RICE UNIVERSITY

Injectable Cell-Laden Hydrogel Composites for Osteochondral Tissue Engineering

by

Xuan Guo

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APPROVED, THESIS COMMITTEE:

Antonios G. Mikos, Louis Calder Professor, Bioengineering, Chemical and Biomolecular Engineering, Rice University

F. Kurtis Kasper, Faculty Fellow, Bioengineering, Rice University

K. Jane Grande-Allen, Associate Professor, Bioengineering, Rice University

Kyriacos Zygourakis, A.J. Hartsook Professor, Chair, Chemical and Biomolecular Engineering, Rice University

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ABSTRACT

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This work investigated an injectable, biodegradable hydrogel composite of oligo(poly(ethylene glycol) fumarate) (OPF) and gelatin microparticles (MPs) as a cell and growth factor carrier for osteochondral tissue engineering applications. An in vitro study first investigated chondrogenic differentiation of rabbit marrow mesenchymal stem cells (MSCs) encapsulated in single-layer hydrogel composites of different swelling ratios with or without transforming growth factor-β1 (TGF-β1). The results showed that hydrogel composites containing TGF-β1-loaded MPs and of higher swelling ratios supported MSC chondrogenic differentiation. When implanted in a rabbit osteochondral defect, these hydrogel composites containing MSCs facilitated subchondral bone formation in the presence of TGF-β1. However, the delivery of MSCs either with or without TGF-β1 did not improve cartilage morphology. Accordingly, a bilayered OPF/MP hydrogel composite consisting of a chondrogenic layer and an osteogenic layer was fabricated. In vitro culture of the construct demonstrated that MSCs encapsulated in the chondrogenic layer differentiated into chondrocyte-like cells in the presence of TGF-β1-loaded MPs. In the osteogenic layer, osteogenically precultured MSCs
maintained their osteoblastic phenotype, and synergistically enhanced chondrogenic differentiation of the MSCs in the chondrogenic layer with TGF-β1. In a following study investigating similar hydrogel composites, TGF-β3-loaded MPs in the chondrogenic layer showed a more effectively stimulatory effect on MSC chondrogenic differentiation than TGF-β1-loaded MPs. Furthermore, encapsulated cells of different degrees of osteogenic differentiation in the osteogenic layer were found to significantly influence the chondrogenic gene expression of co-cultured MSCs in both the presence and absence of TGF-β3. Overall, this study demonstrated the fabrication of hydrogel composites that mimic the structure and function of osteochondral tissue, along with the application of these composites as cell and growth factor carriers for osteochondral tissue engineering.
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CHAPTER I: SPECIFIC AIMS

The objective of this work focuses on the development of a biodegradable hydrogel composite as a cell and growth factor carrier for osteochondral tissue engineering applications. In particular, hydrogel composites of oligo(poly(ethylene glycol) fumarate) (OPF) encapsulating gelatin microparticles (MPs) and rabbit marrow mesenchymal stem cells (MSCs) were investigated, towards the following specific aims:

1. Fabricate OPF/transforming growth factor-β1 (TGF-β1)-loaded MP hydrogel composites for chondrogenic differentiation of encapsulated MSCs in vitro, and determine the effect of swelling ratio of the hydrogel composites on cell differentiation;

2. Evaluate the capacity of these hydrogel composites containing MSCs and TGF-β1 for cartilage and bone tissue repair in an osteochondral defect in vivo;

3. Generate a bilayered OPF/MP hydrogel composite consisting of a chondrogenic layer and an osteogenic layer, and evaluate cell differentiation in each layer in vitro;

4. Investigate the combined effect of a chondrogenic growth factor and osteogenic cells of different preculture periods on the chondrogenic differentiation of the co-cultured MSCs in the chondrogenic layer of the bilayered OPF hydrogel composites.
CHAPTER II: BACKGROUND -- APPROACHES FOR OSTEOCHONDRAL TISSUE ENGINEERING

Abstract

Articular cartilage is an avascular tissue in the articular joint with a limited self-repair potential once injured. Injured articular cartilage also leads to degenerative changes in subchondral bone, resulting in severe pain and loss of mobility of the joint. Tissue engineering has become a promising strategy for tissue regeneration, by combining scaffolds, cells, and bioactive molecules. In this review, different scaffold materials, cells and bioactive factors for engineering of both cartilage and bone tissues are discussed. Furthermore, the review emphasizes tissue engineering strategies to fabricate a well-integrated construct consisting of both cartilage-like and bone-like tissues, which can mimic the structure and fulfill the functions of native osteochondral tissue.

Abbreviations

ALP, alkaline phosphatase; BMP, bone morphogenetic protein; dw, dry weight; ECM, extracellular matrix; FGF, fibroblast growth factors; GAG, glycosaminoglycan; IGF, insulin-like growth factor; MMP, matrix metalloproteinase; MP, gelatin microparticles; MPC, mesenchymal progenitor cell; MSC, mesenchymal stem cell; OPF, oligo(ethylene glycol) fumarate; PCL, poly(ε-caprolactone); PEG, poly(ethylene glycol); PEG-DA, poly(ethylene glycol)-diacrylate; PGA, poly(glycolic acid); PLA, poly(lactic acid); PLGA, poly(lactic-co-glycolic acid); PNIPAAm, poly(N-isopropylacrylamide); PPO, poly(propylene oxide); PPF, poly(propylene fumarate); TCP, tricalcium phosphate; TGF, transforming growth factor; VEGF, vascular endothelial growth factor; ww, wet weight; 3D, three dimensional.

Motivation

In the articular joint, articular cartilage and subchondral bone are two distinct tissues that contribute to the unique and important functions of the joint. Articular cartilage protects bone from high stresses and allows for low-friction movements within the joint, while subchondral bone serves as a mechanical support for the articular surface.
However, articular cartilage has limited ability to regenerate once injured due to its complex structure and poor access to progenitor cells [1]. Furthermore, injured articular cartilage can lead to degenerative changes of the subchondral bone, resulting in severe pain and loss of mobility of the affected joint [1, 2]. Osteoarthritis, a disease caused by degeneration of osteochondral tissue, has been reported to affect about 10% United States population older than 30, and most of the people older than 65, leading to a total direct cost of $28.6 billion each year [3].

Current treatments for cartilage injury often involve surgical interventions to remove affected tissues and insert transplanted osteochondral plugs as replacement [1, 3, 4]. The most common methods include: 1) arthroscopic drilling, to get assess to bone marrow for cartilage repair; 2) mosaicplasty, to replace both cartilage and subchondral bone with an osteochondral graft; 3) autologous cell transplantation, to inject pre-expanded cells under an autologous periosteal flap sutured on the cartilage; and 4) prosthetic replacement, to remove the joint and replace it with a mechanical device [1, 3, 4]. While these procedures may provide relief from pain and restore joint mobility, they can present long-term complications. Tissue engineering strategies, integrating the elements of cells, scaffolds and bioactive factors, provide a potential solution for repair and regeneration of native osteochondral tissues [5-7].

**Anatomy of Osteochondral Tissues**

**Articular Cartilage**

Articular cartilage covers the ends of long bones in synovial joints, and this tissue provides elasticity, distribution of load, resistance to compressive forces, as well as a smooth articulating surface during joint movement. Articular cartilage has a highly
specialized composition and structure which is adapted to serve its many important functions [5, 8, 9].

Chondrocytes are the only type of cells in articular cartilage. These specified cells originate from mesenchymal cells during fetal development, and are responsible in generating and maintaining the large amount of matrix. However, chondrocytes were found to be scattered throughout the matrix in mature tissue, occupying less than 10% of the total tissue volume [10].

The extracellular matrix (ECM) in cartilage is composed of approximately 75% water and 25% solid matrix. The solid matrix consists of 50-73% collagens and 15-30% proteoglycans as well as a lesser amount of glycoproteins [11, 12]. Additionally, 90-95% of the collagen in articular cartilage is type II, and the collagen fibers form a complex network that is essential for maintaining the tissue’s volume and shape [11]. Other collagens, such as collagen type IX, X, XI, and VI, also contribute to the unique function of the tissue [13, 14]. Proteoglycans, a diverse group of heterogenous complex macromolecules with one or more covalently bound glycosaminoglycan (GAG), are embedded within the fibrillar network of collagens [10]. The negatively charged GAG chains of proteoglycans interact with water, forming a high molecular weight complex, which generates swelling pressure to counter the high compressive loads at the joint [15].

Previous studies on the morphology and biology of articular cartilage have revealed its elaborate, highly ordered structure, consisting of four distinct zones: superficial, middle, deep and calcified zone [9, 11, 16, 17]. The chondrocytes in each zone demonstrate differences in density, morphology as well as mitotic and metabolic activity [18, 19]. The ECM components they assemble also vary in concentration and
orientation with depth from the articular surface [10, 19, 20]. The superficial zone (first 10-20% of the full-thickness), forming the gliding surface, contains elongated cells and densely packed collagen fibrils (diameter, 20nm) oriented tangentially to the articular surface [21]. The middle zone next (40-60% of the full-thickness) contains less aligned collagen fibers with a larger diameter, and chondrocytes with a more rounded appearance. Directly beneath is the deep zone, in which columns of ellipsoid hypertrophic chondrocytes are distributed between thicker collagen fibers (diameter, 120nm) oriented perpendicular to the articulating surface [21]. Finally, the calcified zone contains rather small cells, an abundance of calcium salts and a thin vasculature network as the tissue transitions from cartilage to mineralized bone. Moving through these zones from the articulating surface towards the subchondral bone, water content decreases linearly, from approximately 84% wet weight (ww) to 40-60% ww, and collagen content falls from 86% dry weigh (dw) to 67% dw, while proteoglycan content increases from 15% dw in the superficial zone to a peak of 25% dw in the middle zone, then falls to 20% dw in the deep zone [9].

Articular cartilage has a poor capacity to repair itself due to many reasons, such as a low cell density, the lack of blood supply, and the lack of access to progenitor cells that can promote tissue repair [1, 3]. Partial thickness defects, which are confined in the cartilage region, do not have access to the progenitor cells from bone marrow, therefore do not heal spontaneously. Full thickness defects penetrate to the subchondral bone and have access to the bone marrow. This triggers a wound healing response with progenitor cell infiltration from the bone marrow, however, the defects heal with hyaline cartilage or fibrocartilage instead of articular cartilage, which undergoes degenerative changes [1, 3].
In general, both lesions do not result in complete tissue regeneration [1, 4].

**Subchondral Bone**

Bone tissue lies directly under the calcified zone of cartilage, and provides mechanical support for the cartilage. The bone tissue contains multiple cell types, including osteoblasts, osteocytes and osteoclasts [22, 23]. Osteoblasts and osteocytes are immature and mature bone-forming cells, respectively, that produce and maintain bony matrix, while osteoclasts are multinucleated cells responsible for bone resorption. It is known that there is a balance between bone formation and resorption regulated by the cells, allowing for bone remodeling [24].

The bony matrix is composed of about 5% water, 25% organic matrix and 70% inorganic matrix [22]. The organic matrix contains mainly collagen type I (90%), although other molecules such as proteoglycans, osteocalcin, osteopontin, and growth factors also serve important roles structurally and biochemically [22]. The inorganic phase contains minerals in the form of hydroxyapatite [22]. They are deposited in the collagen framework, giving the bone stiffness and strength.

Compared to cartilage, bone tissue has a higher healing ability. Due to abundant vascularization and access to progenitor cells, the bony part of any injury at the joint usually heals readily [25].

**Cells**

*Marrow Mesenchymal Stem Cells*

Tissue engineering strategies sometimes involve introducing cells to the site of injury in order to help ECM production and remodeling. This is especially important for articular cartilage, which has limited self-repair ability.
Different cell types are available for osteochondral tissue engineering, including differentiated cells (such as chondrocytes and osteoblasts), and progenitor stem cells (like MSCs) [4, 5]. Previous research has demonstrated that autologous chondrocytes serve as a reliable cell source with no concern of an immune response [4]. However, the sources for chondrocyte harvest are limited, and the harvest process usually involves invasive surgeries and results in further injury to a healthy cartilage [4, 26]. Additionally, the subsequent in vitro expansion of the isolated chondrocytes leads to de-differentiation of the cells. In the monolayer culture, these chondrocytes become fibroblastic and lose their chondrocytic characteristics [27], which affects their success in cartilage repair upon implantation.

