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Biodegradable Hydrogel Composites for Growth Factor and Stem Cell Delivery in Osteochondral Tissue Engineering

by

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ABSTRACT

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Cartilage has a limited endogenous ability for self-repair and current clinical treatments for damaged or diseased cartilage tissue are insufficient. Additionally, there is a biological and mechanical interplay between cartilage and the underlying subchondral bone, linking the pathogenesis/regeneration of both tissues. Thus, this thesis seeks to develop hydrogel composites as growth factor and cell delivery vehicles to study the regeneration of osteochondral tissue. First, we investigated the release of growth factors from acellular hydrogel composites containing gelatin microparticles (GMPs) to stimulate the repair of cartilage tissue in an in vivo osteochondral defect model. Transforming growth factor-β3 (TGF-β3) with varying release kinetics and/or insulin-like growth factor-1 (IGF-1) were delivered from the chondral layer of bilayered hydrogel composites while the subchondral layer remained growth factor-free. Results demonstrated that dual delivery of TGF-β3 and IGF-1 did not synergistically enhance cartilage repair, regardless of release kinetics, and the delivery of IGF-1 alone positively stimulated osteochondral tissue repair. Subsequently, we focused on improving the repair of the subchondral bone. The second part of this thesis investigated the delivery of IGF-1 and bone morphogenetic protein-2 (BMP-2) from the chondral and subchondral layers, respectively, of bilayered scaffolds in vivo. Results showed that BMP-2 enhanced
subchondral bone repair, and that while the dual delivery of both growth factors did not improve cartilage repair, they synergistically enhanced subchondral bone formation over the delivery of IGF-1 alone. Using the results from this study, we also investigated relationships between specific cartilage and bone repair metrics to provide a fuller understanding of the osteochondral repair process. Correlation analysis revealed an intrinsic association between the degree of subchondral bone formation and cartilage surface regularity. Lastly, the third part of this thesis investigated the hydrogel composites as stem cell delivery vehicles. Degradable GMPs were used as temporary adherent substrates for anchorage-dependent mesenchymal stem cells (MSCs). MSCs were seeded onto GMPs and subsequently encapsulated in hydrogels to investigate their role on influencing MSC differentiation and aggregation. Non-seeded MSCs co-encapsulated with GMPs in the hydrogels were used as a control for comparison. Results revealed that MSC-seeded GMPs exhibited more cell-cell contacts, greater chondrogenic potential, and a down-regulation of osteogenic markers compared to the controls. Overall, these hydrogel composites demonstrate potential as growth factor and cell delivery vehicles for the stimulation and study of osteochondral tissue regeneration.
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<table>
<thead>
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<th>3,4,6-O-Bu3GlcNAc</th>
<th>3,4,6-O-tributanylated-N-acetylglucosamine</th>
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<td>AA</td>
<td>ascorbic acid</td>
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<td>aggrecan</td>
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<td>anterior cruciate ligament</td>
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<td>alkaline phosphatase</td>
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<td>basic fibroblast growth factor</td>
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<td>DMEM</td>
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<tr>
<td>Dox</td>
<td>doxorubicin</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>EO</td>
<td>ethylene oxide</td>
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<tr>
<td>ER</td>
<td>estrogen receptor</td>
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<tr>
<td>FBS</td>
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<td>FDA</td>
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<td>GA</td>
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<td>GAG</td>
<td>glycosaminoglycan</td>
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<td>GAM</td>
<td>gene-activated matrix</td>
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<td>GDF-5</td>
<td>growth differentiation factor 5</td>
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<td>GF</td>
<td>growth factor</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<td>GM</td>
<td>general medium</td>
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<tr>
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<td>gelatin microparticle</td>
</tr>
<tr>
<td>GMP10mM</td>
<td>gelatin microparticles crosslinked with 10 mM glutaraldehyde</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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<tr>
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<td>gelatin microparticles crosslinked with 40 mM glutaraldehyde</td>
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<td>hematoxylin and eosin</td>
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<tr>
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<td>hyaluronic acid</td>
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<tr>
<td>HCF</td>
<td>heparin-conjugated fibrin</td>
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<td>hepatocyte growth factor</td>
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<td>hydroxyproline</td>
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<td>IL-1β</td>
<td>interleukin 1β</td>
</tr>
<tr>
<td>IL-1Ra</td>
<td>interleukin 1 receptor antagonist</td>
</tr>
<tr>
<td>LCST</td>
<td>lower critical solution temperature</td>
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<td>LG-DMEM</td>
<td>Low Glucose-Dulbecco’s modified Eagle’s medium</td>
</tr>
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<td>MAETAC</td>
<td>[2-(methacryloyloxy) ethyl]-trimethylammonium chloride</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MP</td>
<td>microparticle</td>
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<tr>
<td>MSC</td>
<td>mesenchymal stem cell</td>
</tr>
<tr>
<td>MW</td>
<td>nominal molecular weight</td>
</tr>
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<td>MW&lt;sub&gt;n&lt;/sub&gt;</td>
<td>number-average molecular weight</td>
</tr>
<tr>
<td>MW&lt;sub&gt;w&lt;/sub&gt;</td>
<td>weight-average molecular weight</td>
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<td>N-vinylpyrrolidone</td>
</tr>
<tr>
<td>OA</td>
<td>osteoarthritis</td>
</tr>
<tr>
<td>ODP</td>
<td>osteopontin-derived peptide</td>
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<tr>
<td>OM</td>
<td>osteogenic medium</td>
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<tr>
<td>o-NB</td>
<td>ortho-nitrobenzyl</td>
</tr>
<tr>
<td>OPC</td>
<td>osteogenic progenitor cell</td>
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<tr>
<td>OPF</td>
<td>oligo(poly(ethylene glycol) fumarate)</td>
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<td>OPN</td>
<td>osteopontin</td>
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<tr>
<td>PBS</td>
<td>phosphate buffer solution</td>
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<td>platelet-derived growth factor</td>
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<td>poly(ethylene glycol)</td>
</tr>
<tr>
<td>PEG-DA</td>
<td>poly(ethylene glycol) diacrylate</td>
</tr>
<tr>
<td>PLGA</td>
<td>poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>PLGA-GCH</td>
<td>poly(lactic-co-glycolic) acid/gelatin/chondroitin sulfate/hyaluronic acid</td>
</tr>
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<td>PPF</td>
<td>poly(propylene fumarate)</td>
</tr>
<tr>
<td>PPG</td>
<td>poly(propylene glycol)</td>
</tr>
<tr>
<td>PPy</td>
<td>polypyrrole</td>
</tr>
<tr>
<td>PRP</td>
<td>platelet-rich plasma</td>
</tr>
<tr>
<td>PTH</td>
<td>parathyroid hormone</td>
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<tr>
<td>PTHrP</td>
<td>parathyroid hormone related peptide</td>
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<tr>
<td>RGDM</td>
<td>Arg-Gly-Asp</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcriptase-polymerase chain reaction</td>
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<td>runt-related transcription factor 2</td>
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<td>SDF-1α</td>
<td>stromal derived factor 1α</td>
</tr>
<tr>
<td>SOX9</td>
<td>(sex determining region Y)-box 9</td>
</tr>
<tr>
<td>TEA</td>
<td>triethylamine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>TEMED</td>
<td>$N,N',N'$-tetramethylethylenediamine</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>transforming growth factor β1</td>
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<td>TGF-β2</td>
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<td>TGF-β3</td>
<td>transforming growth factor β3</td>
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<td>TNF-α</td>
<td>tissue necrosis factor α</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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Chapter 1

Specific Aims

Overview

The overall objective of this thesis is to develop a biodegradable hydrogel composite as a growth factor and stem cell delivery vehicle for the study and regeneration of osteochondral tissue. These hydrogel scaffolds, fabricated from oligo(poly(ethylene glycol fumarate) (OPF) macromers, include gelatin microparticles as digestible porogens, carriers for the controlled release of growth factors, and moieties for stem cell interaction. Accordingly, the work in this thesis is divided into three specific aims.

Specific Aim 1

The first aim investigates the dual delivery of insulin-like growth factor-1 (IGF-1) and transforming growth factor-β3 (TGF-β3) from acellular hydrogel composites on articular cartilage repair in an osteochondral defect. Specifically, the release kinetics of
IGF-1 and TGF-β3 from the chondral layer of OPF hydrogel composites are studied *in vitro* and the effects of dual release on cartilage repair *in vivo* are assessed.

**Specific Aim 2**

The second aim investigates the dual delivery of IGF-1 and bone morphogenetic protein 2 (BMP-2) from bilayered hydrogel composites on osteochondral tissue repair. Specifically, IGF-1 and BMP-2 are incorporated in the chondral and subchondral layers, respectively, of bilayered scaffolds and the influence of the enhanced subchondral bone repair on cartilage regeneration is examined *in vivo*.

**Specific Aim 3**

The third aim investigates the use of degradable GMPs as temporary adherent-substrates for MSCs within hydrogel composites. Specifically, the degradation rate of GMPs is tuned by modulating crosslinking density, and the differentiation and condensation potential of MSCs seeded onto GMPs of varying crosslinking densities and sizes are assessed *in vitro*. 
Chapter 2

Background

Cartilage damage and cartilage-related diseases are the most common cause of disability in the United States today, affecting more than 21% of adults at a cost of $128 billion in medical bills and lost earnings to the economy [1-3]. 27 million adults, more than 10% of the United States adult population, alone have clinical osteoarthritis, and was the fourth most common cause of hospitalization in 2009 [4]. Articular cartilage’s low friction surface and high capacity to bear loads makes it indispensible in joint movement. However, its poor intrinsic capacity for self-repair has necessitated numerous therapeutic strategies, none of which have yet to provide a consistent and sustained healing response. Clinical therapeutic interventions have often resulted in only transient pain relief or inconsistent spontaneous healing [5]. As a result, tissue engineering has emerged as a valuable strategy to recapitulate the functional requirements of native articular cartilage.
2.1. Osteochondral Tissue

2.1.1. Articular Cartilage

2.1.1.1. Function and Composition

Articular cartilage is a multiphasic tissue consisting primarily of 70-80% water by weight [6]. The solid fraction of the tissue is composed of 50-75% collagens, 15-30% proteoglycans, chondrocytes, and other protein macromolecules [3, 6]. Cartilage is an alymphatic, aneural, and avascular tissue, and as a result, has a limited potential for self-repair. Due to its avascularity, the interstitial fluid within the pores of the extracellular matrix (ECM) plays an important role in transporting nutrients and waste within the tissue. In addition, due to its high water content, articular cartilage exhibits viscoelastic properties, allowing it to support high compressive loads [6].

Collagen types present in articular cartilage include type II, VI, IX, X, and XI, of which type II makes up 90-95% of all collagen present in cartilage ECM [7]. Collagen type II fibers form complex networks to provide tensile strength and are essential to the tissue’s shape and volume. In addition, these fibers form a mesh with collage type XI and IX that entrap other macromolecules such as proteoglycans [7]. Proteoglycans are large macromolecules consisting of a protein core and glycosaminoglycan (GAG) side chains. The primary articular cartilage proteoglycan is aggrecan, comprising GAG chains of keratin sulfate and chondroitin sulfate linked to a protein core. Aggrecan, in turn, can bind to a long hyaluronan chain to form large proteoglycan aggregates. Due to the presence of highly negatively charged GAG chains, cartilage ECM has a net negative
charge known as the fixed charge density, which attracts ions from the interstitial fluid to create an osmotic pressure and causes the matrix to swell [6, 8].

Another proteoglycan abundant in articular cartilage is perlecan. Found in the pericellular matrix of cartilage, perlecan bears heparin sulfate chains that have high affinity for growth factor binding [9]. While the mechanistic actions of perlecan in cartilage remains elusive, the presence of perlecan in cartilage condensation, maturation, and homeostasis is essential [10]. Perlecan provides cell-cell and cell-matrix interactions, which suggests its role in mechanotransduction processes, and plays a role as a ligand reservoir for the protection, storage, and release of growth factors [11, 12].

Articular cartilage can be modeled as a tissue with four structurally distinct layers as seen in Figure 2.1, each playing a role in its tensile, shear, and compressive properties [13]. The superficial/tangential layer comprises the upper 10-20% of articular cartilage and is the primary articulating surface with collagen fibers aligned parallel to the joint surface [6, 14]. This layer also has a high water content with compressive strains reaching up to 50% [3]. Due to fluid flow and matrix consolidation, chondrocytes present in this layer are flattened oriented along the collagen fibers [3].

The middle/intermediate layer experiences little strain and occupies 40-60% of the total cartilage thickness [6]. Cell density in this layer is lower than the superficial layer and assume a more spherical morphology. These chondrocytes also produce high amounts of proteoglycans and collagen type II in random orientation [6].

In the deep/radial zone, collagen fibers are packed tightly into large bundles oriented perpendicular to the articular surface and are anchored to the underlying subchondral bone. Proteoglycan content here is lower than the middle layer, and cell
density is the lowest of the three cartilaginous layers [6]. These cells often form a columnar arrangement in groups of four to eight cells and have high metabolic activity [14].

The calcified zone marks the transition from pliable cartilage to stiff bone and is responsible for attaching the non-calcified cartilage to the underlying subchondral bone. Chondrocytes in this region are larger and are referred to as hypertrophic [14].

2.1.1.2. Injury and Repair

Injury to articular cartilage can occur through trauma, mechanical injury, and/or wear and tear. Three main types of injury occur: cartilage microfracture/matrix disruption, chondral defect/partial thickness defect, or osteochondral defect/full thickness defect. If a cartilage microfracture injury is minimal, remaining chondrocytes can increase their synthetic activity to repair the tissue. If the injury is more extensive and the collagen network is disrupted, altered load distribution of the cartilage matrix may occur, resulting in propagation of forces to the bone. This can result in thicker subchondral bone and thinner cartilage, leading to further degeneration of articular cartilage [15]. This type of injury can also lead to chondral fissures.

Chondral fissures are defects contained within the cartilage layer and do not extend to the subchondral bone. These types of defects often lead to an osteochondral defect due to limited metabolic activity for healing. Osteochondral defects comprise both the chondral and subchondral layer due to penetration of the underlying bone. As a result, access to the bone’s vasculature enables a spontaneous healing response by tapping into growth factors and progenitor cells present in the bone marrow [16].
Following the creation of an osteochondral defect, permeation of blood into the defect site forms a fibrin clot and a classic wound healing response ensues [7]. Factors from the fibrin clot recruit MSCs and in the following two weeks, MSCs proliferate and differentiate. In rabbit osteochondral defect models, differentiated cells continue to produce collagen type II and I after two weeks, and by week 4-8, a well demarcated cartilage layer is developed [17-19]. Presence of hyaline cartilage can be seen by week 4-12 depending on the type of defect created: in a femoral patellar groove defect, neo-cartilage with Safranin O staining was seen as early as week 4 [19], whereas in a femoral head defect, hyaline cartilage was observed between 8 and 12 weeks [18]. However, regenerated cartilage all defect types often consists of both hyaline cartilage and fibrocartilage and degeneration of the neo-tissue can be observed as early as 12 weeks [19]. A number of factors may contribute to the degeneration of the newly formed cartilage. Fibrocartilage is mechanically inferior to hyaline cartilage and fissures may occur due to matrix degeneration and GAG loss [6]. In addition, the collagen fibrils of the repair tissue have been seen to be poorly integrated with the native cartilage adjacent to the defect site [19]. This could lead to micromotion and vertical shear stresses between the repair and residual cartilage, exacerbating cartilage degeneration [16]. Between 6 and 12 months, matrix and cells of the repaired tissue continually regress to fibrocartilage, leading to chronic degeneration [16, 17].
2.1.2. Subchondral Bone

2.1.2.1. Function and Composition

Bone is a highly anisotropic material that continually adapts to changes in its physiological and/or mechanical environment through a cyclic process of bone formation and resorption [20, 21]. The primary elements of bone tissue consist of collagen and hydroxyapatite crystals, with collagen type I comprising approximately 95% of the total collagen content and about 80% of the total proteins present in bone [22]. Like articular cartilage, the mechanical properties of bone reflect the inherent material properties of its constituents: collagen grants bone its toughness and capacity to absorb energy while the mineral content helps determine the bone’s stiffness [21]. Three types of cells also exist within bone: osteoclasts which resorb bone, osteoblasts which produce calcified tissue, and osteocytes which continue to calcify surrounding tissue [23].

The subchondral bone itself can be classified into two types: cortical bone/subchondral plate and trabecular bone. Cortical bone is dense, lies adjacent to calcified cartilage, and has low porosity and vascularity while trabecular bone is approximately 80% porous [23]. Due to differences in bone structure and organization, these two tissues adapt to mechanical loads in different ways and have distinct mechanical properties.

2.1.2.2. Injury and Repair

Penetrative injuries to subchondral bone often result from continued degeneration of chondral fissures that reach the underlying bone as mentioned above. However, degenerative changes in subchondral bone can occur prior to the appearance of
an osteochondral defect. Certain studies have shown that a biphasic phenomenon is seen with early and late stage osteoarthritis. Early osteoarthritis is associated with thinning of the subchondral plate (the cortical bone) and overall decrease in bone volume [24, 25], whereas late stage osteoarthritis is associated with a thickening of the subchondral plate, increased stiffness, and increased density [26, 27]. An increase in trabecular bone volume also occurs with an increase in trabecular number and reduced separation between trabeculae [20]. However, while apparent (overall bone tissue) density increases, the matrix itself is not well mineralized leading to a reduced material density [20, 28]. Research has indicated that 6-12 months are required before new bone is fully mineralized [20, 29]. An increase in denatured collagen content has also been seen in subchondral bone in osteoarthritis [28]. Understanding these changes in subchondral bone during injury and repair can help interpret the mechanisms involved in the overall repair of osteochondral tissue.

2.1.3. Interaction between articular cartilage and subchondral bone

Given the contact between cartilage and bone, total joint homeostasis will ultimately rely on the functional restoration of both tissues. Injury to articular cartilage can propagate to the underlying bone, and the health of cartilage tissue has been linked to the maintenance of normal biomechanics of the subchondral bone [30, 31]. It has been hypothesized that changes in subchondral bone may precede cartilage deterioration [32], and many studies have showed that changes in underlying bone occur early in osteoarthritis [33]. However, both cartilage and bone respond to biological and mechanical signals at different paces [33, 34]: bone has the capacity to rapidly remodel
its matrix, while chondrocytes in cartilage have a much lower synthetic activity [33]. The mechanisms of osteochondral tissue degeneration are complex and still unclear. Yet, with these challenges in mind, numerous tissue engineering strategies are being developed to address the biological, mechanical, and temporal interplay between articular cartilage and subchondral bone.

### 2.2. Osteochondral Tissue Engineering

The regeneration of osteochondral tissue is a unique, albeit complicated endeavor; one that involves two anisotropic structures, both with distinct layers and biological constituents. Reviews on the tissue engineering of either cartilage or bone are extensive and are listed as follows [3, 5, 7, 16, 35-46]. While the generation of osteochondral tissue involves many of the same cell types, bioactive molecules, and scaffolds used for cartilage and bone tissue repair, osteochondral engineering requires more intricate designs to target the two layers. A general overview of these strategies are outlined below.

#### 2.2.1. Scaffold Design

Currently, there are four different approaches in designing scaffolds for osteochondral tissue as seen in Figure 2.2: (A) a scaffold for the subchondral layer but no scaffold for the chondral layer, (B) different scaffolds for the chondral and subchondral
layer which are combined at the time of implantation, (C) a single, heterogeneous composite scaffold, or (D) a single, homogenous scaffold for both layers [13, 47, 48]. Type (A) scaffolds generally involve seeding of cells on top of the subchondral component. For Type (B) scaffolds, cartilage-like and/or bone-like tissues are engineered in vitro and then combined through suturing or adhering. In contrast, Type (C) scaffolds are heterogeneous with two distinct, yet integrated, layers. These scaffold types also include continuous material gradient designs [49]. Type (D) scaffolds involve a single homogenous scaffold that may or may not incorporate distinct chondrogenic and osteogenic cells/bioactive molecules. Each scaffold type has its pros and cons and have shown promise in regenerating osteochondral tissue.

2.2.2. Scaffold Materials

Osteochondral tissue engineering often requires a biocompatible and biodegradable scaffold to provide structural support for neo-tissue formation [50]. These scaffolds can also be designed to match the mechanical properties of surrounding tissue as well as control the delivery of cells and/or bioactive molecules [51]. In addition, these three-dimensional scaffolds can be highly porous with an interconnected pore network to facilitate cell migration and transport of nutrients and waste [52]. Scaffold materials for the subchondral layer usually fit in one of three categories: natural polymers, synthetic polymers, and inorganic materials. These materials in turn can be used to make a variety of matrices with unique cell/bioactive molecule delivery properties [50]. Similarly, natural and synthetic polymers have been used as materials to develop numerous three-
dimensional scaffolds, hydrogels, micro- or nanoparticles, and/or composites for the chondral layer [50].

2.2.3. Cells

Cells are another key component of tissue engineering. While cells are necessary for the in vitro generation of osteochondral tissue, an exogenous cell source is not essential for in vivo regeneration. This is especially true for osteochondral defects which penetrate the subchondral bone and access progenitor cells in the marrow space. However, studies have shown that delivering exogenous cells to chondral and osteochondral defects accelerates regeneration [6]. A number of cell types exist for osteochondral repair, the most prevalent being MSCs. These cells are capable of differentiating down a number of mesenchymal tissue lineage pathways including bone and cartilage [41], making it the most versatile cell type for osteochondral tissue engineering. Chondrocytes have also been used for osteochondral tissue repair, not only because of their cartilage-specific phenotype, but due to their ability to re-differentiate into osteoblasts [14]. Similarly, osteoblasts, the primary bone-forming cells, have been used for bone repair. However, both chondrocytes and osteoblasts must be isolated from an autologous biopsy, which can cause damage to the harvest site. Additionally, availability of such cells are often limited and in the case of chondrocytes, ex vivo expansion can result in de-differentiation [47]. Embryonic stem cells have also been investigated due to their pluripotent nature and capacity for unlimited proliferation. However, results have been tenuous and the political, legal, and ethical questions surrounding the use of embryonic stem cells deters their use [6, 51].
2.2.4. Bioactive Factors

The growth and development of osteochondral tissue heavily relies on biochemical signals, of which the intensity, temporal, and spatial stimulation all play a role in determining specific ECM synthesis and tissue formation. Among these biochemical signals include growth factors which control a wide variety of cellular responses through transmembrane receptor binding [46]. A summary of the most common growth factors used for bone and cartilage tissue engineering are listed in Table 2.1 with their effects on MSCs. BMPs, particularly BMP-2, are the most extensively studied osteogenic factors due to their powerful osteoinductive properties. On the other hand, TGF-β has been shown to be effective at inducing chondrogenic differentiation of MSCs and retaining the chondrogenic phenotype of chondrocytes in vitro [36]. However, mixed results have been reported on TGF-β delivery to osteochondral defects in vivo with potential deleterious effects [36].

The effects of TGF-β, BMP, and other growth factors such as vascular endothelial growth factor, platelet-derived growth factor, fibroblast growth factor, and insulin-like growth factor all have cartilage- and bone-specific responses. For example, BMP-2 can reverse chondrocyte de-differentiation and can increase cartilage-specific ECM production while decreasing collagen type I expression [36, 39]. Yet, BMP-2 also stimulate MSCs towards an osteogenic lineage and are an important osteoinductive factor [45, 50]. Harnessing the tissue-specific effects of these growth factors can be an important tool in osteochondral tissue engineering.
2.3. Background Summary

Due to articular cartilage’s low propensity for self-repair and the shortcomings of clinical therapies, tissue engineering strategies have emerged as an alternative to regenerating articular cartilage. In studying the injury and repair of articular cartilage, research has shown a close functional relationship with the underlying subchondral bone. These two distinct tissues comprise the osteochondral unit. As such, unique osteochondral tissue engineering constructs have been implemented, combining both cartilage and bone tissue engineering strategies. One of these constructs is the oligo(poly(ethylene glycol) fumarate) (OPF) hydrogel. OPF hydrogels have been utilized for a myriad of regenerative medicine applications, notably the regeneration of cartilage and bone. Previous research has validated the use of bilayered OPF hydrogels containing GMPs to create composites for the generation of osteochondral tissue. Most recently, these composites have dual delivered IGF-1 and TGF-β3 in vitro and investigated their combined effects on MSCs. In addition, MSCs have been encapsulated in OPF hydrogel composites to evaluate the potential of cellular composites for osteochondral tissue repair. Taking these previous results into account, the following Specific Aims will outline growth factor and cell delivery strategies in bilayered OPF hydrogel composite systems for osteochondral tissue engineering.
Figure 2.1 A diagram depicting the different layers and components of articular cartilage and subchondral bone.
Figure 2.2 A schematic of the different scaffolding strategies used for the fabrication of osteochondral grafts [47].

Table 2.1 Summary of the effect of growth factors on MSCs [36, 45, 53]

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>Source</th>
<th>Effect on MSCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transforming growth factor beta (TGF-β)</td>
<td>Platelets, bone ECM, cartilage matrix</td>
<td>Pleiotropic factor</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increases proliferation and ECM production</td>
</tr>
<tr>
<td>Bone morphogenetic proteins (BMP)</td>
<td>Osteoprogenitor cells, osteoblasts, bone ECM</td>
<td>Promotes differentiation of MSCs into chondrocytes and osteoblasts</td>
</tr>
<tr>
<td>Fibroblast growth factors (FGF)</td>
<td>Macrophages, MSCs, chondrocytes, osteoblasts</td>
<td>Mitogenic factor</td>
</tr>
<tr>
<td>Insulin-like growth factors (IGF)</td>
<td>Bone matrix, osteoblasts, chondrocytes</td>
<td>Mitogenic factor</td>
</tr>
<tr>
<td>Platelet-derived growth factor (PDGF)</td>
<td>Platelets, osteoblasts</td>
<td>Chemotaxis factor</td>
</tr>
</tbody>
</table>
Chapter 3

Fumarate-based hydrogels in regenerative medicine applications

The past several years have seen the development of novel fumarate-based hydrogels for tissue engineering applications. These biocompatible and biodegradable hydrogels include poly(propylene fumarate-co-ethylene glycol), oligo(poly(ethylene glycol) fumarate), and poly(lactide-co-ethylene oxide-co-fumarate). They have the capacity to be injectable and crosslinked in situ, and each hydrogel has its own unique set of physical properties that can be tailored to meet the requirements of a specific function. Such versatile functionality situates fumarate-based hydrogels as excellent scaffolds for numerous regenerative medicine applications.

*This chapter was published as Lu S, Kim K, Lam J, Kasper FK, Mikos AG. Fumarate-based hydrogels in regenerative medicine applications. In Biomaterials and Regenerative Medicine, Ma PX, editor: Cambridge University Press, New York, 279-294, 2014.*
3.1. Introduction

Hydrogels are an excellent scaffold structure for numerous applications in tissue engineering and regenerative medicine. In particular, they can be used as cell and drug carriers to deliver such therapeutic components directly and locally [54]. Hydrogels can be injectable and crosslinked in situ, reducing the need for risky invasive surgeries [55]. In addition, hydrogels can mimic the natural extracellular matrix (ECM) environment and allow for control of cellular and tissue functions as well as the transport of nutrients and bioactive molecules [56, 57].

Fumarate-based hydrogels are synthetic polymers, allowing for flexible control of physical, mechanical, and degradative properties for a desired application [57]. Fumaric acid, the fundamental component of these hydrogel scaffolds, is an unsaturated organic acid commonly found in the human body and can be metabolized through the Krebs cycle [58-60]. Polymer chains that contain fumarate units crosslink easily via the unsaturated double bonds and degrade through hydrolysis of the ester bonds in the fumarate group [59-62]. Furthermore, the biodegradable nature of these hydrogels allows for neotissue ingrowth and eliminates the need for another surgery to remove the implanted scaffold [58, 63].

Current research on fumarate-based hydrogels as tissue engineering scaffolds for regenerative medicine applications include poly(propylene fumarate-co-ethylene glycol) (P(PF-co-EG)), oligo(poly(ethylene glycol) fumarate) (OPF), and poly(lactide-co-ethylene oxide-co-fumarate) (PLEOF). These hydrogel scaffolds have been well characterized with a number of in vitro and/or in vivo studies proving them to be non-cytotoxic and biocompatible to surrounding tissues [64-67]. In addition, the ease in
crosslinking modulation of fumarate-based hydrogels is a testament to their versatility. As biodegradable and biocompatible materials, each hydrogel has its unique physical and mechanical properties that can be tailored to meet the needs of specific regenerative medicine applications including drug delivery and regeneration of bone, cartilage, and nerve tissue. This chapter will illustrate the chemistry and characterization behind these fumarate-based hydrogels in addition to their applications in regenerative medicine.

3.2. Poly(propylene fumarate-co-ethylene glycol) hydrogel

Macroporous hydrogels based on poly(propylene fumarate-co-ethylene glycol) (P(PF-co-EG)) have been investigated over the past decade as an injectable, biodegradable scaffolding material for the modulation of cellular behavior [68-71].

3.2.1. Chemistry

P(PF-co-EG) is a block copolymer of poly(propylene fumarate) (PPF) and poly(ethylene glycol) (PEG). It is formed via a multistep reaction that begins with the synthesis of PPF, a biodegradable polyester previously characterized and investigated for applications in orthopaedic and craniofacial tissue engineering [72-75]. First, diethyl fumarate and propylene glycol are reacted in the presence of catalytic zinc chloride to create bis(2-hydroxypropyl) fumarate, a diester intermediate. The intermediate can then be transesterified under vacuum to yield a linear unsaturated form of PPF [76]. Once PPF
is obtained, it can be reacted with PEG or methoxy-PEG to form the P(PF-co-EG) copolymer via a transesterification reaction as seen in Figure 3.1 [61, 69]. The use of methoxy-PEG allows for a more controlled synthesis of P(PF-co-EG) and ensures that at most two PEG block attach to the linear PPF chain [61].

Due to the presence of fumarate double bonds in the PPF blocks, P(PF-co-EG) is a highly unsaturated copolymer. As a result of this intrinsic property, the raw copolymer material can undergo crosslinking to produce amphiphilic hydrogels [64]. The amphiphilic nature of P(PF-co-EG) also permits the copolymer to undergo reversible thermal gelation via changes in intermolecular behavior upon fluctuations in temperature [61]. The thermal initiation system for crosslinking can leverage a crosslinker, such as poly(ethylene glycol) diacrylate (PEG-DA), in combination with a radical initiator (e.g., the redox radical initiators ammonium persulfate (APS) and ascorbic acid (AA)) [77]. Sodium bicarbonate can be added to the reaction to interact with AA to generate carbon dioxide gas. The generated gas allows the production of macroporous hydrogel materials [78, 79].

3.2.2. Characterization

3.2.2.1. Swelling and degradation

P(PF-co-EG) consists of alternating hydrophobic PPF and hydrophilic PEG polymer blocks. Due to the hydrophilic nature of the PEG component, P(PF-co-EG) materials can retain water to form amphiphilic hydrogels after crosslinking. By altering
the amounts of the hydrophilic and hydrophobic components, the swelling properties of P(PF-co-EG) hydrogels can be tuned [70]. Indeed, previous swelling studies have demonstrated that equilibrium swelling ratios of P(PF-co-EG) hydrogels are increased with higher PEG amounts and decreased with higher PPF amounts [70]. Additionally, physical properties such as compressive and tensile moduli can be modulated by adjusting the PPF:PEG ratio [70]. Because PPF retains the bulk structural integrity, higher PPF amounts are correlated with materials of greater mechanical strength. For example, hydrogels prepared with a 1:2 weight ratio of PPF to PEG had a tensile modulus of 1.90 ± 0.67 MPa while the same hydrogels prepared with a 2:1 weight ratio of PPF to PEG had a significantly higher tensile modulus of 20.66 ± 2.42 MPa [16].

With regard to the degradation characteristics of P(PF-co-EG) hydrogels, several studies have observed the mechanism by which these amphiphilic hydrogels degrade [64, 79]. Due to the presence of hydrolytically cleavable ester bonds in PPF, P(PF-co-EG) hydrogels are expected to undergo bulk degradation following the absorption of water. Typically, bulk degrading materials maintain their shape and volume but lose mechanical strength as their internal polymeric networks undergo hydrolysis. However, this may not apply to hydrogels that undergo bulk degradation, as increased water penetration will affect the shape and volume of these materials over time. The gradual loss of mechanical strength over time was observed in both in vitro and in vivo conditions as measured by decreases in complex dynamic flexural and tensile moduli [64, 79].

Like the swelling properties, the degradation rate of P(PF-co-EG) can be altered as well. For instance, greater amounts of mass loss are observed in hydrogels with higher PPF content [64]. Although PPF is responsible for a majority of the mechanical strength
attributed to P(PF-co-EG) hydrogels, the ester linkages make it most susceptible to hydrolytic cleavage [79]. As a result, increasing weight percentages of PPF initially increase tensile strength of the hydrogel but ultimately increase the rate of bulk degradation. Since the current system uses PEG-DA as the crosslinker, the crosslinking density can be modified by changing the P(PF-co-EG):PEG-DA ratio to also affect the degradation of P(PF-co-EG) hydrogels. Purportedly, smaller P(PF-co-EG):PEG-DA ratios, or higher crosslinking densities, result in slower degradation [79]. This is because more ester linkages need to be cleaved in hydrogels with greater crosslinking densities, ultimately leading to a slower degradation rate. Unlike other polyester scaffolds, however, the degradation of P(PF-co-EG) hydrogels is not heavily influenced by porosity [79]. While hydrophobic scaffolds may not swell in aqueous environments, P(PF-co-EG) hydrogels are already swollen with water and as a result, the rate of hydrolytic degradation for these materials is not influenced by an increase in surface area due to porosity.

3.2.2.2. Cytocompatibility and biocompatibility

Investigations of the in vitro cytotoxicity and in vivo biocompatibility of P(PF-co-EG) hydrogels revealed favorable biological responses. Specifically, cytotoxicity was evaluated by exposing monolayer endothelial cell cultures to leachable materials from P(PF-co-EG) hydrogel films and then measuring their levels of viability and proliferation [80]. As determined by an MTT assay, the viability of exposed monolayers to gel compositions with high PEG amounts was not significantly different from that of negative controls [80]. Since P(PF-co-EG) based hydrogels are intended for injectable, biodegradable applications within the body, in vivo biocompatibility is also crucial. A
cage implant system was employed as the in vivo model to assess biocompatibility [80]. Briefly, dehydrated gel films were placed inside sterilized small cylindrical cages and implanted subcutaneously into Sprague Dawley rats [80]. Biochemical and histological analyses of exudates and extracted copolymer films revealed that the biocompatibility of P(PF-co-EG) hydrogels is dependent on the copolymer composition. In particular, increasing amounts of uncrosslinked PPF elicited more toxic responses in vivo [80]. Hence, cytotoxicity and inflammation can be attenuated with higher weight percentages or molecular weights (MWs) of the PEG component. Overall, P(PF-co-EG) hydrogels exhibited excellent biocompatibility upon implantation, demonstrating their suitability as effective tissue engineering scaffolds.

