage interface in Chapter 9. Although tri-culture of all three zonal populations, regardless of organization, is sufficient to suppress mineralization within uncalcified cartilage, layered cartilage scaffolds also regulate mineralization within the calcified cartilage zone. Within uncalcified cartilage, surface zone chondrocytes suppress deep zone chondrocyte mineralization, although all three zones are required for optimal matrix homeostasis (Chapter 10).

11.2 Future Directions

The findings of this thesis demonstrate the feasibility of using polymer-ceramic composite scaffolds to regenerate the osteochondral interface. However, to realize the clinical translation of the scaffold system, several areas of study are needed and are described below.

11.2.1 Mesenchymal Stem Cells

Mesenchymal stem cells are a clinically relevant cell source; autologous cells can be harvested from a patient, cultured in vitro and then implanted back into the defect site. This procedure has been
previously used for repair cartilage defects and osteochondral defects in patients[259-261]. Although mesenchymal stem cells have not been previously used for calcified cartilage regeneration, Mueller et al. investigated the differentiation of mesenchymal stem cells into hypertrophic chondrocytes[262]; findings from this study indicate that the combination of thyroid hormone stimulation and dexamethasone withdrawal promotes the hypertrophic phenotype in vitro. Preliminary results in a hydrogel model demonstrate that prolonged dexamethasone treatment in fact promotes the highest ALP activity (Fig. 11.1). On the other hand, dexamethasone withdrawal promotes collagen deposition, and the combination of dexamethasone withdrawal and T3 stimulation upregulates GAG deposition. Future studies will investigate the response of stem cells within the hydrogel-ceramic composite, in order to determine whether scaffold optimization parameters vary from those derived with terminally differentiated chondrocytes. Furthermore, stem cell response within the scaffold system is pertinent for understanding in vivo healing.
Figure 11.1. Hypertrophic stem cell differentiation. Mesenchymal stem cells were seeded in agarose scaffolds (10 million/mL) and cultured in a variety of treatment conditions and compared to ITS media-only controls. All other groups were stimulated with TGF-β3 (10 ng/mL) with dexamethasone (0.02 ug/mL), and the effects of dexamethasone withdrawal and addition of T3 (2.5 nM) were investigated. A significant decrease in cell number was measured over time for all groups (#p<0.05). Higher ALP activity was detected for all groups except control at Day 14 as compared to Day 1 (#p<0.05), and significantly higher ALP activity was measured for the Dex group as compared to -Dex and -Dex+T3 groups (*p<0.05). Increases in GAG and collagen deposition were measured for all groups during the first two weeks of culture (#p<0.05). Higher GAG content was found for the -Dex+/+T3 group as compared to control and -Dex groups (*p<0.05), and higher collagen content was found for the -Dex group as compared to control, -Dex, and +T3 groups at Day 28 (*p<0.05).
11.2.2 Large Animal Model

Although the rabbit model has been shown to closely approximate healing response in humans, it is still crucial to evaluate scaffold designs in large animal models[263]. Compared to the rabbit, larger animal models such as goat[19,25,42,264-266] or sheep[26,147,267-269] would provide a more physically demanding environment, and these higher loads more closely approximate those experienced by patients. Furthermore, the cartilage thickness of large animal models is sufficient to make full thickness defects and position the interface scaffold at the cartilage-to-bone interface without excessive drilling into bone.

11.2.3 Extension of Nanofiber Cup Design

The nanofiber cup design developed in this thesis was designed to augment the agarose hydrogel interface scaffold. However, it is envisioned that this scaffold platform can be expanded in the future to other hydrogel systems or even explants to improve the in vivo implementation of other cartilage scaffolds.
REFERENCES