In contrast, the use of pluripotent stem cells for tissue repair is a viable alternative for tissue engineering. Although there are political and ethical concerns with the use of embryonic stem cells, there are no such worries concerning adult stem cells. Mesenchymal stem cells (MSCs) are self-renewable and multipotent stem cells that can differentiate into a variety of cell types of connective tissues, such as cartilage, bone, tendon, and muscle, when given specific environmental cues [28-30]. Although MSCs reside in many tissues throughout the adult organism, bone marrow is considered to be the most accessible and enriched source [31, 32]. Recent research has demonstrated that MSCs can be easily isolated from bone marrow and expanded in vitro without losing their pluripotency [33-35]. Therefore, MSCs have become a popular cell source for regeneration of connective tissues, especially an osteochondral tissue consisting of both cartilage and bone.
MSC Differentiation during Skeletal Development

During skeletal development in vivo, cartilage and bone are formed by a complex and sequential event, where chondrogenesis first results in the formation of a cartilage intermediate, and then long bone is developed from the cartilage by endochondral ossification [24, 26, 36, 37]. More specifically, endochondral development begins with condensation of MSCs, which results in chondrogenesis of MSCs to chondrocytes. The differentiating chondrocytes proliferate, secrete a cartilaginous matrix, and subsequently progress to a more mature phenotype, hypertrophic chondrocytes. At this stage, cartilaginous matrix becomes calcified, and the chondrocytes undergo apoptosis. However, these hypertrophic chondrocytes produce vascular endothelial growth factor (VEGF) before apoptosis, which stimulates vascular invasion into the cartilaginous matrix. Followed by chondrocyte apoptosis and angiogenesis, osteoblasts differentiated from osteoprogenitor cells invade into the cartilaginous matrix and lay down a collagen matrix. The matrix is eventually mineralized while osteoclasts resorb the original calcified cartilage, resulting in bone formation. The whole process is regulated by numerous growth factors, transcription factors, as well as matrix molecules [38, 39].

MSC Chondrogenic Differentiation

As mentioned above, chondrogenic differentiation of MSCs passes through sequential stages, including MSC condensation, chondrocyte differentiation, and maturation. Each stage is characterized by specific gene expression and ECM production [14, 36, 37, 40]. The undifferentiated MSCs produce ECM rich in collagen type I, hyaluronan and fibronectin. As the cells differentiate into chondrocytes, they begin to lay down a cartilage specific ECM, including collagen type II, IX and XI, large chondroitin
sulfate rich proteoglycans, aggrecans and link proteins. Meanwhile, the expression of collagen type I is turned off. After further maturation and hypertrophy, these cells express another cadre of ECM components including type X collagen and matrix metalloproteinase (MMP)-13. The expression of collagen type II decreases at the maturation stage.

Recent studies on MSC chondrogenic differentiation in vitro demonstrated that the chondrogenesis process depends on coordinated activities of many factors, including the protein-based growth factors, extracellular matrix, nonproteinaceous chemicals, biophysical parameters, mechanical stimuli, and cell density [41]. For example, a serum-free medium condition is commonly used to enhance MSC chondrogenic differentiation [41, 42]. Some chemical compounds supplemented to the medium, such as dexamethasone and ascorbic acid have also been shown to induce chondrogenesis and to encourage cartilaginous matrix production [41, 42]. The delivery of exogenous cytokines and growth factors also play crucial roles for directing cell differentiation, which will be detailed in a later section. Other culture conditions conductive for MSC chondrogenesis include a high cell density. Many studies have pelleted MSCs to mimic their condensation in vivo, and cultured these pellets in defined medium for chondrogenesis [42-44]. However, the disadvantages of the pellet culture system are its small size and poor mechanical properties, making it impractical for articular cartilage repair. On the other hand, polymer scaffolds can provide a three-dimensional (3D) environment for cells to grow and differentiate, synthesize ECM and other biological molecules. Therefore, in vitro and in vivo studies of chondrogenic differentiation of MSCs in a polymer scaffold with bioactive molecules have been increasingly active [45-48].
**MSC Osteogenic Differentiation**

Similar to chondrogenic differentiation, osteogenic differentiation of MSCs also contains a sequence of three principle stages, including proliferation, matrix maturation and mineralization [49]. In the first stage, cells are actively proliferating, with expression of cell-cycle and cell-growth- regulated genes (such as histone), as well as genes associated with ECM formation (such collagen type I, fibronectin and transforming growth factor-β (TGF-β)). As the cells start to differentiate, their proliferation rate slows down. Cells in this stage increasingly secrete alkaline phosphatase (ALP), a protein associated with the bone phenotype. ALP usually peaks during the ECM maturation stage prior to the onset of mineralization. Meanwhile, the ECM undergoes a series of modifications in composition and organization that renders it competent for mineralization. With the onset of mineralization, other bone-related genes, such osteopontin and osteocalcin, become induced. Both of the proteins contribute to hydroxyapatite binding and are maximally expressed during the mineralization period.

Induction of osteogenic differentiation of MSCs *in vitro* has also been extensively investigated recently [50]. Similar to chondrogenic differentiation, it requires the combined use of a supplemented culture medium, a 3D environment, the presence of growth factors and mechanical stimuli [50].

Varying the culture period of MSCs in an osteogenic medium has resulted in the development of osteogenic cells of different maturity stages [49, 51]. Many studies have underscored the importance of investigating the stages of MSC osteogenic differentiation, since they found that the *in vitro* osteogenic preculture period of MSCs significantly affected their osteogenic potential when implanted *in vivo* [51-53]. This is probably
related to various components and amount of extracellular matrix and soluble growth factors secreted by the cells at different stages.

**Scaffolds**

Scaffolds provide a 3D environment for cell attachment and growth, however, for cartilage and bone tissue engineering the scaffold should fulfill the requirements below [54]. First, the scaffold should be biocompatible, meaning that the material and its degradation products (if degradable) must not elicit an unresolved inflammatory response nor demonstrate extreme immunogenicity or cytotoxicity. Second, the material is preferably biodegradable so that a second surgery will not be required for removing the implant. Third, the scaffold should also have the ability to promote cartilage and bone regeneration. The scaffold should be porous with high surface to volume ratio, which allows for cell attachment, extracellular matrix growth as well as nutrient exchange. The architecture of the scaffold should mimic natural ECM in order to enhance cell growth. Furthermore, the scaffold material should offer sufficient temporary mechanical support as cartilage and bone tissues regenerate. More specifically for articular cartilage, the scaffold will need to withstand not only compressive loads but also shear forces at the joint surface. In addition to these, ease of handling and sterilization is also important for clinical use.

Usually, scaffold materials can be classified as naturally based and synthetically based [54, 55]. Natural materials, such as collagen/gelatin, fibrin, alginate, hyaluronate, and chitosan, have demonstrated their applications in a variety of orthopedic tissues due to their adequate biocompatibility and biodegradability. However, they generally possess low mechanical properties, which limit their use in clinical applications [54, 55].
Ceramics, such as β-tricalcium phosphate (TCP), hydroxyapatite, are natural materials specific for bone tissue engineering. Although they are stronger than many other natural materials, studies have found that their tensile strengths are still below those of native bone [54].

In contrast, synthetic materials are controllable in formulations, and therefore have adjustable mechanical and degradation properties [54, 55]. Many synthetic materials, such as poly(esters), poly(anhydrides), poly(acrylamides) and derivatives of poly(ethylene glycol) (PEG), have been successfully utilized in cartilage and bone tissue engineering in a variety of physical forms (fibers, meshes, gels) [54, 55]. Based on implantation method, scaffolds can be divided as preformed scaffolds and injectable scaffolds.

Preformed Scaffolds

Preformed scaffolds can be fabricated via various methods, including phase separation, solvent casting/particulate leaching or electrospinning [56]. Interconnected pore structures formed in these scaffolds are important to provide sites for cell attachment and also void spaces for nutrient exchange [57, 58].

Poly(α-hydroxy esters), such as poly(glycolic acid) (PGA), poly(lactic acid) (PLA), and their copolymer poly(lactic-co-glycolic acid) (PLGA) are commonly used for preformed scaffolds in both cartilage and bone tissue engineering [54, 57, 59]. These polymers undergo bulk degradation via hydrolytic cleavage of the ester bonds. By varying the ratio of lactic acid and glycolic acid in copolymer formulations, the mechanical and degradation properties of the material can be tailored [54]. Recent work has demonstrated the efficacy of using such biomaterials, in cell-based cartilage and bone
tissue engineering. For example, PGA porous nonwoven scaffolds seeded with bovine chondrocytes and cultured for 12 weeks showed hyaline-like cartilage formation [54]. Similarly, electrospun PLA scaffolds supported osteogenic differentiation of osteoprogenitor cells in vitro, and the fiber diameter of the scaffolds was found to affect cell spreading and proliferation [60]. Additionally, the implantation of PLGA scaffolds with or without cells in a rabbit osteochondral defect resulted in both cartilage and bone tissue regeneration [61, 62].

Poly(ε-caprolactone) (PCL) is a semicrystalline polymer, which is biodegradable and bioresorbable. Compared to PLGA, PCL has a slower degradation rate [63]. Recently, PCL was used to fabricate three-dimensional nano- or micro- fibrous scaffolds with structural similarity to natural collagen networks using the electrospinning technique [64]. These scaffolds have large surface area, which leads to a faster degradation time than the homopolymer, about 6 months [65]. Furthermore, they support the attachment, proliferation and differentiation of many cell types, including chondrocytes, osteoblasts, and MSCs, suggesting their potential in both soft and hard tissue engineering [47, 58, 66].

Injectable Scaffolds

Compared to preformed scaffolds, injectable materials enable the transplantation of cells and bioactive factors within a polymer mixture in a minimally invasive manner. This type of materials are especially convenient in dealing with irregular-shaped defects commonly found in articular cartilage [6, 7, 67, 68]. Hydrogels, which are insoluble hydrophilic polymer networks formed by crosslinking water soluble monomers, have great potential among various injectable scaffolds. The aqueous environment of
hydrogels not only allows diffusion of nutrients and bioactive factors [69, 70], but also mimics the natural extracellular matrix of soft tissues, thus supporting the growth of cells encapsulated in the hydrogels [7].

Many natural polymers mentioned previously, such as fibrin and alginate, are widely used as hydrogel scaffolds for cartilage tissue engineering [6, 67]. A variety of synthetic hydrogels have also been developed. Among those, poly(acrylamides), such as poly(N-isopropylacrylamide) (PNIPAAm), and PEG derivatives, have been actively investigated for cartilage tissue engineering [54, 55, 71].

PNIPAAm and a triblock copolymer of PEG and poly(propylene oxide) (PEG-PPO-PEG) (also know as Pluronic), can form thermally reversible gels without any permanent crosslinks, thus demonstrating good biocompatibility for cell encapsulation [54]. A study by Cao et al. compared the use of different polymers for autologous chondrocyte implantation in a porcine model, and found that Pluronic resulted in more cartilage-like tissue formation than either alginate or PGA [72]. However, both PNIPAAm and Pluronic are not degradable [54].

Acrylated or methacrylated PEG allows for the formation a hydrogel via chemical crosslinking, either by radical initiation or photo initiation [7, 55]. For example, Williams et al. encapsulated both MSCs and TGF-β1 in a poly(ethylene glycol)-diacrylate (PEG-DA) photopolymerizing hydrogel [7, 45]. In vitro culture of the constructs after 6 weeks showed marked increases in cellularity and cartilaginous matrix production, suggesting chondrogenesis of the cells in the hydrogel. Similarly, Elisseeff et al. injected a PEG-dimethacrylate/chondrocytes solution subcutaneously in mice. The hydrogel that was formed by photopolymerization resulted in cartilage tissue formation after 7 weeks in
Another PEG-derived injectable material, oligo(poly(ethylene glycol) fumarate) (OPF) has been developed in our laboratory recently [73, 74]. OPF is synthesized from fumaryl chloride and PEG. The fumarate double bonds in the macromer chains allow this material to chemically crosslink to form a hydrogel network, and the hydrolysis of ester linkages results in the degradation of the crosslinked hydrogel [73]. Previous work in our laboratory has demonstrated the cytocompatibility of OPF hydrogels with various cell types, including fibroblasts, chondrocytes and MSCs [74-78]. For example, rat MSCs encapsulated in OPF hydrogels and cultured in an osteogenic medium have been shown to be viable and to differentiate down the osteogenic lineage. The cells produced various amounts of calcium after 4 weeks, depending on the swelling and degradation properties of the hydrogels [76]. Additionally, the encapsulation of both cells and particulate drug delivery systems within the hydrogels demonstrated the potential of the hydrogels for cartilage tissue engineering applications [70, 74, 75]. Hydrogel composites containing TGF-β1-loaded microparticles (MPs) enabled controlled release of the growth factor [69, 79], and enhanced the chondrogenesis of the co-encapsulated bovine chondrocytes or rabbit MSCs in vitro [74, 75]. In vivo, these hydrogel composites underwent biocompatible degradation and supported neo-tissue formation in a rabbit osteochondral defect after 14 weeks [80, 81]. These results suggested the potential of OPF as a cell and growth factor carrier for orthopedic tissue engineering.