3.2.2.3. Modification with biomimetic peptides

With regard to biocompatibility, P(PF-co-EG) hydrogels were able to accommodate varying levels of cell adhesion. Copolymer modifications via changing the PEG amount can influence the physicochemical properties of hydrogels and thus cell adhesion. Tanahashi et al. reported that the adhesion of smooth muscle cells and human endothelial cells was dependent on the hydrophilicity of the hydrogels; less hydrophilic P(PF-co-EG) hydrogels elicited smaller amounts of cell adhesion [68]. However, cells bound to macroporous P(PF-co-EG) hydrogels alone do not display any morphological signs of activity [68]. In order to affect changes in cellular behavior, ECM adhesion peptides such as the arginine-glycine-aspartic acid (RGD) sequence have been incorporated into the bulk macroporous hydrogel, imparting properties that are biomimetic of the natural ECM [71]. Behravesh et al. incorporated RGD adhesion peptides into P(PF-co-EG) hydrogels by crosslinking P(PF-co-EG) with acryloyl-PEG-
GRGDS, which was synthesized by reacting the oligopeptide GRGDS with acryloyl-poly(ethylene glycol) N-hydroxysuccinimide (Ac-PEG-NHS) [15]. Adhesion and migration assays studying the behavior of bone marrow stromal cells, also referred to as mesenchymal stem cells (MSCs), on macroporous P(PF-co-EG) hydrogels demonstrated that cell specific binding can be achieved and that the cells bound are biologically active [71]. For instance, marrow-derived osteoblasts have been shown to bind, spread, and migrate on RGD-functionalized P(PF-co-EG) hydrogels, revealing the ability of these scaffolds to activate anchorage-dependent behaviors in this particular cell type [71]. By incorporating RGD peptide sequences into P(PF-co-EG) copolymers using PEG spacers and PEG blocks of specific MWs, only cell types with receptors for the RGD sequence are selected for binding. Additionally, biomimetic P(PF-co-EG) hydrogels can sustain the differentiation of progenitor cells [71]. Utilizing RGD-functionalized P(PF-co-EG) hydrogels as three-dimensional scaffolds for culture of bone marrow-derived MSCs, the biomimetic hydrogel was used as a vehicle for osteoblastic differentiation [81]. One can imagine the utility of such technologies for the ex vivo modulation of bone marrow-derived MSCs to develop cell-inspired therapeutics for orthopedic tissue engineering applications.

Another way P(PF-co-EG) hydrogels were modified involved the tethering of positively charged agmatine to the PEG copolymer blocks [82]. Agmatine is a compound naturally synthesized from the amino acid arginine and is naturally metabolized by the body to form putrecine and urea [82]. Its cationic properties can be used to enhance the adhesion of vascular smooth muscle cells via the adsorption of anionic ECM molecules. To incorporate agmatine into P(PF-co-EG) hydrogels, Tanahashi et al. first generated
PEG-tethered fumarate (PEGF) groups by reacting PEG with diethyl fumarate in the presence of catalysts [29]. Next, PEGF was reacted with succinic anhydride to generate succinylated PEGF, which was further reacted with N-hydroxysuccinimide (NHS) to generate NHS-PEGF. The NHS group was then leveraged for the attachment of agmatine sulfate to PEGF via the NHS ester linkage to yield agmatine-PEGF. Finally, agmatine-PEGF was crosslinked with P(PF-co-EG) to fabricate agmatine modified P(PF-co-EG) hydrogels in the presence of ascorbic acid [29]. Interestingly, P(PF-co-EG) hydrogels modified in the bulk with agmatine possess the ability to accommodate cell binding while maintaining high levels of hydrophilicity [83]. This property, along with the ability for these cationic hydrogels to biodegrade, makes agmatine modified P(PF-co-EG) hydrogels very suitable for cell carrier applications in tissue engineering.

3.2.2.4. Thermoreversibility

Hydrogels based on certain compositions of P(PF-co-EG) copolymers possess thermoreversible properties. Such thermoreversible behavior, as indicated by the presence of a lower critical solution temperature (LCST), arises from the amphiphilic nature of the P(PF-co-EG) copolymers. Behravesh et al. reported the appearance of LCSTs for P(PF-co-EG) copolymer solutions that were synthesized using PEG of low MWs [61]. Indeed, the block length of the PEG components in the copolymer played a crucial role in determining the LCST and sol-gel transition temperatures. Copolymers comprising PEG with high MWs may not exhibit any thermoreversible properties due to their high hydrophilicity. P(PF-co-EG) hydrogels have been formulated to achieve LCSTs that increase from 25 °C (near room temperature) to 35 °C (near body temperature) by decreasing the salt concentration of aqueous sodium chloride solutions in
which the P(PF-co-EG) hydrogels are immersed [77, 78]. The presence of salts alters the hydrogen bonding between the hydrophobic PPF and hydrophilic PEG blocks to affect changes in LCST [8]. These properties make P(PF-co-EG) hydrogels advantageous for the minimally invasive delivery of cells or other therapeutics for tissue engineering applications. Furthermore, these hydrogels can be chemically crosslinked utilizing a water-soluble redox initiator system that incorporates a basic and an acidic initiator to form macroporous P(PF-co-EG) hydrogels [78].

3.2.3. Regenerative medicine applications

3.2.3.1. Bone regeneration

Using several in vitro models, P(PF-co-EG) based hydrogels have been investigated for potential applications in bone tissue engineering [68, 71, 81]. One such study seeded marrow-derived osteoblasts within biomimetic P(PF-co-EG) hydrogels and observed their subsequent adhesion and migration [71]. From the results, incorporating RGDS into P(PF-co-EG) hydrogels permitted the adhesion of up to 84% of the initial seeding density of marrow-derived osteoblasts [71]. Using a megacolony migration assay, Behravesh et al. detected increasing cell surface area coverage that occurred as a result of cell migration on RGD and not proliferation of cells [71]. By employing such behavior, it becomes feasible to apply biomimetic P(PF-co-EG) based hydrogels for the guided regeneration of orthopaedic or dental tissues. Cells would mechanistically bind to implanted biomimetic scaffolds and migrate according to biological cues (like RGD peptides) engineered to therapeutically direct these cells to the defect site. It was also
demonstrated that MSCs seeded in P(PF-co-EG) based hydrogels could undergo osteoblastic differentiation when treated with osteogenically conditioned media [81]. Specifically, MSCs seeded in macroporous three-dimensional P(PF-co-EG) hydrogels exhibited substantial calcium deposition [81]. Using macroporous P(PF-co-EG) hydrogels that are biomimetic of the bone environment, marrow-derived progenitor cells may be harvested from patients with minimal donor site morbidity and expanded ex vivo for subsequent autologous cellular therapies.

### 3.2.3.2. Cartilage regeneration

Injectable and thermoreversible P(PF-co-EG) based hydrogels have also been investigated for cartilage regeneration. Due to the hydrophilicity of the PEG blocks, synthetic scaffolds based on P(PF-co-EG) copolymers inherently retain water to yield water-laden materials. Such properties are biomimetic of natural cartilage tissues. In an effort to assess the potential of applying P(PF-co-EG) hydrogels for cartilage regeneration, Fisher et al. encapsulated bovine articular chondrocytes within thermoreversibly gelled P(PF-co-EG) hydrogels and examined their viability [84]. Accordingly, the chondrocytes maintained satisfactory levels of proteoglycan and collagen type II syntheses. The proliferation of these cells was also increased with the addition of bone morphogenetic protein-7 [84].
3.3. Oligo(poly(ethylene glycol) fumarate) hydrogel

While P(PF-co-EG) showed promise as a hydrogel scaffold for regenerative medicine applications, oligo(poly(ethylene glycol) fumarate) (OPF) macromers were developed for the fabrication of hydrogels with improved control over hydrogel parameters. P(PF-co-EG) macromers contain multiple PPF blocks, with each PPF block comprising several fumarate groups [70, 76, 77]. However, these multiple fumarate groups can result in varying MW between crosslinks and mesh sizes in fabricated hydrogel networks [77]. OPF macromers consist of fumarate groups separated by PEG chains of defined molecular weight. As a result, hydrogels with different mesh sizes can be easily fabricated by varying the molecular weight of the PEG employed in the synthesis of the macromer [62]. OPF hydrogels with tailored and controlled physical properties have great potential in a myriad of tissue engineering applications.

3.3.1. Chemistry

OPF macromers consist of alternating units of fumarate groups and PEG chains, with the macromers capped by end PEG chains [59, 85]. These macromers are synthesized in a one-pot reaction, as seen in Figure 3.2, through the addition of distilled fumaryl chloride (FuCl) and triethylamine (TEA) dropwise to distilled PEG in a molar ratio of 1:0.9 PEG:FuCl. The molar ratio of FuCl to TEA is 1:2 in order to remove chlorine from the ends of FuCl. Upon completion of the reaction, the mixture is filtered, purified, and dried to obtain the macromer in powdered form [59].
OPF macromers have been crosslinked in a variety of ways to form hydrogels through its fumarate groups. Crosslinking can occur by radical polymerization through photo-initiation or thermal initiation. Photo-crosslinking has been achieved with a commercialized photo-initiator such as Irgacure 2959 and N-vinylpyrrolidone (NVP) as a comonomer [86]. Thermal crosslinking has been implemented in the presence of either APS and AA or APS and \(N,N,N',N'\)–tetramethylethylenediamine (TEMED) as seen in Figure 3.3. While both initiator systems have previously been used, the APS/TEMED initiator mix remains near neutral pH whereas the APS/AA system is acidic [66, 87]. Poly(ethylene glycol)-diacrylate and \(N,N'\)-methylene bisacrylamide (BISAM) have been used as crosslinkers in combination with the initiator systems just described to form OPF hydrogels [62, 88].

3.3.2. Characterization

3.3.2.1. Swelling and degradation

Numerous swelling and degradation studies have been performed to investigate changes in physical properties as the OPF macromer length is varied [55, 62, 65]. Since OPF macromers consist of alternating units of fumarate groups and PEG chains, it is expected that longer PEG chains incorporated into the macromers would increase the hydrophilicity of the hydrogels, and thus greater swelling would occur. As the PEG chain MW increased from 1K to 35K, equilibrium swelling of chemically crosslinked hydrogels increased [62, 89, 90]. In addition, molecular weight between crosslinks and mesh size also increased as the initial PEG MW in OPF macromers increased [62]. For
example, as the PEG MW increased from 1K to 10K, mesh size of the resulting hydrogel increased from 76 to 160 Å respectively [62].

As previously mentioned, degradation of OPF hydrogels is induced by cleavage of ester bonds within the polymer network by hydrolysis. The degree of degradation is extrapolated through mass loss and swelling ratio data over time and has been shown to be a function of PEG chain length in OPF macromers as well as crosslinking density [65]. There is an inverse relationship between crosslinking density and mass loss with greater mass loss occurring over time with a lower crosslinking density [65, 89].

When OPF hydrogels were crosslinked through photo-initiation, increasing the concentration of the comonomer NVP decreased the swelling ratio [86]. Over a period of 21 days, a significant increase in sol fraction for low NVP concentrations in comparison to high NVP concentrations was also seen [86].

Composite OPF hydrogels have also been fabricated that have their own distinct swelling and degradation characteristics. Composite hydrogels comprising OPF and gelatin microparticles (GMPs) have been investigated using GMPs as growth factor (GF) delivery vehicles and an enzymatically digestible porogen [88]. When comparing OPF hydrogels and OPF-GMP composite hydrogels, no significant difference in swelling ratio and mass loss was observed by day 28 in PBS [55]. However, a significant increase in swelling ratio of composite hydrogels over simple hydrogels was observed by day 28 in collagenase-PBS [55]. This increase in degradation highlights the potential enzymatic effects of an in vivo environment on OPF-GMP composite hydrogels.

Mixed-mode OPF hydrogels crosslinked with PEG-dithiol produced hydrogels with distinct degradation and swelling properties. Hydrogels containing 20 wt% PEG-
dithiol had increased swelling and decreased degradation time in comparison to 10 wt% PEG-dithiol and 0 wt % PEG-dithiol containing OPF hydrogels [91]. Due to the complex nature of these mixed-mode hydrogels involving chain and step polymerization, 20 wt% PEG-dithiol hydrogels may have a lower crosslinking density than 10 wt% or 0 wt % PEG-dithiol containing OPF hydrogels [91]. In addition, the presence of sulfide bonds located near the ester groups may promote faster ester hydrolysis [91, 92].

### 3.3.2.2. Cytocompatibility and biocompatibility

Before utilizing OPF hydrogels in tissue engineering, evaluation of its in vitro cytotoxicity and in vivo biocompatibility need to be investigated. Cytotoxicity of hydrogel constituents including the linear macromer, crosslinking agents, and initiators were examined. In addition, leachable substances from crosslinked hydrogels (i.e., sol fraction) were tested. Results showed that while OPF macromers were found to be non-cytotoxic with no significant difference between high and low MW OPF macromers, shorter chain PEG-DA was more cytotoxic than longer chain PEG-DA [66]. Unreacted leachable components from crosslinked hydrogels were also found to be non-cytotoxic [66]. Also, the chemical initiators used to fabricate crosslinked hydrogels exhibited favorable cytocompatibility at concentrations relevant to hydrogel formation [66, 93].

Since the intended applications for OPF hydrogels include injectable scaffolds, studies investigating in vivo biocompatibility are essential. Research on the bone and soft tissue behavior of OPF hydrogels in rabbit models demonstrated positive results [65, 94]. Cranial and subcutaneous implants resulted in formation of a thin fibrous capsule regardless of varying hydrogel fabrication parameters [65]. Also, in vivo degradation products from hydrogels with 8K PEG MW elicited a minor inflammatory response,
whereas hydrogels with 1K PEG MW had limited degradation [65]. Overall, minimal cytotoxicity and favorable biocompatibility establish OPF hydrogels as excellent synthetic scaffolds for tissue engineering applications.

### 3.3.2.3. Modification with biomimetic peptides

OPF hydrogels have been functionalized with biomimetic peptides with the intent to modulate cellular functions in guided tissue regeneration [95]. These peptides, including RGD and osteopontin-derived peptide (ODP), were incorporated in OPF hydrogels through bulk modification: by coupling the peptides to an Ac-PEG-NHS spacer, the resulting acrylated peptides could be crosslinked within the OPF network [96, 97]. In a comparison between hydrogels modified with ODP and Gly-Arg-Gly-Asp-Ser (GRGDS), osteoblasts migrated faster on ODP modified hydrogels [98]. Also, increased migration occurred with increasing peptide concentration. In addition, bone marrow-derived MSCs showed greater alkaline phosphatase (ALP) activity and greater OPN secretion on ODP-modified hydrogels in comparison to RGD-modified hydrogels [99]. These peptide modification techniques support the versatility of OPF hydrogels to enhance cellular functions and direct tissue-specific regeneration.

### 3.3.3. Regenerative medicine applications

#### 3.3.3.1. Bone regeneration

In a number of experiments, OPF hydrogels have been used as biomaterial scaffolds to support osteogenic differentiation of bone marrow-derived MSCs. In
particular, OPF hydrogels modified with RGD and ODP peptides exhibited greater OPN production and calcium deposition by seeded bone marrow-derived MSCs in comparison to unmodified hydrogels [97]. In addition, bone marrow-derived MSC adhesion, migration, and differentiation into osteoblasts are regulated by the concentration of incorporated adhesion peptides [98, 99]. Encapsulated bone marrow-derived MSCs also exhibit osteogenic differentiation when cultured in osteogenic media with greater OPN and calcium deposition in higher swelling hydrogels [54, 87]. Similarly, OPF gels with greater pore sizes enhanced osteogenic differentiation of bone marrow-derived MSCs [100].

3.3.3.2. Cartilage regeneration

Besides osteogenesis, OPF hydrogels have been used to mimic a native articular cartilage environment to support cartilage tissue formation. Several in vitro studies investigated chondrogenesis of encapsulated cells in hydrogels through a myriad of cell and GF combinations. By combining marrow-derived MSCs or chondrocytes with transforming growth factor-β1 (TGF-β1) released from GMPs, greater chondrogenesis was observed with the release of TGF-β1 in comparison to the absence of TGF-β1 [101, 102]. An insulin-like growth factor-1 (IGF-1) and TGF-β1 dual growth factor release system was also investigated with encapsulated MSCs. Results showed upregulation of chondrocyte-specific genes for MSCs with TGF-β1 and promotion of cell aggregation with IGF-1 [103]. The physical properties of OPF hydrogels also affect chondrogenesis with a larger mesh size correlating to a larger PEG chain in OPF macromers increasing chondrogenesis of encapsulated MSCs [89]. In addition, a hydrogel co-culture system utilizing osteogenic cells in the bottom layer and MSCs in the top layer of bilyared OPF
constructs suggests a synergistic effect between the two layers to promote chondrogenesis in the top layer [104, 105]. In vivo studies utilizing OPF hydrogels to repair a full-thickness osteochondral defect have also been carried out. In these experiments, successful regeneration of subchondral tissue was seen employing OPF hydrogel scaffolds [106-108]. In addition, OPF hydrogels comprising GFs, including IGF-1 and TGF-β1, as well as MSCs show great potential for the repair of cartilage tissue.

3.3.3.3. Tendon/ligament regeneration

In a series of studies delivering fibroblasts for tendon/ligament regeneration, OPF based hydrogels were used as a template biomaterial to investigate cellular responses from mechanical stimuli [91, 109, 110]. Fibroblasts harvested from cruciate ligaments and patellar tendons of bovine knee joints have been encapsulated in OPF/PEG-DA/PEG-dithiol mixed-mode hydrogels [91]. This research revealed that incorporation of 20 wt% PEG-dithiol can promote cell clustering and aggregation within hydrogel environments. Additionally, the differentiation of encapsulated human MSCs in OPF hydrogels into fibroblasts can be induced under cyclic tensile culture conditions [109]. Upregulated expression levels of tenascin-C, collagen type I and collagen type III were exhibited over 21 days in the presence of mechanical stimuli. Another study also investigated spatial controllability of photo-lithographically patterned OPF hydrogels with high spatial fidelity and thickness [110]. By utilizing serial steps of photocrosslinking and lamination in a simple, inexpensive microfluidic device, tissue-scale (1-2 mm in thickness) OPF hydrogels encapsulating multiple primary cell types could be patterned with high spatial control.
3.3.3.4. Lens regeneration

One of the recent investigations of OPF hydrogels for tissue engineering applications is pigment epithelial cell encapsulation for lens regeneration [111]. Newt iris pigment epithelial cells were encapsulated in OPF hydrogels for implantation into lentectomized newts in vivo. Histological examination after 30 days indicated that encapsulated cells in hydrogel beads (1 mm in diameter) trans-differentiated into lens tissues. This was confirmed by lens fiber tissue formation without adverse side effects by hydrogel degradation [111].

3.3.3.5. Nerve tissue regeneration

Fumarate-based biomaterials have also been investigated for spinal cord and nerve regeneration in neural tissue engineering [112-115]. In order to overcome the drawbacks of autologous nerve grafts for the treatment of segmental peripheral nerve defects, such as malfunction in donor nerve site and size mismatch between a nerve graft and an implanted site, a variety of hydrogel systems have recently been investigated [112]. Among these degradable synthetic biomaterials, OPF hydrogels with the aid of electrically charged reagents have been developed for preliminary applications in neural tissue engineering [112, 114]. By copolymerization with [2-(methacryloyloxy) ethyl]-trimethylammonium chloride (MAETAC), OPF hydrogels can be positively charged to support dorsal root ganglion neuron attachment and differentiation in a MAETAC monomer amount-dependent manner [112]. Another study investigated the incorporation of an electrically conductive polymer, polypyrrole (PPy) [114]. In order to achieve high conductivity in a nerve guidance conduit and to stabilize the positive charge, three anions
were incorporated with the OPF-PPy hydrogel system; naphthalene sulfonic acid, docecyl benzene sulfonic acid, and dioctyl sulfosuccinate. This composite hydrogel system was not cytotoxic to PC12 cells over 7 days in vitro. More importantly, neurite extension analysis after 24 hrs indicated that OPF-PPy hydrogels with naphthalene sulfonic acid promoted PC12 cell attachment and neurite length extension [114]. These recent studies demonstrate the potential use of electrically conductive fumarate-based hydrogel composites for nerve regeneration [113, 115].

3.3.3.6. Delivery of bioactive molecules

The delivery of bioactive molecules from OPF hydrogel composites has been researched for medicinal and regenerative applications. Using an OPF hydrogel carrier composite, the delivery of a chemotherapeutic anti-cancer drug, doxorubicin (Dox), was investigated [116]. An ionic monomer, sodium methacrylate, was crosslinked with OPF to form negatively charged hydrogels which could then couple with positively charged Dox through electrostatic interactions. This research demonstrated that controlled release kinetics of Dox from OPF hydrogels depended on the charge density of the hydrogel as well as the ionic strength of the surrounding environment [116]. In addition, released Dox maintained its anti-cancer activity to MG63 cells. Precise control in the concentration of incorporated drugs could be achieved by modulation of OPF hydrogel chemical composition, subsequent changes in polyelectrolyte properties, and interactions with the ionic strength and pH of the surrounding environment [116].

With the aid of poly(lactic-co-glycolic acid) (PLGA) microparticles, therapeutic proteins and drugs can be delivered in vivo in a controlled manner using OPF hydrogel platforms [117]. One such therapeutic protein, dibutyryl cyclic adenosine monophosphate
(dbcAMP), has been encapsulated in PLGA microparticles in OPF hydrogels for spinal cord transection studies [117]. This study demonstrated that sustained delivery of dbcAMP reduced capillary formation and the volume of cyst and scar formation in the presence of Schwann cells and MSCs. A functional recovery of motor skills in an animal model was significantly improved when dbcAMP was delivered with only MSCs [117].

Therapeutic proteins have also been released from OPF hydrogels and OPF-GMP composites. Due to innate charge differences, IGF-1 and TGF-β1 with isoelectric points (IEPs) of 7.5 and 9.5 respectively can bind to acidic GMPs with an IEP of 5.0 through ionic complexion at physiologic pH [118]. By complexing TGF-β1 to GMPs, burst release of the GF was reduced in comparison to release from simple OPF hydrogels. Dual GF delivery systems have also been employed using OPF-GMP composites with no deleterious effects of one GF release on the other [118]. Such systems offer spatial and temporal control on GF release for regenerative medicine applications [102-104].

Plasmid DNA can also be delivered using OPF hydrogels [119, 120] and OPF-GMP composites [121-123]. The release of plasmid DNA from an OPF hydrogel can be controlled by the PEG chain MW and subsequent degradation profile of the hydrogel [120, 121]. A study involving co-encapsulation of plasmid DNA and cells in OPF hydrogels demonstrated that bone tumor cells in hydrogels could be transfected by plasmid DNA encoding green fluorescent protein (GFP) and the transfected cells continuously expressed GFP protein over 21 days [119]. In the same study, estrogen receptor (ER)-negative human fetal osteoblasts were transfected by plasmid DNA encoding ER, and increased ALP activity and estrogen-dependent luciferase activities with the estrogen treatment were seen [119]. When plasmid DNA-GMP complexes were
encapsulated in OPF hydrogels, a sustained release of plasmid DNA over 42 days was observed in mice \textit{in vivo} relative to a direct injection of DNA solution and the delivery of non-embedded DNA [122]. Another study also investigated the potential of OPF composite hydrogels encapsulating plasmid DNA encoding human bone morphogenetic protein-2 (BMP-2) complexed to GMPs to enhance bone regeneration in a critical sized rat cranial defect model [123].

### 3.4. Poly(lactide-co-ethylene oxide-co-fumarate) hydrogel

In addition to OPF, another fumarate-based hydrogel has been developed with controlled physical properties as an alternative for specific regenerative medicine applications. Poly(lactide-\textit{co}-ethylene oxide-\textit{co}-fumarate) (PLEOF) comprises an ultralow molecular weight poly(t-lactide) (PLA) block that offers greater tunability of the fabricated hydrogel water content and degradation rate [124]. Preliminary studies show PLEOF hydrogels have potential as a fumarate-based tissue engineered scaffold with such added modifications.

#### 3.4.1. Chemistry

PLEOF is also a PEG-based terpolymer modified using a fumarate unit to control crosslinkability [60]. \textit{In situ} crosslinkable PLEOF macromers can be synthesized with a
co-polymer of PLA and diethylene glycol (produced by ring opening polymerization of L-lactide with diethylene glycol as an initiator), PEG blocks, and FuCl by condensation polymerization [60, 124]. The degradation products of the terpolymer include lactic acid and fumaric acid that can be metabolized, as well as PEG which can be excreted by the body [60].

For the crosslinking reaction, BISAM was used as a crosslinker while an acidic initiator, APS, and basic radical catalyst, TEMED, were utilized for a neutral redox initiation [60]. Crosslinking properties of PLEOF hydrogels are dependent on the concentrations of initiator/accelerator. As such, increasing APS/TEMED concentrations increased the rate of the crosslinking reaction, decreased gelation time, and increased the ultimate storage modulus of PLEOF hydrogels [60]. Increasing the concentration of BISAM also increased the ultimate modulus of the gels [60]. An increase in crosslinking density of PLEOF hydrogels was achieved by increasing the density of fumarate groups in PLEOF [60, 67]. In addition to chemical crosslinking, PLEOF can also be photo-crosslinked with the aid of NVP, a photo-initiator, and UV radiation [125]. Like P(PF-co-EG) and OPF macromers, PLEOF precursor chains can be crosslinked through the C=C double bond in the fumarate group. Crosslinking density and degradation of photo-crosslinked PLEOF hydrogels are dependent of the composition (e.g., NVP concentration, PLA to PEG ratio, and PLEOF concentration) as well as the time and intensity of UV radiation [125].

In addition to chemically crosslinked hydrogels using BISAM, a peptide crosslinker was also investigated to develop both hydrolytically and enzymatically degradable PLEOF hydrogels [67]. In this study, matrix metalloproteinase (MMP)-13
degradable peptide sequence QPQGLAK with acrylate end-groups functioned as the crosslinker polymerized with PLEOF macromers. Peptide crosslinked PLEOF hydrogels showed higher water content and sol fraction compared to BISAM crosslinked hydrogels due to the relatively higher MW of the peptide crosslinker [67]. Using both peptide and BISAM crosslinkers, the degradation rate of PLEOF hydrogels were modulated by the ratio of the two crosslinkers, the amount of MMP-13 to cleave the peptide sequences, and the incubation time to expose to MMP-13 [67].

3.4.2. Characterization

A number of studies have been done to characterize the physical properties of this versatile terpolymer macromer. The swelling ratio of PLEOF can be adjusted by varying the ratio of hydrophobic PLA blocks to hydrophilic PEG blocks as well as the PEG chain length [124, 126]. In addition, the degradation rate is controlled by the ratio of PLA to PEG blocks and the hydrogel mesh size is modified through the density of fumarate groups in the terpolymer [60]. In experiments with photo-crosslinked PLEOF hydrogels, hydrogels with greater PLA:PEG ratio exhibited decreased swelling and increased sol fraction. Degradation experiments indicated that the PLA:PEG block ratio, and ultimately the ratio of lactide to fumarate units, affected the degradation rate of PLEOF hydrogels [124].

The cytocompatibility of PLEOF hydrogels was also tested with MSCs. MSCs cultured in a medium containing BISAM crosslinker had a marked decrease in cell viability in comparison to those cultured in medium containing a peptide crosslinker [67].
The peptide crosslinked hydrogel network was shown to be cytocompatible with encapsulated MSCs (e.g., 90% viability), however, the sol fraction exhibited some adverse affects (e.g., 65% viability) [67].

### 3.4.3. Regenerative medicine applications

#### 3.4.3.1. Bone regeneration

Peptide incorporation in PLEOF hydrogels can enhance their osteoconductivity [127]. In order to conjugate various bioactive peptides in PLEOF hydrogels, acrylamide-terminated RGD peptides were first crosslinked with fumarate groups in PLEOF macromers and PEGylated peptides derived from BMP-2 were grafted to the hydrogel surface by specific click chemistry between the azide group of the peptides and the propargyl group of the hydrogels [127]. As osteoblastic differentiation of encapsulated MSCs was observed in peptide-modified PLEOF hydrogels [67], MSCs seeded onto hydrogels conjugated with both RGD and BMP-2 derived peptides exhibited increased ALP activity and mineralization over 21 days of *in vitro* culture compared to hydrogels with single incorporation of either RGD or BMP-derived peptides [127]. Specifically, calcium deposition on PLEOF hydrogels with both peptides was significantly higher than solely RGD or BMP-2 derived peptide grafted hydrogels.

Hydroxyapatite (HA) nanoparticles have also been incorporated for the fabrication of PLEOF nanocomposite hydrogels [128-130]. Interaction between HA nanoparticles with uncrosslinked PLEOF macromer chains [129] or incorporated peptides
influenced the viscoelastic properties of PLEOF composite hydrogels. Moreover, PLEOF/HA hydrogels conjugated with RGD peptides were utilized to coat electrospun PLA fiber-meshes to form multilayered fiber-reinforced laminated composite hydrogels [130]. In this study, MSCs seeded onto laminated PLEOF hydrogels with HA incorporation and RGD peptide conjugation exhibited enhanced osteoblastic differentiation, as evidenced by the expression of OPN and osteocalcin marker genes, and mineralization over 21 days of in vitro culture.

3.4.3.2. Delivery of bioactive molecules

Recently, the release kinetics of stromal derived factor-1α (SDF-1α) from PLEOF hydrogels and its effect on the migration of MSCs were investigated [126]. Increasing the PLA content in PLEOF macromers resulted in a decreased mesh size, increased SDF-1α loading efficiency, and sustained release of SDF-1α [126]. In addition, the migration rate of MSCs was dependent on the release kinetics of SDF-1α from the PLEOF hydrogels.

3.5. Conclusion

The multitude of human tissues, each uniquely developed with specific function, calls upon versatile tissue engineered scaffolds with tailored and controlled properties. The synthetic hydrogels described here have been conceived to possess fumarate units. These fumarate units contain double bonds that allow the formation of a crosslinked
network, and ester groups that potentiate these hydrogels to degrade hydrolytically into biocompatible elements. P(PF-co-EG), OPF, and PLEOF have been synthesized with their own distinct physical properties and the development of these fumarate-based biomaterials has led to hydrogels with further adaptability over their physicochemical properties for specific tissue type regeneration. Future developments can illustrate the robust potential of these fumarate-based hydrogels as injectable, biodegradable, and biocompatible tissue engineered scaffolds.
3.6. Tables and Figures

Figure 3.1 A schematic of the transesterification reaction between PEG and PPF to form the copolymer P(PF-co-EG) [69]
Figure 3.2 A schematic showing the synthesis of the OPF macromer from PEG and FuCl

[59]
Figure 3.3 Chemical crosslinking of OPF with PEG-DA in the presence of initiators to form a hydrogel [62]
The delivery of biologics is an important component in the treatment of osteoarthritis and the functional restoration of articular cartilage. Numerous factors have been implicated in the cartilage repair process, but the uncontrolled delivery of these factors may not only reduce their full reparative potential and can also cause unwanted morphological effects. It is therefore imperative to consider the type of biologic to be delivered, the method of delivery, and the temporal as well as spatial presentation of the biologic to achieve the desired effect in cartilage repair. Additionally, the delivery of a single factor may not be sufficient in guiding neo-tissue formation, motivating recent research towards the delivery of multiple factors. This review will discuss the roles of

4.1. Introduction

The avascular and relatively acellular nature of articular cartilage complicates its natural capacity for regeneration upon damage. While numerous clinical therapies, such as microfracture, autologous chondrocyte implantation, and osteochondral grafts, have been developed for the treatment of cartilage injuries, they have been hampered by inferior cartilage repair and significant donor site morbidity [131]. Indeed, given the extensive literature presently highlighting the shortcomings of current clinical techniques for the management of chondral and osteochondral injuries, it is clear that the field of cartilage repair remains an area in critical need of innovative alternative therapies [132-134]. In light of the disadvantages hindering the efficacy of currently available cartilage treatment options, the discipline of tissue engineering provides promising alternatives. Particularly, the area of osteochondral tissue engineering leverages the controlled combination of carefully engineered scaffolds, progenitor cells, and biochemical cues for replacing or restoring lost articular cartilage and subchondral bone function. While the selection of an optimal cell type and appropriate scaffold is necessary to reconstruct
specific tissues with a particular configuration and function, successful regeneration is greatly influenced by the cellular microenvironment in which cells and tissues grow [135]. Inspired by physiological events that occur during fetal development and long bone formation [136-138], the concept of growing articular cartilage has led to the integration of a wide variety of soluble cues in an effort to mimic natural signaling cascades in the wound healing environment.

In cartilage tissue repair, the goal of eliciting the desired phenotypic responses from host and/or co-delivered progenitor cells remains. Yet, techniques for the delivery of soluble cues and other biologics for the treatment of cartilage and osteochondral defects have evolved over the years from simple bolus injections into the defect to more sophisticated and controlled multi-functional delivery systems. These engineered strategies, which permit localized drug delivery with controlled release kinetics, utilize delivery platforms that typically leverage at least one of several main delivery schemes developed for controlled release. The simplest approach involves the direct intra-articular injection of growth factors or palliative agents into the synovial space. While such injections represent an attractive and relevant option due to the lack of surgery, the frequencies of injections required and the supra-physiological dosages employed often complicate therapeutic efficacy. Additionally, the recapitulation of natural signaling cascades for proper healing and regeneration of damaged tissues would be near impossible with single injections [134, 138-141]. Nevertheless, the motivation to recapture the complexity of endogenous healing cascades into simplified and controlled forms has driven the evolution of materials-based delivery systems towards a range of modalities involving drug release from microspheres, bulk scaffolds, or a combination of
both. Although the type of modality employed for drug delivery can determine the mechanism of release, the method of agent incorporation also offers a significant form of modulation for controlled release. Conventional strategies for the incorporation of signaling molecules into engineered delivery systems typically involve either the physical entrapment or the chemical immobilization of factors into or onto a polymer matrix. Recent reviews by Mehta et al. and Santo et al. provide excellent summaries of modern controlled release strategies utilizing such means for bone and orthopedic therapies [142, 143]. By carefully combining the physical entrapment of bioactive agents with the chemical conjugation of other bioactive factors, innovative and smart delivery systems can be engineered to sustain the release of single or multiple biologics in a spatiotemporally controlled fashion for effective cartilage therapy.

This review begins with an overview of the various bioactive factors that have been utilized in delivery and controlled release strategies for cartilage repair. The aim is to first briefly enable an understanding of the different biological approaches available for both *in vitro* and *in vivo* cartilage repair applications. The following sections will discuss the methods of delivery and assess the current state of recent controlled release strategies developed for cartilage tissue engineering and cartilage repair. Special emphasis will be placed on combining materials-driven and biologically-driven strategies for cartilage repair in order to provide an outlook for future developments that are aware of the needs for both.
4.2. Cartilage Regenerative Factors

The homeostasis and repair of articular cartilage is regulated by a number of growth factors, differentiation factors, systemic factors, and other biologics. The ultimate response from a specific biologic depends on its identity, and so a critical component in designing a controlled delivery system is the selection of an appropriate factor.

4.2.1. Growth Factors

Growth factors are a group of soluble signaling molecules that can stimulate cellular division, growth, and differentiation through specific binding of transmembrane receptors on target cells [144]. Among the biologics involved in cartilage repair, growth factors remain the most extensively studied due to their powerful proliferative, proanabolic, and/or anticatabolic properties [36]. These include select members of the transforming growth factor-β (TGF-β) superfamily, fibroblast growth factor (FGF) family, and insulin-like growth factor-1 (IGF-1). Another major growth factor, platelet-derived growth factor (PDGF), plays a role in the wound healing of cartilage defects, but the direct effect of PDGF delivered to a cartilage defect has yet to be investigated [145]. It is most often used within platelet-rich plasma (PRP), which will be discussed in a later section.
4.2.1.1. Transforming Growth Factor-β Superfamily

The TGF-β superfamily includes over 30 structurally related members and serves an important role in regulating embryogenesis as well as adult homeostasis [146]. Among the TGF-β superfamily, the most heavily investigated growth factors for cartilage repair include prototypic members TGF-β1, 2, and 3 and bone morphogenetic proteins (BMP) 2, 4, and 7. Growth differentiation factors (GDFs), particularly GDF-5, have shown chondrogenic potential in vitro, but their ability to promote in vivo cartilage repair has yet to be evaluated [147-150].

Transforming Growth Factor-βs

TGF-β1, 2, and 3 are considered to be potent stimulators of chondrogenesis, inducing Sox9 expression and increasing cartilaginous extracellular matrix (ECM) production in bone marrow-derived mesenchymal stem cells (MSCs) as well as stimulating synthetic activity in chondrocytes [36, 138, 151]. In animal models, TGF-βs are highly expressed during MSC condensation in the growth plate of long bones [138] and are also implicated in the early stages of cartilage repair [136]. As a result, TGF-β isoforms, particularly TGF-β1 and 3 have been used in a number of promising in vivo studies exploring the ability of TGF-βs to promote the repair of cartilage defects [152]. In a rabbit full-thickness cartilage defect model, poly(lactic-co-glycolic acid) (PLGA)/fibrin gel scaffolds loaded with MSCs and TGF-β1 resulted in better cartilage restoration than PLGA/fibrin gel scaffolds loaded with MSCs alone [153]. Additionally, TGF-β1 in calcium alginate beads improved osteochondral tissue repair after 12 weeks compared to alginate scaffolds alone [154]. However, the effects of TGF-β1 on cartilage repair in vivo...
are not always consistent [107]. Guo et al. demonstrated that blank oligo(poly(ethylene glycol) fumarate) (OPF)-based scaffolds in a rabbit osteochondral defect model resulted in cartilage repair that was equal to or improved relative to OPF scaffolds with MSCs or OPF scaffolds with MSCs and TGF-β1 [108]. Intra-articular injections of TGF-β have also caused synovial fibrosis and endochondral ossification [155, 156]. Several reasons for the observed negative effects of TGF-β delivery have been suggested, including the supra-physiological levels of growth factor employed as well as the relatively non-specific effects of TGF-β on MSC differentiation [108]. As a result, it is important to consider the dosage and presentation of TGF-βs when delivered in vivo, understanding that TGF-βs are multifunctional and induce gene responses in different cell types for proliferation and ECM synthesis [157].

**Bone Morphogenetic Proteins**

Similar chondrogenic effects of TGF-β have also been observed for several BMP molecules, likely due to the extensive crosstalk between TGF-β and BMP signaling pathways [158]. Different BMPs, particularly BMP-2, 4, 6, 7, and 9, have been shown to stimulate chondrogenic differentiation of MSCs and/or adipose-derived stem cells (ASCs) either individually or with a TGF-β prototypic member [159]. In a comparison between TGF-β1 and BMP-2 delivery from PLGA microspheres in alginate gel, both groups resulted in greater histological scores for osteochondral repair compared to blank scaffolds at 12 and 24 weeks in a rabbit patellar groove defect [160]. BMPs also play a role throughout the MSC chondrogenic differentiation process, from MSC condensation to proliferation, differentiation, maturation, and calcification. In particular, BMP-2 is
expressed throughout the entire chondrogenic process, from proliferation to calcification [138], and hence, a long-term delivery of BMP-2 can be beneficial and has been shown to result in a higher quality repair of cartilage as opposed to short-term delivery [161]. Different BMPs also have different effects on osteochondral repair. In a study delivering BMP-2 or BMP-4 in alginate gels to a rabbit femoral condyle defect, delivering BMP-2 alone resulted in better subchondral bone restoration while BMP-4 alone gave better cartilage tissue repair [162]. BMP-7 has also shown efficacy in vivo: BMP-7 delivered from a collagen sponge in conjunction with microfracture led to thicker repair cartilage, superior matrix and superior cell distribution compared to a collagen sponge plus microfracture alone in a rabbit chondral defect [163]. With the ability to induce both cartilage and bone formation, BMPs, particularly BMP-2, are attractive growth factors for the regeneration of the osteochondral tissue unit.

4.2.1.2. IGF-1

Within articular cartilage, IGF-1 is the main anabolic growth factor and plays a key role in cartilage homeostasis, balancing proteoglycan synthesis and breakdown by chondrocytes [145]. IGF-1 can decrease catabolic responses as well as increase proliferation and cartilaginous ECM production in MSCs and chondrocytes in vitro [36, 164]. In vivo, IGF-1 has demonstrated the ability to improve filling of chondral defects [165], improve quality of cartilage repair [166, 167], and decrease the postoperative inflammatory response [168]. IGF-1 has also demonstrated an additive effect when combined with other growth factors such as TGF-β1, BMP-2, and BMP-7 [164, 169, 170]. However, achieving similar synergistic results when delivering IGF-1 with other growth factors in vivo is an area of much interest. IGF-1 delivered from gelatin
microparticles (MPs) in an OPF-based scaffold resulted in higher quality cartilage repair compared to OPF composites alone in a rabbit medial femoral condyle defect at 12 weeks. But the benefits of IGF-1 were not maintained when co-delivered with TGF-β1 [107]. Similarly, the single delivery of IGF-1 from OPF composites showed an improvement in cartilage morphology over blank scaffolds alone, but the dual delivery of IGF-1 with TGF-β3 did not have a synergistic effect [171]. Further study on combining IGF-1 with other growth factors as well as the appropriate presentation of IGF-1 in a multiple growth factor delivery strategy in vivo is needed.

4.2.1.3. FGF-2

Like IGF-1, the FGF family also plays an important role in the homeostasis of cartilage. In particular, FGF-2, or basic FGF (bFGF), has a potent mitogenic effect on MSCs and chondrocytes [172]. In addition, treatment with FGF-2 increased both the proliferative and chondrogenic potential of MSCs in vitro [164, 173, 174]. In vivo, the delivery of FGF-2 has been shown to improve both cartilage repair as well as the underlying subchondral bone [175, 176]. However, FGF-2 may have contraindications: FGF-2 induced chondrocyte proliferation in a cartilage explant, but resulted in chondrocyte clonal cluster formation, which is a histopathological feature of osteoarthritis (OA) [177]. Additionally, evidence suggests that FGF-2 may antagonize proteoglycan synthesis and upregulate matrix metalloproteinases (MMPs) [36]. Proper delivery and presentation of FGF-2 may help mitigate potential contraindicator effects on cartilage repair.
4.2.2. Anti-Angiogenic Factors

Full-thickness lesions that perforate the subchondral bone and bone-marrow spaces may trigger the onset of angiogenic and osteogenic processes in the cartilage layer, leading to conditions unfavorable for chondrogenesis. As a result, there has been interest in introducing anti-angiogenic factors to inhibit blood vessel growth and restore cartilage tissue to its natural state of avascularity [178]. This class of factors includes endostatin [179-181], suramin [182, 183], Flt-1 [184, 185], and bevacizumab [186]. While these factors demonstrate the ability to block vascularization and inhibit the activity of vascular endothelial growth factors (VEGFs), a clear benefit of this approach in a cartilage defect model remains to be seen.

4.2.3. Systemic Factors and Notable Pharmaceuticals

The therapeutic role of anti-inflammatory cytokines, chemokines, hormones, and other drugs have also been considered in the repair of cartilage. These biologics do not serve the primary role as a mitogenic or anabolic factor, but mediate the wound healing response through other mechanisms. Stromal cell-derived factor-1 (SDF-1) is a key chemokine in cell trafficking and homing of CD34+ stem cells, particularly MSCs [187], and has the potential to enhance cartilage repair through increased MSC migration to the site of a cartilage defect without the need for additional cell transplantation [188, 189]. In the parathyroid hormone (PTH) family, peptide segments of PTH have been shown to inhibit the progression of OA and advance the repair of shallow chondral defects [190, 191]; and PTH-related proteins (PTHrP) are synthesized by chondrocytes and can
suppress induction of hypertrophy [192, 193]. However, the timing of PTH or PTHrP administration to cartilage defects remains an important parameter in affecting treatment outcome. Inhibitory factors on necrosis, apoptosis, MMPs, and aggrecanases have also shown potential in the treatment of cartilage defects [194-197]. Other notable systemic factors and pharmaceuticals are listed in Table 4.1.

4.3. Biologic Delivery Methods

Almost as important as the selection of an appropriate biologic is choosing a suitable delivery mechanism to enable an appropriately controlled release and elicit the intended response. The proper delivery of the biologic can affect the dosage as well as the release rate and ultimately determine whether or not a therapeutically effective pharmacokinetic release profile was achieved.

4.3.1. Injection Delivery

The delivery of a biologic through an intra-articular injection or systemic injection is perhaps the simplest method for minimally invasive administration. The ability to treat not only the articulating cartilage but also the entire joint is a relevant delivery strategy, particularly for the management of pain and degenerative processes in OA. Intra-articular injection of a chondroprotective agent, high-molecular-weight crosslinked hyaluronic acid (HA), was seen to improve joint lubrication and retard the
progression of OA in a rabbit anterior cruciate ligament transection model over lower molecular weight HA or a saline solution [198]. The benefit and potential of intra-articular modalities for the presentation of chondroprotective agents has also been demonstrated in other studies [198-203]. In addition to palliative treatments, anti-inflammatory agents have also been delivered through intra-articular means to treat OA inflammation [204-206]. By treating the synovial fluid and lining in addition to articular cartilage, the expression of major pro-inflammatory cytokines such as interleukin (IL)-1 and tumor necrosis factor-α (TNF-α) can be addressed and reduced [207-213]. However, the delivery of chondrogenic growth factors such as TGF-β and IGF-1 in an injection manner can effect unwanted changes in the host tissue due to uncontrolled presentation of these bioactive factors [214-218]. Growth factors have a short half-life in vivo and can result in rapid clearance when delivered systemically, hindering their potent mitogenic and/or anabolic effects [219-221]. Additionally, a bolus injection of growth factors gives supra-physiological doses, potentially resulting in pathological and non-specific effects, and an absence of a physiologically effective release profile.

4.3.2. Bulk Phase Delivery

Although the direct injection of bioactive agents has been used with some success for the treatment of OA, the rapid clearance of such drugs from the synovial capsule generally hinders therapeutic efficacy. Incorporation of biologics within a biomaterial carrier can address this issue by delivering the biologic in a concentrated and controlled fashion. Three-dimensional matrices and porous scaffolds are the most common delivery
vehicles, particularly for growth factors. By using a drug-delivering scaffold, focal chondral or osteochondral defects can be treated by releasing the factors to the surrounding tissue or promoting cell infiltration into scaffold. Many different techniques have been developed to regulate the release kinetics of soluble factors as well as retain the molecular bioactivity.

One of the most common approaches for bulk phase delivery is a simple dispersion of the biologic within the matrix. The release of the bioactive factor is then dependent on the interaction between the factor and the matrix, either mediated by encapsulation [153, 175, 222], electrostatic interactions [163, 166, 176, 223-225], immobilization/tethering [226-228], or ECM affinity [229-231]. Biologics encapsulated or entrapped within bulk matrices often have a large burst release, which can be tuned by scaffold crosslinking density and pore size. However, particular natural materials have innate physical properties that can control the release of growth factors. Yang et al. delivered BMP-2 from fibrin gels and heparin-conjugated fibrin gels in a full-thickness trochlear groove defect in rabbits combined with microfracture [161]. An electrostatic interaction with heparin resulted in a sustained release of BMP-2 (82% over 13 days) compared to a burst release of BMP-2 from normal fibrin gels (88% in first 3 days). This long-term delivery of BMP-2 resulted in greater filling of cartilage as well as a higher quality of cartilage repair as opposed to short-term delivery [161].

While many growth factors can adsorb onto scaffolds made of natural polymers through electrostatic interactions, hydrogen bonding, and/or van der Waals forces, non-covalent binding methods may not enable a long-term sustained delivery. Fan and colleagues achieved stable localization of growth factors by crosslinking TGF-β3 onto
PLGA/gelatin/chondroitin sulfate/HA scaffolds through a condensation reaction between the carboxyl group of the hybrid scaffold and amine group of TGF-β3 [226]. Results indicated that the cumulative release of TGF-β3 was reduced to 29.5% over 28 days and that TGF-β3-immobilized scaffolds could induce similar levels of chondrogenic differentiation of seeded MSCs and in vivo cartilage repair compared to non-immobilized scaffolds cultured in medium with TGF-β3. However, caution should be taken when tethering factors to monolithic scaffolds. Kopesky and colleagues demonstrated that adsorption of TGF-β1 onto self-assembling peptide hydrogels stimulated chondrogenesis of seeded MSCs in vitro whereas biotin-streptavidin tethered TGF-β1 hydrogels did not [232]. The authors suggested that the biotin-streptavidin affinity may exceed the strength of some covalent bonds and that tethering TGF-β1 may have prevented internalization of the receptor-ligand complex [227, 228].

The release of growth factors can also be controlled through their innate affinity to certain ECM epitopes [229]. The use of an alginate-sulfate scaffold enhanced TGF-β1 attachment to the scaffold via heparin-like affinity interactions and resulted in a more sustained release of TGF-β1 over 7 days as opposed to a >90% burst release from regular alginate scaffolds after 1 day [230]. Implantation of similar scaffolds with affinity bound TGF-β1 and BMP-4 demonstrated the ability to induce endogenous regeneration of the osteochondral unit [231].
4.3.3. Microparticle and Nanoparticle Delivery

Another method of delivery is releasing the selected biologic from micro- or nano-sized carriers. MPs and nanoparticles (NPs) are attractive drug delivery vehicles due to their small dimension, high surface area to volume ratio, high drug loading efficiency, and the ability to quickly respond to environmental stimuli such as temperature, pH, magnetic fields, or ultrasounds [138, 233, 234]. While MPs have been investigated for several decades, NPs as delivery vehicles for cartilage repair have been gaining interest [235-239]. NPs can be endocytosed by cells, allowing for the accumulation of NP-encapsulated drugs, and their exceptionally high surface area to volume ratio enhances their affinity to therapeutic drugs and external stimuli [240]. However, a high surface area to volume ratio may also reduce the stability of the nano-sized delivery vehicle, and the tendency for certain NPs to aggregate into microscale particles may mitigate the advantages of a NP system [240].

MPs have traditionally been used as a delivery vehicle, either by dispersing the MPs in a continuous phase or using the MPs as building blocks without a surrounding matrix to form macroscopic scaffolds [138]. In particular, introducing MPs in a bulk scaffold, particularly hydrogels, adds a level of complexity and allows greater control over the growth factor release profile, spatial delivery, and leads to greater stability and bioactivity of encapsulated proteins [241, 242].

Similar to bulk phase delivery, biologics can also be incorporated in MPs and NPs through entrapment, ionic interactions, or a combination of both. Ultimately, the subsequent release depends on the composition of the carrier as well as the factor
incorporation method. One popular material for the construction of MPs is synthetic PLGA due to its tunable degradation into lactic and glycolic acid [243], its ease of fabrication, and its established safety in other FDA-approved applications [244]. Several research groups have employed PLGA MPs for the delivery of anabolic growth factors to stimulate chondrogenesis/cartilage repair either in a hydrogel [242, 245-247] or as a building block to make PLGA MP plugs [248-250]. By dispersing growth factor-delivering PLGA MPs in a hydrogel, near zero-order release kinetics of TGF-β1 and BMP-2 were observed in vivo [245, 247]. However, despite the appeal of PLGA microcarriers for controlled drug release, the use of such hydrophobic materials can adversely affect the bioactivity of encapsulated factors [235, 251]. Growth factor delivery particulates have also been fabricated with other synthetic polymers, including heparin-poly(L-lysine) NPs loaded with TGF-β3 [237], Pluronic F68/heparin NPs for TGF-β2 immobilization [252], and poly(N-isopropylacrylamide) NPs for TGF-β1 release [238]. Yet, different carrier matrices may be better suited for different biologics. Wang et al. demonstrated that PLGA MPs were better for releasing IGF-1 while silk MPs were more efficient in delivering BMP-2 [253].

Many natural polymers, notably gelatin [85, 103, 118, 234, 254-260], alginate [261], HA [261-264], chondroitin sulfate [236], and silk [253], have been used to create growth factor delivering MPs. Gelatin is a natural polymer derived from collagen, and by subjecting collagen precursors to either alkaline or acidic processing during the production of gelatin, basic or acidic gelatin with different isoelectric points can be obtained [254, 260]. Resultant gelatin carrier matrices can then achieve either a net negative or positive charge at physiological pH depending on the isoelectric point,
allowing for the ionic complexation of various growth factors during drug loading. Acidic gelatin MPs, which can be complexed with positively charged growth factors, were previously used for the controlled delivery of TGF-β1 [258, 259], TGF-β3 [256], IGF-1 [118], and BMP-2 [260] for cartilage and bone tissue engineering applications. However, since natural materials like gelatin and alginate are not native to articular cartilage, new ventures are exploring the use of more chondromimetic carriers with the aim of leveraging potential synergistic effects between growth factors and orthotopically relevant materials.

Ansboro and colleagues recently described a layer-by-layer approach for the fabrication of hollow HA microspheres as carriers of TGF-β3 [264]. In their work, the authors reported that the delivery of TGF-β3 from HA microspheres to human MSC pellets enhanced the expression of chondrogenic genes (type II collagen and aggrecan) while inhibiting the expression of hypertrophic markers (type X collagen) in vitro. Lim and colleagues reported the development of nanoscale and microscale particles using chondroitin sulfate, a negatively charged glycosaminoglycan (GAG) found in articular cartilage [236], where they were able to show the controlled release of TGF-β1 from these particles. An interesting study by Bajpayee and colleagues modeled the effect of NP charge and size on particle uptake and binding in articular cartilage [265]. Using Avidin, a highly glycosylated protein with a high positive charge and a diameter of ~7 nm, as a model protein for the development of NPs as drug carriers, the authors demonstrated that while particles less than 10 nm in diameter were able to penetrate through the full-thickness of bovine cartilage explants, particles that were 15 nm in diameter were confined only to the superficial cartilage layer. Of note, the presence of a positive fixed
charge density facilitated the rapid uptake of Avidin via electrostatic partitioning within 24 hrs when compared to neutrally charged NeutrAvidin and enhanced Avidin retention time to over 15 days [265]. Such results indicate that charge properties can be leveraged in innovative nanoparticle designs for the rapid uptake and retention of nanocarriers throughout the entire cartilage layer. Within the context of anti-inflammatory strategies for cartilage repair, the use MP carriers as the controlled delivery vehicles for small anti-inflammatory agents is also a subject of great interest [213, 235, 266-268].

4.4. Strategies for Controlling Delivery of Biologics

The plethora of studies on single growth factor delivery to induce chondrogenesis and/or facilitate neo-cartilage growth have been indispensible in showing the complexity of the cartilage wound healing environment. The current research on biologics for cartilage repair sees a trend towards multiple growth factor delivery to mimic the numerous signaling cascades involved. Yet the lack of an overwhelming improvement for multiple growth factor delivery over single growth factor delivery exemplifies the need to modulate the temporal expression and spatial distribution of biologics for not only cartilage regeneration, but for the repair of the osteochondral unit as a whole. The following sections will discuss the recent progress in the delivery of multiple biologics and spatiotemporally controlled delivery strategies for cartilage repair applications, and
will highlight several advanced options for the design of biologic delivery platforms with potential for precise spatial and/or temporal control over the release of bioactive factors.

4.4.1. Multiple Biologics Delivery

Musculoskeletal development is an intricate process governed precisely by the activation and interplay of numerous biochemical cell-signaling pathways. In particular, the process of chondrogenesis alone involves the activation of chondrogenic adhesion molecules (integrins) and the up-regulation of chondrogenic growth factors (TGF-β superfamily) and their signal regulators, followed by the up-regulation of anabolic factors (bFGF, IGF-1, VEGF) during hypertrophy [269]. While technically simple, it is extremely unlikely that the delivery of a single factor can stimulate the recapitulation of these signaling pathways for cartilage regeneration. As a result, strides have been made towards the development of controlled release systems for the delivery of multiple growth factors for cartilage repair in the hopes of increasing therapeutic potency. Since the work of Richardson and colleagues [270], which successfully demonstrated the controlled dual release of both VEGF and PDGF for the rapid formation of a mature vascular network, the field of controlled release has seen the advent of many sophisticated delivery systems for multiple bioactive agents. Indeed, by regulating the delivery of several bioactive factors, the induction of various biological responses in a fashion that promotes optimal cartilage repair becomes theoretically possible.

A common chondrogenic strategy involves the sequential release of TGF-β1 or TGF-β3 to first chondrogenically stimulate a synthetic response by host/delivered
progenitor cells, followed by the release of an anabolic or maturation factor like IGF-1 to encourage cartilage matrix production. Our laboratory, as well as others, has previously established that the dual delivery of TGF-β1 and IGF-1 can be achieved with distinct release kinetics [118, 271], and that the released growth factors stimulate the chondrogenic gene expression of MSCs in vitro [103]. In the case of bulk hydrogel and NP/MP composite scaffolds, varying the phase of incorporation can modulate the release kinetics of multiple growth factors. By loading TGF-β1 directly into the bulk OPF hydrogel phase while loading IGF-1 into highly crosslinked GMPs encapsulated within the OPF hydrogel, Holland and colleagues were able to attain a burst release of the former combined with the simultaneous but sustained release of the latter [118]. Another approach for the sequential delivery of TGF-β1 and IGF-1 involves the use of biodegradable PLGA MPs [272]. Jaklenec and colleagues fused IGF-1 containing and TGF-β1 containing PLGA microspheres using dichloromethane vapor in order to generate three-dimensional drug-eluting scaffolds [271]. Incorporating bovine serum albumin (BSA) into the organic phase during microsphere fabrication improved IGF-1 internalization into the microspheres and hence, delayed the release of IGF-1. The release of TGF-β1 was controlled by either capping or uncapping PLGA with a carboxylic acid chain, where uncapped PLGA led to delayed release of the growth factor due to increased secondary interactions between the PLGA, TGF-β1, and BSA [271]. The development of such a modular design allowed for the combination of PLGA microspheres containing various growth factors or bioactive agents in order to generate custom dual or multiple biologic delivery systems. Indeed, a similar system utilizing PLGA microspheres of
different co-polymer ratios and spherical sizes was able to achieve multiple burst releases of encapsulated chondroitin sulfate for potential applications in treating OA [273].

A more recent system leveraged a NP-laden hydrogel composite for the dual delivery of BMP-7 and TGF-β2. TGF-β2-immobilized NPs were created by first mixing Pluronic F68 with heparin to permit hydrogen bonding between the two components [252]. TGF-β2 was subsequently immobilized via ionic complexation with the heparin component. Chitosan and polyvinyl alcohol were then added to promote the formation of individual TGF-β2 immobilized NPs, which were embedded into a BMP-7 immobilized alginate hydrogel bulk phase. The authors were able to show fast release of BMP-7 (up to 80% cumulative release) coupled with the sustained release of TGF-β2 (up to 30% cumulative release) over 21 days *in vitro* [252]. Interestingly, the delivery of both BMP-7 and TGF-β2 from the hydrogel phase, as opposed to delivery from the encapsulated NPs, was slower due to the potential aggregation between growth factors during hydrogel fabrication, highlighting the need to examine the potential interplay between biologics in multiple growth factor delivery systems. Nevertheless, these systems coupling the delivery of BMPs with TGF-β are especially useful in applications involving MSCs derived from sources other than the bone marrow (i.e., adipose tissue), which require such growth factor combinations for chondrogenesis [274-277]. Despite our ability to tailor the release of multiple therapeutic agents in order to affect a desired cellular responses *in vitro*, one must consider the challenges of accurate preclinical translation *in vivo*. For instance, the dual delivery of TGF-β2 and BMP-7 to trochlear groove defects in rabbits, even with the co-implantation of adipose derived MSCs, failed to elicit any histological improvement in osteochondral tissue repair over controls [225].
Analogously, the co-delivery of TGF-β1 and IGF-1 via OPF/gelatin MP hydrogel composites did not offer any additional benefits over the delivery of IGF-1 alone for osteochondral tissue regeneration *in vivo* [257]. Recently, it was investigated whether the lack of additive or synergistic effects could be due to differences in TGF-β release kinetics between the *in vivo* environment and what was observed *in vitro* [171]. Using a similar OPF composite system, IGF-1 and TGF-β3 were co-delivered to an osteochondral defect site to evaluate tissue repair, where changing the loading phase of TGF-β3 varied its release kinetics. However, the results seemed to confirm a lack of synergy between these two growth factors in affecting a favorable healing response *in vivo* [171].

Bian and colleagues recently described an alternative strategy with high potential for cartilage repair [261]. Their approach entailed the initial transient exposure of MSCs encapsulated in a HA hydrogel to TGF-β3 released from co-encapsulated alginate microspheres to induce chondrogenesis, followed by the exposure to PTHrP to prevent hypertrophy. While the release of TGF-β3 induced the chondrogenesis of MSCs *in vivo* (in a subcutaneous mouse model), the uncontrolled and rapid delivery of PTHrP was unable to inhibit hypertrophic calcification [261]. Another strategy described the combined use of anti-angiogenic agent suramin and TGF-β1 for the generation of hyaline cartilage from periosteal cells on an agarose hydrogel implanted within a subperiosteal space [183]. Together, these studies highlight the complexity of the native joint environment and indicate that caution must be taken when combining multiple growth factors with similar chondrogenic stimulatory effects for controlled delivery *in vivo*. Indeed, it may prove advantageous to instead combine factors with varying anti-
inflammatory, anti-angiogenic, chondrogenic, or anabolic biological effects in order to simulate biomimetic cascades and processes for more effective cartilage repair *in vivo*.

Within the context of multiple growth factor delivery, an emerging field of interest comprises the application of PRP. PRP is an enriched blend of growth factors that can be autologously derived through the centrifugation of patient blood. Several protocols exist for the isolation and preparation of PRP for specific applications [143, 278]. In contrast to the rising potential of PRP for cartilage repair, information regarding its composition and mechanisms of action remain relatively scarce. Additionally, it remains unclear how different processing techniques and donor-to-donor variability affect the composition and effectiveness of PRP. Using protein antibody membrane arrays, Krüger and colleagues recently profiled the growth factor composition of human PRP and revealed a plethora of chondrogenic and anabolic growth factors including various BMPs, FGFs, PDGFs, IGFs, TGF-βs and VEGFs [279]. Indeed, several studies of late have reported the positive effects of PRP on cartilage repair in both diseased and acute defect models [280].

Sundman and coworkers treated osteoarthritic synovium and cartilage explants with PRP *ex vivo* and showed that PRP decreased the gene expression of inflammatory markers including TNF-α and MMP-13 while enhancing endogenous HA production [281]. Raeissadat and colleagues evaluated the effects of PRP injections on functional improvement and quality of life of OA patients in a clinical study and found that even the direct intra-articular administration of PRP ameliorated joint pain and knee stiffness, and improved patients’ quality of life in the studied time frame of 6 months [282], which corroborates the results from similar investigations [283-285]. However, the lack of
proper controls necessitates further investigations regarding the clinical use of PRP for OA. In an acute femoral defect model, the treatment of osteochondral grafts with PRP prior to implantation actually led to the improvement of graft-host integration when compared to grafts treated with saline solution [286], further indicating that PRP can decrease cartilage degeneration via the inhibition of inflammatory signals and the induction of neo-cartilage integration. Given the multifarious properties of PRP, future combinatory strategies can aim to leverage specific PRP effects through the co-delivery of synergistic or inhibitory factors. One potential strategy could explore the possibility of releasing the anti-angiogenic drug Avastin [287] following the delivery of PRP in order to promote a hypoxic environment, which has been shown to be vital for non-hypertrophic chondrogenesis [288] for articular cartilage repair.

### 4.4.2. Spatially Controlled Delivery

It is well known that the extracellular matrix structure of articular cartilage represents an intricate hierarchy of distinct layers that function together to meet the osmotic and viscoelastic demands of the tissue. While advances toward the utilization of multiple bioactive agents are beginning to address some of the complexities of cartilage regeneration *in vivo*, the presence of undefined and potentially negative cross-effects suggests that the method of growth factor presentation to defect sites still requires significant fine-tuning. Indeed, it is recognized that bioactive factors should ideally be delivered in a spatially and temporally controlled fashion in order to elicit maximum therapeutic efficacy. By first reviewing strategies for spatial control followed by
strategies for temporal control, this section primarily highlights recent advances made toward the development of technologies or platforms that can allow precise control over spatiotemporal release conditions for cartilage repair.

For cartilage applications, spatial control over drug delivery can be mainly achieved via two distinct approaches: the conditional/permanent immobilization of chondrogenic growth factors to desired regions within a scaffold, or the directional release of chondrogenic growth factors from a reservoir. Within the context of osteochondral tissue repair, growth factor concentration gradients and multiphasic scaffolds are often applied. Out of the strategies available for spatial control, the physical entrapment or chemical conjugation of factors to a scaffold represents the most technically simple approach. Such methods for protein sequestration commonly offer several advantages including localized growth factor presentation, dosage control, and preservation of protein bioactivity. McCall and colleagues recently investigated a strategy for TGF-β1 immobilization using clinically relevant materials [289]. TGF-β1 was first thiolated by a reaction with 2-iminothiolane (via primary amine groups on the N-terminus), and was subsequently functionalized into poly(ethylene glycol) (PEG)-diacrylate hydrogels using mixed-mode photopolymerization. By changing the initial concentration of thiolated TGF-β1 prior polymerization, it was shown that the growth factor dosage as well as bioactivity could be precisely controlled. Interestingly, co-encapsulated human MSCs, when exposed to the lower dosages of immobilized TGF-β1, actually exhibited equal or greater levels of chondrogenesis when compared to soluble TGF-β1 in culture medium [289].
Implications for the utility of spatially tethered chondrogenic growth factors to act as chondrogenic stimulants or chemoattractants for host progenitor cells in cartilage defects in vivo were further provided by Griffin and colleagues, who recently reported the synthesis of photodegradable macromers for the conjugation and release of biologics [290]. In their work, the authors synthesized a class of photosensitive ortho-nitrobenzyl (o-NB) macromers that could be functionalized with different reactive groups at the benzylic position. These reactive groups, which included alcohols, alkyl halides, amines, carboxylic acids, N-hydroxysuccinyl ester, and biotin, permitted the conjugation of essentially any type of therapeutic agent for cartilage repair. The mechanistic release of such therapeutics could then be externally controlled by light exposure. Additionally, it was previously reported by the same group that o-NB macromers could be designed with different photodegradation rates, hence yielding external control over the multistaged release of multiple bioactive factors [291]. As a proof of concept, Griffin and coworkers incorporated o-NB macromers that were conjugated with TGF-β1 into PEG hydrogels and demonstrated that photoreleased TGF-β1 maintained high bioactivity and was able to effectively induce the chondrogenic differentiation of human MSCs in vitro. The development of such platforms confers researchers with not only options for the precise external control over the spatial presentation of bioactive factors in potential strategies for cartilage repair, but also the temporal patterning of bioactive factor delivery.

While many systems have been designed for the spatially controlled delivery of growth factors to cartilage tissues as a whole, few studies if any have employed spatial gradients with high enough resolution to target the subtle inhomogeneity of the hierarchical articular cartilage makeup. However, as aforementioned, recent innovations
using Avidin as a model protein for articular cartilage drug uptake [265] indicate that the particle diameter and fixed charge density of drug-loaded NP systems could be finely tuned to affect NP uptake depth and retention time in order to target distinct zones within the cartilage layer. Currently, spatial concentration gradients usually consist of lower resolution biphasic or multiphasic systems built to stimulate the simultaneous repair of cartilage and subchondral bone in osteochondral composite tissues. In such cases, bilayered composites can easily be used to bias the local delivery of chondrogenic factors to the cartilage layer and osteogenic factors to the subchondral layer in osteochondral defects. As discussed earlier in the section describing the delivery of multiple biologics for cartilage repair, our laboratory has previously evaluated the utility of bilayered OPF composite systems for the spatially controlled delivery of chondrogenic factors IGF-1 and TGF-β1 or TGF-β3 only to the chondral regions of an osteochondral defect [171, 257].

In line with the need for high spatial resolution scaffolds and interfacial considerations between the cartilage and bone layers of osteochondral composite tissues, Wang and colleagues developed a BMP-2 and IGF-1 gradient biopolymer system with the ability to elicit the corresponding osteogenic and chondrogenic response of MSCs [253]. Using an aqueous-derived silk porous scaffold, silk microspheres loaded with BMP-2 or IGF-1 were differentially mixed together via a gradient maker to generate either single BMP-2/IGF-1 gradients or dual reverse gradients of both growth factors in a single scaffold. However, it was found that only silk scaffolds delivering BMP-2 gradients (i.e., single BMP-2 gradients or reverse BMP-2/IGF-1 gradients) were effective at promoting a gradient response [253], indicating that bioactive agents must be
compatible with the delivery platform. More recently, Dormer and coworkers described the creation of a PLGA microsphere-based bioactive plug with a continuous gradient transition between chondrogenic TGF-β1 and osteogenic BMP-2 for osteochondral tissue repair \textit{in vivo} [248]. When implanted into medial femoral defects in rabbits, it was shown that the presence of a reverse continuous gradient of chondrogenic and osteogenic growth factors led to improved histological repair over blank controls. While results highlight the potential of growth factor gradient design for osteochondral tissue regeneration, the lack of controls that test specifically the effect of the gradient on tissue repair necessitates future studies evaluating such an effect.

In contrast to growth factor gradient designs, Li and coworkers developed a unique spatial control strategy based on inhomogeneous bilayered collagen scaffolds that could bias the direction of growth factor release [292]. The described construct comprised a dense collagen layer and a loose collagen layer sandwiching a reservoir of chitosan-heparin NPs loaded with growth factors for the directional release of the anabolic factor bFGF [292]. bFGF was preferentially released through the loose collagen layer, thereby making the loose layer a better cell-adhesive and proliferative substrate with potential for tissue repair \textit{in vivo}. The same release of bFGF was not observed for the dense layer. When this bFGF release platform was utilized for articular cartilage repair in osteochondral defects \textit{in vivo}, the authors showed that the directional release of bFGF toward the subchondral bone (i.e., loose layer facing subchondral bone) stimulated the early up-regulation of endogenous TGF-βs, BMPs, and VEGFs as detected in the synovial fluid [293]. Additionally, the controlled and directional release of bFGF toward the subchondral bone in an osteochondral defect improved histological scores for
cartilage repair. Furthermore, such a spatially oriented approach limits the release of growth factors into the synovial space and hence, preserves the supply and longevity of growth factors for optimal therapeutic efficacy.

4.4.3. Temporally Controlled Delivery

Most, if not all, controlled release strategies enabling spatial control over the delivery of biologics also confer some degree of temporal control. Hence, the following will highlight several recent advances specifically emphasizing precise temporal control for the release of bioactive agents in the context of cartilage-related applications. Accordingly, one class of biomaterials that can offer improved temporal control over traditional delivery systems is self-assembling peptides. Kopesky and coworkers recently reported the slow and sustained release of TGF-β1 from acellular self-assembled peptide formed from AcN-(KLDL)₃-CNḤ₂ custom peptide sequences [232]. Such KLD peptides were able to efficiently uptake growth factors, which could be loaded into the equilibrium peptide solution before or after self-assembly, with five times greater uptake before self-assembly. This provided the ability to precisely modulate the dosage of growth factors to be released. It was shown that by day 3, only 18% percent of the total TGF-β1 loaded was released, indicating the avoidance of a burst release [232]. By day 21, only 44% of cumulative TGF-β1 was achieved, suggesting the utility of KLD hydrogels for long-term controlled growth factor release and for avoiding the dosage limitations of other single growth factor delivery systems. Furthermore, KLD hydrogels could theoretically maintain growth factors in their bioactive macromolecular form efficiently through
designed electrostatic interactions during peptide sequencing. Despite the advantages of such long-term release strategies, the efficacy of delivered peptides and proteins commonly face issues related to short half-lives. To address this problem, Ashley and colleagues developed a generic drug delivery platform based on a tetra-PEG porous hydrogel incorporating β-eliminative linkers that could undergo β-eliminative cleavage in the presence of an electron withdrawing modulator for the release of covalently tethered drugs [294]. The β-eliminative linkers, which were not prone to enzymatic degradation, could be designed to have highly predictable half-lives and could be used to simultaneously conjugate bioactive factors and crosslink PEG hydrogels. The covalent fixation of therapeutics to and their subsequent release from the β-eliminative linkers could then be directly controlled by the half-life of the linker, which can be designed to range from a few hours to over a year [294]. Hence, by combining β-eliminative linkers with short half-lives for drug tethering and those with longer half-lives for hydrogel crosslinking, one can ensure the complete release of growth factors prior to hydrogel degradation. Such a strategy for osteochondral defect repair would allow one to take full biomechanical advantage of having hydrogel structural support while mitigating any form of release kinetics limitations associated with bulk material degradation. Additionally, the ability to fine-tune the temporal release of biologics and to potentially decouple spatial and temporal modulation should inspire future innovations toward the design of growth factor delivery patterns that mimic the cartilage signaling cascades during fetal development in order to stimulate robust cartilage regeneration. Table 4.2 lists various strategies that have leveraged the use of spatial and/or temporal control for the delivery of biologics for cartilage repair applications.
Other approaches toward achieving temporal control over growth factor presentation typically involve more indirect means via the use of gene therapy. Indeed, many pharmacotherapy strategies that currently exist in the literature describe the use of gene therapy, or the viral/non-viral conditioning of cells into endogenous growth factor depots, for cartilage and osteochondral repair. However, it is not clearly known how the effectiveness of such strategies compares with those that rely on the controlled delivery of exogenous factors. Using bovine articular chondrocytes, Shi and coworkers compared the effectiveness of exogenously and endogenously delivered IGF-1 and TGF-β1 for *in vitro* chondrogenesis [295]. The authors showed no difference between exogenously or endogenously delivered IGF-1, but found that exogenously delivered TGF-β1 elicited greater chondrogenic gene expression when compared to endogenously delivered TGF-β1. This was confirmed to be due to the non-covalent complexation of a latency-associated peptide with TGF-β1 during endogenous production to form a small latent complex, which was then bound by latent TGF-β1 binding protein to form a large latent complex [295]. While this preserved growth factor bioactivity, the complex was shielded from TGF-β1 receptors on the chondrocyte surfaces. These results suggest that while the endogenous delivery of certain bioactive factors via gene therapy may prove beneficial for cartilage repair, not all factors are suitable with this form of delivery. The current state of gene therapy as a means for temporally controlled chondrogenic growth factor delivery is aptly reflected by the work of Lu and colleagues, who recently reported the utility of a chitosan-based gene-activated matrix encapsulating chitosan/HA NPs carrying plasmids for the prolonged delivery of plasmid genes for transfection [296]. In their application, the authors leveraged a hybrid NP system for the delivery of plasmids
encoding TGF-β1, where the presence of HA theoretically improved the transfection efficiency and also provided a substrate with which cells could interact. It was shown that the release of plasmids was sustained for over 120 days \textit{in vitro}, and that the released plasmids stimulated the proliferation of seeded chondrocytes. These results demonstrate that the main advantage of sustained gene therapy when compared to conventional exogenous delivery is the ability to control the timely administration of growth factors in an endogenously relevant fashion, therefore highlighting the potential of such strategies for effective cartilage repair.