Composite Scaffolds for Osteochondral Tissue Engineering

Due to the significant difference in the composition and properties of articular cartilage and subchondral bone, composite scaffolds containing a chondrogenic and an
osteogenic compartment have been recently developed in order to mimic the native osteochondral tissue structurally and functionally. Different approaches have been reported in the literature involving the use of both preformed and injectable materials [3, 26, 82].

In one of the approaches, an osteochondral construct was fabricated from the same or similar biomaterials, but having a porosity/pore size gradient with depth. For example, Woodfield et al. reported the fabrication of a poly(ethylene glycol)-terephthalate-poly(butylene terephthalate) copolymer scaffold with a pore size gradient with depth (200-1650 μm), using a 3D fiber deposition technique [21]. The culture of chondrocytes on these scaffolds demonstrated a zonal distribution of cells and extracellular matrix similar to those in native articular cartilage. Similarly, Sherwood et al. utilized a 3D printing process to fabricate an osteochondral scaffold of varied composition and properties between layers [83]. The cartilage region made of PLGA/PLA had a higher porosity of 90%, which allowed cartilage formation after the culture of chondrocytes for 6 weeks. The bone portion made of PLGA/TCP had a porosity of 55%, which demonstrated similar tensile strength as native bone tissue.

Another approach involves independent fabrication or culture of chondrogenic and osteogenic layers, and a subsequently joining of the two layers by suturing or gluing [48, 84, 85]. For example, in some studies, an engineered cartilage cultured from chondrocytes seeded on a PGA scaffold, was sutured on an engineered bone to form a composite. These composites showed characteristic architectural features of an osteochondral tissue in both in vitro co-culture and upon implantation in vivo, with GAG production in the chondrogenic layer and mineralization in the osteogenic layer [48, 84].
However, the integration between layers was dependent on the maturity/preculture period of each layer, and poor integration with host cartilage was generally observed in the *in vivo* cases [3, 48].

In order to improve the integration between layers, other researchers have combined the use of both preformed scaffolds and injectable scaffolds for the fabrication of an osteochondral composite [86, 87]. More specifically, a polymer solution with cells is loaded on a porous scaffold as an osteogenic base. Crosslinking of the solution forms a chondrogenic hydrogel layer on top the osteogenic layer, resulting in an integrated osteochondral composite. Cao et al. reported that fibrin glue containing chondrocytes was able to coagulate on a precultured PCL scaffold containing osteoblasts, to form a composite [86]. Scanning electron microscopy on the co-cultured constructs revealed cell proliferation and matrix production within each compartment, as well as matrix integration at the interface. However, in a similar study by Lima et al., the incorporation of devitalized bone under a chondrocyte-seeded-agarose was found to reduce the biochemical and mechanical properties of the chondrogenic layer, although good integration between layers was observed [87].

Integrated osteochondral composites have also been fabricated using hydrogels in both layers [80, 81, 88, 89]. Compared to other approaches, this technique is advantageous because it can achieve spatial control of cell types and bioactive factor release in a minimally invasive way. A previous study in our laboratory has demonstrated the successful fabrication of a laminated hydrogel composite with OPF macromer, using a two-step crosslinking procedure [90]. Mechanical testing on these hydrogels revealed that the presence of an interfacial area did not significantly alter their tensile properties,
indicating a good integration between layers. Similar hydrogel composites encapsulating gelatin microparticles in both layers were implanted in a rabbit osteochondral model [80, 81]. The results demonstrated degradation of the composites at 14 weeks after implantation, and the formation of cartilaginous and bony tissue in the respective region of the defects. In another study, an osteochondral construct was fabricated in the shape of an articular condyle using PEG-DA hydrogels [88, 89]. *In vitro* cultured chondrogenic cells and osteogenic cells were encapsulated in the cartilage and bone compartments, respectively. The implantation of the construct *in vivo* demonstrated not only stratified layers of cartilaginous and osseous tissues, but also a mutual infiltration of the tissues into each other.

Despite promising results from the *in vivo* studies above, challenges still remain for the *in vitro* culture of an osteochondral construct. Further investigations are required to construct a well-integrated bilayered scaffold that can guide the chondrogenic and osteogenic differentiation of cells in different regions of the same scaffold. Additionally, a single set of cell culture conditions that can maintain the appropriate chondrogenic and osteogenic phenotype need to be further explored.

**Bioactive Factors**

*Bioactive Molecules*

In native osteochondral tissue, both insoluble and soluble components of the ECM play important roles in regulating cell attachment, proliferation and differentiation [36, 37]. Insoluble ECM can interplay with the embedded cells in many ways. Some components signal to the cells directly. For example, previous studies have showed that collagen type II, a major cartilaginous matrix component, is superior to collagen type I
for inducing chondrogenesis of stem cells, when added to culture medium or used as a hydrogel scaffold [91, 92]. This again suggests it is important to mimic the native ECM structurally and functionally when we design a scaffold. In addition to direct signaling, ECM can also regulate cellular behavior by binding, storing, and releasing soluble mediators. In fact, studies in our lab have demonstrated the presence of different growth factors in the ECM generated by in vitro culture of MSCs on a polymeric scaffold [93]. After decellularization the ECM showed a great potential in inducing osteogenic differentiation of reseeded MSCs on the scaffolds [94-96].

Soluble growth factors, such as transforming growth factors (TGFs), bone morphogenetic proteins (BMPs), and insulin-like growth factor (IGF) are known to play important roles in cartilage and bone formation during embryonic development and during tissue repair in vivo [97-99].

The transforming growth factor (TGF) family includes a number of polypeptides involved in the regulation of cell growth and differentiation in wound healing [11]. TGF-βs have been widely investigated and shown to exert various effects on different cell types [11]. In vitro, TGF-β1 is crucial to promote the chondrogenic differentiation of MSCs and to enhance cartilaginous matrix synthesis, in combination with a serum-free and chemically defined medium condition [42, 75]. In vivo, the delivery of TGF-β1 to a chondral or osteochondral defect has been shown to improve neo-cartilage and bone morphology in many studies [80, 100, 101]. However, a higher concentration of TGF-β1 resulted in side effects, such as inflammatory synovitis, cartilage erosion, and osteophyte formation [100, 102]. Recently, other isoforms of TGF-βs, such as TGF-β2 and -β3, have also been investigated [103, 104]. Due to a similar amino acid sequence as TGF-β1, both
TGF-β2 and TGF-β3 also demonstrated a stimulatory effect on MSC chondrogenic differentiation [103, 105]. In a comparative study, TGF-β2 and -β3 led to an earlier and greater GAG and collagen type II production for human MSCs than TGF-β1 in vitro [103]. The effects of TGF-βs on osteogenic differentiation are controversial. While some researchers reported TGF-β1 induced and accelerated osteogenic differentiation of bone marrow cells [106, 107], others showed that TGF-βs inhibited osteogenesis of MSCs or MSC derived osteoblasts in vitro [104, 108].

BMPs, a subfamily of the TGF superfamily, have been shown to have the ability to induce both chondrogenesis and osteogenesis [109-111]. For example, BMP-2, -4, -6, and -7 were shown to enhance the chondrogenesis of MSCs with an increase in type II collagen and proteoglycan synthesis. BMP-2, -6, and -9 also significantly promoted osteogenic differentiation of MSCs, as evidenced by increased alkaline phosphatase activity, osteocalcin expression and calcium deposition [111].

Some other growth factors, such as IGFs, fibroblast growth factors (FGFs), and vascular endothelial growth factor (VEGF) also play important roles in cartilage and bone formation [11, 112, 113]. Although these growth factors may not directly promote MSC chondrogenic or osteogenic differentiation [102], the combination of these growth factors with TGF-βs was shown to have a synergistic effect on the chondrogenic differentiation of MSCs and on cartilage-like matrix synthesis [43, 44, 114-116]. The synergistic effect could be due to regulatory cross-talks between different signaling pathways.

The challenge in the application of signaling molecules to optimize cartilage and bone regeneration is that their regulatory effects depend on the dose and timing of administration, therefore, different drug delivery systems have been investigated for
controlled release of the growth factors [70]. Particulate drug delivery systems, such as microparticles (MPs) made of gelatin and PLGA, are of great attention recently [117]. Previous work in our lab has shown that growth factors, like TGF-β1, BMP-2, IGF-1, can be loaded on gelatin MPs through polyionic complexation [69, 70, 79]. The encapsulation of growth factor-loaded MPs in OPF hydrogels enabled a sustained release of the growth factors in vitro, where the release kinetics can be tailored simply by varying the crosslinking density of the gelatin MPs and formulation of the OPF hydrogels [69, 70, 79]. This system has been successfully used for both cartilage and bone tissue engineering. Studies in our laboratory by Park et al. have shown that OPF hydrogel composites encapsulating TGF-β1-loaded MPs supported the proliferation and matrix synthesis of co-encapsulated bovine chondrocytes [74]. Similar hydrogel composites releasing TGF-β1 or releasing both TGF-β1 and IGF-1, enhanced chondrogenic differentiation of co-encapsulated rabbit MSCs [75, 114]. For bone tissue engineering, Patel et al. investigated dual delivery of VEGF and BMP-2 from a poly(propylene fumarate) (PPF)-MP composite for in vivo bone regeneration [118]. Although a similar amount of bone growth was observed in both BMP-2 release and dual release groups at 12 weeks, bone formation appeared most abundant in the dual release group at 4 weeks, indicating an interplay between these growth factors for early bone regeneration.

**Mechanical Stimuli**

Both articular cartilage and subchondral bone reside in a dynamic environment with various mechanical stimuli [119, 120]. Therefore, to mimic this environment, a variety of bioreactors such as spinner flasks, rotating wall vessel reactors and flow perfusion bioreactors, have been designed for in vitro generation of cartilage and bone
tissues [119, 120]. These bioreactors not only provide relevant mechanical signals for cell proliferation and differentiation, but also allow for more uniform distribution of cells throughout the scaffold and better mass transfer between the cells and culture medium.

The effects of mechanical stimuli, including direct compression, shear stress and hydrostatic pressure, on chondrocyte proliferation and ECM production have been examined using different types of bioreactors [119]. In general, moderate levels of mechanical loading show a positive stimulus to chondrogenesis [120]. Compression has been found to increase cartilage-like ECM synthesis through a mechanism that potentially involves increased localized fluid flow [121]. Hydrostatic pressure helps maintain chondrocyte phenotype and upregulate the expression of genes associated with chondrogenesis [120]. Studies with low shear, generated either by flow perfusion or rotating wall bioreactors, demonstrated enhanced cartilage-specific ECM deposition [120]. There is also increasing evidence that biomechanical stimuli can promote the differentiation of progenitor cells into a chondrogenic phenotype. Cyclic compressive loading was found to induce the synthesis of TGF-β1 and promote the chondrogenesis of MSCs and proteoglycan synthesis in vitro [122, 123]. Hydrostatic pressure was also shown to enhance the chondrogenic differentiation of MSCs in the presence of other bioactive factors in vitro, as evidenced by increased proteoglycan production and chondrogenic gene expression [124].

Similarly, different bioreactors have been utilized for bone tissue engineering. In our laboratory, a perfusion bioreactor has been developed [125], in which MSCs were able to undergo osteogenic differentiation under shear stress [126, 127]. By changing the amount of shear stress applied or scaffold architecture, the differentiation of MSCs into
Osteoblasts can be modulated [126, 128]. In addition, bioactive molecules introduced to the bioreactor have been shown to have a synergistic effect with flow shear on the osteogenic differentiation of MSCs. For example, direct addition of dexamethasone into culture media or the use of a biomimetic scaffold containing bone-like ECM in the bioreactor, both showed an enhanced osteogenic differentiation of rat MSCs [95, 127].