\textbf{4.5. Conclusion}

Despite its perceived simplicity, the consistent repair of articular cartilage still remains a significant clinical challenge. However, the advent of innovative tissue engineering technologies is beginning to address many of the major shortcomings of current clinical approaches. Specifically, tissue engineering approaches leveraging the use of release platforms that offer spatiotemporal control over the delivery of bioactive factors are eliciting favorable outcomes \textit{in vitro} and \textit{in vivo}. In a relatively short time span, release systems for the delivery of biologics have evolved from simple intra-articular modalities into complex multifunctional and modular delivery platforms. While such systems have generally employed the benefits of chondrogenic growth factors in cartilage repair applications, new approaches are now finding utility in the use of
biologics with primary effects on progenitor cells other than chondrogenic or anabolic stimulation. As highlighted, therapeutic treatment of cartilage repair could also be garnered from the delivery of various anti-inflammatory, anti-angiogenic, or chondroprotective agents. The delivery of multiple factors simultaneously or in a spatiotemporally designed fashion for cartilage repair applications, as evidenced by the use of biphasic delivery scaffolds or growth factor gradients, is also becoming a popular area of research. Yet, as new findings show, the delivery of more factors may not necessarily elicit additive or synergistic effects in cartilage regeneration. Additionally, studies are now beginning to show that certain bioactive factors may only be compatible with certain delivery platforms. Hence, future efforts should aim to identify these key biologic-to-biologic and biologic-to-platform interactions as such information will be critical to the success of future strategies leveraging the delivery of various biologics for cartilage repair.
### 4.6. Tables and Figures

Table 4.1 Biologics delivered for *in vitro* and *in vivo* cartilage repair applications

<table>
<thead>
<tr>
<th>Biologics</th>
<th>Biological Effect</th>
<th>Biologic Type</th>
<th><em>In vitro</em>/<em>In vivo</em> Model</th>
<th>Comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTHrP</td>
<td>Anti-Hypertrophy</td>
<td>Growth Factor</td>
<td><em>In vitro</em> cultures; <em>In vivo</em> OC defect model</td>
<td>Inhibited hypertrophy of chondrocytes and MSCs during differentiation <em>in vitro</em>; Reduced, but did not prevent, calcification <em>in vivo</em></td>
<td>[261]</td>
</tr>
<tr>
<td>S-(-)-ibuprofen</td>
<td>Anti-inflammatory</td>
<td>Small Molecule</td>
<td><em>Ex vivo</em> OA model</td>
<td>Reduced prostaglandins synthesis at 50 µM. When released from PLGA-PEG microspheres, reduced cartilage degradation at 1 mM <em>ex vivo</em></td>
<td>[209, 266]</td>
</tr>
<tr>
<td>Sulforaphane</td>
<td>Anti-inflammatory</td>
<td>Small Molecule</td>
<td><em>In vitro</em> cultures; <em>In vivo</em> OA model</td>
<td>Inhibited inflammatory markers including COX-2, ADAMTS-5, and MMP-2 <em>in vitro</em>; Delayed progression of OA <em>in vivo</em></td>
<td>[213]</td>
</tr>
<tr>
<td>IL-1Ra</td>
<td>Anti-inflammatory</td>
<td>Antigen</td>
<td><em>In vitro</em> cultures; <em>In vivo</em> OA model</td>
<td>Bound to IL-1 surface receptors of synoviocytes <em>in vitro</em>; Retained in joint without inducing degenerative changes <em>in vivo</em>; Specific chondroprotective effects need to be evaluated</td>
<td>[235]</td>
</tr>
<tr>
<td>3,4,6-O-Bu3GlcNAc</td>
<td>Anti-inflammatory/Chondroprotective</td>
<td>Small Molecule</td>
<td><em>In vitro</em> cultures</td>
<td>Decreased the expression of IL-1β-stimulated NFkB target genes and increased GAG production and chondrogenic gene expression of IL-1β challenged chondrocytes</td>
<td>[297]</td>
</tr>
<tr>
<td>SDF-1</td>
<td>Cell Homing</td>
<td>Chemokine</td>
<td><em>In vitro</em> cultures; <em>In vivo</em> OC model and intraperitoneal cell migration model</td>
<td>Did not influence proliferation/chondrogenesis of MSCs in OC defect but resulted in ectopic cartilage formation when delivered with TGF-β1 at 4 weeks <em>in vivo</em></td>
<td>[188, 298]</td>
</tr>
<tr>
<td>PRP</td>
<td>Chondrogenic/Anabolic/Anti-inflammatory</td>
<td>Growth Factor Cocktail</td>
<td>In vitro and ex vivo cultures; In vivo OA and OC defect models</td>
<td>Decreased the expression of inflammatory markers including TNF-α and MMP-13 and enhanced endogenous HA production ex vivo; OC graft pre-treatment with PRP enhanced graft-host integration in vivo</td>
<td>[281, 286]</td>
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<tr>
<td>HA</td>
<td>Chondroprotective</td>
<td>Viscosupplement</td>
<td>In vitro cultures; In vivo OA models</td>
<td>High molecular weight HA resulted in improved histological scores and lower cartilage friction coefficients when compared to low molecular weight HA in vivo</td>
<td>[198]</td>
</tr>
<tr>
<td>Proteoglycan 4</td>
<td>Chondroprotective</td>
<td>Viscosupplement</td>
<td>In vitro cultures</td>
<td>Supplementation of OA synovial fluid with proteoglycan 4 restored lubricating ability by reducing friction coefficient in cartilage-on-cartilage tests</td>
<td>[299]</td>
</tr>
<tr>
<td>Prostaglandins E2</td>
<td>Inflammatory/Anabolic</td>
<td>Small Molecule</td>
<td>In vitro cultures</td>
<td>Low concentrations (10^{-9} M to 10^{-6} M) stimulated increased chondrogenic gene expression of articular chondrocytes in 3D</td>
<td>[210]</td>
</tr>
</tbody>
</table>
Table 4.2 Selected spatiotemporally controlled delivery strategies for *in vitro* and *in vivo* cartilage repair applications

<table>
<thead>
<tr>
<th>Biologics Delivered</th>
<th>Delivery Platform</th>
<th><em>In vitro</em>/<em>In vivo</em> Model</th>
<th>Spatial Strategy</th>
<th>Temporal Strategy</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β1, IGF-1</td>
<td>Gelatin microparticles encapsulated in bilayered OPF hydrogels</td>
<td>Rabbit femoral medial condyle osteochondral defect</td>
<td>Growth factors delivered from the chondral layer of bilayered OPF construct</td>
<td>Burst release of TGF-β1 with sustained release of IGF-1</td>
<td>[107]</td>
</tr>
<tr>
<td>TGF-β3, IGF-1</td>
<td>Gelatin microparticles encapsulated in bilayered OPF hydrogels</td>
<td>Rabbit femoral medial condyle osteochondral defect</td>
<td>Growth factors delivered from chondral layer of bilayered OPF construct</td>
<td>Burst release or sustained release of TGF-β3 with sustained release of IGF-1</td>
<td>[171]</td>
</tr>
<tr>
<td>TGF-β1, IGF-1</td>
<td>PLGA microsphere-fused 3D scaffold</td>
<td><em>In vitro</em></td>
<td>N.A.</td>
<td>Burst or sustained release of TGF-β1; Burst or sustained release of IGF-1</td>
<td>[271]</td>
</tr>
<tr>
<td>BMP-7, TGF-β2</td>
<td>Pluronic F68-heparin-chitosan nanoparticles encapsulated in alginate hydrogel</td>
<td>Rabbit trochlear groove osteochondral defect</td>
<td>N.A.</td>
<td>Burst release of BMP-2 with sustained release of TGF-β2</td>
<td>[252]</td>
</tr>
<tr>
<td>TGF-β3, PTHrP</td>
<td>Alginate microspheres encapsulated in HA hydrogel</td>
<td>Mouse subcutaneous model</td>
<td>N.A.</td>
<td>Burst release of TGF-β3 with simultaneous release of PTHrP</td>
<td>[261]</td>
</tr>
<tr>
<td>BMP-2, IGF-1</td>
<td>Silk microspheres incorporated into aqueous-derived silk porous scaffold</td>
<td><em>In vitro</em></td>
<td>Single BMP-2 gradient; Single IGF-1 gradient; Continuous BMP-2/IGF-1 transitional reverse gradient</td>
<td>N.A.</td>
<td>[253]</td>
</tr>
<tr>
<td>BMP-2, TGF-β1</td>
<td>PLGA microsphere-based bioactive plug</td>
<td>Rabbit femoral condyle osteochondral defect model</td>
<td>Continuous BMP-2/TGF-β1 transitional reverse gradient</td>
<td>N.A.</td>
<td>[248]</td>
</tr>
<tr>
<td>Growth Factor/Technique</td>
<td>Matrix/Release Pattern</td>
<td>Model</td>
<td>Release Pattern</td>
<td>Reference(s)</td>
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<tr>
<td>bFGF</td>
<td>Chitosan-heparin nanoparticles in loose/dense bilayered collagen scaffold</td>
<td>Rabbit trochlear groove osteochondral defect model</td>
<td>Directional bFGF release via the loose collagen layer - Compared release toward or away from subchondral bone</td>
<td>[292, 293]</td>
<td></td>
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<tr>
<td>BMP-2, microfracture</td>
<td>Heparin-conjugated fibrin (long-term delivery) or fibrin (short-term delivery)</td>
<td>Rabbit trochlear groove osteochondral defect model</td>
<td>Burst or sustained release of BMP-2 with bone marrow exposure</td>
<td>[161]</td>
<td></td>
</tr>
<tr>
<td>TGF-β1, BMP-4</td>
<td>Affinity binding bilayered alginate sulfate</td>
<td>Rabbit trochlear groove osteochondral defect model</td>
<td>TGF-β1 affinity-bound into chondral layer; BMP-4 affinity-bound into subchondral layer</td>
<td>[230]</td>
<td></td>
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<tr>
<td>BMP-2 or TGF-β1</td>
<td>PLGA microspheres encapsulated in alginate matrix overlaid on porous PLGA cylinder</td>
<td>Rabbit trochlear groove osteochondral defect model</td>
<td>Burst release followed by sustained release of TGF-β1 or BMP-2</td>
<td>[160]</td>
<td></td>
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</table>
Osteochondral tissue regeneration using a bilayered composite hydrogel with modulating dual growth factor release kinetics in a rabbit model* ,^ 

Biodegradable oligo(poly(ethylene glycol) fumarate) (OPF) composite hydrogels have been investigated for the delivery of growth factors (GFs) with the aid of gelatin microparticles (GMPs) and stem cell populations for osteochondral tissue regeneration. In this study, a bilayered OPF composite hydrogel that mimics the distinctive hierarchical structure of native osteochondral tissue was utilized to investigate the effect of

^ The candidate was involved in designing and executing the in vitro swelling and release kinetics studies, the design and animal surgery of the in vivo study, and writing and editing of the manuscript.
transforming growth factor-β3 (TGF-β3) with varying release kinetics and/or insulin-like growth factor-1 (IGF-1) on osteochondral tissue regeneration in a rabbit full-thickness osteochondral defect model. The four groups investigated included (i) a blank control (no GFs), (ii) GMP-loaded IGF-1 alone, (iii) GMP-loaded IGF-1 and gel-loaded TGF-β3, and (iv) GMP-loaded IGF-1 and GMP-loaded TGF-β3 in OPF composite hydrogels. The results of an *in vitro* release study demonstrated that TGF-β3 release kinetics could be modulated by the GF incorporation method. At 12 weeks post-implantation, the quality of tissue repair in both chondral and subchondral layers was analyzed based on quantitative histological scoring. All groups incorporating GFs resulted in a significant improvement in cartilage morphology compared to the control. Single delivery of IGF-1 showed higher scores in subchondral bone morphology as well as chondrocyte and glycosaminoglycan amount in adjacent cartilage tissue when compared to a dual delivery of IGF-1 and TGF-β3, independent of the TGF-β3 release kinetics. The results suggest that although the dual delivery of TGF-β3 and IGF-1 may not synergistically enhance the quality of engineered tissue, the delivery of IGF-1 alone from bilayered composite hydrogels positively affects osteochondral tissue repair and holds promise for osteochondral tissue engineering applications.
5.1. Introduction

Articular cartilage is a flexible connective tissue that facilitates the articulation of bone in major synovial joints via the dissipation of friction and physiological compressive forces [7, 15, 300-302]. With a limited endogenous ability for self-repair, damaged cartilage as a result of disease or trauma oftentimes leads to premature arthritis. Although current clinical methods are insufficient for long-term treatment [303], tissue engineering strategies provide promising alternatives for cartilage repair. To date, many research groups have adapted a wide variety of natural or synthetic polymers for the fabrication of scaffolds for cartilage tissue engineering. In particular, hydrogel scaffolds derived from these materials can be used as a vehicle to deliver biochemical factors that stimulate the chondrogenic differentiation of host progenitor cells within a tissue defect site [3, 304]. Our laboratory has developed a novel class of water-soluble synthetic macromers based on oligo(poly(ethylene glycol) fumarate) (OPF) that can be chemically crosslinked to yield hydrolytically degradable hydrogels. Injectable and biodegradable hydrogels formed from OPF have been leveraged for the controlled delivery of chondrogenic growth factors (GFs) with the aid of gelatin microparticles (GMPs), which serve as GF delivery vehicles and enzymatically digestible porogens [88, 89, 101-105, 118]. Previously, such composite hydrogel systems have been utilized to deliver chondrogenic GFs for the elicitation of osteochondral tissue repair within osteochondral defect sites in animal models [106-108]. However, the simultaneous delivery of multiple GFs and how these GFs interact *in vivo* to repair osteochondral tissue remains an area of investigation.
In the present work, OPF composite hydrogels are used to deliver transforming growth factor-β3 (TGF-β3) and/or insulin-like growth factor-1 (IGF-1) to an osteochondral defect to facilitate cartilage regeneration and subchondral tissue formation. TGF-β3 is a potent GF that can induce the chondrogenic differentiation of progenitor cell populations \textit{in vitro} \cite{305-308} as well as augment cartilage tissue formation \textit{in vivo} \cite{152, 261, 309, 310}. IGF-1 primarily acts as an anabolic maturation factor to stimulate the cellular synthesis of proteoglycans and type II collagen \cite{36, 311}. Previously, OPF composite systems were employed to deliver TGF-β1, an isoform of TGF-β3 with similar chondrogenic effects, to the chondral space of an osteochondral defect \cite{106}. Although the presence of TGF-β1 alone did confer some therapeutic advantage, such as the improvement of joint surface regularity over controls at 4 and 14 weeks, the GF did not effect a different overall healing response when compared to controls \cite{106}. To achieve an overall improvement in osteochondral regeneration and to study the effects of a dual GF delivery system in a wound healing environment, the delivery of TGF-β1 with IGF-1 on osteochondral tissue repair was evaluated \cite{107}. This study was based on results in literature demonstrating the synergistic effects of TGF-β1 with IGF-1 on increased chondrogenic gene expression and proteoglycan synthesis by articular chondrocytes \cite{312, 313} and mesenchymal stem cells (MSCs) \textit{in vitro} \cite{314}. The \textit{in vivo} study however, showed that an initial burst release of TGF-β1 during early stages of cartilage healing followed by a sustained release of IGF-1 was not effective at regenerating osteochondral tissue under the conditions investigated \cite{107}. Despite the lack of synergy between TGF-β1 and IGF-1, the controlled delivery of multiple GFs merits further investigation when one considers the complex interplay of GFs during different stages chondrogenesis and
cartilage regeneration [37, 39, 315]. Indeed, several *in vitro* studies have demonstrated that the anabolic effects of TGF-β isoforms on progenitor cells are dependent on the time of delivery [316, 317]. Recently, it was shown that the sequential exposure of umbilical cord-derived MSCs to TGF-β3 followed by IGF-1 enhanced their production of cartilage-like extracellular matrix (ECM) components *in vitro* [318]. Moreover, our laboratory has found that the TGF-β3 isoform was more effective than TGF-β1 at inducing the chondrogenic differentiation of rabbit MSCs encapsulated within bilayered OPF composite hydrogels [104]. Therefore, we hypothesize that the delivery of TGF-β3, when combined with the sustained release of IGF-1, may influence the differentiation of host progenitor cell populations and thereby affect the degree of osteochondral tissue repair.

It has been demonstrated that release kinetics of GFs from OPF composite hydrogels can be modulated by hydrogel construction parameters, which include the degree of crosslinking of GMPs, the molecular weight of the poly(ethylene glycol) block in the OPF macromer, the resultant mesh size of such crosslinked OPF hydrogels, and the method of GF incorporation within the composite hydrogel [88, 106, 107, 118]. Such a level of tunability with OPF composite hydrogels may be leveraged for the timed delivery of multiple chondrogenic GFs to progenitor cell populations in the host tissues to repair an osteochondral defect. Indeed, the release kinetics of TGF-β3 when delivered together with IGF-1 from OPF composite hydrogels might be an important parameter to regulate for the induction of high quality cartilage tissue remodeling and regeneration *in vivo*. The present study investigates how different release kinetics of TGF-β3, in the presence of IGF-1, from OPF composite hydrogels affect the tissue response in an osteochondral defect.
The global hypothesis of this study is that the release of TGF-β3, when combined with the sustained release of IGF-1, from OPF composite hydrogels will synergistically facilitate osteochondral tissue regeneration in a rabbit osteochondral defect model relative to the delivery of IGF-1 alone. Furthermore, it is hypothesized that the release kinetics of TGF-β3, when delivered together with a sustained release of IGF-1, will influence the degree and quality of osteochondral tissue repair. To this end, the specific objectives of this study are: (1) to characterize the in vitro release kinetics of IGF-1 and TGF-β3 from composite hydrogels over 28 days, (2) to investigate the synergistic effect of a dual delivery of TGF-β3 with IGF-1 on osteochondral tissue regeneration, and (3) to evaluate the effect of modulated TGF-β3 release kinetics with sustained IGF-1 release on the quality of cartilage and subchondral bone tissue regeneration in a rabbit full-thickness osteochondral defect model.

5.2. Materials and Methods

5.2.1. OPF synthesis

According to a method developed in our laboratory [59, 85], poly(ethylene glycol) with a number average molecular weight (M_n) of 35,000 (Sigma-Aldrich, St. Louis, MO) was utilized to synthesize OPF. The resulting OPF with an M_n of 42,500 ± 180 and a weight average molecular weight (M_w) of 120,300 ± 6,500 was used
throughout the study. Prior to hydrogel fabrication, OPF was sterilized by exposure to ethylene oxide (EO) for 12 hrs according to established methods [102, 103, 108].

5.2.2. Gelatin microparticle fabrication

Gelatin microparticles (GMPs) were fabricated using acidic gelatin with an isoelectric point of 5.0 (Nitta Gelatin INC., Osaka, Japan) and were crosslinked with 10 or 40 mM glutaraldehyde (Sigma-Aldrich, St. Louis, MO) according to previously established methods [118]. After lyophilization, GMPs with a diameter of 50 – 100 µm were selected by sieving and sterilized with EO prior to hydrogel fabrication. Sterile GMPs were swollen with phosphate buffered saline (PBS) or GF solutions at 4°C for 15 hrs, according to previously described methods [88]. For equilibrium swelling of GMPs, 55 µL of PBS or GF solution was applied to 11 mg of dried GMPs. For the degradation study, blank GMPs were swollen with PBS alone. For the in vitro release study and in vivo implantation, 40 mM GMPs were loaded with an IGF-1 solution with a concentration of 24.42 µg of human recombinant IGF-1 (R&D Systems, Minneapolis, MN) per mL PBS to achieve a concentration of 2000 ng IGF-1 per mL crosslinked OPF composite hydrogel prior to equilibrium swelling. Similarly, 10 mM GMPs were loaded with a TGF-β3 solution with a concentration of 21.97 µg of human recombinant TGF-β3 (R&D Systems) per mL PBS to achieve 1800 ng TGF-β3 per mL crosslinked OPF composite hydrogel prior to equilibrium swelling. 10 and 40 mM GMPs, which have different degradation rates, were selected to deliver dual GFs in a fashion that first induces chondrogenic differentiation of progenitor cells by TGF-β3, followed by IGF-1
induced stimulation of the ECM production [107]. Furthermore, a 2.5 times higher concentration for both IGF-1 and TGF-β3 solutions was used in the preparation of additional groups for the in vitro release study.

5.2.3. Bilayered composite hydrogel fabrication

Bilayered composite hydrogels to deliver GFs were fabricated via a two-step crosslinking procedure as previously described [104, 105, 107]. The subchondral layer was partially crosslinked first and the chondral layer was fabricated on top of the subchondral layer. Specifically, 100 mg of sterile OPF and 50 mg of sterile poly(ethylene glycol) diacrylate (PEG-DA, Glycosan, Alameda, CA) with a molecular weight of 3400 were dissolved in 468 µL PBS and mixed with 110 µL of blank GMPs swollen with PBS (GMP/PBS). Equal parts (46.8 µL) of the thermal radical initiators, 0.3 M of ammonium persulfate (APS, Sigma-Aldrich) and 0.3 M of N,N,N’,N’-tetramethylethylenediamine (TEMED, Sigma-Aldrich), were then mixed into the polymer solution. The polymeric mixture was quickly injected into a cylindrical Teflon mold (2.2 mm in diameter and 2.2 mm in thickness) and partially crosslinked at 37°C for 4 min. Subsequently, the polymer-GMP mixture for the chondral layer was prepared. 100 mg of sterile OPF and 50 mg of sterile PEG-DA were dissolved in 468 µL PBS and mixed with 110 µL of blank GMP solution swollen with PBS for the control group, 55 µL of GMP/PBS and 55 µL of GMP solution swollen with IGF-1 (GMP/IGF-1) for Group 1, and 55 µL of GMP/PBS and 55 µL of GMP solution swollen with TGF-β3 (GMP/TGF-β3) for Group 3 (Table 5.1). For Group 2 (Gel phase loading), the same amount of OPF/PEG-DA was dissolved.
in 413 µL PBS, mixed with 55 µL of TGF-β3 solution, and subsequently mixed with 46.8 µL of 0.3 M of APS and 46.8 µL of 0.3 M of TEMED. The mixture for the chondral layer was then injected onto the partially crosslinked subchondral layer, and crosslinked at 37°C for 8 min.

5.2.4. Degradation

For the swelling and degradation studies, two different sizes of bilayered composite hydrogels containing only GMP/PBS for both chondral and subchondral layers were fabricated as described above. The composite hydrogels were placed in either 3 mL of PBS or collagenase-PBS (370 ng collagenase 1A per mL PBS) in a 12 well plate, and incubated at 37°C for 28 days on a shaker table (70 RPM). At days 1, 4, 7, 14, 21, and 28, the swelling ratio, sol fraction, and mass remaining for OPF hydrogel composites were determined (n=4) using the following equations; swelling ratio = \((W_s - W_d) / W_d\) %, sol fraction = \((W_i - W_d) / W_i \times 100\) (%), and mass remaining = \(W_d / W_i \times 100\) (%), where \(W_i\), \(W_s\), and \(W_d\) represent the weight of dried hydrogel immediately after fabrication prior to swelling, the weight of wet hydrogel after swelling at each time point, and the weight of dried hydrogel after swelling at each time point, respectively.

5.2.5. Quantification of in vitro release kinetics

Release kinetics of the total amount of GFs from bilayered composite hydrogels was assessed by measuring the radioactivity of \(^{125}\text{I}\)-labeled GFs [106, 118]. Briefly, \(^{125}\text{I}-\)
labeled IGF-1 and TGF-β3 (Perkin Elmer Life Sciences, Boston, MA) were incorporated with unlabeled GFs (Peprotech, Rocky Hill, NJ) for GMP swelling. 3% of the required GF solution volume for GMP swelling was replaced with $^{125}$I-labeled GF solution of the same concentration. Bilayered OPF composite hydrogels were fabricated as described above. For the IGF-1 release study, 40 mM GMPs were loaded with $^{125}$I-labeled IGF-1 and unlabeled IGF-1 for the hydrogels in all groups, while unlabeled TGF-β3 was used for hydrogel samples in Group 2 (Gel phase loading) and Group 3 (GMP loading) (Table 5.1). For the TGF-β3 release study, 10 mM GMPs were loaded with $^{125}$I-labeled TGF-β3 and unlabeled TGF-β3 in the presence of unlabeled IGF-1 was used for the samples in Group 2 (Gel phase loading) and Group 3 (GMP loading). In addition, a 2.5 times higher GF amount (i.e., 2.5 times higher concentration of GF solution with same volume for GMP swelling or gel phase loading) was loaded in hydrogels to investigate the effect of GF concentration on release kinetics. After fabrication, hydrogels were placed in 3 mL of PBS or collagenase-PBS in a 12 well plate, and incubated at 37°C for 28 days on a shaker table (70 RPM). At 2 hrs, 12 hrs, days 1, 2, 4, 7, 10, 14, 18, 21, 24, and 28, the supernatant of each hydrogel was collected and replaced with fresh buffer solution. The amount of released GF was determined by the correlation of measured radioactivity to a standard curve using a gamma counter (Cobra II Autogamma, Packard, Meridian, CT) (n=5).
5.2.6. Bioactivity of released growth factors

Release kinetics of biologically active GFs were assessed by cellular assays, as described in previous studies [271, 319]. Bilayered composite hydrogels 3 mm in diameter and 3 mm in thickness were utilized. For the IGF-1 activity assay, hydrogels in Group 1 were fabricated as described above. Hydrogels delivering both IGF-1 and TGF-β3 were not examined with the cellular assays, as the presence of TGF-β3 may confound the results. Four hydrogels were placed in 1 mL of collagenase-PBS in a 12 well plate, and incubated at 37°C for 28 days on a shaker table (70 RPM). At 2 hrs, 12 hrs, days 1, 2, 4, 7, 10, 14, 18, 21, 24, and 28, the supernatant of each hydrogel was collected and replaced with fresh buffer solution. Collected supernatants were stored at -80°C until the assay time. A MCF-7 cell line proliferation assay was used to measure the biologically active fraction of released IGF-1 from the composite hydrogels [271]. 5,000 MCF-7 cells (HBT-22, ATCC, Manassas, VA) in serum-free DMEM:F12 medium (Life Technologies, Grand Island, NY) were plated in a 96 well plate. 25 µL of collected supernatant or 25 µL of an IGF-1 standard (0.15 – 20 ng/mL range) were then added. The cells were cultured in an incubator (37°C, 5% CO₂) for 72 hrs. Proliferative activity of plated cells was measured by a CellTiter Blue assay (Promega, Madison, WI) according to the manufacturer’s protocol. Fluorescence intensity was measured using a microplate reader (excitation 560 nm/emission 590 nm). A standard curve was generated by a 4 parameter fit and the active amount of released IGF-1 was calculated at each time point (n=4) [271]. The cumulative % active release was plotted based on the released amount of IGF-1 as measured with ¹²⁵I-labeled GFs in the “Quantification of in vitro release kinetics” section. For the TGF-β3 activity assay, composite hydrogels in Group 2 and Group 3 were
fabricated as described above. Similarly, IGF-1 was not included in these composite hydrogels as its presence may confound the results of the TGF-β3 activity assay. Three gels in each group were placed in 1 mL of collagenase-PBS in a 12 well plate, and incubated at 37°C for 28 days on a shaker table (70 RPM). Sample collection and storage were the same as described above. A Mink lung epithelial cell line (CCL-64, ATCC, Manassas, VA) inhibition assay was used to measure the biologically active fraction of released TGF-β3 from OPF composites [319]. 10,000 CCL-64 cells (HBT-22, ATCC, Manassas, VA) in DMEM media (Life Technologies, Grand Island, NY) were plated in a 96 well plate. 25 µL of collected supernatant or 25 µL of a TGF-β3 standard (0.005 – 5 ng/mL range) were then added. The cells were cultured in an incubator (37°C, 5% CO₂) for 72 hrs. The level of inhibition, the active amount of released TGF-β3, and the cumulative % release of active TGF-β3 were analyzed as described above.

5.2.7. Animal surgery

A total of 24 skeletally mature (i.e., 6 months old) male New Zealand white rabbits were utilized in this study based on a previously established full-thickness osteochondral defect model [106-108]. The number of defects and animals per each group were determined by power analysis and consideration of previous studies [106-108]. All surgical procedures were approved by the Institutional Animal Care and Use Committees of Rice University and University of Texas Health Science Center at Houston. Animal surgeries for implantation in bilateral defects were performed as previously described [106-108]. Prior to surgery, anesthesia was induced by
subcutaneous injection of Ketamine (25-40 mg/kg) and Acepromazine (1-2 mg/kg). General anesthesia was then maintained through ventilator administration of a mixture of isoflurane and oxygen. Osteochondral defects (3 mm in diameter and 3 mm in thickness) were created on the medial femoral condyles. Pre-fabricated bilayered composite hydrogels were swollen in sterile PBS for 30 minutes and these swollen composite hydrogels with the same dimensions as the defect were press-fitted into the osteochondral defect. Then, the muscle and skin were closed. This procedure was repeated for the contralateral knee using a hydrogel of the same formulation. Therefore, each animal received two hydrogels (one per knee) and a total of 12 hydrogels (n=12) were implanted in 6 animals per experimental group. To minimize post-operative discomfort, Carprofen (4 mg/kg) was administrated for 2 days post-operatively.

5.2.8. Tissue Processing

Rabbits were euthanized by intravenous administration of Beuthanasia (0.22 mL/kg) at 12 weeks post-surgery. The tissue surrounding the medial femoral condyle was retrieved en bloc, fixed in 10% buffered formalin (pH 7.4) for 1 week, decalcified in EDTA solution for 6 weeks, dehydrated through a graded series of ethanol baths, and then embedded in paraffin. Longitudinal sections of 6 µm thickness were taken from the center (within the central 1 mm), lateral edge (within the lateral 1 mm), and medial edge (within the medial 1 mm) of each defect using a microtome. Two sections from each location were stained with hematoxylin and eosin (H&E), Safranin O/Fast Green, and van Gieson’s Picrofuchsins.
5.2.9. Histological Scoring

Histological sections were blindly and independently scored by three evaluators (J.L., P.P.S., and F.K.K.) using a previously established scoring system for osteochondral repair, as shown in Table 5.2 [108]. A total of 11 parameters to evaluate osteochondral repair were used to analyze the whole defect for both chondral (within the upper 1 mm of the defect) and subchondral (within the bottom 2 mm of the defect) regions.

5.2.10. Statistical Analysis

The data from the in vitro assays were analyzed by one-way analysis of variance (ANOVA) and Turkey’s multiple-comparison test. The means and the standard deviations were reported in each figure. For the histological analysis, ordered logistic regression of histological scores was performed to analyze the potential effect of experimental group, location within the defect, and knee joint [106-108]. p<0.05 was considered to indicate a significant difference.
5.3. Results

5.3.1. Degradation of bilayered OPF composite hydrogels

The swelling ratio of bilayered OPF composite hydrogels remained stable over 28 days in PBS, but started to increase after 7 days in collagenase-PBS (Figure 5.1A). The sol fraction of composite hydrogels in PBS was also stable (about 40%) over 28 days (Figure 5.1B). However, the sol fraction in collagenase-PBS started to increase after 7 days of incubation and maintained a gradual increase until day 28. Similarly, the fraction of the initial mass remaining continuously decreased over time in collagenase-PBS (Figure 5.1C).

5.3.2. In vitro IGF-1 release kinetics

The % cumulative release profiles of IGF-1 (as measured by radiolabeled IGF-1) over 28 days for Groups 1-3 are shown in Figure 5.2A-C, respectively, while the % cumulative release profile of the active amount of released IGF-1 for Group 1 (as measured by cellular assays) is shown in Figure 5.2D. The release kinetics of IGF-1 were similar between groups in both PBS and collagenase-PBS conditions as seen in Figure 5.2A-C. Initial burst release of IGF-1 within the first 4 days in collagenase-PBS was 43.9 ± 3.4 % in Group 1 (GMP-loaded IGF-1 only), 46.2 ± 1.7 % in Group 2 (GMP-loaded IGF-1 and gel-loaded TGF-β3), and 40.8 ± 3.7 % in Group 3 (GMP-loaded IGF-1 and GMP-loaded TGF-β3). 28-day % cumulative IGF-1 release in collagenase PBS was 83.2 ± 1.4 % in Group 1, 85.0 ± 0.9 % in Group 2, and 85.2 ± 3.0 % in Group 3. In addition, the IGF-1 release rate (% release of IGF-1 per day) is shown in Table 5.3A. No statistical
difference was observed between groups in collagenase-PBS during all phases. For all groups, approximately 30% of IGF-1 was released in phase 1 (up to 24 hrs), while approximately 1% of IGF-1 was released each day in phase 4 (days 18 - 28) (Table 5.3A). Biologically active IGF-1 released from the OPF composites (i.e., the released IGF-1 to actively enhance the proliferation of the MCF-7 cell line) was analyzed by using the same formulation in Group 1 in collagenase-PBS. Figure 5.2D showed that 36.6 ± 8.8 % of active IGF-1 was released within the first 4 days and that a total cumulative release of 66.6 ± 15.5 % was achieved by day 28. Release rate of active IGF-1 in collagenase-PBS (Table 5.3B) was similar to release rate determined by gamma counter (Table 5.3A) in all phases.

5.3.3. In vitro TGF-β3 release kinetics

The % cumulative release profile of TGF-β3 over 28 days is shown in Figure 5.3A-B, while the % cumulative release profile of the active amount of TGF-β3 is shown in Figure 5.3C. When comparing Group 2 and Group 3, a significantly higher burst release of TGF-β3 is seen within the first 2 days in Group 2 in collagenase-PBS (Figure 5.3A). Specifically, the 2-day burst release of TGF-β3 is 26.4 ± 3.3 % in Group 2 (GMP-loaded IGF-1 and gel-loaded TGF-β3) and 19.5 ± 3.5 % in Group 3 (GMP-loaded IGF-1 and GMP-loaded TGF-β3), although the 4-day release for both groups has no statistical difference (32.2 ± 3.3 % in Group 2 and 36.5 ± 4.0 % in Group 3). From day 7 to day 28, the % cumulative release is higher in Group 3 than in Group 2 at each time point (Figure 5.3A). The 28-day % cumulative TGF-β3 release in collagenase-PBS is 71.4 ± 9.2 % in
Group 2 and 84.8 ± 2.6 % in Group 3. Similar release kinetics were found in the standard PBS condition (Figure 5.3B). The release rate of TGF-β3 (% release per day) in Table 5.4A also indicates the difference between Groups 2 and 3. The release rate in Group 2 is higher than that in Group 3 in phase 1, but the release rate in Group 3 is higher than that in Group 2 in phases 2 and 3 in collagenase-PBS. The release of a higher (2.5 times) concentration of TGF-β3 in composite hydrogels featured similar % cumulative release profiles and the release rate (data not shown).