**Co-culture**

In addition to the delivery of bioactive factors and application of mechanical stimuli, co-culture has become another important tool to regulate cell growth and differentiation. Understanding how cells of different types interact with each other and how signaling cascades exist and contribute to cell proliferation, differentiation, and distribution are important to generate a complex tissue. Recently, different co-culture models have been developed for osteochondral tissue engineering applications [82, 129].

Two dimensional models are utilized to investigate the interactions between different cell types, such as chondrocytes, osteoblasts and progenitor cells [130]. One simple method is to mix two or more types of cells together, allowing their direct contact. The results are commonly compared to those of an insert/trans-well system, which prevents direct contact of the cells but allows for exchange of growth factors secreted by them. In one study involving the co-culture of chondrocytes and osteoblasts, Nakaoka et al. found active interactions between the two cell types with or without direct contact (in plates or trans-well) [130]. Osteoblasts produced growth factors that enhanced chondrocyte proliferation, and the differentiation level of the osteoblasts significantly influenced the differentiation level of the co-cultured chondrocytes. On the other hand, chondrocytes regulated osteogenic differentiation of the osteoblasts through both soluble
growth factors and direct contact. Similarly, in another study by Ahmed et al., rat MSCs embedded in alginate beads were cultured with cartilage chips in trans-wells without addition of growth factors [39]. Chondrogenesis of the MSCs was induced by paracrine factors released from the cartilage, as evidenced by a sustained expression of an early chondrogenic marker, Sox9. Additionally, the co-culture was found to suppress collagen X expression of MSCs, indicative of a hypertrophic phenotype.

Based on these studies, some three-dimensional co-culture systems have been developed. Different types of cells were either pelleted, or seeded in a scaffold (preformed or injectable), and subsequently co-cultured to generate an osteochondral construct.

Jiang et al. has developed a co-culture system with a high density chondrocyte micromass and a monolayer of osteoblasts adhering to it [131]. Their results showed that chondrocytes in the co-culture significantly reduced proteoglycan production; while osteoblasts maintained their ALP activity, but delayed in mineralization. However, the authors were not sure whether the regulatory interactions were due to cell-cell contact or paracrine signaling. Tuli et al. press-coated a mesenchymal progenitor cell (MPC) pellet on a PLA scaffold and induced chondrogenesis using a chondrogenic medium [132]. After 2 or 5 weeks, osteogenic precultured mesenchymal progenitor cells were loaded on the PLA scaffold and co-cultured with the pellet in a cocktail medium. Analyses on the osteochondral construct showed the formation of cartilage-like and bone-like tissues in the respective layers. The co-culture did not negatively affect cell differentiation in the other layer, and furthermore supported the formation an interface resembling the native osteochondral junction.
In another study by Mahmoudifar et al., chondrocytes seeded on PGA scaffolds were co-cultured with either seeded chondrocytes or osteoblasts, or with ex vivo cartilage or bone, to investigate the effect of co-culture on osteochondral tissue formation [133]. Cartilage and bone compartments were sutured together and then cultured in a recirculation bioreactor. The work revealed that the engineered bone (seeded with osteoblasts) had the most stimulatory effect on GAG and collagen synthesis of the chondrocytes in the engineered cartilage compared to the other formulations. Additionally, they reported that the presence of chondrocytes did not prevent osteoblast differentiation and mineralization in the engineered bone during the co-culture. In contrast, some other studies involving the co-culture of chondrogenic and osteogenic cells showed that the co-culture with chondrogenic cells inhibited osteocalcin expression or calcium deposition of the osteogenic cells in an osteochondral construct [84, 86].

Co-culture has also been performed in layered hydrogel systems. Compared to other 3D systems, layered hydrogels demonstrated better integration between layers, and additionally prevented significant cell migration within the system [20, 134]. For example, Lee et al. constructed a layered PEG-DA hydrogel by photopolymerization of polymer and cell solution [134]. The hydrogels consisted of a top-layer encapsulating embryonic stem cells, an acellular layer in the middle, and a bottom layer encapsulating hepatic cells. The co-culture showed enhanced chondrogenic differentiation of embryonic stem cells, probably due to growth factors secreted by the hepatic cells. The same group also reported the co-culture of chondrocytes with MSCs, osteoblasts and fibroblasts in a similar bilayered hydrogel [7]. Their results showed that the cells maintained their phenotypes in each layer during the co-culture, however, they interacted with each other
through paracrine signaling. In the top layer, chondrocytic matrix production was enhanced by the co-cultured MSCs, but not by the co-cultured osteoblasts or the fibroblasts; whereas in the bottom layer, MSC mineralization was suppressed by the chondrocytes in the adjacent layer.

Although various results were found from the studies mentioned above due to different co-culture systems selected, all the cases indicate active interactions between different cell types. For osteochondral tissue engineering, we should take advantage of co-cultures, not only to include both chondrogenic and osteogenic cells, but also to guide and support cartilage formation through cellular interactions with other cell types.

**Summary**

Tissue engineering, integrating the use of cells, scaffolds and bioactive factors, provides a promising solution for osteochondral tissue repair. Exciting results have been shown using MSCs along with different biomaterials to generate both cartilage-like and bone-like tissues. Further research should focus on the development of a well-integrated composite scaffold that can induce and support chondrogenic and osteogenic differentiation of MSCs in the respective layers. Furthermore, 3D co-culture systems which allow for cellular interactions between different cell types should also be further investigated and utilized to improve the quality of an engineered osteochondral tissue.
CHAPTER III: EFFECT OF SWELLING RATIO OF INJECTABLE HYDROGEL COMPOSITES ON CHONDROGENIC DIFFERENTIATION OF ENCAPSULATED RABBIT MARROW MESENCHYAL STEM CELLS IN VITRO†

Abstract

An injectable, biodegradable hydrogel composite of oligo(poly(ethylene glycol) fumarate) (OPF) and gelatin microparticles (MPs) has been investigated as a cell and growth factor carrier for cartilage tissue engineering applications. In this study, hydrogel composites with different swelling ratios were prepared by crosslinking OPF macromers with poly(ethylene glycol) (PEG) repeating units of varying molecular weights from 1,000 ~ 35,000. Rabbit marrow mesenchymal stem cells (MSCs) and MPs loaded with transforming growth factor-β1 (TGF-β1) were encapsulated in the hydrogel composites in order to examine the effect of the swelling ratio of the hydrogel composites on the chondrogenic differentiation of encapsulated rabbit marrow MSCs both in the presence and absence of TGF-β1. The swelling ratio of the hydrogel composites increased as the PEG molecular weight in the OPF macromers increased. Chondrocyte-specific genes were expressed at higher levels in groups containing TGF-β1-loaded MPs and varied with the swelling ratio of the hydrogel composites. OPF hydrogel composites with PEG repeating units of molecular weight 35,000 and 10,000 with TGF-β1-loaded MPs exhibited a 159 ± 95 and a 89 ± 31 fold increase in type II collagen gene expression at day 28, respectively, while OPF hydrogel composites with PEG repeating units of molecular weight 3,000 and 1,000 with TGF-β1-loaded MPs showed a 27 ± 10 and a 17

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± 7 fold increase in type II collagen gene expression, respectively, as compared to the composites with blank MPs at day 0. The results indicate that chondrogenic differentiation of encapsulated rabbit marrow MSCs within OPF hydrogel composites could be affected by their swelling ratio, thus suggesting the potential of OPF composite hydrogels as part of a novel strategy for controlling the differentiation of stem cells.

Abbreviations

APS, ammonium persulfate; DMEM-LG, Dulbecco’s modified Eagle’s medium-low glucose; DMMB, dimethylmethylene blue; DMSO, dimethyl sulfoxide; dsDNA, double-stranded DNA; FBS, fetal bovine serum; GA, glutaraldehyde; GAG, glycosaminoglycan; GAPDH, glyceraldehyde-3-phosphatase dehydrogenase; GPC, gel permeation chromatography; IEP, isoelectric point; MP, gelatin microparticle; MSC, mesenchymal stem cell; OPF, oligo(poly(ethylene glycol) fumarate); PBS, phosphate buffered saline; PEG, poly(ethylene glycol); PEG-DA, poly(ethylene glycol)-diacrylate; RT-PCR, reverse transcriptase-polymerase chain reaction; TEMED, N,N,N',N'-tetramethylethylenediamine, TGF, transforming growth factor.

Introduction

Articular cartilage tissue often lacks the ability of self-repair and is difficult to fully regenerate with currently available treatments [2]. Therefore, tissue engineering strategies have been investigated as an alternative to treat articular cartilage defects [2, 135]. The success of a tissue engineering approach relies on the proper selection of cells, bioactive molecules, and scaffolding materials [2]. One attractive candidate for cells in this approach is mesenchymal stem cells (MSCs), since MSCs may be easily isolated from the bone marrow and expanded without losing their capacity to differentiate into cells of various mesenchymal lineages, including chondrocytes [136]. Numerous studies have demonstrated the successful transplantation of autologous MSCs for cartilage and bone tissue engineering applications [137-139].
Recently, various hydrogel materials have been developed for use as delivery vehicles of bioactive molecules and cells in tissue engineering [140-143]. Hydrogels are physically or chemically crosslinked three-dimensional polymers swollen in water that allow the controlled release of bioactive molecules [55]. Among those, injectable hydrogel materials, which can gel under physiological conditions, have held great potential. Cells and bioactive factors can be easily incorporated in the system and injected to the defect site, which allows minimally invasive procedures for tissue repair [67, 71]. Moreover, depending on the selection of crosslinking molecules, the mechanical properties of hydrogels can be easily tailored [144].

A novel, degradable fumarate-based macromer, oligo(poly(ethylene glycol) fumarate) (OPF), has been developed in our laboratory as an injectable hydrogel carrier for growth factors for orthopedic tissue engineering. Previous studies have demonstrated the potential of degradable OPF hydrogels for in vitro osteogenesis by rat MSCs. OPF hydrogels with two different OPF formulations of varying poly(ethylene glycol) (PEG) molecular weight were prepared for encapsulation of rat marrow mesenchymal stem cells and cultured for 4 weeks both with and without osteogenic supplements [76]. That study demonstrated that the molecular weight of the PEG repeating unit in the macromer affected the hydrogel swelling ratio and that changes in the swelling ratio of OPF hydrogels resulted in different amounts of calcium deposition by encapsulated rat marrow MSCs. In addition, OPF hydrogels have been recently investigated as a cell and growth factor delivery system for cartilage tissue engineering [75]. In that study, rabbit marrow MSCs encapsulated with TGF-β1-loaded MPs in OPF hydrogels showed an increase in gene expression of type II collagen and aggrecan, demonstrating that the incorporation of
gelatin microparticles into a hydrogel matrix is a promising strategy for localized and sustained release of a growth factor. Additional studies have demonstrated the degradation of OPF hydrogel composites with encapsulated MPs both in vitro and in vivo [79, 80].

The present study asked whether the swelling ratio of OPF hydrogel composites affected the proliferation and chondrogenic differentiation of encapsulated rabbit marrow MSCs. OPF hydrogel composites of different swelling ratios encapsulating rabbit marrow MSCs and gelatin MPs loaded with or without TGF-β1 were prepared and cultured for 4 weeks to investigate the effect of hydrogel swelling on the chondrogenic differentiation of encapsulated rabbit marrow MSCs as measured by gene expression of two chondrogenic markers, collagen type II and aggrecan.

**Materials and Methods**

*OPF Synthesis and Characterization*

OPF was synthesized from fumaryl chloride and PEG according to a previously established method [73]. PEG of four different nominal molecular weights (35,000 g/mol, 10,000 g/mol, 3,300 g/mol and 1,000 g/mol) was used to prepare OPF macromers of four different repeating units (namely OPF 35K, OPF 10K, OPF 3K and OPF 1K). Macromer molecular weight was determined by gel permeation chromatography (GPC; Model 410; Waters, Milford, PA) using a refractive index detector (n=3). The purified macromer was stored at −20°C and sterilized prior to use by exposure to ethylene oxide for 14 h.