Biologically active released TGF-β3 (i.e., the released TGF-β3 to actively inhibit the proliferation of the CCL-64 cell line) was analyzed by using the same formulations for Groups 2 and 3 (Figure 5.3C) in collagenase-PBS. Initial burst release of active TGF-β3 as measured by a cell assay is similar within the first 2 days for both formulations. The 28-day % cumulative release of active TGF-β3 is 58.4 ± 11.9 % in Group 2 and 69.0 ± 18.6 % in Group 3. The release rate of active TGF-β3 in Group 3 is higher than that in Group 2 for phases 2 and 3 from days 1 to 18 (Table 5.4B).

5.3.4. Histological observation and scoring

Sections from three locations (lateral edge, medial edge, and center) per sample were obtained for histological evaluation. Representative images from each group are shown in Figure 5.4 – Figure 5.7, respectively. Figure 5.8 shows the histological scores for the 11 parameters listed in Table 5.2.
In all groups, partially degraded OPF gels were observed in the subchondral layer (Figure 5.4 – Figure 5.7). No significant difference was observed in both overall tissue filling and overall hydrogel degradation (Figure 5.8A). In all formulations, a mean score of about 2 was recorded in overall filling at the 12 week time point. This observation was closely related to a mean score of 2 in the percent degradation of the implant. Partially degraded gels in the subchondral layer in all formulations contributed to this score. In addition, statistical analysis revealed that location in the defect (lateral/medial/center) was a significant factor in overall degradation. Specifically, degradation in the lateral edge was greater than that in the center, which is correlated with the observation of remaining hydrogel in the center area.

Although mean scores for overall hydrogel degradation in Figure 5.8A are similar in all groups, none of the samples in the control group (Figure 5.4), which lacked GF incorporation, showed complete degradation, while groups incorporating GF showed at least one sample with complete degradation (i.e., all three sections per sample with a score of 3; one sample in Group 1, one in Group 2, and two in Group 3). In particular, the size of partially degraded gels in the subchondral region in Group 1 was generally smaller than those found in the control (i.e., approximately less than 30% of subchondral area in all sections), as shown in Figure 5.5. Specimens in groups delivering both IGF-1 and TGF-β3 (Groups 2 and 3) showed similar subchondral characteristics to samples seen in the control group, such as the frequency of partially degraded gels, the presence of inflammatory cells, and the area covered with newly formed bone tissues in the defect site (Figure 5.6E and Figure 5.7E).
In the evaluations for subchondral bone regeneration including percent bone filling, bone morphology, and bonding between newly formed bone tissue and adjacent bone (Table 5.2), the mean score for bone filling was slightly above 2 (Figure 5.8B) in all groups. However, the frequency of complete bone filling in Group 1 is greater than the other groups (data not shown). Although inflammatory cells and surrounding fibrous tissue were also observed in samples from Group 1 (Figure 5.5E), the quality and frequency of trabecular bone formation was higher than the specimens in the control group. Location in the defect was also a significant factor in bone filling; scores for the lateral edge were significantly higher than those for the center.

In subchondral bone morphology, a mean score (1.67 ± 0.96) for Group 1 (GMP-loaded IGF-1 only) was higher than that (1.11 ± 0.62) for Group 3 (GMP-loaded IGF-1 and GMP-loaded TGF-β3). In the blank control, the morphological characteristics of newly formed subchondral tissue in the defect were generally a mixture of compact bone with fibrous tissues (Figure 5.4 and Figure 5.9A). As indicated by the score distribution in Figure 5.9A, sections in Group 1 showed higher levels of trabecular bone regeneration as well as less compact bone with fibrous tissue formation than other groups. However, the presence of partially degraded OPF gels that were surrounded by fibrous tissues maintained a mean score between 1 and 2 for each group. Despite this result, bone bonding between newly formed tissue and adjacent native bone within the subchondral region was found to be complete on both edges of the defect and received a score of 3 for all sections in all experimental groups (Figure 5.8B).

Cartilage regeneration was evaluated in the categories of cartilage morphology, thickness of newly formed cartilage, surface regularity, chondrocyte clustering,
chondrocyte and glycosaminoglycan (GAG) amount in newly formed cartilage, and chondrocyte and GAG amount in adjacent cartilage tissue (Table 5.2). Histological analysis of the cartilage region indicated that the formulation significantly affected the cartilage morphology as well as the cell and GAG content in adjacent cartilage tissue (Figure 5.8C). However, location (lateral/medial/center) within the defect was not a significant factor. For cartilage morphology, all GF groups (Group 1, 2, and 3) received statistically higher mean scores when compared to the blank control (without GF loading). The score distribution for cartilage morphology (Figure 5.9B) indicated that higher levels of hyaline cartilage regeneration as well as less fibrous tissue formation were more frequent in Groups 1, 2, and 3 than the blank control. The cartilage layer in samples in the control group (Figure 5.4) was usually composed of thick fibrous tissue and fibrocartilage and the complete disruption of neo-cartilage surface or deep fissures in the cartilage layer was also observed in some samples. Neo-cartilage tissue in the chondral layer in GF groups (Figure 5.5 - Figure 5.7) was usually fibrocartilage (Figure 5.9B). However, it should be noted that the frequency of hyaline cartilage accompanied with a smooth surface and zonal organization was higher in GF groups than the control (Figure 5.9C). Group 2 (GMP-loaded IGF-1 and gel-loaded TGF-β3) received the highest mean score (2.17 ± 0.61) (Figure 5.8C) and frequency of hyaline-like cartilage tissue formation (Figure 5.9B), which indicated the presence of a mixture of newly formed hyaline cartilage and fibrocartilage within the cartilage defect space. Samples with hyaline cartilage formation generally demonstrated intensive GAG staining and less chondrocyte clustering, as shown in Figure 5.6D. Overall cartilage morphology observed
in Group 3 was similar to that in Group 2, except with less GAG staining (Figure 5.7B and Figure 5.7D).

Although no statistical differences were observed in cartilage thickness, surface regularity, and chondrocyte clustering (Figure 5.8C), all GF incorporating groups (Groups 1, 2, and 3) received higher mean scores for these parameters than the blank control. Mean scores for surface regularity (between 1 and 2) and chondrocyte clustering (around 1) reflected that newly formed cartilage tissues in all formulations contained some fissures and clustered chondrocyte populations, respectively. Scores for cell and GAG amount in neo-cartilage did not show any statistical difference between groups (Figure 5.8C). In cell and GAG amount in adjacent cartilage tissue, normal chondrocyte cellularity and normal Safranin O staining (i.e., a mean score close to 3) were observed for all formulations. Specifically, the mean score (3.00 ± 0.00) in Group 1 (GMP-loaded IGF-1 only) for this parameter was statistically higher than that (2.75 ± 0.50) in Group 2 (GMP-loaded IGF-1 and gel-loaded TGF-β3).

5.4. Discussion

The main objective of this study was to determine the effect of dual GF delivery using IGF-1 and TGF-β3 in bilayered OPF composite hydrogels for osteochondral tissue regeneration. Specifically, we investigated (1) how the in vitro release kinetics of TGF-β3 could be modulated by differing the incorporation method in OPF composite hydrogels,
in the presence of IGF-1, (2) whether the delivery of TGF-β3 in the presence of IGF-1 would enhance the osteochondral tissue regeneration over delivery of IGF-1 alone, and (3) whether varied release kinetics of TGF-β3 when delivered with IGF-1 would affect the degree of osteochondral tissue regeneration in a rabbit defect model. While we have previously studied the effects of TGF-β1 delivery alone and the dual delivery of TGF-β1 and IGF-1 in a rabbit osteochondral model [106, 107], questions regarding how interactions between multiple GFs and how varying the release kinetics of these GFs affect osteochondral tissue regeneration in vivo remain to be investigated.

An increase in GF release (Figure 5.2 and Figure 5.3), especially in phase 3 (Day 4-18) was related to GMP degradation by collagenase and subsequent mass loss (Figure 5.1). The release profile demonstrated that the release kinetics of IGF-1 were not influenced by the incorporation of the other GF (i.e., TGF-β3) in the same layer of a bilayered hydrogel construct, while the release kinetics of TGF-β3 could be modulated by loading the GF in the GMP or OPF phase of the hydrogel composites (Figure 5.2 – Figure 5.3 and Table 5.3, Table 5.4). In addition, the release kinetics of TGF-β3 were not influenced by the concentration of GF solution used to swell the GMPs. It was hypothesized that the ionic complexation of GFs with GMPs would reduce any loss of their activity [320]. Indeed, higher amounts of active TGF-β3 released were detected in Group 3 (GMP-loaded TGF-β3) compared to Group 2 (gel-loaded TGF-β3) using a Mink lung epithelial cell line, especially in phase 2 and 3 (Table 5.4).

Histological data demonstrated that improved cartilage morphology in the defect by GF incorporation was observed when compared to the control (Figure 5.8C), indicating a beneficial effect of GF delivery (TGF-β3 and/or IGF-1) using OPF
composite hydrogels. The released bioactive GFs may have contributed to the migration of host cell populations to the defect site, stimulated the chondrogenic differentiation of progenitor stem cells, and subsequently improved cartilage-like tissue regeneration. Histological data also demonstrated that TGF-β3 delivery in the presence of IGF-1 (specifically, Group 2) resulted in a greater frequency of hyaline-like cartilage formation (Figure 5.8C and Figure 5.9B). No major degenerative effect on surrounding host tissues was observed throughout the samples in the dual delivery groups (i.e., Group 2 and 3). Highly localized TGF-β3 delivery in the present study did not influence fibrous tissue formation in both chondral and subchondral layers when histological sections in Group 2 and 3 were compared to those in the blank control. Although a higher frequency of hyaline cartilage formation (Figure 5.9B) was observed in Group 2, any synergistic effect of dual GF delivery of TGF-β3 with IGF-1 over a single delivery of IGF-1 was limited. Both dual GF delivery groups (i.e., Group 2 and 3) did not show higher levels of osteochondral tissue regeneration over delivery of IGF-1 alone (Figure 5.8C). The significant differences between GF loading groups were found in bone morphology (Figure 5.8B) and adjacent GAG (Figure 5.8C). Delivery of IGF-1 alone (Group 1) showed an improved subchondral bone morphology over Group 3 (GMP-loaded IGF-1 and GMP-loaded TGF-β3) as well as a higher cell and GAG amount in adjacent cartilage tissue over Group 2 (GMP-loaded IGF-1 and gel-loaded TGF-β3).

These results show that delivery of IGF-1 alone for osteochondral tissue repair improves the subchondral bone morphology and the interaction with the surrounding chondral tissue over a dual GF delivery with IGF-1 and TGF-β3. Since TGF-β3 has been shown to have an inhibitory effect on the osteogenic differentiation of MSCs and
osteoblastic cells *in vitro* [104, 321, 322], it is possible that the amount of TGF-β3 released in this study suppressed subchondral bone formation. Indeed, greater amounts of active TGF-β3 were released in Group 3 as compared to Group 2, potentially resulting in the significantly lower bone morphology score observed in Group 3 than in Group 1. Further research could help elucidate the effects of TGF-β3 on *in vivo* subchondral bone repair. Additionally, other *in vivo* studies using the dual delivery of IGF-1 and TGF-β1 in a rabbit full-thickness defect model for osteochondral tissue regeneration also demonstrated a lack of synergy despite the fact that different hydrogel materials and different types and doses of GF were applied [107, 323]. One study using self-assembled peptide hydrogels demonstrated that the incorporation of chondrogenic factors including IGF-1 and TGF-β1 did not significantly improve cartilage regeneration over hydrogels without chondrogenic factors [323]. In a previous *in vivo* study from our laboratory [107], the single sustained delivery of IGF-1 using OPF composite hydrogels showed significantly improved cartilage regeneration over the single burst delivery of TGF-β1 within an osteochondral defect. IGF-1 alone also resulted in a significantly higher score for chondrocyte clustering when compared to the dual delivery of IGF-1 and gel-loaded TGF-β1 [107]. Furthermore, several *in vivo* studies showed a positive result of IGF-1 delivery in cartilage tissue regeneration using other animal models [145, 165, 168].

Taken together, the delivery of TGF-β3 (at the varying release kinetics examined) with IGF-1 did not improve osteochondral tissue regeneration over the delivery of IGF-1 alone, suggesting a lack of synergy between these two growth factors. Further research investigating the dose-dependent effect of IGF-1 alone on osteochondral tissue regeneration is warranted.
A comparison between Group 2 and Group 3 reveals no statistical differences in histological scores for subchondral bone evaluation and cartilage regeneration. While *in vitro* results demonstrate distinct release kinetics between gel-loaded and GMP-loaded TGF-β3, *in vivo* release kinetics may have been different. Indeed, previous studies have shown differences between *in vitro* and *in vivo* release kinetics of GFs from composite scaffolds using microparticles as delivery vehicles [324-326]. In one study, the release of BMP-2 from a poly(DL-lactic-co-glycolic acid)/calcium phosphate composite was found to be faster *in vivo* than from similar composites *in vitro* [326, 327]. This highlights the complexity of an *in vivo* environment and suggests that the release kinetics of TGF-β3 *in vivo* in this study may not have been sufficiently different to elicit a change in osteochondral regeneration. As a result, with the amount of TGF-β3 incorporated within the OPF composite hydrogels for Groups 2 and 3, differing release kinetics of TGF-β3 together with the presence of IGF-1 did not significantly influence the quality of regenerated osteochondral tissue in a full-thickness defect model.

Compared to a series of previous *in vivo* studies using OPF composite hydrogels [106-108], PEG with a higher molecular weight (Mₙ of 35,000) was utilized to synthesize OPF in the present study. As the molecular weight of the PEG chain between crosslinks increases, one would expect that the crosslinking density of the hydrogels would decrease, thus resulting in an increased level of hydrogel degradation [89, 96, 97]. In addition to the faster degradation, it has also been reported that larger network mesh sizes and better transport of nutrients/signaling molecules as modulated by higher molecular weight of PEG enhanced the chondrogenic differentiation of encapsulated MSCs in OPF composite hydrogels [89]. Furthermore, such modulation in hydrogel properties offers a
mode of control over the diffusion of soluble components within hydrogel composites, which is a critical factor for determining the release kinetics of GFs that are incorporated in hydrogels. For instance, when compared to a previous study that used OPF synthesized from PEG with $M_n$ of 25,000 and methylene bisacrylamide as a crosslinker [107], a higher cumulative release of IGF-1 on day 28 (Figure 5.2) was observed in the present study with the same crosslinking density of GMPs (67.4 % vs 83-85%).

However, the in vivo degradation of implanted gels using OPF synthesized from PEG with a $M_n$ of 35,000 and PEG-DA as a crosslinker in this study was not complete after 12 weeks. One possible factor to affect the degradation is the molar crosslinking ratio of OPF and PEG-DA. When compared to using a lower PEG block $M_n$ at the same OPF:PEG-DA weight ratio and total polymer content for hydrogel fabrication [108], the current system had a higher molar crosslinking ratio of OPF and PEG-DA which explains the prolonged in vivo degradation. Although the newly formed subchondral bone tissue and neo-cartilage tissue at the site of implant edges were well integrated with the surrounding native tissues, partially degraded gels were often observed in the center of the subchondral region of the defect, regardless of GF loading formulation (Figure 5.4 – Figure 5.7). The observation suggests that bone infiltration begins from the defect margin toward the center of the implantation region in the subchondral layer, but bone remodeling in the core area is limited. In addition, the presence of inflammatory cells and fibrous tissue in the subchondral area resulted in low histological scores in bone morphology.
5.5. Conclusions

IGF-1 and TGF-β3 were independently released from bilayered OPF composite hydrogels and the release kinetics of TGF-β3 could be modulated by the incorporation method. Higher amounts of active TGF-β3 were released when it was incorporated with GMPs as compared to gel phase loading. Single delivery of IGF-1 and dual delivery of both IGF-1 and TGF-β3 significantly enhanced cartilage morphology over a blank hydrogel control in a full-thickness osteochondral defect model after 12 weeks. Although IGF-1 delivery alone contributed to enhanced cartilage repair compared to the dual delivery of IGF-1 and TGF-β3, there was no significant effect of the TGF-β3 release kinetics on osteochondral tissue repair. The lack of synergy between IGF-1 and TGF-β3, regardless of TGF-β3 kinetics, demonstrates that the dual delivery of GFs does not necessarily confer an improved healing response over the single delivery of GFs in certain tissue engineering applications in vivo.
Figure 5.1 Degradation profile of bilayered OPF composite hydrogels. Swelling ratio (A), sol fraction (B), and mass remaining (C) were measured over 28 days in collagenase containing PBS (■) and PBS (○) (n=4). Error bars correspond to standard deviation.
Figure 5.2 % cumulative IGF-1 release from a bilayered OPF composite hydrogels in Group 1 (A), Group 2 (B), and Group 3 (C) over 28 days (n = 5). Solid line indicates collagenase-PBS condition while dashed line indicates PBS condition. % cumulative active IGF-1 release from a hydrogel in Group 1 (IGF-1 only) in collagenase-PBS is shown in (D) (n = 4). Error bars correspond to standard deviation. (*) indicates a significant difference between groups (p < 0.05).
Figure 5.3 % cumulative TGF-β3 release from a bilayered OPF composite hydrogels in Group 2 (solid line) and Group 3 (dashed line) in collagenase-PBS (A) and PBS (B) over 28 days (n = 5). % cumulative active TGF-β3 release from a hydrogel in Group 2 (solid line) and Group 3 (dashed line) in collagenase-PBS is shown in (C) (n = 4). Error bars correspond to standard deviation. (*) indicates a significant difference between groups (p < 0.05).
Figure 5.4 Representative histological sections of osteochondral tissue formation after 12 weeks of implantation of OPF composite hydrogels in control (blank without GFs). Sections were stained with H&E (A), Safranin-O/Fast Green (B), and van Gieson's Picrofuchsin (C) (scale bar: 1000 μm). Images indicate a thick fibrous layer with a discontinuity in morphology compared to adjacent host cartilage tissue and incomplete degradation of hydrogels in the subchondral area along with inflammatory cell infiltration. Boxed regions are shown with a higher magnification (D, E) (scale bar: 250 μm). (*) indicates some partially degraded OPF composite hydrogels remaining in the defect site.
Figure 5.5 Representative histological sections of osteochondral tissue formation after 12 weeks of implantation of OPF composite hydrogels in Group 1 (IGF-1 only). Sections were stained with H&E (A), Safranin-O/Fast Green (B), and van Gieson's Picrofuchsin (C) (scale bar: 1000 μm). Images with higher magnification indicate hyaline-like cartilage morphology with a hierarchical aligning of chondrocytes in the cartilage region (D) and a small fraction of partially degraded OPF composite hydrogels (indicated by *) in the subchondral area (E) (scale bar: 250 μm).
Figure 5.6 Representative histological sections of osteochondral tissue formation after 12 weeks of implantation of OPF composite hydrogels in Group 2 (IGF-1 and gel-loaded TGF-β3). Sections were stained with H&E (A), Safranin-O/Fast Green (B), and van Gieson's Picrofuchsin (C) (scale bar: 1000 μm). Images with higher magnification indicate hyaline-like cartilage morphology with GAG staining in the cartilage region (D) and partially degraded OPF composite hydrogels (indicated by *) that are surrounded by regenerated bone tissue in the subchondral area (E) (scale bar: 250 μm).
Figure 5.7 Representative histological sections of osteochondral tissue formation after 12 weeks of implantation of OPF composite hydrogels in Group 3 (IGF-1 and GMP-loaded TGF-β3). Sections were stained with H&E (A), Safranin-O/Fast Green (B), and van Gieson's Picrofuchsin (C) (scale bar: 1000 μm). Images with higher magnification indicate fibrocartilage formation in the cartilage region (D) and a similar subchondral bone morphology with large volume of partially degraded OPF composite hydrogels (indicated by *) compared to the control specimen (E) (scale bar: 250 μm).
Figure 5.8 Histological scoring for overall defect (A), subchondral region (B), and cartilage region (C). Overall evaluation (A) includes overall tissue filling and overall implant degradation; subchondral evaluation (B) includes bone filling, bone morphology, and bonding to adjacent tissue; cartilage evaluation (C) includes cartilage morphology, cartilage thickness, surface regularity, chondrocyte clustering, chondrocyte and GAG amount in neocartilage, and chondrocyte and GAG amount in adjacent cartilage. Data are shown as average scores with error bars representing standard deviation. (*) indicates a significant difference between groups (p < 0.05).
Figure 5.9 Histological score distribution for subchondral bone morphology (A) and cartilage morphology (B) for the four groups tested.
Table 5.1 Bilayered OPF composite design for the four experimental groups tested in this study. Growth factor loading concentration is shown as ng of incorporated growth factor per mL of crosslinked OPF composite hydrogels before swelling.

<table>
<thead>
<tr>
<th>Cartilage layer (Top 1mm)</th>
<th>Experimental groups</th>
<th>Control</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OPF Hydrogel Loading Solution</td>
<td>PBS</td>
<td>PBS</td>
<td>TGF-β3</td>
<td>PBS</td>
</tr>
<tr>
<td></td>
<td>10 mM GMPs mg per 100 mg OPF</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Loading solution</td>
<td>PBS</td>
<td>PBS</td>
<td>PBS</td>
<td>TGF-β3</td>
</tr>
<tr>
<td></td>
<td>40 mM GMPs mg per 100 mg OPF</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Loading solution</td>
<td>PBS</td>
<td>IGF-1</td>
<td>IGF-1</td>
<td>IGF-1</td>
</tr>
<tr>
<td></td>
<td>TGF-β3 loading (ng TGF-β3/mL composite gel)</td>
<td>0</td>
<td>0</td>
<td>1800</td>
<td>1800</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Subchondral layer (Bottom 2 mm)</th>
<th>Experimental groups</th>
<th>Control</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OPF Hydrogel Loading Solution</td>
<td>PBS</td>
<td>PBS</td>
<td>PBS</td>
<td>PBS</td>
</tr>
<tr>
<td></td>
<td>10 mM GMPs mg per 100 mg OPF</td>
<td>22</td>
<td>22</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Loading solution</td>
<td>PBS</td>
<td>PBS</td>
<td>PBS</td>
<td>PBS</td>
</tr>
<tr>
<td></td>
<td>Growth Factor Loading</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>TGF-β3 loading (ng TGF-β3/mL composite gel)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Description

White: Blank 10 mM GMPs, Gray: Blank 40 mM BMPs, Green: IGF-1 loaded 40 mM GMPs, Red: TGF-β3 loaded 10 mM GMPs, Brown: TGF-β3 loaded OPF hydrogel
Table 5.2 Histological scoring system for evaluation of (A) overall tissue evaluation, (B) subchondral bone evaluation, and (C) cartilage evaluation in rabbit osteochondral defects.

<table>
<thead>
<tr>
<th>(a) Overall defect evaluation (throughout the entire defect depth)</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Percent filling with newly formed tissue</td>
<td></td>
</tr>
<tr>
<td>100%</td>
<td>3</td>
</tr>
<tr>
<td>&gt;50%</td>
<td>2</td>
</tr>
<tr>
<td>&lt;50%</td>
<td>1</td>
</tr>
<tr>
<td>0%</td>
<td>0</td>
</tr>
<tr>
<td>2. Percent degradation of the implant</td>
<td></td>
</tr>
<tr>
<td>100%</td>
<td>3</td>
</tr>
<tr>
<td>&gt;50%</td>
<td>2</td>
</tr>
<tr>
<td>&lt;50%</td>
<td>1</td>
</tr>
<tr>
<td>0%</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(b) Subchondral bone evaluation (within the bottom 2 mm of defect)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>3. Percent filling with newly formed tissue</td>
<td></td>
</tr>
<tr>
<td>100%</td>
<td>3</td>
</tr>
<tr>
<td>&gt;50%</td>
<td>2</td>
</tr>
<tr>
<td>&lt;50%</td>
<td>1</td>
</tr>
<tr>
<td>0%</td>
<td>0</td>
</tr>
<tr>
<td>4. Subchondral bone morphology</td>
<td></td>
</tr>
<tr>
<td>Normal, trabecular bone</td>
<td>4</td>
</tr>
<tr>
<td>Trabecular bone, with some compact bone</td>
<td>3</td>
</tr>
<tr>
<td>Compact bone</td>
<td>2</td>
</tr>
<tr>
<td>Compact bone and fibrous tissue</td>
<td>1</td>
</tr>
<tr>
<td>Only fibrous tissue or no tissue</td>
<td>0</td>
</tr>
<tr>
<td>5. Extent of new tissue bonding with adjacent bone</td>
<td></td>
</tr>
<tr>
<td>Complete on both edges</td>
<td>3</td>
</tr>
<tr>
<td>Complete on one edge</td>
<td>2</td>
</tr>
<tr>
<td>Partial on both edges</td>
<td>1</td>
</tr>
<tr>
<td>Without continuity on either edge</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(c) Cartilage evaluation (within the upper 1 mm of defect)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>6. Morphology of newly formed surface tissue</td>
<td></td>
</tr>
<tr>
<td>Exclusively articular cartilage</td>
<td>4</td>
</tr>
<tr>
<td>Mainly hyaline cartilage</td>
<td>3</td>
</tr>
<tr>
<td>Fibrocartilage (spherical morphology observed with ≥ 75% of cells)</td>
<td>2</td>
</tr>
<tr>
<td>Only fibrous cartilage (spherical morphology observed with &lt; 75% of cells)</td>
<td>1</td>
</tr>
<tr>
<td>No tissue</td>
<td>0</td>
</tr>
<tr>
<td>7. Thickness of newly formed cartilage</td>
<td></td>
</tr>
</tbody>
</table>
Similar to the surrounding cartilage | 3
Greater than the surrounding cartilage | 2
Less than the surrounding cartilage | 1
No cartilage        | 0

8. Joint surface regularity
   Smooth, intact surface | 3
   Surface fissures (<25% of new surface thickness) | 2
   Deep fissures (≥25% of new surface thickness) | 1
   Complete disruption of the new surface | 0

9. Chondrocyte clustering
   None at all | 3
   <25% chondrocytes | 2
   25-100% chondrocytes | 1
   No chondrocytes present (no cartilage) | 0

10. Chondrocyte and GAG content of new cartilage
    Normal cellularity with normal Safranin O staining | 3
    Normal cellularity with moderate Safranin O staining | 2
    Clearly less cells with poor Safranin O staining | 1
    Few cells with no or little Safranin O staining or no cartilage | 0

11. Chondrocyte and GAG content of adjacent cartilage
    Normal cellularity with normal Safranin O staining | 3
    Normal cellularity with moderate Safranin O staining | 2
    Clearly less cells with poor Safranin O staining | 1
    Few cells with no or little Safranin O staining or no cartilage | 0
Table 5.3 (A) Release kinetics (% release per day) of IGF-1 \((n = 5)\) and (B) release kinetics (% release per day) of active IGF-1 in Group 1 in collagenase-PBS \((n = 4)\) from OPF composite hydrogels in phase 1 \((\sim 24 \text{ h})\), phase 2 \((1-4 \text{ days})\), phase 3 \((4-8 \text{ days})\), and phase 4 \((18-28 \text{ days})\). (*) indicates a significant difference between groups in the same phase \((p < 0.05)\).

(A)

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>Phase 1 (%/day) ((\sim 24 \text{ hrs}))</th>
<th>Phase 2 (%/day) ((\text{Days 1 - 4}))</th>
<th>Phase 3 (%/day) ((\text{Days 4 - 18}))</th>
<th>Phase 4 (%/day) ((\text{Days 18 - 28}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Collagenase-PBS</td>
<td>PBS</td>
<td>Collagenase-PBS</td>
<td>PBS</td>
</tr>
<tr>
<td>Group 1</td>
<td>31.1 ± 2.8</td>
<td>27.3 ± 2.8</td>
<td>4.3 ± 0.4</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td>Group 2</td>
<td>31.7 ± 1.8</td>
<td>31.7 ± 1.8*</td>
<td>4.8 ± 0.3</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>Group 3</td>
<td>28.1 ± 2.3</td>
<td>27.6 ± 2.3</td>
<td>4.2 ± 0.6</td>
<td>2.7 ± 0.2</td>
</tr>
</tbody>
</table>

(B)

<table>
<thead>
<tr>
<th>Active IGF-1 Release ((\text{Group 1, collagenase-PBS}))</th>
<th>Phase 1 (%/day) ((\sim 24 \text{ hrs}))</th>
<th>Phase 2 (%/day) ((\text{Days 1 - 4}))</th>
<th>Phase 3 (%/day) ((\text{Days 4 - 18}))</th>
<th>Phase 4 (%/day) ((\text{Days 18 - 28}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>26.2 ± 13.4</td>
<td>3.5 ± 2.3</td>
<td>1.7 ± 0.6</td>
<td>0.6 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>
Table 5.4 (A) Release kinetics (% release per day) of TGF-β3 (n = 5) and (B) release kinetics (% release per day) of active TGF-β3 (n = 4) from OPF composite hydrogels in phase 1 (~ 24 h), phase 2 (1–4 days), phase 3 (4–8 days), and phase 4 (18–28 days). (*) indicates a significant difference between Group 2 (Gel loading) and Group 3 (GMP loading) in the same phase (p < 0.05).

(A)

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>Phase 1 (%/day) (~ 24 hrs)</th>
<th>Phase 2 (%/day) (Days 1 - 4)</th>
<th>Phase 3 (%/day) (Days 4 - 18)</th>
<th>Phase 4 (%/day) (Days 18 – 28)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Collagenase-PBS</td>
<td>PBS</td>
<td>Collagenase-PBS</td>
<td>PBS</td>
</tr>
<tr>
<td>Group 2</td>
<td>25.0 ± 3.1*</td>
<td>28.8 ± 3.3*</td>
<td>2.5 ± 0.4</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td>Group 3</td>
<td>17.1 ± 3.2</td>
<td>19.5 ± 1.0</td>
<td>6.5 ± 1.2*</td>
<td>1.7 ± 0.3</td>
</tr>
</tbody>
</table>

(B)

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>Phase 1 (%/day) (~ 24 hrs)</th>
<th>Phase 2 (%/day) (Days 1 - 4)</th>
<th>Phase 3 (%/day) (Days 4 - 18)</th>
<th>Phase 4 (%/day) (Days 18 – 28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 2</td>
<td>25.6 ± 2.7</td>
<td>1.6 ± 0.6</td>
<td>1.0 ± 0.3</td>
<td>1.4 ± 0.6</td>
</tr>
<tr>
<td>Group 3</td>
<td>24.4 ± 5.1</td>
<td>3.1 ± 0.6*</td>
<td>1.8 ± 0.1*</td>
<td>1.0 ± 0.1</td>
</tr>
</tbody>
</table>
Chapter 6

Dual growth factor delivery from bilayered, biodegradable hydrogel composites for spatially-guided osteochondral tissue repair*

The present work investigated the use of biodegradable hydrogel composite scaffolds, based on the macromer oligo(poly(ethylene glycol) fumarate) (OPF), to deliver growth factors for the repair of osteochondral tissue in a rabbit model. In particular, bilayered OPF composites were used to mimic the structural layers of the osteochondral unit, and insulin-like growth factor-1 (IGF-1) and bone morphogenetic protein-2 (BMP-2) were loaded into gelatin microparticles and embedded within the OPF hydrogel matrix in a spatially controlled manner. Three different scaffold formations were implanted in a

medial femoral condyle osteochondral defect: 1) IGF-1 in the chondral layer, 2) BMP-2 in the subchondral layer, and 3) IGF-1 and BMP-2 in their respective separate layers. The quantity and quality of osteochondral repair was evaluated at 6 and 12 weeks with histological scoring and micro-computed tomography (micro-CT). While histological scoring results at 6 weeks showed no differences between experimental groups, micro-CT analysis revealed that the delivery of BMP-2 alone increased the number of bony trabecular islets formed, an indication of early bone formation, over that of IGF-1 delivery alone. At 12 weeks post-implantation, minimal differences were detected between the three groups for cartilage repair. However, the dual delivery of IGF-1 and BMP-2 had a higher proportion of subchondral bone repair, greater bone growth at the defect margins, and lower bone specific surface than the single delivery of IGF-1. These results suggest that the delivery of BMP-2 enhances subchondral bone formation and that, while the dual delivery of IGF-1 and BMP-2 in separate layers does not improve cartilage repair under the conditions studied, they may synergistically enhance the degree of subchondral bone formation. Overall, bilayered OPF hydrogel composites demonstrate potential as spatially-guided, multiple growth factor release vehicles for osteochondral tissue repair.
6.1. Introduction

The osteochondral unit is a multiphasic tissue comprised of two main tissue types: the articulating cartilage responsible for nearly frictionless movement and shock-absorbance, and the subchondral bone indispensable for underlying mechanical support [328-330]. Within the knee, the health of cartilage tissue has been linked to the maintenance of the natural biomechanics of the subchondral bone, and given the contact between cartilage and bone, total joint homeostasis will ultimately rely on the functional restoration of both tissues [331-334]. As a result, tissue engineering strategies to regenerate the osteochondral unit have evolved over the years from monolithic structures to gradient/bilayered scaffolds as a means of controlling treatment to repair the cartilage-bone interface or to target each tissue separately [335-337]. In addition to selecting an appropriate scaffold design, successful regeneration of osteochondral tissue is greatly influenced by the microenvironment in which cells and tissues grow [135]. A number of soluble cues have been implicated in osteochondral wound healing environment [337], and the goal of eliciting the desired phenotypic response from co-delivered and/or host progenitor cells \textit{in vivo} remains a difficult challenge. Due to the numerous signaling pathways involved in osteochondral repair, the delivery of multiple growth factors to improve therapeutic potency is an area of great interest, particularly when both cartilage and bone tissues are considered [338].

The present work seeks to repair osteochondral tissue through the dual delivery of growth factors from acellular, bilayered hydrogel composites. These hydrogel composites are fabricated from a synthetic macromer, oligo(poly(ethylene glycol) fumarate) (OPF), which is synthesized by the esterification of poly(ethylene glycol) (PEG) and fumaryl
chloride. The resulting macromer can be crosslinked via the double bonds of the fumarate group to form a hydrogel as well as degrade through hydrolysis of the ester groups [59, 339]. Gelatin microparticles (GMPs) are also embedded within the crosslinked hydrogel matrix to act as both an enzymatically digestible porogen and a delivery vehicle for growth factors [258, 259, 340]. These OPF-based hydrogel composites have been used in a number of in vitro and in vivo studies as growth factor delivery vehicles, and allows for comparisons between growth factor types and delivery methods for cartilage and osteochondral tissue repair applications [171, 257-259, 340, 341]. In an effort to mimic the osteochondral unit, these hydrogels are laminated to form two distinct layers as a means of tailoring scaffold composition and drug loading to the cartilage and bone areas of the defect [256, 341, 342]. With this design, the current study incorporates a chondroinductive factor and an osteoinductive factor into the bilayered hydrogels in separate layers to facilitate simultaneous cartilage and subchondral tissue repair within an osteochondral defect.

The strategy of spatially incorporated biologics for simultaneous in vivo cartilage and bone repair is a growing area of research: Chen et al. delivered plasmids encoding for transforming growth factor-β1 (TGF-β1) and bone morphogenetic protein-2 (BMP-2) in separate layers of a chitosan-gelatin scaffold [343], Re'em et al. spatially presented TGF-β1 and BMP-4 in an alginate-sulfate hydrogel [231], and Mohan et al. and Dormer et al. created sintered poly(D,L-lactic-co-glycolic acid) microsphere scaffolds with reverse gradients of TGF-β1 and BMP-2 [249, 344]. However, an optimal combination of chondrogenic and osteogenic growth factors has yet to be achieved for osteochondral regeneration.
As a result, the main hypothesis of this study is that insulin-like growth factor-1 (IGF-1) delivered from the chondral layer and BMP-2 delivered from the subchondral layer of bilayered OPF hydrogel composites can act synergistically to promote osteochondral tissue repair over the delivery of either growth factor alone. Additionally, it is hypothesized that earlier subchondral bone repair, stimulated by the release of BMP-2, will affect the degree of cartilage repair. IGF-1 is the main anabolic growth factor in articular cartilage and plays a key role in homeostasis by balancing synthesis and breakdown of proteoglycans by chondrocytes [218]. Additionally, previous studies have demonstrated similar or improved cartilage repair with the single delivery of IGF-1 over the dual delivery of IGF-1 with TGF-β1 or TGF-β3 from bilayered OPF composites in a medial femoral condyle osteochondral defect model [171, 257]. BMP-2 is a potent osteogenic growth factor for inducing de novo bone formation in ectopic and orthotopic sites and has been shown to improve subchondral bone formation when delivered to osteochondral defects [219, 222, 345-349]. Additionally, BMP-2 is expressed throughout the entire chondrogenic process of mesenchymal stem cells (MSCs), from condensation to proliferation, differentiation, maturation, and calcification [337]. With the ability to induce the growth of both cartilage and bone tissue, several research groups have delivered BMP-2 from monolithic scaffolds to osteochondral defects and have improved cartilage repair over blank scaffolds [348-350]. However, the current study isolates the incorporation of BMP-2 to the subchondral layer to maximize its osteoinductive potential and reduce its direct morphogenetic effects on progenitor cell chondrogenesis in the chondral layer.
To test the two hypotheses aforementioned, the current study 1) investigates the repair of cartilage and subchondral bone tissue in an osteochondral defect facilitated by bilayered hydrogel composites delivering IGF-1 and/or BMP-2 incorporated in a spatially controlled manner, 2) evaluates the degree of subchondral bone repair with micro-computed tomography (micro-CT) and osteochondral tissue repair through histological scoring, and 3) compares the extent of cartilage and subchondral bone repair at 6 week and 12 week time points.

6.2. Materials and Methods

6.2.1. Oligo(poly(ethylene glycol) fumarate) synthesis

OPF was synthesized from PEG (Sigma-Aldrich, St. Louis, MO) with a nominal molecular weight (MW) of 35,000 according to methods previously developed in our laboratory [59, 339]. Gel permeation chromatography was used to characterize the OPF macromer to give a number average molecular weight of 50,400 ± 900 Da and a weight average molecular weight of 127,400 ± 3,200 Da. Prior to the fabrication of hydrogel implants, OPF was sterilized for 12 hours by exposure to ethylene oxide (EO) according to established protocols [171, 351].
6.2.2. Gelatin microparticle fabrication and growth factor loading

Using established methods, GMPs were fabricated using acidic gelatin (Nitta Gelatin, Osaka, Japan) with an isoelectric point (IEP) of 5.0 and crosslinked in 40 mM glutaraldehyde (Sigma-Aldrich, St. Louis, MO), followed by glycine quenching of unreacted glutaraldehyde [340, 352]. Following lyophilization, GMPs of 50-100 μm in diameter were obtained through sieving and EO sterilized for 12 hours prior to GF loading. GMPs were then partially swelled with either phosphate buffered saline (PBS) or a GF solution at a ratio of 5 μL solution to 1 mg dried GMP for 15 hrs at 4°C before hydrogel encapsulation according to previously described methods [258, 259]. In particular, 225 μL of PBS, IGF-1 solution, or BMP-2 solution was combined with 45 mg of GMPs. Recombinant human IGF-1 (Peprotech, Rocky Hill, NJ) was reconstituted with PBS to a GF loading concentration of 7.95 μg per mL solution and loaded onto GMPs to give a total of 5.6 ng IGF-1 per hydrogel construct. Recombinant human BMP-2 (Peprotech, Rocky Hill, NJ) was reconstituted with a mixture of ddH₂O and PBS to a GF loading concentration of 712 μg per mL solution to give a total of 1 μg BMP-2 per hydrogel construct.

6.2.3. Bilayered hydrogel composite fabrication

Bilayered hydrogel composites were fabricated via a two-step crosslinking procedure similar to methods previously described [171, 257]. Using a 2 mm x 2 mm cylindrical teflon mold, the subchondral layer was first partially crosslinked, then the chondral layer was crosslinked on top of the subchondral layer. Specifically, to prepare
the OPF precursor solution, 113 mg of OPF and 37 mg of PEGDA (3,400 Da MW, Laysan Bio, Arab, AL) were dissolved in 353 μL of PBS and set at room temperature for 45 minutes to eliminate air bubbles. 46.8 μL each of the thermal radical initiators, 0.3 M ammonium persulfate (APS, Sigma-Aldrich, St. Louis, MO) and 0.3 M N,N,N',N'-tetramethylethylenediamine (TEMED, Sigma-Aldrich, St. Louis, MO), were then mixed into the polymer solution. The addition of APS and TEMED at the concentrations used are expected to have minimal toxic effects on the surrounding tissue post-implantation, particularly in consideration of previous studies showing high viability of encapsulated cells in OPF-based hydrogels following the fabrication process [87, 256, 353]. Lastly, 45 mg of GMPs swollen with either 225 μL of PBS or a GF solution were added to the solution and mixed carefully to avoid the formation of bubbles. The polymer-GMP mixture was then injected into the bottom two-thirds of the teflon mold for the subchondral layer and partially crosslinked at 37°C for 5 minutes. A similar polymer-GMP mixture was then prepared for the chondral layer and injected on the subchondral layer and crosslinked at 37°C for 25 minutes. Table 6.1 highlights the three groups used in the study with BMP-2 delivered from the subchondral layer and IGF-1 delivered from the chondral layer of the bilayered hydrogel composites. Prior to implantation in an osteochondral defect, hydrogels were transferred with a drop of sterile PBS to prevent the hydrogel composites from drying. Bilayered hydrogel composites at equilibrium swelling were cylinders 3 mm x 3 mm in size (data not shown).
6.2.4. Animal surgery

A total of 36 male, skeletally mature (i.e., 6 months old) New Zealand White rabbits were used as determined by a power analysis and in consideration of previous studies [171, 257, 341, 351]. Each animal received bilateral implants (one per knee) and a total of 10-12 hydrogels were implanted in 5-6 rabbits per group per time point. It should be noted that one rabbit for the 6 week BMP-2 group had complications on the surgery table due to previously undetected Pasteurella infection and was excluded from the study. All surgical procedures and subsequent animal care were approved by the Institutional Animal Care and Use Committees of Rice University and The University of Texas Health Science Center at Houston. Briefly, anesthesia was induced prior to surgery by subcutaneous injections of ketamine (25-40 mg/kg) and acepromazine (1-2 mg/kg). General anesthesia was maintained by inhalation of isoflurane (2-3%) and oxygen. Rabbits were immobilized on their backs and the hair from both hind legs was shaven. The medial femoral condyle was then exposed through a medial parapatellar longitudinal incision, incision of synovial capsule, and lateral luxation of the patella. An osteochondral defect (3 mm in diameter and 3 mm in depth) was then created through serial drilling (2 mm → 2.75 mm → 3 mm) with a dental drill under irrigation (Osseo Scientific, Toms River, NJ). The defect was then flushed with saline, dried with gauze, and the hydrogel composite was implanted to allow for swelling to the same dimensions of the defect. This procedure was then repeated for the contralateral knee using a hydrogel of the same formulation. The synovial capsule and skin on both knees were then closed with 3-0 and 4-0 Vicryl sutures, respectively. To minimize post-operative
discomfort, Carprofen (4 mg/kg) was administered subcutaneously for two days postoperatively.

6.2.5. Tissue processing and histological scoring

Rabbits were euthanized at 6 or 12 weeks post-surgery with an intravenous injection of Beuthanasia (0.22 mL/kg). The tissue surrounding the medial femoral condyle defect was retrieved en bloc, cut with a low-speed diamond saw to isolate the medial femoral condyle, fixed in 10% neutral buffered formalin for no more than 72 hours, decalcified in EDTA solution for 6 weeks, dehydrated through a graded series of ethanol baths, and embedded in paraffin. Longitudinal sections of 5 μm thickness were taken from the center (within the central 1 mm) and the peripheral edges (within the medial and lateral 1 mm) with a microtome (Leica RM2165). Sections from each location were stained with hematoxylin and eosin (H&E), Safranin O/Fast Green (Saf.O), and van Gieson's Picrofuchsin (VGP). Histological sections were blindly and independently scored by three evaluators (J.L., J.T., F.K.K.) using a previously established histological scoring system for osteochondral defects as shown in Table 6.2 [171, 257, 351]. H&E and VGP sections were primarily used for overall defect and subchondral bone evaluation parameters while Saf.O sections were primarily used for cartilage evaluation parameters. Independent of the histological scoring system, the presence of complete subchondral bone plate bridging within the defect and neo-surface depressions below the tidemark were also evaluated for each histological section. It should be noted that sections from one sample in the 12 week BMP-2 group had large artifacts that resulted in the inability to accurately score the histological images and were excluded in the histological analysis.
6.2.6. Micro-computed tomography

All femoral condyles were scanned using a SkyScan 1172 micro-CT imaging system (Bruker, Kontich, Belgium) prior to EDTA decalcification. A voltage of 100 kV and a current of 100 μA with a nominal resolution of 10 μm pixel\(^{-1}\) was used. Image data were reconstructed using NRecon v1.6 and analyzed with CTAn v1.10, where thresholding levels of gray values (70-255) were set for all samples. A circular region of interest 3 mm in diameter was selected for a top-down analysis and transverse images were used to position the upper boundary of the volume of interest (VOI) at the surface of the cartilage layer of the cylindrical defect. Due to the presence of two distinct bone morphologies in the subchondral tissue, the bone plate and trabecular bone, two VOIs were analyzed separately: the upper .85 mm for the cartilage and cortical region (C&C) and the lower 2.15 mm for the trabecular region. Due to the potential for the subchondral bone plate to migrate upward during osteochondral repair, the cartilage region was also included for the C&C VOI [354]. Parameters analyzed for the C&C and trabecular VOI include bone mineral density (BMD), percent bone volume (BV/TV), intersection surface (i.S.), bone specific surface (BS/BV), and object number (Obj.N.). Table 6.3 further explains each of these parameters. Independent of the micro-CT parameter analysis, the presence of complete subchondral bone plate filling and bridging was also evaluated for each sample.
6.2.7. Statistical analysis

A significance level of $p < 0.05$ was used for all statistical analysis through JMP Pro v10.0.2. For histological scoring analysis, a one-way analysis was performed to analyze the potential effect of experimental group, time point, location within the defect, and knee joint. The Kruskal-Wallis test of variance was used and post-hoc analysis done with the Steel-Dwass All Pairs test. For micro-CT analysis, a one-way analysis was performed to analyze the potential effects of experimental group and time point. A one-way ANOVA was used to test variance and Tukey HSD All Pairs test was used to compare means.

6.3. Results

6.3.1. Histological Observations and Scoring

Sections from three locations (lateral edge, medial edge, and center) were obtained per sample for histological evaluation. Representative images for all groups at week 6 and week 12 are shown in Figure 6.1 and Figure 6.2, respectively. Figure 6.3 displays the mean histological scores from the 11 parameters examined as described in Table 6.2, and the distribution of scores for bone morphology, cartilage morphology, and cartilage thickness are shown in Figure 6.4.

Following histological scoring of all images, the potential effects of experimental group, time point (week 6 vs. week 12), location within the defect (edges vs. center), and
knee joint (right vs. left) were evaluated. Location within the defect and knee joint did not have any statistical impact on the 11 histological parameters scored, indicating that there was no major intra-defect variability nor bilateral implant variability. However, it should be noted that neo-tissue formation was seen to develop most commonly from the defect margins in the subchondral and chondral regions. The effect of time point for all samples for the histological parameters scored is shown in Table 6.4.

6.3.1.1. Overall Evaluation

In the overall evaluation of the defect, the histological scores for overall tissue filling generally mirrored those of overall implant degradation (Figure 6.3a). At week 6, there were no differences between groups in overall filling and degradation and none of the samples were seen to have complete degradation of the hydrogel composite implant as seen by representative images in Figure 6.1. Additionally, the remaining hydrogel was located in the subchondral region of the osteochondral defect for all samples. At week 12, greater degradation of the implants was seen with 44%, 63%, and 41% of histological images showing no presence of remaining hydrogel composite for IGF-1, BMP-2, and Both groups, respectively. More specifically, when all three sections from one sample had no presence of the remaining implant, 3/12 IGF-1 samples, 4/11 BMP-2 samples, and 0/12 Both samples showed complete degradation. When all samples at week 12 were compared against week 6 samples, there was a significant effect of time point, with week 12 samples having greater scores for overall filling and degradation than week 6 samples.
6.3.1.2. Subchondral Evaluation

Similar to overall evaluation, evaluation of the subchondral bone revealed no differences between experimental groups at 6 weeks. In the majority of the samples, the implant was surrounded by a fibrous capsule (Figure 6.1F), giving the histological image a score of 1 for bone morphology. Hypertrophic and calcified cartilage were also seen in many of the sections (Figure 6.1G-I), indicative of cartilage resorption and bone formation processes. At 12 weeks, while there were no differences for bone filling between groups, the BMP-2 group had better bone morphology scores compared to the Both group as seen in Figure 6.3B. The BMP-2 group also had the greatest percentage of sections with trabecular bone at 36%, whereas 17% and 12% of sections for IGF-1 and Both, respectively, showed trabecular bone for the entire subchondral region (Figure 6.4A). However, the majority of all samples at both week 6 and 12 had cortical and/or trabecular bone formation from the subchondral defect margins and all received a score of 3 for new tissue bonding with the adjacent bone (Figure 6.3B). The repair of the subchondral bone plate was also assessed, with 22%, 22%, and 50% of histological sections for IGF-1, BMP-2, and Both groups at week 12, respectively, showing repaired bone plate tissue in line with the adjacent bone plate. More specifically, 0/12, 2/11, and 3/12 samples for IGF-1, BMP-2, and Both groups respectively showed complete bone plate bridging with all three sections per samples having presence of a continuous bone plate. No repair of the bone plate was seen in any of the 6 week samples. In a comparison between 6 week and 12 week samples for subchondral bone histological scores, 12 week samples had statistically greater bone filling and bone morphology scores than 6 week samples.
6.3.1.3. Cartilage Evaluation

Cartilage repair was evaluated with the parameters of cartilage morphology, cartilage thickness, surface regularity, chondrocyte clustering, neocartilage glycosaminoglycan (GAG) and cell content, and adjacent GAG and cell content (Figure 6.3C). For neo-cartilage morphology, there was no difference between time points nor between groups. However, the 12 week Both group had the greatest percentage of samples with articular cartilage repair while many 12 week BMP-2 samples had fibrocartilage, and the 12 week IGF-1 group had the most sections with fibrous cartilage formation (Figure 6.4B). In having the most sections with articular cartilage, the 12 week Both group also had the best scores for chondrocyte clustering and a significant difference from the 12 week BMP-2 group (Figure 6.3C). Although harvested at an earlier time point, many of the 6 week samples had hyaline cartilage formation with intense GAG staining. However, these samples also tended to have hypercellularity in the neo-cartilage tissue with cell clustering or unorganized cells (Figure 6.1G-I). As a result, 6 week samples overall had statistically greater neocartilage GAG and cellularity scores than 12 week samples.

The thickness of neo-cartilage was also greater at 6 weeks compared to 12 weeks with approximately 80% of all 6 week samples having similar or thicker neo-cartilage tissue with respect to surrounding cartilage (Figure 6.4C). In contrast, approximately 70% of all 12 week samples had similar or thinner neo-cartilage tissue. In examining surface regularity, there were no differences among all groups. However, the nature of the surface regularity differed between 6 and 12 week samples. 12 week samples generally exhibited more irregularities on the repaired tissue surface (e.g., small fissures, bumps,
etc.), while 6 week samples had more depressions in the cartilage compared to the surrounding tissue (Figure 6.1). 30%, 21%, and 34% of IGF-1, BMP-2, and Both sections, respectively, at 6 weeks had the surface of the neo-tissue below that of the surrounding tidemark. On the other hand, 8%, 16%, and 21% of IGF-1, BMP-2, and Both sections, respectively, at 12 weeks had surface depressions that dipped below the adjacent cartilage. In evaluating the GAG and cell content of the adjacent cartilage, normal GAG staining and cellularity was seen in most 12 week samples, with some sections demonstrating normal GAG staining on one side of the defect, and reduced staining on the other side. This was more apparent for 6 week samples as seen in Figure 6.1G. As a result, 12 week samples overall had statistically greater scores for adjacent GAG and cell content compared to their 6 week counterparts.

6.3.2. Micro-Computed Tomography Analysis

The degree of subchondral bone repair was measured through quantitative analysis of two different VOIs (Figure 6.5A), C&C and trabecular, for five morphometric bone parameters: BMD, BV/TV, i.S., BS/BV, and Obj.N. (Figure 6.6). The effects of time point for all samples was also analyzed (Table 6.4). No differences were seen among the three groups at week 6 for BMD, BV/TV and i.S. However, group BMP-2 had greater BS/BV in the C&C region and a higher Obj.N. in both VOIs than group IGF-1 at week 6 (Figure 6.6D-E). Figure 6.5B-C show representative images of the incomplete subchondral tissue repair for a 6 week sample (6 week Both). By 12 weeks, micro-CT analysis showed that the delivery of both growth factors resulted in greater BMD, BV/TV, and i.S. compared to that of the IGF-1 only group for the C&C region.
Additionally, the 12 week Both group had greater i.S. and lower BS/BV than the 12 week IGF-1 group for the trabecular VOI. Comparison between 6 week and 12 week samples revealed that 12 week samples had a higher BMD, BV/TV ratio, and greater i.S. area than the 6 week samples, and conversely, 6 week samples had greater BS/BV and Obj.N. than 12 week samples in both C&C and trabecular regions. The repair of the subchondral bone plate was also assessed through observation of the micro-CT images. In accordance with the histological images, no bone plate was fully repaired for the 6 week samples and 0, 2, and 3 samples at 12 weeks for the IGF-1, BMP-2, and Both groups respectively showed complete bridging and filling of the defect area with compact bone. However, the regeneration of the bone plate was not complete as seen in Figure 6.5D, with the repair bone in the defect showing a marked difference in mineral density compared to the adjacent compact bone.

**6.4. Discussion**

The primary objective of this study was to determine the effect of dual growth factor release using IGF-1 from the chondral layer and BMP-2 from the subchondral layer of bilayered OPF hydrogel composites on osteochondral tissue repair. The degree of osteochondral repair was evaluated at 6 and 12 weeks and histological scoring and micro-CT analyses were used. Specifically, we investigated 1) if early subchondral bone repair, as measured at 6 weeks, would result in better bone and cartilage repair at 12 weeks and 2) whether the dual delivery of IGF-1 and BMP-2 in separate layers would act
synergistically to improve osteochondral tissue regeneration over the delivery of either growth factor alone.

As a potent morphogenetic factor, BMP-2 has been delivered for a number of ectopic and orthotopic bone regeneration applications and has been used to stimulate chondrogenesis of MSCs \textit{in vitro} as well as improve both cartilage and bone repair in osteochondral defects \textit{in vivo} [249, 344, 345, 355-357]. Since it can directly affect osteogenesis and chondrogenesis of MSCs, this study loaded BMP-2 onto GMPs that were localized in the subchondral layer of bilayered scaffolds in order to focus the morphogenetic potential of BMP-2 on bone repair. However, we acknowledge that BMP-2 may diffuse into chondral space, or conversely, IGF-1 may diffuse into the subchondral space over the course of 6 - 12 weeks. Further research tracking the diffusion of growth factors or confining the delivery of growth factors to their respective regions could help address this limitation. Even so, previous studies studying subchondral bone repair in a trochlear groove defect show that upward migration of the subchondral bone plate and thinning of cartilage is the natural course of osteochondral repair [354, 358], and that the presence of BMP-2 in the chondral region of the defect does not exacerbate this process [348, 349]. In this study, only two samples at week 12, one in the BMP-2 group and the other in the Both group, had repair of the subchondral bone extending beyond the tidemark of the adjacent tissue but below the joint surface.

In order to examine early subchondral bone repair, we evaluated the defects at 6 weeks with both histological scoring and micro-CT analysis. We saw that there were no differences in the quantity of bone formation at 6 weeks, as evaluated by bone filling, BMD, and BV/TV. This was most likely due to the incomplete degradation of the
implants, which was present in all 6 week samples. Bone morphology scoring, bond bonding scoring, and i.S. measurements also showed no differences between the experimental groups. However, micro-CT analysis revealed a difference with an increase in BS/BV in the C&C VOI and a higher Obj.N. count for group BMP-2 in both VOIs over group IGF-1 at 6 weeks. This could signify an increase in the number of woven bone islets in the subchondral space, which is a commonly observed phenomenon in early bone repair for segmental defects [345, 355, 359]. Other studies delivering BMP-2 to osteochondral defects have also demonstrated accelerated subchondral bone formation with the delivery of BMP-2 [348, 349]. In particular, Sellers et al. found that when BMP-2 was delivered in a collagen sponge to a trochlear groove defect, most of the new-bone formation took place between two and four weeks, and new subchondral bone tended to be more sclerotic between four and eight weeks [348]. Thus, the single delivery of BMP-2 may have enhanced subchondral ossification over that of the single delivery of IGF-1, resulting in a greater number of isolated bony trabeculae at the 6 week time point.

Histological and micro-CT analysis revealed greater differences in subchondral bone repair at 12 weeks. With histological scoring, the delivery of BMP-2 alone resulted in better scores for bone morphology than the dual delivery of IGF-1 and BMP-2. This could potentially be due to the increased percentage of samples with complete implant degradation in the BMP-2 only group (4/11 samples) compared to the Both group (0/12 samples), resulting in more sections with trabecular bone and no fibrous tissue present. With micro-CT, in-depth scrutiny of the repair bone was provided in both the trabecular and cortical regions. Analysis of BMD, BV/TV, i.S., and BS/BV revealed that the delivery of both BMP-2 and IGF-1 resulted in better subchondral bone repair than IGF-1
alone. Additionally, group Both at 12 weeks had the highest bone mineral density, percent bone volume, and bone intersection surface in the cortical region than all other groups, regardless of the time point. Compounded with the fact that the dual delivery group had the most sections with complete bone plate bridging with the adjacent bone plate at 50% suggests that the delivery of IGF-1 and BMP-2 synergistically improved subchondral bone plate repair over either growth factor alone. Indeed, the dual delivery of IGF-1 and BMP-2 has been shown to enhance alkaline phosphatase expression and calcium deposition from periodontal ligament fibroblasts over either growth factor alone [360]. The combination of both growth factors has also been shown to improve bone formation in a rabbit femoral osteotomy defect over BMP-2 alone at 4 and 8 week time points [361].

When the extent of cartilage repair at 12 weeks was examined through histological scoring, the dual delivery of IGF-1 and BMP-2 resulting in less chondrocyte clustering than the single delivery of BMP-2. While group Both had the highest percentage of sections with articular cartilage and well-organized chondrocytes in a columnar fashion, there were no statistical differences with the other groups. Interestingly, the delivery of IGF-1 alone resulted in better adjacent GAG and cell content at 12 weeks than all 6 week groups. A similar result was achieved in previous studies where the delivery of IGF-1 alone also gave the highest scores for adjacent GAG and cell content [171, 257]. This may highlight the importance of IGF-1 in regulating the homeostasis of native cartilage tissue and balancing proteoglycan synthesis and breakdown.
Overall, with minimal differences in cartilage repair at 12 weeks it is difficult to identify a correlation between early bone repair and improved cartilage repair at 12 weeks. Additionally, the delivery of IGF-1 together with BMP-2 from bilayered OPF hydrogel composites did not improve cartilage repair over either growth factor alone. This could be due to the different layers from which IGF-1 and BMP-2 were delivered. Previous in vitro research has demonstrated the synergistic effects of BMP-2 and IGF-1 on chondrocytes seeded in agarose gels in increasing aggregate modulus and GAG production over either growth factor alone [356]. However, in a porous silk scaffold with reverse gradients of BMP-2 and IGF-1, seeded human MSCs exhibited chondrogenic and osteogenic differentiation along the BMP-2 gradient, with the greatest changes in human MSC differentiation occurring in the region with the highest BMP-2 concentration [362]. In contrast, cells in the region with the highest IGF-1 and lowest BMP-2 concentration retained MSC morphology (spindle-shaped). With this in mind, the improved subchondral bone repair with IGF-1 and BMP-2 and the lack of statistical differences in cartilage repair between experimental groups suggests that the BMP-2 delivered from the subchondral layer of bilayered OPF composites had a greater direct effect on bone repair than on cartilage repair.

6.5. Conclusion

IGF-1 and BMP-2 were loaded into GMPs in the chondral layer and subchondral layer, respectively, of bilayered OPF hydrogel composites either separately or together.
These three formulations were tested for osteochondral repair in a rabbit model at 6 and 12 weeks. While histological scoring showed no differences in cartilage and bone repair at 6 weeks, micro-CT revealed that the single delivery of BMP-2 increased bone specific surface and the number of bony trabecular islets over the single delivery of IGF-1. At 12 weeks, micro-CT also showed an increase in bone mineral density, percent bone volume, and intersection surface, and a decrease in bone specific surface for the dual delivery of IGF-1 and BMP-2 over the single delivery of IGF-1. However, there was no significant difference between the three groups on cartilage morphology at 12 weeks. These results demonstrate that the delivery of BMP-2 can accelerate bone formation at an early time point and that the delivery of both growth factors in separate layers has a synergistic effect on subchondral bone repair, but not on cartilage repair, in an osteochondral defect. Finally, the outcomes of this study can help inspire future spatially-controlled, multiple growth factor delivery strategies for osteochondral tissue regeneration.
6.6. Tables and Figures

Figure 6.1 Representative histological sections of osteochondral tissue repair after 6 weeks of implantation for all three groups. Sections were stained with hematoxylin and eosin (H&E) (A-C), van Gieson's Picrofuchsin (VGP) (D-F), or Safranin O/Fast Green (Saf.O) (G-I). All 6 week samples had incomplete degradation of the implant, evidenced by a light gray staining of the hydrogel surrounded by a yellow fibrous capsule in the VGP stains. The neo-cartilage formed was generally thicker than the surrounding cartilage with intense Saf.O staining and was present in the subchondral region. (scale bar: 1000 μm)
Figure 6.2 Representative histological sections of osteochondral tissue repair after 12 weeks of implantation for all three groups. Sections were stained with hematoxylin and eosin (H&E) (A-C), van Gieson's Picrofuchsin (VGP) (D-F), or Safranin O/Fast Green (Saf.O) (G-I). 12 week samples generally had greater degradation than 6 week samples and a smaller fibrous capsule. Group IGF-1 had the most sections with fibrous tissue (D,G), group BMP-2 had the most sections with fibrocartilage (E,H), and group Both had the most sections with articular cartilage (F,I). (scale bar: 1000μm)
Figure 6.3 Histological scores for the overall defect evaluation (A), subchondral bone evaluation (B), and articulating cartilage evaluation (C). Data are shown as average scores with error bars representing standard deviation. (*) indicates a significant difference between groups regardless of time point and (&) indicates a significant difference between a 12 week group and all 6 week groups (p < 0.05).
Figure 6.4 Distribution of histological scores for subchondral bone morphology (A), cartilage morphology (B), and cartilage thickness (C) for the three groups investigated at both 6 and 12 weeks.
Figure 6.5 Micro-CT images showing a transverse section of the osteochondral defect in a medial femoral condyle (A), sections of a 6 week Both sample in the cortical (B) and trabecular (C) region, and sections of a 12 week BMP-2 sample in the cortical (D) and trabecular (E) region. (A) also demonstrates the two volumes of interest used, cartilage and cortical (C&C) and trabecular, when analyzing the subchondral bone. (scale bar: 3 mm)
Figure 6.6 Morphological parameter analysis for both trabecular and cartilage and cortical (C&C) volumes of interest (VOI). Bone mineral density (A), percent bone volume (B), intersection surface (C), bone specific surface (D), and object number (E) were measured for all groups at both time points. Data are shown as average scores with error bars representing standard deviation. (*) indicates a significant difference between groups regardless of time point, (&) indicates a significant difference between a 12 week group and all 6 week groups, and (#) indicates a significant difference between a 6 week group and all 12 week groups (p < 0.05).
Table 6.1 Experimental groups investigated in this study. Growth factors were loaded into gelatin microparticles prior to encapsulation within bilayered OPF hydrogels.

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>IGF-1</th>
<th>BMP-2</th>
<th>Both</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chondral Layer (Top 1mm)</td>
<td>IGF-1</td>
<td>Blank</td>
<td>IGF-1</td>
</tr>
<tr>
<td>Subchondral Layer (Bottom 2mm)</td>
<td>Blank</td>
<td>BMP-2</td>
<td>BMP-2</td>
</tr>
<tr>
<td>6 Week Repetitions</td>
<td>12</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>12 Week Repetitions</td>
<td>12</td>
<td>11</td>
<td>12</td>
</tr>
</tbody>
</table>
Table 6.2 Histological scoring system for the evaluation of (a) overall tissue, (b) subchondral bone, and (c) cartilage in rabbit osteochondral defects.

<table>
<thead>
<tr>
<th>Description</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(a) Overall defect evaluation (throughout the entire defect depth)</strong></td>
<td></td>
</tr>
<tr>
<td>1. Percent filling with newly formed tissue</td>
<td></td>
</tr>
<tr>
<td>100%</td>
<td>3</td>
</tr>
<tr>
<td>&gt;50%</td>
<td>2</td>
</tr>
<tr>
<td>&lt;50%</td>
<td>1</td>
</tr>
<tr>
<td>0%</td>
<td>0</td>
</tr>
<tr>
<td>2. Percent degradation of the implant</td>
<td></td>
</tr>
<tr>
<td>100%</td>
<td>3</td>
</tr>
<tr>
<td>&gt;50%</td>
<td>2</td>
</tr>
<tr>
<td>&lt;50%</td>
<td>1</td>
</tr>
<tr>
<td>0%</td>
<td>0</td>
</tr>
<tr>
<td><strong>(b) Subchondral bone evaluation (within the bottom 2 mm of defect)</strong></td>
<td></td>
</tr>
<tr>
<td>3. Percent filling with newly formed tissue</td>
<td></td>
</tr>
<tr>
<td>100%</td>
<td>3</td>
</tr>
<tr>
<td>&gt;50%</td>
<td>2</td>
</tr>
<tr>
<td>&lt;50%</td>
<td>1</td>
</tr>
<tr>
<td>0%</td>
<td>0</td>
</tr>
<tr>
<td>4. Subchondral bone morphology</td>
<td></td>
</tr>
<tr>
<td>Normal, trabecular bone</td>
<td>4</td>
</tr>
<tr>
<td>Trabecular bone, with some compact bone</td>
<td>3</td>
</tr>
<tr>
<td>Compact bone</td>
<td>2</td>
</tr>
<tr>
<td>Compact bone and fibrous tissue</td>
<td>1</td>
</tr>
<tr>
<td>Only fibrous tissue or no tissue</td>
<td>0</td>
</tr>
<tr>
<td>5. Extent of new tissue bonding with adjacent bone</td>
<td></td>
</tr>
<tr>
<td>Complete on both edges</td>
<td>3</td>
</tr>
<tr>
<td>Complete on one edge</td>
<td>2</td>
</tr>
<tr>
<td>Partial on both edges</td>
<td>1</td>
</tr>
<tr>
<td>Without continuity on either edge</td>
<td>0</td>
</tr>
<tr>
<td><strong>(c) Cartilage evaluation (within the upper 1 mm of defect)</strong></td>
<td></td>
</tr>
<tr>
<td>6. Morphology of newly formed surface tissue</td>
<td></td>
</tr>
<tr>
<td>Exclusively articular cartilage</td>
<td>4</td>
</tr>
<tr>
<td>Mainly hyaline cartilage</td>
<td>3</td>
</tr>
<tr>
<td>Fibrocartilage (spherical morphology observed with $\geq 75%$ of cells)</td>
<td>2</td>
</tr>
<tr>
<td>Only fibrous cartilage (spherical morphology observed with $&lt; 75%$ of cells)</td>
<td>1</td>
</tr>
<tr>
<td>No tissue</td>
<td>0</td>
</tr>
</tbody>
</table>
7. Thickness of newly formed cartilage
   - Similar to the surrounding cartilage: 3
   - Greater than the surrounding cartilage: 2
   - Less than the surrounding cartilage: 1
   - No cartilage: 0

8. Joint surface regularity
   - Smooth, intact surface: 3
   - Surface fissures (<25% of new surface thickness): 2
   - Deep fissures (≥25% of new surface thickness): 1
   - Complete disruption of the new surface: 0

9. Chondrocyte clustering
   - None at all: 3
   - <25% chondrocytes: 2
   - 25-100% chondrocytes: 1
   - No chondrocytes present (no cartilage): 0

10. Chondrocyte and GAG content of new cartilage
    - Normal cellularity with normal Safranin O staining: 3
    - Normal cellularity with moderate Safranin O staining: 2
    - Clearly less cells with poor Safranin O staining: 1
    - Few cells with no or little Safranin O staining or no cartilage: 0

11. Chondrocyte and GAG content of adjacent cartilage
    - Normal cellularity with normal Safranin O staining: 3
    - Normal cellularity with moderate Safranin O staining: 2
    - Clearly less cells with poor Safranin O staining: 1
    - Few cells with no or little Safranin O staining or no cartilage: 0
Table 6.3 Explanation of 3D morphometric parameters analyzed within two volumes of interest (VOI): the cartilage and cortical region and the trabecular region.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone mineral density (g/cm³) BMD Volumetric density of bone within a mixed bone-soft tissue VOI</td>
<td></td>
</tr>
<tr>
<td>Percent bone volume (%) BV/TV Proportion of VOI occupied by binarised solid objects (e.g., bone)</td>
<td></td>
</tr>
<tr>
<td>Intersection surface (mm²) i.S. Surface of VOI intersected by bone, useful for evaluating bone growth at a defined boundary</td>
<td></td>
</tr>
<tr>
<td>Bone specific surface (mm⁻¹) BS/BV Ratio of bone surface to bone volume within the VOI, useful for characterizing the relative complexity of structures</td>
<td></td>
</tr>
<tr>
<td>Object number Obj.N. Total number of discreet binarised objects within the VOI, where a discreet object is a connected cluster of solid (white) voxels fully surrounded by space (black) voxels</td>
<td></td>
</tr>
</tbody>
</table>
Table 6.4 Main effects analysis of time point (week 6 vs. week 12) for histological scoring and micro-CT morphometric parameters (p < 0.05).

<table>
<thead>
<tr>
<th>Histological scoring parameters</th>
<th>Micro-CT morphometric parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall Filling 12wk &gt; 6wk</td>
<td>Bone Mineral Density 12wk &gt; 6wk</td>
</tr>
<tr>
<td>Overall Degradation 12wk &gt; 6wk</td>
<td>Percent Bone Volume 12wk &gt; 6wk</td>
</tr>
<tr>
<td>Bone Filling 12wk &gt; 6wk</td>
<td>Intersection Surface 12wk &gt; 6wk</td>
</tr>
<tr>
<td>Bone Morphology 12wk &gt; 6wk</td>
<td>Bone Specific Surface 6wk &gt; 12wk</td>
</tr>
<tr>
<td>Bone Bonding --</td>
<td>Object Number 6wk &gt; 12wk</td>
</tr>
<tr>
<td>Cartilage Morphology --</td>
<td></td>
</tr>
<tr>
<td>Cartilage Thickness 6wk &gt; 12wk</td>
<td></td>
</tr>
<tr>
<td>Surface Regularity --</td>
<td></td>
</tr>
<tr>
<td>Chondrocyte Clustering --</td>
<td></td>
</tr>
<tr>
<td>Neocartilage GAG &amp; Cell 6wk &gt; 12wk</td>
<td></td>
</tr>
<tr>
<td>Adjacent GAG &amp; Cell 12wk &gt; 6wk</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 7

Technical Report: Correlation between the repair of cartilage and subchondral bone in an osteochondral defect using bilayered, biodegradable hydrogel composites*

The present work investigated correlations between cartilage and subchondral bone repair, facilitated by a growth factor-delivering scaffold, in a rabbit osteochondral defect model. Histological scoring indices and micro-computed tomography morphological parameters were used to evaluate cartilage and bone repair, respectively, at 6 and 12 weeks. Correlation analysis revealed significant associations between specific cartilage indices and subchondral bone parameters that varied with location in the defect.