*Gelatin Microparticle Preparation*

Gelatin microparticles were fabricated from acidic gelatin (Nitta Gelatin Inc., Osaka, Japan) following established procedures [74]. Briefly, a gelatin solution was
prepared by dissolving 5 g of gelatin in 45 ml of distilled, deionized water (ddH₂O) at 60°C. Then, this solution was added dropwise to 250 ml of chilled olive oil while stirring at 500 rpm. After 30 min, 100 ml of chilled acetone (4°C) was added to the emulsion. After an additional 60 min, the microspheres were collected by filtration and washed with acetone. These microparticles were then crosslinked in 0.1 wt % Tween 80 (Sigma, St. Louis, MO) solution with 10 mM glutaraldehyde (GA) (Sigma, St. Louis, MO) while stirring at 500 rpm at 15°C. After 15 h, crosslinked microparticles were collected by filtration, washed with ddH₂O, and then agitated in 25 mM glycine solution for 1 h to inactivate any unreacted GA. These microparticles were collected by filtration, washed with ddH₂O, and then lyophilized overnight. Finally, dried microparticles were sieved to obtain particles of 50-100 µm in diameter and sterilized with ethylene oxide for 14 h.

**OPF Hydrogel Composite Swelling and Degradation Experiment**

For swelling studies, OPF 35K, OPF 10K, OPF 3K and OPF 1K hydrogel composites encapsulating gelatin microparticles were prepared in the same manner as would be used for cell encapsulation. Briefly, 0.1 g of OPF and 0.05 g of the crosslinking agent poly(ethylene glycol) diacrylate (PEG-DA; nominal MW 3400, Nektar Therapeutics, Huntsville, AL) and 0.0219 g of microparticles were combined in 578 µl of phosphate buffered saline (PBS). Equal volumes (46.8 µl) of the thermal radical initiators, 25 mM ammonium persulfate (APS) and 25 mM N,N,N′,N′-tetramethylethlenediamine (TEMED) in PBS, were then added. After gentle mixing, the suspension was quickly injected into Teflon molds (6 mm diameter, 1 mm thickness) followed by incubation at 37°C for 8 min. Hydrogel composites were transferred to PBS and cultured statically at
37°C for 4 weeks. At day 1, 7, 14, 21 and 28, the swelling ratio and sol fraction of OPF hydrogel composites were then determined by the following equations.

\[
\text{Swelling ratio} = \frac{W_s - W_d}{W_d}
\]

\[
\text{Sol fraction} = \frac{W_i - W_d}{W_i}
\]

Here, \(W_i\), \(W_s\), and \(W_d\) represent the weight of dried hydrogel composites after crosslinking, the weight of hydrogel composites after swelling in PBS, and the weight of dried hydrogel composites after swelling, respectively. The swelling ratio is defined as the fractional increase in the weight of the hydrogel due to water absorption. The sol fraction represents the fraction of the polymer following a crosslinking reaction that is not part of a crosslinked network. A decrease in sol fraction over time reflects polymer loss and characterizes the extent of hydrogel degradation.

**Rabbit Marrow MSC Isolation and Pre-culture**

Rabbit marrow MSCs were isolated from the tibias of 4 month old New Zealand white rabbits as previously described [75]. Briefly, after anesthesia, rabbit bone marrow was collected into a 10 ml syringe containing 5000 U of heparin. The bone marrow was then filtered through a cell strainer (40 μm) and cultured in Dulbecco's modified Eagle's medium-low glucose (DMEM-LG) supplemented with 10% v/v fetal bovine serum (FBS; Gemini, Calabasas, CA), 250 μg/l fungizone, 100 mg/l ampicillin, and 50 mg/l gentamicin (Invitrogen) for 2 weeks. In an effort to reduce any interanimal variation, a pool of rabbit marrow MSCs from a total of 6 rabbits was collected together, placed in medium containing 20% FBS and 10% dimethyl sulfoxide (DMSO), and cryopreserved in liquid nitrogen prior to use. For experiments, cryopreserved cells were thawed at 37°C,
seeded in T-75 flasks, and expanded for 14 days of culture in DMEM supplemented medium containing 10% v/v FBS, 250 μg/l fungizone, 100 mg/l ampicillin, and 50 mg/l gentamicin. Cells from one preparation were used in all experiments and were cultured up to passage three before the encapsulation process.

**Microparticle and Rabbit Marrow MSC Encapsulation**

Before encapsulation, OPF 35K, OPF 10K, OPF 3K and OPF 1K were combined with PEG-DA at a 2:1 ratio of OPF to PEG-DA by weight. These polymer mixtures and gelatin microparticles were sterilized with ethylene oxide for 14h. Then, sterilized MPs were loaded with TGF-β1 (R&D Systems, Minneapolis, MN) by immersing them in aqueous TGF-β1 solution at pH 7.4 and incubating them at 4°C for 15h according to established methods [69]. At this pH, there is ionic complexation of gelatin microparticles and TGF-β1 due to the negative charge of the acidic gelatin (isoelectric point (IEP) of 5.0) and positive charge of TGF-β1 (IEP of 9.5) [145]. The total TGF-β1 loading of the microparticles in each gel was 25 ng, which resulted in a concentration of 10 ng TGF-β1/ml relative to the medium (2.5 ml) used for culturing each gel. TGF-β1-free MPs were also prepared for comparison.

For encapsulation of isolated rabbit marrow MSCs and gelatin microparticles in OPF hydrogel composites, a combination of OPF and PEG-DA were dissolved in 300 μl PBS and mixed with the 110 μl microparticle swelling solution. Equal volumes (46.8 μl) of the thermal radical initiators, 0.3 M APS and 0.3 M TEMED in PBS, were then added. After this mixture was vortexed, a 168 μl PBS suspension containing 6.7 million cells was added to achieve a cell concentration of 10 million cells/ml in the final suspension. After gentle mixing, the suspension was quickly injected into Teflon molds (6 mm
diameter, 1 mm thickness) followed by incubation at 37°C for 8 min. Final gel constructs
were transferred into 12 well tissue culture plates. Each well contained one gel construct
and 2.5 ml chondrogenic medium, which was 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, Franklin Lakes,
NJ), 1 mM sodium pyruvate, 50 µg/ml ascorbate 2-phosphate (Sigma-Aldrich), 10⁻⁷ M
dexamethasone (Sigma-Aldrich), 250 mg/l fungizone, 100 µg/l ampicillin and 50 µg/l
gentamicin. The medium was changed every 3 days. For the comparison with
TGF-β1-loaded MP groups (noted as +), the same OPF cell-hydrogel composites with
blank MPs (noted as -) were also prepared and cultured exactly as described above. At
day 7, 14, and 28, samples were collected from each group for biochemical assays (n=4)
and quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) (n=4).
Samples at day 0 were collected immediately after the encapsulation process, including
four samples from each OPF formulation (OPF 35K, OPF 10K, OPF 3K and OPF 1K) for
biochemical assays and a total of four samples from TGF-β1-free groups used as a
control for all groups for RT-PCR analysis. In addition, cell-free hydrogel composites
(n=3) were also prepared following the same methods. These cell-free hydrogel
composites were analyzed with samples to establish any background contribution from
the hydrogel to fluorescence and absorbance measurements in the biochemical assays.

Biochemical Assays for Cell Proliferation and Glycosaminoglycan Production

At each time point, samples and cell-free hydrogels were removed from medium,
rinsed in 2 ml PBS, homogenized with a pellet grinder (Fisher Scientific) and digested in
500 µl of a proteinase K solution (1 mg/ml proteinase K (Sigma-Aldrich), 10 µg/ml
pepstatin A (Sigma-Aldrich), and 185 µg/ml iodoacetamide (Sigma-Aldrich) in tris-EDTA solution (6.055 mg/ml tris(hydroxymethyl aminomethane) (Sigma-Aldrich), 0.372 mg/ml EDTA (Sigma-Aldrich), pH 7.6 adjusted by HCl)) at 60°C for 16 h. After collection and digestion of all samples and cell-free hydrogels, specimens underwent three repetitions of a freeze/thaw/sonication cycle (1 h at −80°C, 30 min at room temperature, 30 min of sonication) for complete extraction of DNA from the cell cytoplasm. DNA and glycosaminoglycan (GAG) assays were then run for samples at each time point (n=4).

DNA content was calculated by measuring double-stranded DNA (dsDNA) content using the PicoGreen assay (Molecular Probes, Eugene, OR) according to the manufacturer’s instructions. The PicoGreen dye binds to dsDNA and results in fluorescence corresponding to the concentration of dsDNA, which was measured by a plate reader (FLx800, Bio-Tek Instrument, Winooski, VT) at a wavelength of 490 nm. The fluorescence of negative, cell-free hydrogels was subtracted from the fluorescence values of experimental groups to account for fluorescence of the material alone.

Similarly, glycosaminoglycan content was also determined using a biochemical assay, the dimethylmethylene blue dye (DMMB) assay (Sigma-Aldrich), as previously described [146]. Upon DMMB binding to GAG, a pink color is produced, allowing for quantification of GAG by measuring absorbance at 520 nm. GAG content in hydrogels was calculated by comparison to a curve generated from standards of known amounts of porcine chondroitin sulfate B (Sigma-Aldrich). A microplate reader (BIO-TEK Instrument, Winooski, VT) was utilized for the absorbance measurements.
**Real-time PCR**

Total RNA was extracted from hydrogel composites at each time point via the RNeasy Mini Kit (Qiagen, Valencia, CA). Briefly, hydrogels were transferred into RNA lysis buffer solution and homogenized by gentle pipetting. The homogenized solution was purified using a Qiagen shredder column and total RNA was extracted with the RNeasy Mini Kit. RNA samples were then reverse-transcribed to cDNA using Oligo dT primers and superscript III transcriptase. The final cDNA transcripts were then subjected to real-time PCR (7300 Real-Time PCR System, Applied Biosystems, Foster City, CA) to determine the expression of genes for type II collagen and aggrecan. Gene expression data were analyzed using the $2^{-\Delta\Delta CT}$ method as reported previously [137, 147]. Briefly, all gene expression data were normalized to the expression of the house-keeping gene, glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) and expressed as the fold ratio as compared with those of a control group. For this study, the control group contained rabbit marrow MSCs embedded in OPF with blank MPs ($n=4$, one sample from each OPF formulation) that were analyzed immediately after encapsulation (day 0). The sequence of primers for GAPDH, type II collagen, and aggrecan were as follows [75]:

**GAPDH:**

5’-TCACCATCTTCCAGGAGCGA-3’

5’-CACAATGCGCAAGTGTCGT-3’

**Type II collagen:**

5’-AACACTGCGAACCAGCAGAT-3’

5’-CTGCAGCAGGTATAGGTGA-3’

**Aggrecan:**

5’-GCTACGGGAGACAGGATGAGTTC-3’

5’-CGTAAAGACCTCACCCTCCAT-3’
Statistical Analysis

Swelling ratio, sol fraction, DNA, GAG, and gene expression were reported as means ± standard deviation. Repetitive ANOVA and Tukey’s multiple comparison test were used to determine possible significant differences (p < 0.05) in the DNA, GAG, and gene expression between groups.

Results

OPF Characterization

The number average molecular weight ($M_n$) and weight average molecular weight ($M_w$) of OPF macromers and the parent PEG for the synthesis were determined by GPC and are shown in Table III-1.

OPF Hydrogel Composite Characterization

Swelling studies indicated that equilibrium swelling of the hydrogel composites had been reached after being immersed in PBS overnight. Swelling ratios of the hydrogels remained the same afterwards for the 28 day culture period (data not shown). The equilibrium fold swelling of OPF hydrogel composites is shown in Figure III-1 (A). OPF 35K had a swelling ratio of 15.1 ± 0.3, which was statistically higher than that of all the other OPF formulations. In addition, OPF 10K hydrogel composites had a statistically higher swelling ratio (13.9 ± 0.2) than either OPF 3K (13.0 ± 0.4) or OPF 1K (12.7 ± 0.3). There was no statistical difference between OPF 3K and 1K. The results indicated that swelling ratio increased as the PEG molecular weight in the OPF formulation increased. Sol fraction of OPF hydrogel composites over time is shown in Figure III-1 (B). For each OPF formulation, there was little change in sol fraction over time. However, OPF 10K
had a significantly lower sol fraction than OPF 35K, OPF 3K and OPF 1K at each time point.

**Biochemical Assays**

DNA content at each time point is depicted in Figure III-2. All groups showed a trend of decreasing DNA content over the culture period. A significant decrease in DNA content was seen for all the treatments at day 28 as compared to day 0. Differences were also found between groups with different swelling ratios at the same time point. More specifically, when blank MPs were encapsulated, a statistically higher DNA content was observed in both OPF 35K- and OPF 10K- hydrogel composites compared with OPF 3K- or OPF 1K- hydrogel composites at day 7, 14 and 28. While in groups with TGF-β1-loaded MPs, significantly higher DNA content was observed in samples for OPF 35K+ at day 28 as compared to those for OPF 3K+ or OPF 1K+.

As shown in Figure III-3, for all of the eight treatments, there was a higher GAG/DNA content at later time points (day 7, 14 and 28) as compared to day 0. Especially in the TGF-β1 treated groups, a significant increase in GAG/DNA content was observed in OPF 35K+ samples at day 14 and day 28, OPF 10K+ samples at day 7, OPF 1K+ samples at day 28 as compared to day 0. No significant difference was found in the groups loaded with blank MPs during the culture period except for OPF 10K- at day 28. Additionally, swelling ratio was determined to have an influence on GAG/DNA content, as evidenced by the significantly higher GAG/DNA values for OPF 35K+ hydrogel composites than both OPF 3K+ and OPF 1K+ hydrogel composites at day 14.
Real-time PCR

Results of collagen type II gene expression over time are presented in Figure III-4. Statistical analysis revealed significantly higher collagen type II gene expression levels for the OPF 35K+ and OPF 10K+ samples (158.8 ± 95.4 and 89.1 ± 31.5 fold increase, respectively) at day 28 than their corresponding TGF-β1-free groups OPF 35K- and OPF 10K- (62.2 ± 34.4 and 29.9 ± 11.3 fold increase, respectively). However, there was no significant change in collagen type II gene expression level for the OPF 3K and 1K samples over the culture period regardless of the presence of TGF-β1. At day 28, the OPF 35K+ samples were determined to have a significantly higher level of collagen type II gene expression than other treatments. OPF 10K+ samples had a significantly higher collagen type II gene expression level than OPF 3K+ and OPF 1K+ samples at day 28.

Similar to collagen type II gene expression, aggrecan gene expression for all the treatments showed an increasing trend during the culture period, as shown in Figure III-5. The results demonstrated a significantly higher aggrecan gene expression level for the OPF 35K+ (23.7 ± 12.0 fold increase), OPF 10K+ (14.2 ± 7.4 fold increase) and OPF 35K- (12.8 ± 6.4 fold increase) samples at day 28 compared with day 0. Additionally, significant differences were also seen between OPF 35K+ samples and OPF 3K+ or OPF 1K+ samples in the presence of TGF-β1-loaded MPs at day 28, while there was no significant change between the groups with blank MPs.

Discussion

The objective of this study was to examine the effect of the swelling ratio of OPF hydrogel composites with gelatin MPs on the chondrogenic differentiation of encapsulated rabbit marrow MSCs. This study was designed to assess how OPF hydrogel
composites with gelatin MPs of four different swelling ratios (OPF 35K, OPF 10K, OPF 3K, and OPF 1K) affected chondrocyte-specific gene expression of encapsulated rabbit marrow MSCs. For each OPF formulation, composites encapsulating MSCs and TGF-β1-loaded MPs (noted as ‘+’) and composites encapsulating MSCs and blank MPs (noted as ‘-’) were included.

The synthesis of OPF from PEG was characterized by GPC. The results indicated a decrease in the number of PEG chains incorporated in the macromer as the PEG molecular weight increased, which was probably due to steric hindrance of large PEG molecules thus affecting the addition of a fumarate unit to the end of the chain [73].

Although many factors, such as molecular weight of macromer, concentration of macromer, crosslinker and initiator as well as crosslinking extent, can influence the swelling ratio of a crosslinked composite, in the current study the swelling ratio of OPF hydrogel composites was adjusted by changing the initial PEG molecular weight for OPF synthesis based on previous reports [76, 78]. Theoretically, as the PEG molecular weight increases, the spacing between the crosslinks becomes larger and therefore the resulting hydrogel will have a larger mesh size, exhibiting as a higher swelling ratio. A previous study has proved that OPF 10K hydrogels had a significantly larger swelling ratio (17.5 ± 0.2) than OPF 3K hydrogels (13.4 ± 0.4) [78]. The results of this study, as expected, demonstrated a significant difference in composite swelling ratio between OPF formulations even though MPs were present. Specifically, OPF 35K and OPF 10K hydrogel composites were shown to have a statistically higher swelling ratio than that of OPF 3K and OPF 1K composites.
Sol fraction of the hydrogel composites represents the fraction of the polymer that is not involved in the crosslinked network. The sol fraction of OPF hydrogel composites was measured over time to evaluate the degradation of hydrogel composites and its potential influence on cell behavior. The results revealed no significant change in sol fraction over a 28-day culture period for each OPF formulation, suggesting little hydrogel degradation occurred. The results further indicated that hydrogel degradation may not be a factor that would affect cell behavior in the present study. OPF 10K hydrogel composites had a lower sol fraction as compared to that of other OPF formulations at all the time points, which was probably due to the varied molar ratios of fumarate double bonds to acrylate double bonds for the OPF formulations examined because the same weight ratios of OPF to PEG-DA were used. However, this result did not present any concern since the goal of this study was to examine hydrogel composites with different swelling ratios.

Cell encapsulation was performed using a thermal radical initiation system (APS/TEMED), which enables crosslinking in situ. Compared to photoinitiation, a thermal initiation system would be beneficial in areas where there is limited light penetration. Although it is known that the radical initiators may elicit some toxicity to the cells, the initiator concentration used in the present study has been previously shown to be cytocompatible for MSCs in OPF hydrogels [76].

DNA assay results showed a trend of decreasing DNA content in all groups during the culture period. As we previously reported, the cell loss may be attributed to the encapsulation process which resulted in some cell death, especially over the first few days of cell culture [75, 76]. Additionally, the decrease in DNA content may also be due
to the culture of cellular constructs in chondrogenic medium, which is chemically defined and serum free. The absence of serum in the medium was shown to be beneficial for chondrogenic differentiation of MSCs but not for cell attachment and growth [42, 148]. In fact, a previous study involving encapsulation of bovine chondrocytes in similar OPF/MP composites showed a significant increase in DNA content after culture in a serum-containing medium for 14 days [74]. OPF swelling ratio was determined to have a significant influence on DNA content in the hydrogel composites. At day 7, 14 and 28, a significant difference in DNA content was observed between higher swelling ratio composites (OPF 35K- and 10K-) and lower swelling ratio composites (OPF 3K- and 1K-). In addition, in the presence of TGF-β1-loaded MPs, OPF 35K hydrogel composites (OPF 35K+) also had a higher DNA content than OPF 3K as well as OPF 1K hydrogel composites (OPF 3K+ and 1K+) at day 28. OPF 35K and 10K had comparatively higher swelling ratios, indicative of larger mesh sizes, which possibly facilitated nutrient and growth factor diffusion and resulted in higher DNA content.

GAG is a marker of chondrocytic phenotype, and therefore GAG deposition in the hydrogel composites was measured to evaluate MSC chondrogenic differentiation. The results revealed that GAG/DNA content increased after day 0 and remained similar after day 7 for all the formulations (Figure III-3), which provided evidence of MSC chondrogenic differentiation in the hydrogel composites. Significant increase in GAG production as compared to day 0 was seen more frequently in the groups with TGF-β1 (OPF 35K+ at day 14 and 28, OPF 10K+ at day 7 and OPF 1K+ at day 28) than those without TGF-β1 (only OPF 10K- at day 28), suggesting the stimulative effect of TGF-β1 on chondrogenic differentiation. However, a generally low GAG production by rabbit
marrow MSCs encapsulated in a hydrogel composite was seen and this may be explained by the tight polymer network surrounding cells, thus limiting the deposition of GAGs only in the pericellular space. Alternatively, rabbit marrow MSCs may need additional signaling to sustain GAG production following chondrogenic differentiation. Further study is needed to elucidate the relationship between encapsulated rabbit marrow MSC differentiation and extracellular matrix production.

To further characterize the in vitro chondrogenesis by rabbit marrow MSCs as a function of the swelling ratio of hydrogel composites, gene expressions of two chondrogenic-specific markers, collagen type II and aggrecan, were measured by quantitative RT-PCR [137, 147].

The RT-PCR results showed that the presence of TGF-β1 in the hydrogel composites significantly promoted collagen type II gene expression. For each OPF formulation, samples cultured with TGF-β1-loaded MPs expressed more collagen type II than those with blank MPs. Additionally, significant increases were found in OPF 35K+ and OPF 10K+ samples compared with OPF 3K+ or OPF 1K+ samples at day 28, indicating that chondrogenic differentiation was accelerated in hydrogel composites with higher swelling ratios. This result may be due to better diffusion of signaling molecules and nutrients in hydrogel samples with a higher swelling ratio. However, it could also be due to the different local concentrations of TGF-β1 in the hydrogel phase after its release from the gelatin MPs. A previous study investigating the release kinetics of TGF-β1 from OPF 10K and OPF 3K hydrogel composites prepared with different crosslinking agents in the absence of encapsulated cells showed that both composites exhibited a burst release after 3 days followed by a sustained release for a period of 25 days. However, the
relative amount of TGF-β1 released from each hydrogel composite was different, suggesting a different local concentration of TGF-β1 inside the corresponding hydrogel [79].

Aggrecan gene expression demonstrated a similar trend as that for collagen type II. Upregulation of the gene expression was seen over time for all the treatments, especially when TGF-β1 was present. Samples in OPF 35K+, with a higher swelling ratio, resulted in a significantly higher level of aggrecan gene expression at day 28 than all the other formulations. Interestingly, some extent of chondrogenic differentiation was also observed in OPF 35K- hydrogel composites since both collagen type II and aggrecan gene expression were upregulated at day 28. This could be due to the chondrogenic potential of chemically defined medium. Previous literature reports indicated that chondrogenic differentiation was achieved in only 25% of the samples cultured in chondrogenic medium without addition of TGF-β1, while in the presence of TGF-β1 chondrogenic differentiation was induced for all the samples [42].

Both collagen type II and aggrecan gene expressions suggested that hydrogel composites of higher swelling ratios, including OPF 35K and OPF 10K hydrogel composites, promoted chondrogenic differentiation of encapsulated rabbit marrow MSCs. This result was consistent with the findings of a previous study concerning the osteogenic differentiation of rat marrow MSCs encapsulated in OPF hydrogels. OPF 10K hydrogels promoted osteopontin production and calcium deposition over OPF 3K hydrogels in the presence of dexamethasone [76]. Both studies suggested that the swelling ratio of hydrogel composites, which is related to the hydrogel mesh size, may affect nutrient transport and drug delivery throughout the hydrogels and thus influence the proliferation
and differentiation of encapsulated cells. Although hydrogel degradation may also affect MSC differentiation, this was not the case in the present study since equilibrium swelling of the hydrogel composites was reached within one day and little change in swelling ratio or degradation was seen during the culture period.

Conclusions

Rabbit marrow MSCs were encapsulated in hydrogel composites of crosslinked OPF and MPs of different swelling ratios and cultured over a 28-day culture period. Real-time RT-PCR demonstrated chondrocyte-specific gene expression in hydrogel composites containing TGF-β1-loaded microparticles. Additionally, enhanced upregulation of chondrocyte-specific genes such as collagen type II and aggrecan was observed in OPF hydrogel composites with higher swelling ratios, which is indicative of larger mesh sizes. The results suggest that chondrogenic differentiation in this system was affected by the swelling ratio (or mesh size) of surrounding hydrogels. Taken together, these results indicate that OPF hydrogel composites of tailored swelling characteristics can be used as part of a novel strategy for controlling the differentiation of MSCs.
Figure III-1: Swelling ratio (A) of OPF 35K, 10K, 3K and 1K hydrogel composites after swelling in PBS overnight and sol fraction (B) of OPF hydrogel composites after immersion in PBS for up to 28 days. The symbol (#) indicates a significant difference as compared to other OPF formulations ($p<0.05$). Error bars represent means ± standard deviation for $n=4$. 