(cortical vs trabecular region), time point (6 vs 12 weeks), and experimental group (insulin-like growth factor-1 only, bone morphogenetic protein-2 only, or both growth factors). In particular, significant correlations consistently existed between cartilage surface regularity and bone quantity parameters. Overall, correlation analysis between cartilage and bone repair provided a fuller understanding of osteochondral repair and can help drive informed studies for future osteochondral regeneration strategies.
7.1. Introduction

The regeneration of osteochondral tissue remains a difficult challenge due to the inability for articular cartilage to self-regenerate and the need to consider the multiphasic nature of the osteochondral unit.[328, 329] Although the osteochondral unit comprises different tissues, cartilage and bone, homeostasis is predicated by an innate biochemical and biomechanical interplay between these two tissue types.[331, 332, 334, 363, 364] There is an increasing body of knowledge in the fields of osteoarthritis and osteochondral tissue engineering on the importance of the subchondral bone on the pathogenesis, as well as regeneration, of the articulating cartilage.[333, 365-370] Yet, while many studies have examined the associated structural changes of subchondral bone with changes in cartilage tissue morphology in an osteoarthritic model,[300, 371-373] less is known about how the repair of subchondral bone and its associated structural changes are related to the repair of articular cartilage in an osteochondral defect.

Previously, Qiu et al.[374] and Orth et al.[354] investigated the healing of subchondral bone and articular cartilage in an empty rabbit osteochondral defect. In particular, it was found that there was no significant correlation between upward subchondral bone migration and cartilage degeneration over time.[354] Additionally, Zhang et al. characterized cartilage and bone repair using a biphasic hydrogel/ceramic scaffold in a critical-sized rabbit osteochondral defect in a year-long study and saw that the gross appearance of cartilage positively correlated with subchondral bone volume.[375] However, the rise of tissue engineering strategies for osteochondral repair necessitates further research on the associated changes in bone and cartilage repair, especially when facilitated by an implanted biomaterial scaffold. To determine these
associations, correlation modeling offers a statistical analysis for these relationships. By understanding how specific morphological parameters for bone and cartilage are correlated, valuable information can be gathered on the repair process.

As a result, the current report utilized growth factor-delivering, bilayered hydrogel composites to facilitate osteochondral repair in a rabbit model. Specifically, the hydrogel composites were based on the synthetic macromer oligo(poly(ethylene glycol) fumarate) (OPF) and contained gelatin microparticles (GMPs) as drug delivery vehicles. Insulin-like growth factor-1 (IGF-1) and bone morphogenetic protein-2 (BMP-2) were delivered separately or together to facilitate tissue formation. In a previous publication, the effect of growth factor treatment option on osteochondral repair was presented as a comparison of means between experimental groups, as is typically done in an outcome-based analysis.[376] In this technical report, correlation analysis is used on the same raw data to present direct associations between cartilage and bone repair. While causality cannot be determined through correlation analysis (e.g., better bone repair causes better cartilage repair), specific relationships between cartilage and bone parameters can be discovered. In turn, these specific relationships can be the foundation for future mechanistic studies investigating cause-and-effect.

Thus, the main objective of this report was to examine correlations between cartilage indices as determined by histological scoring and subchondral bone parameters as determined by micro-computed tomography (micro-CT). Additionally, correlations between cartilage and bone repair were compared at 6 and 12 week time points, and the influence of growth factor delivery treatment on trends in tissue repair was investigated.
7.2. Materials and Methods

7.2.1. Experimental study design

The materials and methods used for the current report have previously been published and can be found in Chapter 5. Briefly, dried GMPs (50-100 µm in diameter) were loaded with a growth factor solution, IGF-1 or BMP-2, and encapsulated in bilayered OPF hydrogel composites. Three different growth factor-loaded GMP combinations were encapsulated in bilayered hydrogel composites: 1) IGF-1 only in the chondral layer, 2) BMP-2 only in the subchondral layer, or 3) IGF-1 and BMP-2 in the chondral and subchondral layer, respectively. The hydrogel composites were then implanted in skeletally mature New Zealand White rabbits using a medial femoral condyle osteochondral defect model (3 mm in diameter and 3 mm in thickness). A total of 36 rabbits were used, with each rabbit receiving bilateral implants and 10-12 hydrogels for each group at each time point (6 or 12 weeks).

Following 6 or 12 weeks, rabbits were euthanized and condyle samples including the defect site underwent micro-CT imaging and histological preparation. Due to the presence of two distinct bone morphologies in the subchondral bone, two volumes of interest (VOIs) were analyzed separately for the bone plate and the trabecular bone. The upper 0.85 mm of the defect was selected as the cartilage and cortical region (C&C) and the lower 2.15 mm was selected as the trabecular region. The cartilage region was included with the cortical region to cover all possible neo-bone formation as well as due to the potential for upward subchondral bone plate migration during osteochondral repair. Morphological parameters analyzed for both the C&C and trabecular VOI
include bone mineral density (BMD), percent bone volume (BV/TV), intersection surface (i.S), bone specific surface (BS/BV), and object number (Obj.N). Additional parameters analyzed for the trabecular VOI include trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular separation (Tb.Sp). Each of these parameters are further explained in Supplementary Table 7.1. For histology, sections from the center and peripheral edges were stained with hematoxylin and eosin, Safranin O/Fast Green, and van Gieson’s Picrofuchsin. 1 center and 2 opposing peripheral histological sections were then blindly and independently scored by three evaluators (J.L., J.T., F.K.K.) using a previously established histological scoring system for osteochondral defects as seen in Supplementary Table 7.2.[171, 376, 377]

7.2.2. Statistical analysis

A significance level of $p < 0.05$ was used for all statistical analysis through JMP Pro v11.0.0. To determine the presence and strength of correlation between key micro-CT parameters and histological scoring indices, Spearman’s rank correlation test was used due to the non-parametric and ordinal nature of the histological scoring data sets. For each sample, three histological sections were scored (1 center and 2 peripheral) for each of the six cartilage indices. The three histological scores were then averaged for each cartilage index ($n = 70$), and the mean scores for each cartilage index were compared with the associated micro-CT measurements for both C&C ($n = 70$) and trabecular VOIs ($n = 70$). As a result, correlation analysis was performed between 6 cartilage scores and 5 (C&C) or 8 (trabecular) bone morphological measurements, at each time point ($n = 34$ or 36), and for each growth factor group ($n = 10$ or 12). Correlation
coefficients with a significance level of $p < 0.05$ were graded on a scale from .00 to |1.00| as seen in Table 7.1.[378, 379]

7.3. Results

7.3.1. Correlations between micro-CT and histological scoring parameters for all samples

Except for cartilage thickness, all other histological scoring parameters had significant correlations with one or more micro-CT parameters as seen in Table 7.2. Cartilage morphology had a weak correlation with BMD and Tb.Sp in the trabecular VOI. Additionally, neocartilage cell & glycosaminoglycan (GAG) content had a weak correlation with Obj.N in the C&C VOI. Notably, cartilage regularity exhibited moderate correlations with a $p < 0.0001$ with BMD, BV/TV, and i.S in the trabecular VOI.

7.3.2. Correlations between micro-CT and histological scoring parameters for 6 and 12 week samples

In the C&C VOI, differences in correlations among 6 and 12 week samples could be seen (Figure 7.1). Significant 6 week correlations existed between micro-CT parameters BMD, BV/TV, i.S, and BS/BV and histological scoring parameters cartilage morphology and cartilage regularity. In particular, cartilage morphology had a moderate correlation with BMD, and cartilage regularity had moderate correlations with BMD,
BV/TV, and BS/BV (p < 0.001). At 12 weeks, neocartilage cell & GAG, had weak correlations with all micro-CT parameters, except for Obj.N, and chondrocyte clustering had a moderate correlation with Obj.N.

The trabecular VOI displayed similar differences with the C&C VOI in correlations among 6 and 12 weeks samples, with more significant correlations present at 6 weeks compared to 12 weeks (Figure 7.2). At 6 weeks, cartilage morphology and regularity had moderate correlations with BMD and BV/TV, with cartilage regularity also having a significant correlation with i.S. Trabecular-specific parameters, Tb.N and Tb.Sp, were seen to have significant associations with cartilage morphology, cartilage regularity, and neocartilage cell & GAG. At 12 weeks, cartilage morphology exhibited a significant correlation with BMD, and cartilage regularity was moderately correlated with BMD, BV/TV, and i.S. (p < 0.01). This trend can also be seen in Figure 7.3 and Figure 7.4 where the regularity of the neo-cartilage surface is even with the adjacent cartilage in the presence of repaired trabecular bone (Figure 7.4), and a depression in the neo-cartilage surface is seen with minimal subchondral bone repair (Figure 7.3). Chondrocyte clustering was also weakly correlated with BS/BV and Tb.Th, and similar to the C&C VOI, was moderately correlated with Obj.N at 12 weeks.

7.3.3. Correlations between micro-CT and histological scoring parameters for each experimental group

At 6 weeks in the C&C VOI, differences in correlations among the three experimental groups are seen (Table 7.3). In general, histological scoring indices had positive correlations with BMD, BV/TV, i.S, and Obj.N and negative correlations with
BS/BV. Interestingly, there were no correlations between cartilage indices and bone parameters for the group delivering IGF-1 only. On the other hand, associations were seen only in BMP-2 and Both groups. Cartilage morphology, cartilage regularity, and neocartilage cell & GAG had significant correlations with BMD, BV/TV, i.S, and BS/BV for groups with BMP-2 only. Additionally, group Both had significant correlations between cartilage regularity and BMD, BV/TV, i.S, and BS/BV. Adjacent cell & GAG also had significant correlations with BS/BV and Obj.N in the Both group. At 12 weeks in the C&C VOI, most correlations occurred in the IGF-1 and BMP-2 groups with the exception of a positive correlation between cartilage morphology and i.S in the Both group (p < 0.01) (Table 7.4). Additionally, there existed strong negative correlations with a p < 0.01 between neocartilage cell & GAG and BMD, BV/TV, and i.S in the BMP-2 group.

At 6 weeks in the trabecular VOI, strong correlations between cartilage scores and micro-CT parameters existed predominantly in the BMP-2 group, similar to the C&C VOI at 6 weeks (Table 7.5). In particular, cartilage morphology, cartilage regularity, and neocartilage cell & GAG were strongly correlated with BMD, BV/TV, and Tb.N in the BMP-2 group (p < 0.01). Cartilage regularity was also strongly correlated with i.S and Tb.Sp in the BMP group. At 12 weeks in the trabecular VOI, strong correlations are seen between cartilage regularity and BMD, B/TV, and i.S. for IGF-1 and Both groups (Table 7.6). Additionally, adjacent cell & GAG was strongly negatively correlated with Tb.Sp in the Both group.
7.4. Discussion

The primary objective of this report was to examine correlations between the repair of articular cartilage and the repair of subchondral bone in an osteochondral defect, facilitated by a hydrogel composite scaffold. Osteochondral repair was evaluated at 6 and 12 weeks with histological scoring indices for cartilage evaluation and micro-CT structural parameters for subchondral bone repair. Specifically, we investigated 1) if the degree of cartilage repair could be associated with the degree of subchondral bone repair through correlation analysis, 2) whether the correlations between cartilage and bone would change from an early to a later time point, and 3) if the delivery of different growth factor combinations would impact trends between cartilage and bone repair.

When all samples at both time points were analyzed, significant correlations were found between cartilage histological scoring indices and subchondral bone micro-CT parameters. Although most correlations were negligible to weak in strength (< |.50|), cartilage surface regularity was moderately correlated with bone volume and bone formation at the defect edges in the trabecular region (p < 0.0001). This can be seen in histological images where cartilage depressions were seen in samples with low bone formation (Figure 7.3), and smooth, intact surfaces were seen with greater bone repair (Figure 7.4). Indeed, the subchondral bone provides a mechanical support for the overlying cartilage and without sufficient bone formation, fissures can develop in the surface cartilage.[349]

When this association was examined separately at 6 and 12 weeks, moderate correlations still existed between surface regularity and bone formation parameters in the trabecular region (p < 0.01). However, similar correlations were only present in the C&C
region at 6 weeks. Additionally, significant correlations were present between bone formation parameters and cartilage morphology and regularity in the C&C region at 6 weeks, but not at 12 weeks. These changes in correlations from an early to a later time point may be a result of the different rates at which bone and cartilage remodel, as has been seen previously.[354, 380] Indeed, the more rapid appearance of skeletal changes due to abnormal mechanical loading on cartilage highlights the differential adaptive capacity and metabolic activity between bone and cartilage.[300]

Interestingly, cartilage thickness did not correlate with any bone parameters, possibly indicating that the thickness of neo-cartilage tissue was not related to the volume of bone formed or the extent of bone remodeling. However, thinning of neo-cartilage has previously been seen to be affected by thickening and upward migration of the subchondral bone plate.[369, 381] Additionally, thinning of repaired cartilage was seen at 16 weeks, but not 8 weeks, in the healing of an empty osteochondral defect.[374] While the current study saw only two samples with the presence of the subchondral bone plate above the tidemark of the surrounding tissue but below the surface of the articulating cartilage at 12 weeks,[376] further study with longer time points may see more associations of cartilage thickness with subchondral bone parameters.

Correlation analysis for each experimental group at each time point was also performed. While the sample size for these analyses were low (n=10-12), prudent interpretation of these statistically significant results is still relevant. Additionally, greater credence is placed on correlations with a significance level of p < 0.01. However, future studies with larger sample sizes would be needed to confirm the significant correlations, rule out false positives, or find false negatives. In the C&C VOI at 6 weeks, correlations
only existed in the two groups that delivered BMP-2 (i.e., BMP-2 only and both IGF-1 and BMP-2). Group BMP-2 also predominantly had strong correlations between bone parameters and cartilage morphology, regularity, and cell & GAG content in the trabecular VOI at 6 weeks. These strong associations could be attributed to the potent osteoconductive effects of BMP-2 on early bone formation.[349, 376, 382]

On the other hand, in the trabecular VOI at 12 weeks, strong positive correlations were seen between cartilage regularity and bone formation for the two groups delivering IGF-1. Indeed, IGF-1 has been shown to decrease synovial inflammation in comparison to growth factor-free controls when delivered with chondrocytes in an equine model.[383] IGF-1 can also increase proteoglycan synthesis and slow proteoglycan catabolism in a dose-dependent manner.[218, 384, 385] This is corroborated by the strong negative correlations between neocartilage cell & GAG content and bone formation at 12 weeks in the C&C group for the BMP-2 only delivering group. Interestingly, this chondroprotective quality of IGF-1 is not clearly apparent in the outcome-based publication since a comparison of means for cartilage scoring indices revealed no differences between experimental groups at either time point.[376]

Overall, correlation analysis gave valuable insight in the repair process of osteochondral tissue and can be a useful tool in supplementing traditional outcome-based analysis. In the present report, moderate to strong correlations were consistently seen between cartilage regularity and bone formation parameters, especially in the trabecular region. By simply comparing the means of cartilage and bone repair data sets as performed in a previous publication,[22] this unique relationship could not have been construed. Although causality could not be determined with correlation analysis in the
present report (e.g., greater bone formation in the trabecular region improves cartilage regularity), knowing specific correlations can be a preliminary step in inspiring informed studies for future osteochondral repair strategies. For example, scaffold stiffness in the subchondral bone region could be modulated to examine its effects on cartilage regularity. In addition, whether or not early bone repair improves cartilage repair could be directly tested by using minimally invasive techniques for examining bone and cartilage repair, and subsequently performing correlation and/or regression analysis on early and late time point measurements.

7.5. Conclusion

The repair of articular cartilage and subchondral bone, facilitated by a growth factor delivering hydrogel composite scaffold, was evaluated in an osteochondral defect at 6 and 12 weeks. Associations between specific cartilage indices and bone parameters were tested through correlations analysis. Overall, there were significant associations between the repair of cartilage and the repair of subchondral bone. Significant correlations were consistently seen between cartilage surface regularity and bone quantity parameters, which may confirm the role of mechanical support the subchondral bone plays for the overlying articular cartilage. Bone and cartilage correlations also changed from 6 weeks to 12 weeks of repair, an indication of the different rates at which cartilage and bone repair and remodel. Additionally, the three experimental groups had different trends in correlations, revealing stronger correlations between cartilage repair and bone
formation at an earlier time point when BMP-2 was involved, and suggesting the chondroprotective nature of IGF-1 at a later time point, highlighting the differing effects of IGF-1 and BMP-2 on osteochondral repair. Altogether, the repair of cartilage is associated with the repair of subchondral bone for specific structural parameters, and through correlation analysis, a complete understanding of the repair of both tissues can help drive informed studies for future osteochondral repair strategies.
7.6. Tables and Figures

Figure 7.1 Scatterplot matrix showing density ellipses for 95% of the data between cartilage histological scoring indices and bone morphological parameters in the cartilage and cortical VOI. Density ellipses were computed from a bivariate normal distribution fit to the histological scoring and micro-CT measurements and is a function of the means and standard deviations of the variables, as well as the correlation between them. 6 week samples are represented by blue ellipses and 12 week samples are represented by red ellipses. Significant correlations between cartilage and bone parameters are indicated by bolded outlines (p < 0.05), where n = 34 for 6 week correlations and n = 36 for 12 weeks correlations.
Figure 7.2 Scatterplot matrix showing density ellipses for 95% of the data between cartilage histological scoring indices and bone morphological parameters in the trabecular VOI. Density ellipses were computed from a bivariate normal distribution fit to the histological scoring and micro-CT measurements and is a function of the means and standard deviations of the variables, as well as the correlation between them. 6 week samples are represented by blue ellipses and 12 week samples are represented by red ellipses. Significant correlations between cartilage and bone parameters are indicated by bolded outlines (p < 0.05), where n = 34 for 6 week correlations and n = 36 for 12 weeks correlations.
Figure 7.3 Representative histological section with Safranin O/Fast Green staining of osteochondral tissue repair after 12 weeks of implantation (A). (B) and (C) are representative transverse micro-CT images of the bone plate and trabecular bone, respectively. This sample from the IGF-1 only group demonstrated minimal bone formation in both cortical and trabecular regions and had a depressed cartilage surface. (scale bar: 1000 µm)
Figure 7.4 Representative histological section with Safranin O/Fast Green staining of osteochondral tissue repair after 12 weeks of implantation (A). (B) and (C) are representative transverse micro-CT images of the bone plate and trabecular bone, respectively. This sample from the Both group had well-remodeled trabecular structures even with the presence of remaining hydrogel. Additionally, the bone plate has formed and the cartilage surface is regular. (scale bar: 1000 µm)
Table 7.1 Interpretation of correlation coefficient

<table>
<thead>
<tr>
<th>Correlation Coefficient Value</th>
<th>Strength of Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>.00 to</td>
<td>.30</td>
</tr>
<tr>
<td></td>
<td>.30</td>
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<tr>
<td></td>
<td>.50</td>
</tr>
<tr>
<td></td>
<td>.70</td>
</tr>
<tr>
<td></td>
<td>.90</td>
</tr>
</tbody>
</table>

Note: The sign of the correlation coefficient (i.e., positive or negative) indicates the direction of the relationship.
Table 7.2 Correlations between micro-CT morphological and histological scoring parameters for all samples and both VOIs.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
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</tr>
</thead>
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<tr>
<td>Cartilage Morphology</td>
<td>.30</td>
<td>.33</td>
<td>.25</td>
<td>.27</td>
<td>.27</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cartilage Thickness</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cartilage Regularity</td>
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<td>.56</td>
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<td>.50</td>
<td>.33</td>
<td>.57</td>
<td>-.35</td>
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</tr>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Neocartilage Cell &amp; GAG</td>
<td>-.27</td>
<td>-.26</td>
<td>.28</td>
<td>.30</td>
<td>-</td>
<td>-.30</td>
<td>-</td>
<td>-</td>
</tr>
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<td>.30</td>
<td>.27</td>
<td>-.31</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Highlighted cells represent $p < 0.0001$

** Blank cells represent non-significant correlations ($p > 0.05$)

*** $n = 70$
Table 7.3 Cartilage and Cortical VOI, 6 week correlation between micro-CT morphological and histological scoring parameters for each experimental group.

<table>
<thead>
<tr>
<th></th>
<th>BMD</th>
<th>BV/TV</th>
<th>i.S</th>
<th>BS/BV</th>
<th>Obj.N</th>
</tr>
</thead>
<tbody>
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<td>BMP</td>
<td>Both</td>
<td>IGF</td>
<td>BMP</td>
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<td>Cartilage Thickness</td>
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<td>-.71</td>
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</tr>
<tr>
<td>Cartilage Regularity</td>
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<td>.70</td>
<td>.73</td>
<td>.80</td>
<td>.64</td>
</tr>
<tr>
<td>Neocartilage Cell &amp; GAG</td>
<td>.67</td>
<td>.70</td>
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<td></td>
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</tr>
<tr>
<td>Adjacent Cell &amp; GAG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Highlighted cells represent p < 0.01
** Blank cells represent non-significant correlations (p > 0.05)
*** n = 10-12
Table 7.4 Cartilage and Cortical VOI, 12 week correlation between micro-CT morphological and histological scoring parameters for each experimental group.

<table>
<thead>
<tr>
<th></th>
<th>BMD</th>
<th>BV/TV</th>
<th>i.S</th>
<th>BS/BV</th>
<th>Obj.N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IGF</td>
<td>BMP</td>
<td>Both</td>
<td>IGF</td>
<td>BMP</td>
</tr>
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<td>Cartilage Morphology</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cartilage Thickness</td>
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<td></td>
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<td>-.81</td>
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<td>Adjacent Cell &amp; GAG</td>
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</tbody>
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* Highlighted cells represent p < 0.01
** Blank cells represent non-significant correlations (p > 0.05)
*** n = 12
Table 7.5 Trabecular VOI, 6 week correlation between micro-CT morphological and histological scoring parameters for each experimental group.

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<tbody>
<tr>
<td></td>
<td>IGF</td>
<td>BMP</td>
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<td>IGF</td>
<td>BMP</td>
<td>Both</td>
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<td>BMP</td>
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<td>Cartilage Regularity</td>
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<td></td>
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<tr>
<td>Neocartilage Cell &amp; GAG</td>
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<td>.79</td>
<td>.79</td>
<td>.87</td>
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<td></td>
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<td></td>
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<tr>
<td>Adjacent Cell &amp; GAG</td>
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<td>.68</td>
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</tbody>
</table>

* Highlighted cells represent p < 0.01
** Blank cells represent non-significant correlations (p > 0.05)
*** n = 10-12
Table 7.6 Trabecular VOI, 12 week correlation between micro-CT morphological and histological scoring parameters for each experimental group.

<table>
<thead>
<tr>
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<td>Cartilage Thickness</td>
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<td>.69</td>
<td>.81</td>
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<td></td>
</tr>
<tr>
<td>Neocartilage Cell &amp; GAG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adjacent Cell &amp; GAG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Highlighted cells represent p < 0.01
** Blank cells represent non-significant correlations (p > 0.05)
*** n = 12
Supplementary Table 7.1 Explanation of the micro-computed tomography morphometric parameters analyzed within the two volumes of interest (VOI).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bone mineral density</strong></td>
<td>Volumetric density of bone within a mixed bone-soft tissue VOI</td>
</tr>
<tr>
<td>(g/cm³) BMD</td>
<td></td>
</tr>
<tr>
<td><strong>Percent bone volume</strong></td>
<td>Proportion of VOI occupied by binarised solid objects (e.g., bone)</td>
</tr>
<tr>
<td>(%) BV/TV</td>
<td></td>
</tr>
<tr>
<td><strong>Intersection surface</strong></td>
<td>Surface of VOI intersected by bone, useful for evaluating bone growth at a defined boundary</td>
</tr>
<tr>
<td>(mm²) i.S</td>
<td></td>
</tr>
<tr>
<td><strong>Bone specific surface</strong></td>
<td>Ratio of bone surface to bone volume within the VOI, useful for characterizing the relative complexity of structures</td>
</tr>
<tr>
<td>(mm⁻¹) BS/BV</td>
<td></td>
</tr>
<tr>
<td><strong>Object number</strong></td>
<td>Total number of discreet binarised objects within the VOI, where a discreet object is a connected cluster of solid (white) voxels fully surrounded by space (black) voxels</td>
</tr>
<tr>
<td>Obj.N</td>
<td></td>
</tr>
<tr>
<td><strong>Trabecular thickness</strong></td>
<td>True 3D thickness as determined by an average of the local thickness at each point representing bone</td>
</tr>
<tr>
<td>(µm) Tb.Th</td>
<td></td>
</tr>
<tr>
<td><strong>Trabecular number</strong></td>
<td>Implication of the number of traversals across a trabecular or solid structure made per unit length on a random linear path through the VOI</td>
</tr>
<tr>
<td>(mm⁻¹) Tb.N</td>
<td></td>
</tr>
<tr>
<td><strong>Trabecular separation</strong></td>
<td>True 3D thickness as determined by an average of the local thickness at each point representing non-bone</td>
</tr>
<tr>
<td>(µm) Tb.Sp</td>
<td></td>
</tr>
</tbody>
</table>
Supplementary Table 7.2 Histological scoring system for the evaluation of cartilage in rabbit osteochondral defects.

<table>
<thead>
<tr>
<th>Score</th>
<th>Cartilage evaluation (within the upper 1 mm of defect)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.</td>
<td>Chondrocyte and GAG content of adjacent cartilage</td>
</tr>
<tr>
<td></td>
<td>Normal cellularity with normal Safranin O staining</td>
</tr>
<tr>
<td></td>
<td>Normal cellularity with moderate Safranin O staining</td>
</tr>
<tr>
<td></td>
<td>Clearly less cells with poor Safranin O staining</td>
</tr>
<tr>
<td></td>
<td>Few cells with no or little Safranin O staining or no cartilage</td>
</tr>
<tr>
<td>5.</td>
<td>Chondrocyte and GAG content of new cartilage</td>
</tr>
<tr>
<td></td>
<td>Normal cellularity with normal Safranin O staining</td>
</tr>
<tr>
<td></td>
<td>Normal cellularity with moderate Safranin O staining</td>
</tr>
<tr>
<td></td>
<td>Clearly less cells with poor Safranin O staining</td>
</tr>
<tr>
<td></td>
<td>Few cells with no or little Safranin O staining or no cartilage</td>
</tr>
<tr>
<td>4.</td>
<td>Chondrocyte clustering</td>
</tr>
<tr>
<td></td>
<td>None at all</td>
</tr>
<tr>
<td></td>
<td>&lt;25% chondrocytes</td>
</tr>
<tr>
<td></td>
<td>25-100% chondrocytes</td>
</tr>
<tr>
<td></td>
<td>No chondrocytes present (no cartilage)</td>
</tr>
<tr>
<td>3.</td>
<td>Joint surface regularity</td>
</tr>
<tr>
<td></td>
<td>Smooth, intact surface</td>
</tr>
<tr>
<td></td>
<td>Surface fissures (&lt;25% of new surface thickness)</td>
</tr>
<tr>
<td></td>
<td>Deep fissures (≥25% of new surface thickness)</td>
</tr>
<tr>
<td></td>
<td>Complete disruption of the new surface</td>
</tr>
<tr>
<td>2.</td>
<td>Thickness of newly formed cartilage</td>
</tr>
<tr>
<td></td>
<td>Similar to the surrounding cartilage</td>
</tr>
<tr>
<td></td>
<td>Greater than the surrounding cartilage</td>
</tr>
<tr>
<td></td>
<td>Less than the surrounding cartilage</td>
</tr>
<tr>
<td></td>
<td>No cartilage</td>
</tr>
<tr>
<td>1.</td>
<td>Morphology of newly formed surface tissue</td>
</tr>
<tr>
<td></td>
<td>Exclusively articular cartilage</td>
</tr>
<tr>
<td></td>
<td>Mainly hyaline cartilage</td>
</tr>
<tr>
<td></td>
<td>Fibrocartilage (spherical morphology observed with ≥75% of cells)</td>
</tr>
<tr>
<td></td>
<td>Only fibrous cartilage (spherical morphology observed with &lt;75% of cells)</td>
</tr>
<tr>
<td></td>
<td>No tissue</td>
</tr>
<tr>
<td>0</td>
<td></td>
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</tbody>
</table>

**Score**

**Cartilage evaluation (within the upper 1 mm of defect)**

1. Morphology of newly formed surface tissue
   - Exclusively articular cartilage: 4
   - Mainly hyaline cartilage: 3
   - Fibrocartilage (spherical morphology observed with ≥ 75% of cells): 2
   - Only fibrous cartilage (spherical morphology observed with < 75% of cells): 1
   - No tissue: 0

2. Thickness of newly formed cartilage
   - Similar to the surrounding cartilage: 3
   - Greater than the surrounding cartilage: 2
   - Less than the surrounding cartilage: 1
   - No cartilage: 0

3. Joint surface regularity
   - Smooth, intact surface: 3
   - Surface fissures (<25% of new surface thickness): 2
   - Deep fissures (≥25% of new surface thickness): 1
   - Complete disruption of the new surface: 0

4. Chondrocyte clustering
   - None at all: 3
   - <25% chondrocytes: 2
   - 25-100% chondrocytes: 1
   - No chondrocytes present (no cartilage): 0

5. Chondrocyte and GAG content of new cartilage
   - Normal cellularity with normal Safranin O staining: 3
   - Normal cellularity with moderate Safranin O staining: 2
   - Clearly less cells with poor Safranin O staining: 1
   - Few cells with no or little Safranin O staining or no cartilage: 0

6. Chondrocyte and GAG content of adjacent cartilage
   - Normal cellularity with normal Safranin O staining: 3
   - Normal cellularity with moderate Safranin O staining: 2
   - Clearly less cells with poor Safranin O staining: 1
   - Few cells with no or little Safranin O staining or no cartilage: 0
Due to the lack of cell-adhesive moieties in traditional synthetic hydrogels, the present work investigated the use of degradable gelatin microparticles (GMPs) as temporary adherent substrates for anchorage-dependent mesenchymal stem cells (MSCs). MSCs were seeded onto GMPs of varying crosslinking densities and sizes to investigate their role on influencing MSC differentiation and aggregation. The MSC-seeded GMPs were then encapsulated in poly(ethylene glycol)-based hydrogels and cultured in serum-free, growth factor-free osteochondral medium. Non-seeded MSCs co-encapsulated with GMPs in the hydrogels were used as a control for comparison. Over the course of 35

*This chapter was submitted to Annals of Biomedical Engineering as Lu S, Lee EJ, Lam J, Tabata Y, Mikos AG. Evaluation of gelatin microparticles as adherent-substrates for mesenchymal stem cells in a hydrogel composite. (2016)
days, MSC-seeded GMPs exhibited more cell-cell contacts, greater chondrogenic potential, and a down-regulation of osteogenic markers compared to the controls. Although the factors of GMP crosslinking and size had nominal influence on MSC differentiation and aggregation, GMPs demonstrate potential as an adherent-substrate for improving cell delivery from hydrogel scaffold by facilitating cell-cell contacts and improving MSC differentiation.

8.1. Introduction

Articular cartilage has a limited endogenous ability for self-repair, and since current clinical treatments for damaged or diseased cartilage tissue fall short of holistic repair, tissue engineering strategies have emerged as an alternative for physiological cartilage regeneration. In particular, synthetic hydrogels are appealing as scaffolding structures due to their viscoelastic properties, ability to mimic the high water content of native tissues, and tunability for greater control over physical properties [386]. Various biomaterials have consequently been developed into hydrogel structures [387] and are attractive candidates as scaffolds for cell encapsulation and growth [388].

In the field of cartilage tissue engineering, stem cells and/or chondrocytes have typically been incorporated in such synthetic hydrogels to guide cell and tissue growth. Specifically, cell delivery from hydrogels traditionally involves the homogenous suspension of cells within a liquid precursor solution and subsequent curing to form a
cell-laden scaffold. However, as the field evolves, hydrogels have transitioned from merely delivery vehicles to dynamic, bioactive intermediaries of neo-tissue formation [388]. In particular, synthetic hydrogels often lack cell-adhesion moieties, and cell-cell contacts mediated by N-cadherin cell adhesion molecules have increasingly been shown to influence mesenchymal stem cell (MSC) differentiation and cartilage tissue formation through condensation [389, 390]. Condensation, a developmental process during skeletogenesis, is characterized by MSC aggregation following the establishment of cell-cell contacts, which in turn can improve chondrogenic differentiation and cartilaginous extracellular matrix production [391, 392]. Therefore, the current study seeks to improve the differentiation potential of MSCs through the use of enzymatically-degradable gelatin microparticles (GMPs) embedded within a poly(ethylene glycol) (PEG)-based hydrogel matrix.

Synthesized from denatured collagen, gelatin naturally exhibits cell-adhesion moieties and has seen numerous applications in cell delivery in a variety of scaffold forms [393-395]. Thus, gelatin will be fabricated into microparticles as a medium for cell seeding and cell-cell contacts. Specifically, MSCs will first be seeded onto the surface of GMPs, subsequently followed by the encapsulation of MSC-seeded GMPs (MSC-GMPs) within a hydrogel scaffold. It is hypothesized that initially seeding GMPs with MSCs can improve their differentiation potential, and that the GMPs will serve as enzymatically-digestible porogens as well as a temporary adherent-substrate for the MSCs. Additionally, it is hypothesized that the degradation of the GMPs will result in open space for MSC aggregation within the created pores. MSCs are known secretors of a number of matrix metalloproteinases (MMPs) [396], and direct extracellular matrix
contact can modulate MMP activity through specific substrate-protease responses [397]. Moreover, the aggregation of MSCs is modulated by the ratio of cadherin to integrin expression; the lower the availability for substrate adhesion, the greater the affinity for cell-cell aggregation [398]. Thus, it may be possible to form MSC aggregates following degradation of the GMP substrate within the macroporous hydrogel.

To investigate the previously stated hypotheses, this study 1) fabricates GMPs of varying sizes and degrees of crosslinking, 2) investigates GMP size, GMP crosslinking, and seeding method on the activity of MSCs encapsulated within PEG-based hydrogels, and 3) cultures the cell-laden hydrogel composites in a serum-free, growth factor-free medium to elucidate the sole effect of the aforementioned three variables on MSC chondrogenesis, osteogenesis, and condensation.

8.2. Materials and Methods

8.2.1. Experimental design

The main design criteria set for fabricating GMPs was a fast-degrading, temporary adhesive-substrate for MSC delivery. Thus, GMPs with a very low crosslinking density that would still allow for MSC seeding were used. In order to determine which GMPs to use for MSC seeding, GMPs with different crosslinking densities were degraded in phosphate-buffered saline (PBS) with a range of collagenase
1A concentrations (Table 8.1). From this study, select GMPs of different crosslinking density and diameter sizes were then co-encapsulated or seeded with MSCs in hydrogel composites to yield six different experimental groups as outlined in Table 8.2. Cell-laden hydrogel composites were then cultured for 35 days in serum- and growth factor-free osteochondral medium to assess MSC activity, condensation, and differentiation.

8.2.2. Oligo(poly(ethylene glycol) fumarate) synthesis

Oligo(poly(ethylene glycol) fumarate) (OPF) was synthesized from PEG (Sigma-Aldrich) with a nominal molecular weight of 3,350 according to methods previously developed in our laboratory [339]. Gel permeation chromatography was used to characterize the OPF macromer to give a number average molecular weight of 7,500 ± 200 Da and a weight average molecular weight of 36,300 ± 600 Da. Purified OPF was stored at -20°C and sterilized with ethylene oxide for 12 h prior to use following established methods [102].