Figures
Figure III-2: DNA content of OPF 35K, 10K, 3K and 1K hydrogel composites encapsulating rabbit marrow MSCs with TGF-β1-loaded MPs (+) or blank MPs (-) over a 28-day culture period. Samples marked by (*) exhibited significant DNA decrease compared to samples at day 0 (p<0.05). The day 0 samples from each blank formulation served as the controls for all samples (blank and TGF-β1-loaded) associated with the respective OPF (OPF 35K, 10K, 3K or 1K). The symbol (#) indicates a significant difference in DNA content as compared to other OPF formulations at the same time point (p<0.05). Error bars represent means ± standard deviation for n = 4.
Figure III-3: GAG/DNA content of OPF 35K, 10K, 3K and 1K hydrogel composites encapsulating rabbit marrow MSCs with TGF-β1-loaded MPs (+) or blank MPs (-) over a 28-day culture period. Samples marked by (*) exhibited significantly higher GAG/DNA compared to samples at day 0 ($p<0.05$). The day 0 samples from each blank formulation served as the controls for all samples (blank and TGF-β1-loaded) associated with the respective OPF (OPF 35K, 10K, 3K or 1K). The symbol (#) indicates a significant difference in GAG/DNA content as compared to other OPF formulations at the same time point ($p<0.05$). Error bars represent means ± standard deviation for $n = 4$. 
Figure III-4: Quantitative collagen type II gene expression for OPF 35K, 10K, 3K and 1K hydrogel composites encapsulating rabbit marrow MSCs and TGF-β1-loaded MPs (+), or rabbit marrow MSCs and blank MPs (-). Data are presented as a fold ratio after being normalized to GAPDH values. The average expression level of controls (Day 0) is represented as one and is shown with the OPF 35K- group. Within a given hydrogel formulation, significantly higher (p<0.05) gene expression than the day 0 value (control) is noted with (*). Samples indicated with (#) had significantly higher gene expression than other OPF formulations at the same time point (p<0.05). Error bars represent means ± standard deviation for n = 4.
Figure III-5: Quantitative aggrecan gene expression for OPF 35K, 10K, 3K and 1K hydrogel composites encapsulating rabbit marrow MSCs and TGF-β1-loaded MPs (+), or rabbit marrow MSCs and blank MPs (-). Data are presented as a fold ratio after being normalized to GAPDH values. The average expression level of controls (Day 0) is represented as one and is shown with the OPF 35K- group. Within a given hydrogel formulation, significantly higher ($p<0.05$) gene expression than the day 0 value (control) is noted with (*). Samples indicated with (#) had significantly higher gene expression than other OPF formulations at the same time point ($p<0.05$). Error bars represent means ± standard deviation for $n = 4$. 
Tables

Table III-1: Number ($M_n$) and weight ($M_w$) average molecular weights of PEG and OPF as determined by GPC.

<table>
<thead>
<tr>
<th></th>
<th>$M_n$</th>
<th>$M_w$</th>
<th></th>
<th>$M_n$</th>
<th>$M_w$</th>
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<td>PEG 35K</td>
<td>38,380 ± 1,310</td>
<td>62,170 ± 2,110</td>
<td>OPF 35K</td>
<td>41,520 ± 1,570</td>
<td>125,580 ± 4,750</td>
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<td>PEG 10K</td>
<td>8,870 ± 280</td>
<td>11,570 ± 360</td>
<td>OPF 10K</td>
<td>9,230 ± 300</td>
<td>64,970 ± 2,110</td>
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<tr>
<td>PEG 3K</td>
<td>2,900 ± 90</td>
<td>3,390 ± 100</td>
<td>OPF 3K</td>
<td>4,290 ± 140</td>
<td>39,540 ± 1,250</td>
</tr>
<tr>
<td>PEG 1K</td>
<td>860 ± 30</td>
<td>1,000 ± 30</td>
<td>OPF 1K</td>
<td>2,930 ± 90</td>
<td>15,560 ± 490</td>
</tr>
</tbody>
</table>
CHAPTER IV: REPAIR OF OSTEOCHONDRAL DEFECTS WITH BIODEGRADABLE HYDROGEL COMPOSITES ENCAPSULATING MARROW MESENCHYMAL STEM CELLS IN A RABBIT MODEL

Abstract

This work investigated the delivery of marrow mesenchymal stem cells (MSCs), with or without the growth factor transforming growth factor-β1 (TGF-β1), from biodegradable hydrogel composites on the repair of osteochondral defects in a rabbit model. Three formulations of oligo(poly(ethylene glycol) fumarate) (OPF) hydrogel composites containing gelatin microparticles (GMPs) and MSCs were implanted in osteochondral defects, including (1) OPF/GMP hydrogel composites; (2) OPF/GMP hydrogel composites encapsulating MSCs; and (3) OPF hydrogel composites containing TGF-β1 loaded GMPs and MSCs. At 12 weeks, the quality of new tissue formed in chondral and subchondral regions of defects was evaluated based on subjective and quantitative histological analysis. OPF hydrogel composites were partially degraded and the defects were filled with newly formed tissue at 12 weeks with no sign of persistent inflammation. With the implantation of scaffolds alone, newly formed chondral tissue had an appearance of hyaline cartilage with zonal organization and intense staining for glycosaminoglycans, while in the subchondral region hypertrophic cartilage with some extent of bone formation was often observed. The addition of MSCs, especially with TGF-β1 loaded GMPs, facilitated subchondral bone formation, as evidenced by more trabecular bone appearance. However, the delivery of MSCs with or without TGF-β1 at

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the dosage investigated did not improve cartilage morphology. While OPF-based hydrogel composites supported osteochondral tissue generation, further investigations are necessary to elucidate the effects of MSC seeding density and differentiation stage on new tissue formation and regeneration.

**Abbreviations**

APS, ammonium persulfate; DMEM-LG, Dulbecco's modified Eagle’s medium-low glucose; GAG, glycosaminoglycan; GMP, gelatin microparticle; H & E, hematoxylin and eosin; IGF, insulin-like growth factor; MSC, mesenchymal stem cell; OPF, oligo(poly(ethylene glycol) fumarate); PBS, phosphate buffered saline; PEG, poly(ethylene glycol); PEG-DMA, poly(ethylene glycol)-diacrylate; TEMED, N,N,N',N'-tetramethylethylenediamine; TGF, transforming growth factor.

**Introduction**

Although articular cartilage has a complex, highly organized structure responsible for its important function, it lacks an intrinsic capability to repair itself and is difficult to fully regenerate with current treatments [2]. Recently, tissue engineering strategies combining cells, scaffolds and bioactive factors have been investigated for the replacement of structural and functional aspects of native cartilage [2, 135]. Among the different cell populations investigated for cartilage tissue engineering applications, mesenchymal stem cells (MSCs) hold great promise for the generation of constructs as MSCs can be easily isolated from bone marrow and expanded in vitro [136]. Numerous studies have demonstrated the successful transplantation of autologous MSCs for bone and cartilage tissue engineering [138, 149, 150].

Various types of scaffolds made of synthetic and natural polymers have been found to provide a favorable environment for small cartilage lesions [151]. However, the repair of extended osteochondral lesions may require a suitable scaffold that can carry both growth factors and cells [82]. A novel oligomer, oligo(poly(ethylene glycol)
fumarate) (OPF), has been recently developed as a cell carrier and growth factor delivery system for cartilage tissue engineering [74, 76]. OPF can be synthesized by esterification of fumaryl chloride and poly(ethylene glycol) (PEG). The double bonds in the main chain enable this material to crosslink into a hydrogel, and the hydrolysis of ester linkages along the chain leads to degradation of a crosslinked hydrogel [73]. Previous work in our laboratory has shown that incorporation of gelatin microparticles (GMPs) into hydrogels is a promising strategy for controlled release of growth factors [69, 70]. The GMPs encapsulated in the hydrogel composites function as both a digestable porogen and a drug delivery vehicle, which have been shown to improve the sustained release of loaded drug compared to hydrogels or GMPs alone, and to enhance the proliferation of cells co-encapsulated [69, 74, 79]. In vivo studies also demonstrated the therapeutic effect of insulin-like growth factor-1 (IGF-1) released from hydrogel composites implanted into osteochondral defects in rabbits [80, 81]. However, fibrous tissue was observed in the neo-surface with the delivery of transforming growth factor-β1 (TGF-β1) from OPF/GMP hydrogel composites [80, 81].

A previous study in our laboratory also investigated the encapsulation of rabbit marrow MSCs in OPF-based hydrogel composites containing growth factor-loaded GMPs and measured in vitro chondrogenesis [75]. Rabbit MSCs encapsulated in hydrogels combined with TGF-β1-loaded GMPs showed an increase in gene expression of type II collagen and aggrecan, which are characteristic of chondrocytes, indicating that MSCs undergo chondrogenic differentiation in this approach [75]. Therefore, in this study, we hypothesized that the combined delivery of MSCs and TGF-β1 would enhance the quality of new cartilage formation in a rabbit osteochondral defect model by
influencing not only host cells but also implanted cells. More specifically, this study investigated (1) the effect of MSC delivery on the quality of new cartilage and bone tissue formation; and (2) the effect of the combined delivery of MSCs and TGF-β1 on the quality of new cartilage and bone tissue formation, both involving MSCs in a rabbit osteochondral defect model.

Materials and Methods

Three different formulations were designed for the present study and implanted in rabbits, including (1) OPF/GMP hydrogel composites without cells or growth factors (OPF group); (2) OPF hydrogel composites encapsulating blank GMPs and MSCs (10 million cells/ml hydrogel) (OPF/MSC group); and (3) OPF hydrogel composites containing TGF-β1 loaded GMPs (600 ng/ml hydrogel) and MSCs (10 million cells/ml hydrogel) (OPF/MSC/TGF group).

OPF Synthesis

OPF macromer was synthesized from PEG (Sigma, St. Louis, MO) with nominal molecular weight of 10,000 according to a method developed in our laboratory [73]. The resulting OPF was sterilized by exposure to ethylene oxide gas for 14 h.

Gelatin Microparticle Fabrication and Loading

Gelatin microparticles were fabricated from acidic gelatin (Nitta Gelatin Inc., Osaka, Japan) and crosslinked in 10 mM glutaraldehyde (Sigma, St. Louis, MO) according to previously established methods [152]. After lyophilization, the GMPs were sieved to obtain particles 50-100 μm in size and then sterilized with ethylene oxide.

Sterile GMPs were then loaded with recombinant human TGF-β1 (R&D Systems, Minneapolis, MN) by swelling in an aqueous solution of the growth factor at pH 7.4 for
15 hrs before composite fabrication according to established methods [79]. In particular, 110 µl of TGF-β1 loading solution (volume needed for equilibrium swelling of the GMPs) was combined with 22 mg of GMPs. The loading solution had a concentration of 3.6 µg TGF-β1/ml phosphate buffered saline (PBS) to achieve a final loading of 600 ng TGF-β1/ml crosslinked scaffolds. This growth factor concentration has been shown to promote the chondrogenic differentiation of rabbit MSCs encapsulated in hydrogel composites in vitro [75]. Blank GMPs were loaded with PBS in a similar fashion and served as control.

**Rabbit Marrow MSC Isolation and Pre-culture**

Rabbit marrow MSCs were isolated from the tibias of 6-month-old New Zealand White rabbits (specified-pathogen-free, different rabbits from those used for implantation) as previously described [75, 138]. Two isolations were performed in this study, respectively, for the two groups treated with cells (OPF/MSC and OPF/MSC/TGF groups). Each isolation involved 3 rabbits in an effort to minimize any interanimal variation. Specifically, harvested bone marrow from each rabbit was cultured in Dulbecco’s modified Eagle’s medium-low glucose (DMEM-LG; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gemini, Calabasas, CA), 250 µg/l fungizone, 100 mg/l ampicillin, 50 mg/l gentamicin. After 2 weeks, the cells were lifted with a trypsin-EDTA solution (passage one cells) and mixed with cells from the other two rabbits of an isolation for composite fabrication. Previously, rabbitMSCs isolated and cultured using this method have been tested for their multi-potentiality to differentiate into osteoblast-like cells and chondrocyte-like in vitro [75].
Hydrogel Composite Fabrication

Before implantation, hydrogel composites were fabricated according to the formulations shown in Table IV-1 following established methods [74]. Briefly, 0.1 g of sterile OPF and 0.05 g of sterile poly(ethylene glycol) diacrylate (PEG-DA; nominal MW 3400, Nektar Therapeutics, Huntsville, AL) in 300 µl of PBS were transferred to a vial containing the swelling microparticle solution (either blank or TGF-β1-loaded). For crosslinking, equal parts (46.8 µl) of initiator solutions, 0.3 M ammonium persulfate (APS) and 0.3 M N,N,N',N'-tetramethylethylenediamine (TEMED), were first mixed with the polymer solution, followed by the addition of 168 µl of a cell suspension containing 6.7 million rabbit MSCs to reach a final concentration of 10 million cells/ml solution. The solution was then quickly injected into Teflon molds (2.2 mm in diameter x 2.2 mm in depth) and incubated at 37°C for 8 minutes. Cell-free constructs were fabricated in a similar fashion using PBS instead of the cell suspension. After crosslinking, hydrogel constructs with or without MSCs were transferred in PBS before they were implanted in the osteochondral defects in rabbits within 2 h. The final dimensions of the scaffolds after swelling were 3 mm in diameter and 3 mm in height, which match the dimensions of the osteochondral defect.