8.2.3. Gelatin microparticle fabrication

GMPs were fabricated using acidic gelatin (Nitta Gelatin) with an isoelectric point of 5.0 using a modified process of established methods [352, 399]. Briefly, 30 mL of a 10 wt% gelatin solution in distilled, deionized water (ddH2O), preheated to 45°C, was added drop-wise to 250 mL olive oil (Sigma-Aldrich) (containing 0.5 wt% Span 80) stirring at 400 RPM at room temperature. After 10 min, the water-in-oil emulsion was chilled on ice and stirred for 30 more min. Following the emulsion step, 100 mL chilled
acetone (4°C) were added and stirred an additional 60 min. The resulting microparticles were then collected by vacuum filtration and washed with acetone. The collected GMPs were then placed in a 0.1 wt% Tween 80 aqueous solution containing different concentrations of glutaraldehyde (1, 2, 3, 4, and 5 mM) and stirred at 4°C for 15 h. After crosslinking, glycine was added to a concentration of 25 mM to block residual aldehyde groups of unreacted glutaraldehyde and allowed to stir for 1 h. Crosslinked microparticles were then vacuum-filtered, washed with ddH$_2$O and acetone, dried under low-vacuum overnight, and sieved to different size ranges. GMPs of 50-100 µm and 100-150 µm in diameter were sterilized with ethylene oxide for 12 h prior to MSC culture and encapsulation.

8.2.4. Gelatin microparticle degradation

For the degradation study, GMPs of varying crosslinking were placed in PBS with different concentrations of collagenase 1A (Sigma-Aldrich) to facilitate enzymatic digestion (Table 8.1). The collagenase concentrations used were based on estimations of MMP-1 expression from MSCs [400], as well as observed degradation of GMPs when incubated with MSCs. 5 mg of 100-150 µm diameter GMPs were added to the bottom of cell strainers with a 40 µm mesh size. The cell strainers with GMPs were then placed in 6-well plates, with each well containing 10 mL of collagenase-containing PBS (CC-PBS), and incubated at 37°C for up to 35 days (n=3). The CC-PBS was collected and changed at day 1 and every third day thereafter. To measure the amount of gelatin in solution, a bicinchoninic acid assay (Micro BCA Protein Assay Kit, Thermo Scientific) for total protein determination was used with dissolved, uncrosslinked GMPs prepared as
standards. Briefly, 150 µL of standard/sample were combined with 150 µL of the working reagent in a clear 96-well plate and incubated for 1 h at 37°C. The absorbance at 562 nm was then measured with a plate reader (PowerWave x340 Microplate Reader). The GMPs were considered completely degraded when the amount of gelatin detected was within the standard error of the lower detection limit. This was verified via visual observation of the absence of GMPs in the cell strainer.

8.2.5. Rabbit marrow mesenchymal stem cell harvest and culture

Rabbit marrow MSCs were isolated from the tibias of 6-month-old New Zealand white rabbits as previously described [401]. All surgical procedures were approved by the Institutional Animal Care and Use Committee of Rice University. Briefly, after anesthesia, bone marrow was collected into 10 mL syringes containing 3,000 units of heparin. The bone marrow was then plated and cultured in general medium (GM + FBS) containing Low Glucose-Dulbecco’s modified Eagle’s medium (LG-DMEM), 10% v/v fetal bovine serum (FBS), 100 units/mL penicillin, and 100 µg/mL streptomycin. GM + FBS was changed every 3 days and after 2 weeks of culture, the adherent fraction of cells was pooled from 6 rabbits to minimize inter-animal variability and cryopreserved in freezing medium containing LG-DMEM, 20% v/v FBS, 10% v/v dimethyl sulfoxide, 100 units/mL penicillin, and 100 µg/mL streptomycin. Prior to use, MSCs were thawed and expanded with GM + FBS up to passage three.
8.2.6. Mesenchymal stem cell seeding on gelatin microparticles

Prior to MSC seeding, 50 mg dry GMPs (50-100 or 100-150 µm diameter) were swollen in 2 mL GM without FBS (GM – FBS) for 1 h at 37°C. 6 × 10⁶ MSCs and 50 mg GMPs in suspension were then split evenly between two 100 mm ultra-low attachment dishes (Corning) containing 20 mL GM – FBS each. The dish was gently swirled at 120 RPM for 2 min before incubation for 12 h at 37°C. After 12 h culture, aggregates of MSCs and GMPs were broken up with a 1000 µL positive displacement pipette, swirled at 120 RPM for 2 min, and incubated for 24 h at 37°C. For GMPs without seeded MSCs, GMPs were swollen in GM – FBS for 36 h at 37°C in static conditions. After a total of 36 h, the suspension of GMPs or MSC-seeded GMPs (MSC-GMP) was filtered through a 70 µm cell strainer to remove any non-adherent cells and washed with PBS to remove residual media. The GMPs or MSC-GMPs were then loaded in a 1 mL syringe shortly before encapsulation.

8.2.7. Hydrogel composite fabrication

Hydrogel composites were fabricated similar to methods previously described [399, 402]. To prepare the OPF precursor solution, 50 mg of OPF and 25 mg of PEG-diacrylate (3,400 Da, Laysan Bio) were dissolved in 112.5 µL PBS and set at room temperature for 45 min to eliminate air bubbles. 23 µL each of radical initiators, 0.3 M ammonium persulfate (APS) and 0.3 M N,N,N′,N′-tetramethylethylenediamine (TEMED), were then mixed into the polymer solution and vortexed. The concentrations of APS and TEMED used were expected to have minimal cytotoxic effects and allow for
high viability of encapsulated cells in OPF-based hydrogels following fabrication [401, 402]. After 30 seconds, 100 µL PBS or cell suspension (6 × 10⁶ MSCs) were added followed by the MSC-GMPs or blank GMPs, respectively. The solution was mixed carefully to create a homogenous distribution of GMPs and to avoid the formation of bubbles. Lastly, the polymer-GMP solution was injected into Teflon molds (6 mm diameter × 1 mm thickness) and crosslinked at 37°C for 20 min. Teflon molds were flipped at 5 min to prevent GMPs from settling.

8.2.8. Hydrogel composite culture

The resulting hydrogel constructs were transferred to ultra-low attachment 24-well plates (Corning) with 1 mL serum- and growth factor-free osteochondral medium (OM), which contained LG-DMEM supplemented with ITS + Premix (6.25 µg/mL insulin, 6.25 µg/mL transferrin, 6.25 µg/mL selenous acid, 5.35 µg/mL linoleic acid and 1.25 µg/mL bovine serum albumin) (BD Biosciences), 100 units/mL penicillin, and 100 µg/mL streptomycin, 50 mg/L ascorbic acid, 10mM β-glycerophosphate, 10⁻⁷ M dexamethasone, and 1 mM sodium pyruvate [256]. OM was changed every two days and cell-laden composites were cultured up to 35 days.

8.2.9. Confocal fluorescence microscopy

Confocal fluorescence microscopy was used to visualize MSCs encapsulated in the hydrogel composites at day 0 (D0). To assess cell viability, constructs from D0 were rinsed in PBS to remove media and then incubated in a dye solution containing 4 µM
ethidium homodimer-1 and 2 µM calcein acetoxyethyl ester (Invitrogen) for 30 min as reported previously [256]. MSCs complexed with the Live/Dead reagent were then imaged using a Zeiss LSM 510.

To assess the morphology of MSCs either seeded on GMPs or encapsulated in the gel phase, select samples at D7 were stained for nuclei and F-actin. Briefly, samples were fixed in 10% neutral buffered formalin for 10 min at room temperature, washed with PBS, and stored at 4°C until staining. Hydrogels were immersed in 0.3% Triton X-100 for 10 min, 2x, to permeabilize cells, then incubated with DAPI (5 µg/mL) and phalloidin (1:20 dilution, Alexa Fluor 488 Phalloidin, Life Technologies) in 3% BSA diluted in Triton X-100 for 20 min at room temperature. Samples were then washed in PBS for 10 min and imaged with a Nikos A1-Rsi.

8.2.10. Biochemical assays

At each time point (D0, D7, D21, and D35), hydrogel composites were collected (n=4), rinsed in PBS to remove media, cut in half, and stored in -20°C until used for biochemical analysis. To determine DNA, glycosaminoglycan (GAG), and hydroxyproline (HYP) content, 4 hydrogel halves were each digested in 500 µL of proteinase K solution (1 mg/mL proteinase K, 10 µg/mL pepstatin A, and 185 µg/mL iodoacetamide in tris-EDTA solution (6.055 mg/mL tris(hydroxymethyl aminomethane), 0.372 mg/mL EDTA, pH 7.6 adjusted by HCl) at 56°C for 16 h. To determine alkaline phosphatase (ALP) activity and calcium content, 4 hydrogel halves were each thawed in 500 µL of ddH₂O. All samples were then subjected to homogenization with a syringe and needle, three freeze-thaw cycles, and probe sonication.
DNA content was quantified using a Quant-iT PicoGreen dsDNA Assay Kit (Molecular Probes) according to the manufacturer’s instructions. Sample supernatant, assay buffer, and dye solution were combined in a black, opaque 96-well plate, and incubated for 10 min at room temperature. Fluorescence was measured using excitation and emission wavelengths of 485 nm and 528 nm (FL x800 Fluorescence Microplate Reader), respectively. DNA concentrations were determined relative to a lambda DNA standard curve.

GAG content was determined with a dimethylmethylene blue colorimetric assay as previously described [401]. Sample supernatant and color reagent were combined in a clear 96-well plate and the absorbance at 520 nm was measured (PowerWave x340 Microplate Reader). GAG concentrations were determined relative to a chondroitin sulfate standard curve. Synthetic GAG activity was determined by normalizing total GAG content to the DNA content for each sample.

HYP content, an indicator of total collagen amount, was determined via a colorimetric assay as previously described [403]. An aliquot of sample supernatant was combined with an equal volume of 4 N NaOH and hydrolyzed by autoclaving for 15 min, 121°C (approximately 50 min total processing time). The solution was neutralized with HCl and acetic acid to pH 5.5-7.0 and divided into duplicate reactions. Chloramine-T and p-dimethylaminobenzaldehyde solutions were then added sequentially, incubated at 60°C for 30 min, and read at an absorbance of 570 nm with a plate reader. HYP concentrations were determined relative to a trans-4-hydroxy-L-proline standard curve.

ALP enzymatic activity was determined using alkaline buffer solution and phosphatase substrate tablets (Sigma-Aldrich). Sample supernatant and reagents were
combined and incubated for 1 h at 37°C. The reaction was stopped by adding 1 N NaOH, and the absorbance at 405 nm was measured using a plate reader. ALP activity was determined relative to a p-nitrophenol standard curve. Enzymatic activity was normalized to the DNA content for each respective hydrogel composite half.

Calcium content was measured using a colorimetric assay by adding acetic acid to an aliquot of sample supernatant with a final concentration of 0.5 M and incubating at room temperature overnight to dissolve the calcium. Samples were combined with calcium arsenazo III reagent (Genzyme), and the absorbance at 650 nm was measured using a plate reader. Calcium concentrations were determined relative to a CaCl₂ standard curve and normalized to the DNA content for each respective hydrogel composite half.

8.2.11. Real-time reverse transcription polymerase chain reaction

At D7, D21, and D35, hydrogel samples were collected (n=4), rinsed in PBS to remove media, and stored in lysis buffer at -20°C until used for real-time reverse transcription polymerase chain reaction (RT-PCR) analysis as previously described [102]. Additionally, D0 samples were collected from experimental groups containing 4 mM GMPs. Briefly, RNA was isolated from hydrogel samples using the RNeasy Mini Kit (Qiagen), reverse transcribed to cDNA using superscript III transcriptase (Invitrogen) and Oligo dT primers (Promega), and subjected to real-time PCR (Applied Biosystems 7300 Real-Time PCR System) using SYBR Green detection (PerfeCTa SYBR Green FastMix, Rox; Quanta Biosciences) with custom designed primers (Integrated DNA Technologies). Primer sequences used are listed in Table 8.3. All target gene expression were normalized to the housekeeping gene GAPDH and expressed as the fold change relative to the
baseline expression of the Group 1 control at D0. All gene expression data were calculated using the $2^{-\Delta\Delta C_t}$ method [404].

8.2.12. Statistical analysis

A significance level of $p<0.05$ was used for all statistical analysis through JMP Pro v11.0.0. All results are presented as means ± standard deviation. Biochemical assay data were analyzed using one-way ANOVA to test variance followed by Tukey’s HSD All Pairs test, whereas RT-PCR data were analyzed using the Kruskal-Wallis test followed by the Wilcoxon Each Pair test to determine significant differences between groups at each time point and between time points for each group.

8.3. Results

8.3.1. Gelatin microparticle degradation

The main design criterion set for fabricating GMPs was a fast-degrading, temporary adhesive-substrate for MSC delivery. Thus, GMPs with a very low crosslinking density that would still allow for MSC seeding were used. Of the five crosslinking-densities tested, GMPs crosslinked in 1mM glutaraldehyde solution (1mM GMPs) dissolved in 37°C PBS within 24 h while 2, 3, 4, and 5mM GMPs remained stable. In order to model the degradation of GMPs due to MMPs secreted from MSCs,
2mM and 3mM GMPs were initially tested in varying CC-PBS concentrations (Figure 8.1a,b). However, 2mM and 3mM GMPs completely degraded when cultured with MSCs during the seeding period (36 h), whereas 4mM and 5mM GMPs allowed for cell adhesion. As a result, 4mM and 5mM GMPs were only tested at 0, 100, and 200 ng/mL CC-PBS concentrations for up to 35 days. Both 4mM and 5mM GMPs held their spherical morphology in PBS without collagenase during the culture period, and while 4mM GMPs degraded in CC-PBS within 13 and 16 days, 5mM GMPs degraded within 19 and 28 days at the 100 and 200 ng/mL CC-PBS concentrations, respectively.

8.3.2. Mesenchymal stem cell seeding on gelatin microparticles

After MSCs were seeded onto 4mM and 5mM GMPs, the MSC-GMPs were encapsulated in OPF hydrogels, and the viability and morphology of the MSCs were qualitatively examined with confocal fluorescence microscopy. At D0 post-fabrication, the cell-laden hydrogel composites were evaluated with a Live/Dead assay kit, with live cells and dead cells fluorescing green and red, respectively. As seen in Figure 8.2, MSCs are viable following hydrogel encapsulation, whether through direct encapsulation or initial GMP seeding. The minimal cell death seen in the MSC-GMP groups also points to the MSC seeding procedure on the GMPs as cell-friendly. In order to visualize cell morphology in the hydrogel composites, MSCs were stained with DAPI (blue) and phalloidin (red) for cell nuclei and F-actin, respectively (Figure 8.3). As shown in Figure 8.3a, the MSCs exhibit a round morphology, while in Figure 8.3b and Figure 8.3c, MSCs are attached and spread on the surface of GMPs within the hydrogel scaffold.
8.3.3. Biochemical assays

OPF hydrogels containing MSCs + GMPs (Groups 1 and 2) or MSC-GMPs (Groups 3-6) were cultured in a serum-free, growth factor-free OM for 35 days and evaluated for cellularity, synthetic GAG activity, GMP degradation, ALP activity, and mineralization potential (Figure 8.4). Cellularity, as assessed by DNA content, is depicted in Figure 8.4a for each group and time point. At D0, Groups 1 and 2 had significantly greater total DNA content than Groups 3 and 4 (MSC-GMPs, 50-100 µm), which had greater DNA content than Groups 5 and 6 (MSC-GMPs, 100-150 µm). All groups displayed a decrease in total DNA content from D0 to D7. However, the DNA amount remained relatively stable over the course of 35 days following Day 7.

Figure 8.4b represents the synthetic GAG activity as produced by encapsulated MSCs. Groups 3, 5, and 6 (MSC-GMPs) showed a significantly larger normalized GAG amount at D0 compared to the co-encapsulated MSC + GMPs. Additionally, groups with MSCs seeded on 4 mM GMPs showed the greatest GAG synthetic activity at Day 35: Groups 3 and 5 were significantly greater than the control groups and Group 3 was significantly greater than the 5 mM GMP groups. No direct differences were seen between larger and smaller sized GMP groups.

In order to examine the change in collagen content as an estimation of GMP degradation, total HYP content of the hydrogel constructs was determined over time. As seen in Figure 8.4c, total HYP amounts dropped overtime with D21 and D35 values significantly lower than D0 and D7 values for all groups. A difference between groups with 4mM and 5mM GMP groups can be seen at D21 and especially at D35, with 4mM groups having less HYP content than 5mM groups. However, the GMPs were still
present and had a spherical morphology within the hydrogels after the culture period (as verified by light microscopy), which can be seen by remaining HYP content in each group at D35.

Since MSCs were cultured in an OM without factors for specific chondrogenic or osteogenic directed differentiation, ALP enzymatic activity and calcium content were also examined as markers of osteogenic differentiation. Figure 8.4d shows a peak in normalized ALP enzymatic activity at D7 for Groups 1 and 2, while the groups with encapsulated MSC-GMPs decreased in ALP activity over time from D0. MSCs co-encapsulated with 5mM GMPs (Group 2) specifically exhibited the highest normalized ALP activity at D7 among groups. Interestingly, the MSC-GMPs groups with 4mM GMPs (Groups 3 and 5) had greater initial ALP activity at D0 compared to their 5mM GMP counterparts (Groups 4 and 6). Looking at normalized calcium content (Figure 8.4e), no significant differences were observed between groups, and calcium levels remained relatively low over the course of 35 days. However, a significant increase in normalized calcium content was seen from D0 to D21 for Group 1 and from D0 to D35 for Group 2.

8.3.4. Real-time RT-PCR gene expression

In addition to evaluating the synthetic activity of the cell-laden hydrogel composites, real-time RT-PCR analysis was also performed to assess chondrogenic and/or osteogenic differentiation potential. In examining markers for chondrogenic differentiation, certain trends were consistently seen for COL2, ACAN, and SOX9 gene expression. Of note, all MSC-GMP groups exhibited greater chondrogenic gene
expression at D21 than did the MSC + GMP control groups (Figure 8.5). Differences between MSC-GMP (Groups 3-6) and MSC + GMP (Groups 1-2) hydrogel composites were also observed at other time points: MSCs seeded on 5mM GMPs demonstrated higher ACAN and SOX9 fold change at D35 compared to the MSC + 5mM GMP control (Figure 8.5b,c). Similarly, Groups 3 and 5 (4mM MSC-GMPs) had a greater COL2 fold change at D35 compared to the MSC + 4mM GMP control (Figure 8.5a). At D7, MSCs seeded on 5mM GMPs exhibited greater ACAN gene expression than the MSC + 5mM GMP control (Figure 8.5b). Significant differences were also seen between 4mM GMP (Groups 1, 3, and 5) and 5mM GMP (Groups 2, 4, and 6) hydrogel composites. Group 1 had higher COL2, ACAN, and SOX9 expression at D21 and D35 compared to Group 2. Additionally, the larger sized, 5mM MSC-GMP group (Group 6) had higher ACAN and SOX9 gene expression than its 4mM counterpart (Group 5) at D21.

In assessing markers of osteogenic differentiation, COL1 gene expression was consistently down regulated over time for all groups (Figure 8.6a). Moreover, MSC-GMP groups had decreased COL1 fold change compared to the MSC + GMP controls at all time points. RUNX2 expression also revealed similar down regulation for MSC-GMP groups compared to MSC + GMP groups at both D21 and D35 (Figure 8.6b). Interestingly, minimal differences in osteogenic gene expression were observed between smaller and larger GMP size groups. Regarding GMP crosslinking, Group 4 had reduced COL1 and RUNX2 expression at D21 compared to Group 3.

Lastly, the presence and formation of cell-cell contacts through CDH2 expression was investigated (Figure 8.6c). While there was no significant difference between groups with MSCs seeded on GMPs and MSCs homogenously encapsulated in the hydrogel at
early time points, D21 and D35 revealed the impact of the GMPs as a cell substrate: MSC-GMP groups showed greater CDH2 fold change compared to Group 1 at D21. At D35, MSC-5mM GMP groups showed higher N-cadherin expression than the MSC + 5mM GMP control. Similarly, Group 3 exhibited more CDH2 gene expression than Group 1 at D35.

8.4. Discussion

The primary objective of this study was to utilize enzymatically-degradable GMPs as a temporary adherent-substrate for MSCs within a hydrogel matrix. MSCs were seeded onto GMPs of different sizes and crosslinking densities and assessed for chondrogenesis/osteogenesis as well as their condensation behavior over 35 days. Specifically, we investigated 1) whether the GMPs could act as a temporary adherent-substrate for MSCs, allowing for MSC aggregation following GMP degradation, and 2) if the initial seeding of MSCs on the GMPs could improve their differentiation potential.

In order to evaluate GMPs as a temporary adherent-substrate, GMPs of varying crosslinking densities were fabricated and subjected to MSC seeding tests as well as collagenase-containing medium for GMP degradation modeling. Following GMP synthesis, 2 and 3mM GMPs were discovered to be unsuitable for MSC seeding and disappeared before the end of the seeding period. Since 2 and 3mM GMPs did not completely dissolve in the absence of collagenase within 24 h (Figure 8.1a,b), these loosely crosslinked GMPs were likely degraded via enzymes secreted by the MSCs
during the seeding period. Consequently, 4 and 5mM GMPs were chosen as the temporary adherent-substrate for initial MSC seeding. However, this initial enzymatic activity on the GMPs may not have lasted throughout the culture period since microparticles still remained within the hydrogel scaffolds at D35. Indeed, while MSCs are known secretors of MMPs, tissue inhibitors of metalloproteinases (TIMPs) are often produced alongside MMPs, and most TIMPs are highly specific for MMPs, binding in a 1:1 stoichiometric ratio [405]. In a study by Lozito and Tuan, despite secretion of MMP-1 and MMP-2 (a gelatinase) by MSCs cultured in a serum-free, growth factor-free medium, actual MMP activity was significantly lower due to endogenous production of TIMP-1 and TIMP-2 [406]. However, certain chemokines have been found to modulate the expression of MMPs and TIMPs in MSCs: expression of MMP-2 in a serum-free, growth factor-free medium was up-regulated with the exogenous addition of transforming growth factor-β1 while TIMP expression was not affected [407]. As a result, to fully realize the potential of GMPs as a temporary adherent-substrate for cell delivery, the inclusion of serum or growth factors may be necessary.

Due to incomplete GMP degradation, macropores could not form within the hydrogel scaffold, and the space necessary for MSC aggregation was not achieved. Certainly, other methods have been developed to induce cell aggregation within a hydrogel: MSCs seeded within macroproous OPF hydrogels [408], chondrocytes encapsulated within uncrosslinked GMPs and released into a porous hydrogel bulk upon GMP dissolution [409], and MMPs used to degrade crosslinked GMPs as hepatocyte cell carriers within an alginate hydrogel [410]. Nevertheless, an objective of the current study entailed the use of GMPs as temporary adherent-substrates for the anchorage-dependent
MSCs, and it is unclear what the effects of a large dose of MMPs to degrade the GMPs would be on stem cells, as opposed to a differentiated cell phenotype (e.g., hepatocytes).

Despite the lack of MSC aggregation, biochemical and real-time RT-PCR analysis revealed that employing GMPs as an adherent-substrate within OPF hydrogels resulted in greater MSC chondrogenic potential as compared to MSCs co-encapsulated with GMPs. At D35, synthetic GAG activity and type II collagen expression was higher for MSC-4mM GMP groups than the MSC + 4mM GMP control. Furthermore, aggrecan and SOX9 fold changes were larger for the MSC-5mM GMP groups than for the controls at D35. Overall chondrogenic gene expression was higher at D21 for all MSC-GMP groups compared to MSC + GMP groups. While expression of these genes did not necessarily increase over time, the significant differences are corroborated by enhanced N-cadherin expression for MSC-GMP groups at both D21 and D35. Indeed, cell-cell contacts provide additional communication that has been demonstrated to improve adipogenic, osteogenic, and/or chondrogenic differentiation through cadherin signaling [390], and the disruption of such cell-cell adhesion has been shown to reduce MSC chondrogenic potential [389].

While MSC-GMPs exhibited marked chondrogenic differentiation potential, markers of osteogenesis decreased over time for all groups. Interestingly, type I collagen and RUNX2 expression were significantly lower at D35 for MSCs seeded on GMPs compared to MSCs co-encapsulated with GMPs in hydrogels. However, substrate stiffness and the physical environment also play a large role in determining cellular response. Osteoblastic markers such as RUNX2 are often only expressed when the matrix stiffness is close to that of native bone tissue [411]. Although mechanical properties of
the hydrogel composites were not tested, the soft adherent substrate presented by the loosely crosslinked GMPs may have precluded significant osteogenic potential.

Lastly, the extent of GMP crosslinking and GMP size were also investigated as factors for influencing MSC activity in the hydrogel composites. While the variable of GMP size had minimal effects on differentiation and condensation, differences between 4mM and 5mM GMPs were found. Total HYP amount was consistently greater for 5mM GMP groups than 4mM GMP groups at D21 and D35, likely due to the increased crosslinking extent of 5mM GMPs. Additionally, differences between Group 1 and Group 2 were seen: greater normalized ALP expression was seen at D7 for MSCs co-encapsulated with 5mM GMPs compared to 4mM GMPs. This was followed by higher COL2, ACAN, and SOX9 gene expression for Group 1 compared to Group 2 at D21 and D35. Indeed, the microenvironment plays a large role in stimulating the activity of encapsulated MSCs and the relatively softer substrate of 4mM GMPs in Group 1 may have improved the chondrogenic potential of co-encapsulated MSCs.

However, a similar trend was not consistently seen in groups with MSCs-seeded on GMPs. 4mM MSC-GMPs groups displayed greater initial ALP activity compared to 5mM MSC-GMPs groups, yet smaller sized 4mM MSC-GMPs also had greater synthetic GAG activity at D35 compared to 5mM MSC-GMPs. In contrast, larger sized 5mM MSC-GMPs had higher ACAN and SOX9 expression than its 4mM counterpart at D21. Future investigation with a broader range of GMP crosslinking densities would be necessary to clarify the effect of GMP crosslinking. Additionally, the variables of size and crosslinking are anticipated to play a larger role in influencing MSC aggregation following complete degradation of the GMPs. Further studies incorporating serum or
growth factors in the culture medium would be warranted to elucidate the effect of these variables on the differentiation and condensation potential of seeded MSCs.

8.5. Conclusion

In summary, MSCs were seeded onto GMPs of varying crosslinking densities and sizes and subsequently encapsulated in OPF hydrogels. MSCs co-encapsulated with the GMPs were included as controls, and the cell-laden hydrogel composites were cultured in a serum-free, growth factor-free OM for 35 days. Results indicate that the encapsulated GMPs did not completely degrade during the culture period, and crosslinking density and GMP size had nominal influence on MSC differentiation potential. Nonetheless, MSCs seeded on GMPs exhibited greater N-cadherin expression than their MSC and GMP co-encapsulated counterparts. Additionally, MSCs seeded on GMPs showed greater chondrogenic differentiation potential, while osteogenic markers were found to down-regulate over time. Overall, these results demonstrate the potential of GMPs as an adherent-substrate within hydrogel scaffolds for facilitating cell-cell contacts and enhancing MSC differentiation.
Figure 8.1 Degradation profile of GMPs crosslinked with a 2mM (A), 3mM (B), 4mM (C), and 5mM (D) glutaraldehyde solution. Cumulative degradation of GMPs was measured up to 35 days in collagenase-containing PBS. 2_0, 2_20, 2_100, and 2_200 designate 2 mM GMPs incubated with PBS containing 0, 20, 100, or 200 ng/mL collagenase 1A, respectively. Similar shorthand designations apply for 3mM, 4mM, and 5mM GMPs. The day of complete degradation is stated and is indicated by a slight plateau in the cumulative GMP degradation graph. GMPs that did not degrade within the
culture period are denoted with an ND. Error bars correspond to standard deviation for
n=3 samples.
Figure 8.2 Confocal fluorescence microscopy images of MSCs, complexed with live/dead reagents, encapsulated in hydrogel composites at day 0. Green fluorescence designates live cells, whereas red fluorescence indicates dead cells. Round cells can be seen in Groups 1 and 2 (A and B) while flattened cells can be seen in Group 3-6 (C-F). (Scale bar: 200 µm)
Figure 8.3 Confocal fluorescence microscopy images of MSCs, stained with DAPI and phalloidin, encapsulated in hydrogel composites at day 7. Blue fluorescence designates nuclei, whereas green fluorescence indicates F-actin staining of MSCs. Round MSCs encapsulated in the gel phase can be seen in a representative sample from Group 2 (A), while elongated MSCs can be seen in a representative sample from Group 4 (B). A z-axial projection stack of 30 images (10 µm apart) set against differential interference contrast images shows the spread morphology of MSCs seeded on the GMPs (C). (Scale bar: 100 µm)
Figure 8.4 (A) DNA content, (B) GAG content normalized to DNA content, (C) HYP content, (D) ALP activity normalized to DNA content, and (E) calcium content normalized to DNA content for each experimental group at various time points. At each time point, groups connected with different letters are significantly different ($p < 0.05$). Within each group, time points connected by lines and noted with (*) are significantly different. The x-axis labels are denoted by Gel or GMP, representing MSCs co-encapsulated with GMPs vs seeded on GMPs; 4 or 5, representing the crosslinking extent of GMPs at either 4mM vs 5mM; and S or L, representing smaller 50-100µm GMPs vs larger 100-150µm GMPs. Error bars correspond to standard deviation for n=4 samples.
Figure 8.5 (A) Type II Collagen expression, (B) Aggrecan expression, and (C) Sox9 expression for each experimental group at various time points. At each time point, groups connected with different letters are significantly different (p < 0.05). Within each group, time points connected by lines and noted with (*) are significantly different. The x-axis labels are denoted by Gel or GMP, representing MSCs co-encapsulated with GMPs vs seeded on GMPs; 4 or 5, representing the crosslinking extent of GMPs at either 4mM vs 5mM; and S or L, representing smaller 50-100µm GMPs vs larger 100-150µm GMPs. Error bars correspond to standard deviation for n=4 samples.
Figure 8.6 (A) Type I Collagen, (B) Runx2, and (C) N-Cadherin expression for each experimental group at various time points. At each time point, groups connected with different letters are significantly different (p < 0.05). Within each group, time points connected by lines and noted with (*) are significantly different. The x-axis labels are denoted by Gel or GMP, representing MSCs co-encapsulated with GMPs vs seeded on GMPs; 4 or 5, representing the crosslinking extent of GMPs at either 4mM vs 5mM; and S or L, representing smaller 50-100µm GMPs vs larger 100-150µm GMPs. Error bars correspond to standard deviation for n=4 samples.
Table 8.1 Gelatin microparticles (GMP) of varying crosslinking and collagenase-containing phosphate-buffered saline (CC-PBS) concentrations used to model GMP degradation.

<table>
<thead>
<tr>
<th>GMP Crosslinking*</th>
<th>2 mM, 3 mM, 4 mM, 5 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC-PBS Concentration (ng/mL)^</td>
<td>0, 20, 100, 200</td>
</tr>
</tbody>
</table>

* GMPs were crosslinked in a solution of glutaraldehyde at the concentrations indicated above at 4°C for 15 h
^ 20 ng/mL CC-PBS concentration was only tested for 2 mM and 3 mM GMPs

Table 8.2 Experimental groups tested for the encapsulation of MSCs and GMPs in a hydrogel composite.

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Mode of MSC Encapsulation</th>
<th>GMP</th>
<th>Size of Dry GMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Gel Phase</td>
<td>4 mM</td>
<td>50 – 100 µm</td>
</tr>
<tr>
<td>2</td>
<td>Gel Phase</td>
<td>5 mM</td>
<td>50 – 100 µm</td>
</tr>
<tr>
<td>3</td>
<td>GMP-Seeded</td>
<td>4 mM</td>
<td>50 – 100 µm</td>
</tr>
<tr>
<td>4</td>
<td>GMP-Seeded</td>
<td>5 mM</td>
<td>50 – 100 µm</td>
</tr>
<tr>
<td>5</td>
<td>GMP-Seeded</td>
<td>4 mM</td>
<td>100 – 150 µm</td>
</tr>
<tr>
<td>6</td>
<td>GMP-Seeded</td>
<td>5 mM</td>
<td>100 – 150 µm</td>
</tr>
</tbody>
</table>
Table 8.3 Forward (F) and reverse (R) primers used for real-time RT-PCR analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Product length</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Type II Collagen (COL2A1)</strong></td>
<td>F: 5′-AACACTGCCAACGTCCAGAT-3′</td>
<td>201</td>
<td>[401]</td>
</tr>
<tr>
<td></td>
<td>R: 5′-CTGCAGCAGGTTATAGGTA-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Aggrecan (ACAN)</strong></td>
<td>F: 5′-GCTACGGAGACAAGGATGAGT-3′</td>
<td>114</td>
<td>[401]</td>
</tr>
<tr>
<td></td>
<td>R: 5′-CGTAAAGACCTCAACCTCCA-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>(Sex determining region Y)-box 9 (SOX9)</strong></td>
<td>F: 5′-GAGCGAAGAGGACAAGTTCC-3′</td>
<td>72</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>R: 5′-GTCCAGTCTAGCCCTTAGG-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Type I Collagen (COL1A2)</strong></td>
<td>F: 5′-CCCAGAATGGAGCAGTGGTT-3′</td>
<td>270</td>
<td>[401]</td>
</tr>
<tr>
<td></td>
<td>R: 5′-AGCAGACGCATGAGCGCAAG-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Runx-related transcription factor 2 (RUNX2)</strong></td>
<td>F: 5′-CCTTCCACTCAGTAAGAAGA-3′</td>
<td>143</td>
<td>[412]</td>
</tr>
<tr>
<td></td>
<td>R: 5′-TAAGTAAGGCTGGCTAGTATG-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>N-cadherin (CDH2)</strong></td>
<td>F: 5′-CTGCTATTGGATCGGATGAC-3′</td>
<td>96</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>R: 5′-TGAACATGCTGGAGAAGGA-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)</strong></td>
<td>F: 5′-TCACCACCTGCTCCAGGAGGCA-3′</td>
<td>292</td>
<td>[401]</td>
</tr>
<tr>
<td></td>
<td>R: 5′-CACAATGCCGAAGTGGTCG-3′</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Primers without a reference were custom designed and validated
The overall goal of this thesis was to engineer hydrogel composites as a regenerative scaffold and delivery vehicle for growth factors and stem cells, and to investigate their potential for osteochondral tissue regeneration.

In the first specific aim, we utilized OPF hydrogel composites for the spatially controlled incorporation of IGF-1 and TGF-β3 to the chondral layer of bilayered constructs. We showed that the delivery of growth factors enhanced cartilage repair over blank hydrogel controls in an osteochondral defect \textit{in vivo}. Additionally, it was revealed that the single delivery of IGF-1 alone elicited similar levels of osteochondral repair compared to dual delivery. In demonstrating the potential of hydrogel composites as growth factor delivery vehicles for cartilage repair, our next step emphasized improving the repair of the subchondral bone, understanding that the osteochondral unit comprises distinct cartilage and bone layers.
In the second specific aim, IGF-1 and BMP-2 were incorporated in the chondral and subchondral layers, respectively, of bilayered hydrogel composites and the effect of single or dual release of these growth factors on osteochondral repair was studied. BMP-2 enhanced early subchondral bone repair and we found that the dual delivery of both growth factors improved subchondral bone formation over the delivery of IGF-1 alone. Additionally, correlation analysis was performed between specific cartilage and bone repair metrics to provide a fuller understanding of the osteochondral repair process. We found that there exists a significant correlation between the degree of subchondral bone formation and cartilage surface regularity, highlighting the importance of bone as a mechanical support for overlying cartilage.

Although the findings from the first two specific aims demonstrate the utility of growth factor delivery as a strategy to augment repair in the wound healing environment, the use of stem cells also confer powerful potential to regenerate osteochondral tissue. In the third specific aim, we took advantage of GMPs as a temporary adherent-substrate, and the differentiation potential as well as condensation behavior of seeded MSCs were assessed. Non-seeded MSCs co-encapsulated with GMPs in OPF hydrogel composites were used as controls. We discovered that MSCs seeded on GMPs exhibited more cell-cell contacts, greater chondrogenic potential, and a down-regulation of osteogenic markers compared to controls in the absence of exogenous serum or growth factors. Such results demonstrate the significance of physical cues in the microenvironment on progenitor cells and establish the value of GMPs within hydrogel composites as versatile modes for both growth factor and stem cell delivery. Overall, the work in this thesis provides insight into growth factor and stem cell delivery strategies for osteochondral
tissue engineering with the hope that holistic regeneration of osteochondral tissue may one day be realized.
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