Animal Surgery

A total of 18 specified-pathogen-free New Zealand white rabbits (6-month-old) were used for the implantation study based on a well-established rabbit osteochondral (full-thickness) defect model [81, 138]. Rabbits 6 months in age were selected for this study since they are skeletally mature; a previous study has shown histologically and radiographically that their growth plates in the distal femur closed at 19-24 weeks [153].
All in vivo work was conducted in accordance with ISO standards, and protocols of the Radboud University Nijmegen Medical Center, Nijmegen, the Netherlands. National guidelines for the care and use of laboratory animals were observed, and approval of the Experimental Animal Ethical Committee was obtained (RUDEC 2007-019).

Animal surgeries were performed as described previously [81]. Briefly, bilateral osteochondral defects (3 mm in diameter x 3 mm in depth) were first created on the weight-bearing surface of medial femoral condyles. Then a prefabricated hydrogel composite, which had similar dimensions of the defect, was press-fit into the defect. Subsequently, the muscle and skin were closed. Each rabbit received 2 hydrogel composites with the same formulation, one per knee, and the procedure was repeated for 6 rabbits per formulation, resulting in n=12 implants per group. To minimize post-operative discomfort, Fynadyne® was administered for two days postoperatively. The animals were returned to their cages after surgery and allowed unrestricted weight-bearing activity. Signs of pain, infection and proper activity were monitored and carefully recorded.

**Tissue Processing**

Rabbits were euthanized at twelve weeks after surgery by intravenous administration of Nembutal (pentobarbital). The tissue surrounding the medial femoral condyle was retrieved en bloc. Specimens were fixed in 10% buffered formalin (pH 7.4) for 1 week, decalcified in Formical2000 (Decal Corporation, Congers, NY, USA) for 2 weeks, dehydrated through a graded series of ethanol, and then embedded in paraffin. Longitudinal sections of 6 μm 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. Two sections from each region were then stained with hematoxylin and eosin (H&E), Safranin O/Fast green as well as Masson’s trichrome separately, and subsequently scored [80].

**Histological Scoring**

Histological sections were blindly and independently scored by three evaluators (FKK, SY, JDK) using an established scoring system consisting of 11 parameters for osteochondral repair, as shown in Table IV-2 [80, 81, 138, 154-157]. Analysis was done over the whole defect for both chondral (within the upper 1 mm of the defect) and subchondral regions (within the bottom 2 mm of the defect). In particular, the chondral region was scored for its morphology, thickness, regularity, and chondrocyte clustering, as well as cell and glycosaminoglycan (GAG) content. The cell and GAG content of the cartilage tissue adjacent to the defect was also examined to assess possible tissue degeneration near the implant site. Additionally, bone filling, integration, and morphology in the subchondral region of defects were assessed during this evaluation.

**Statistical Analysis**

Prior to the study, the number of defects needed in each group (n=12) was determined by power analysis and consideration of previous studies [80, 81]. For histological data analysis, ordered logistic regression was performed on each of the parameters in the scoring system to determine the potential effects of implant formulation, location within the defect (lateral, medial edges and center) and knee joint (left and right) on tissue regeneration following previous methods [80, 81]. A significance level of 0.05 was used for the statistical analysis.
Results

Macroscopic Observation

All animals regained full movement within one week, and they continued to exhibit normal behavior and movement during the 12-week period. No gross signs of inflammation, infection or swelling were observed upon visual inspection of the joint surface at the time of tissue retrieval. Migration of the hydrogel composites from the defect was not found.

Histological Observation

For all three treatments, complete degradation of the implanted hydrogel composites was seen in approximately half of the specimens. More specifically, the frequency for complete implant degradation was 5/12, 6/12 and 7/12 for the OPF, OPF/MSC and OPF/MSC/TGF groups, respectively. However, in the cases where the hydrogels were not completely degraded, less degradation was seen in the defects when TGF-β1 was not present. Four out of twelve specimens had less than 50% degradation in the OPF and OPF/MSC groups, whereas only one was found in the OPF/MSC/TGF group. A similar extent of implant degradation and tissue filling was seen in the left and right knees of each rabbit.

In the OPF group, the defects were filled with newly formed cartilage tissue in the superficial part and bone tissue in the deep part. In seven out of twelve specimens, cartilage developed in the subchondral bone area and occupied more than 2/3 of the whole defect. Neo-formed cartilage tissue on the surface had an appearance of hyaline cartilage, evidenced by an intense Safranin O staining as shown in Figure IV-1 (b). At higher magnification, the cells were round, clustered, and surrounded by extracellular
matrix, resembling well differentiated chondrocytes [Figure IV-1 (d)]. In some sections, chondrocytes were even arranged with a zonal organization, indicative of true articular cartilage. As also shown in Figure IV-1 (d), slightly elongated cells were located near the articular surface. Below these cells, more rounded cells were clustered in columns, as expected for the cells in middle and deep zones of articular cartilage.

Although trabecular bone was present in some areas of the subchondral region, the bone regeneration seemed incomplete because of incomplete degradation of the hydrogel composites and the presence of cartilage tissue. Hypertrophic cartilage was seen and appeared calcified in some sections, indicative of the process of cartilage resorption and bone formation. In most specimens, tissue in the subchondral region integrated well with the surrounding host tissue and cartilage tissue.

Similarly, in the OPF/MSC group, the subchondral region contained remaining hydrogel composite material, hypertrophic cartilage, and some trabecular bone. Cartilage had an appearance of hyaline cartilage and fibrocartilage with varying thickness, as seen in Figure IV-2.

In the group with TGF-β1, neo-cartilage tissue exhibited a fibrocartilage-like appearance. In particular, a thicker fibrous layer with faint staining for GAG and intense staining for collagen was seen near the articular surface as compared to the other two groups [Figure IV-3 (a-c)]. At higher magnification, the cells in this layer appeared as small dots and were surrounded by fibrillar matrix [Figure IV-3 (d)]. In the subchondral region, although remaining hydrogel and hypertrophic cartilage were still observed in some sections, more trabecular bone formation could be seen as compared to the groups without TGF-β1.
Histological Scoring

Figure IV-4 displays the quantitative scoring of 11 parameters of osteochondral restoration. Statistical analysis was performed to determine the effects of implant formulation, position in the defect site (lateral, center or medial), and knees (left and right) on each of the 11 parameters. The analysis revealed that implant formulation affected significantly the surface morphology, cartilage thickness, surface regularity, and cell and GAG content of the neo-surface. Position within the defect was determined to be a significant factor affecting only cell and GAG content of the adjacent surface, but not for any of the other parameters. No significant difference was found between left and right knees for all the parameters except bone bonding.

Mean scores for overall tissue filling and scaffold degradation were around two [Figure IV-4 (a)], indicating that more than 50% of the implant was degraded and filled with tissue [according to the scoring system, Table IV-2 (a)] over the 12-week period. However, no significant difference existed between the various implant formulations.

Similarly, no significant difference was observed for bone filling, subchondral morphology or bone bonding among the implant formulations [Figure IV-4 (b)]. Bone filling scored slightly above two, indicating that more than 50% of the subchondral region was filled with tissue at 12 weeks. Bone bonding had an average score between two and three, which was consistent with the histological appearance that the neo-formed tissue in the subchondral region was well integrated with the surrounding host tissue. The detailed differences in subchondral bone morphology are shown in Figure IV-5 (a). In the OPF/MSC/TGF group, more sections scored ‘normal, trabecular bone’ (score 4), indicative of the formation of normal trabecular bone, than the other two
groups. These findings corroborate the subjective histological observation that more remodeling of bone tissue was seen in the subchondral region of the defects treated with TGF-β1.

Further, the OPF group had a higher score in cartilage morphology as well as cell and GAG content of neo-cartilage than the other two groups [Figure IV-4 (c)]. Specifically, the score for the cartilage morphology of the OPF group was 2.9 ± 1.3, reflecting the majority of hyaline cartilage near the joint surface. In the OPF/MSC group, the mean score was 2.4 ± 1.3, which was consistent with the histological appearance of a mixture of hyaline cartilage and fibrocartilage. When TGF-β1 was incorporated, the neo-formed tissue had a score of 1.9 ± 1.2 in cartilage morphology, indicating the fibrous nature of the neo-surface. A detailed score distribution of cartilage morphology for each formulation is displayed in Figure IV-5 (b). Scoring for cell and GAG content demonstrated the same trend and additional statistical evaluation revealed that for both of the two markers, significant differences were observed between the OPF and OPF/MSC/TGF groups.

For neo-cartilage thickness, the OPF group had a mean value around two [Figure IV-4 (c)], suggesting thicker neo-cartilage formed than host cartilage according to the scoring system. Although the scores of this group were significantly higher than those of both the OPF/MSC and OPF/MSC/TGF groups, the results may be due to the fact that more hypertrophic cartilage developed in the subchondral area and less remodeling of bone tissue was observed in this group. Additionally, when compared to the group implanted with OPF scaffolds alone, the delivery of cells (OPF/MSC group) significantly improved scores for surface regularity [Figure IV-4 (c)].
No significant difference was seen for chondrocyte clustering or cell and GAG content of adjacent cartilage among the formulations [Figure IV-4 (c)]. The values indicated that no severe degenerative changes occurred in the adjacent cartilage. However, the position within the defect was determined statistically to have an influence on cell and GAG content of adjacent cartilage. Specifically, significant differences in cell and GAG content of adjacent cartilage were seen between the lateral edge and the center of the defect.

Discussion

In this study, we investigated the delivery of MSCs, with or without TGF-β1, from biodegradable hydrogel composites on the repair of osteochondral defects in a rabbit model. Specifically, we investigated (1) whether the implantation of MSCs would influence the quality of new cartilage and bone tissue formation; and (2) whether the combined delivery of MSCs and TGF-β1 would affect the quality of new cartilage and bone tissue formation in a rabbit osteochondral defect model using OPF-based hydrogel composites as cell and growth factor carriers.

At 12 weeks post-surgery, implants were not found to migrate from the defect, as reported previously using OPF constructs in the same model [81]. Hydrogel composites were found to be partially degraded and neo-tissue filling was observed within defects for all three formulations. Although scaffold degradation rate slightly varied among the animals, no signs of prolonged inflammatory response, osteoarthritis, or cartilage destruction were observed, which demonstrated the biocompatibility of OPF-based hydrogel composites and their degradation products.
Tissue formation and integration were seen in the group with the implantation of OPF/GMP scaffolds alone. As shown in Figure IV-1, hyaline cartilage with well-organized chondrocytes and intense GAG staining was seen to fill the chondral portion of the defect at 12 weeks, while hypertrophic cartilage with remodeling matrix was often observed filling the subchondral region. Many previous studies suggested that the post-injury response involves a rapid influx of donor MSCs and growth factors into the defect area and the fabrication of embryonic-like cartilage tissue throughout the defect [150, 158, 159]. This was confirmed by the results of the present study. Although this study did not include an experimental group of untreated defects (empty control), comparison of the current results with the results of a previous study using the same defect model suggests that the quality of neo-tissue is superior to that of untreated defects [81]. The normal cellularity and GAG deposition of neo-cartilage indicated that the OPF/GMP scaffold allowed for the recruitment, infiltration and differentiation of MSCs from the defect site or synovium. It has been reported that gelatin GMPs can bind with growth factors through polyionic interactions [69, 74, 160], therefore we speculate that GMPs incorporated in the hydrogel composites can adsorb and localize the growth factors from the defect site and synovial fluid. The subsequent release of these bioactive factors may contribute to the chondrogenic differentiation of the stem cells.

Many investigators have reported that implantation of MSCs within scaffolds improves bone formation and to a lesser degree cartilage formation [161-164]. However, in the present study, differences in the three markers for subchondral bone formation were not significant among groups. The lack of difference could be due to a comparatively lower cell density in the scaffolds in the present study (10 million cells/ml)
as compared to some other studies using a seeding density of 50 million cells/ml [149, 165].

For cartilage regeneration, implantation of MSCs significantly reduced cartilage thickness, which also provided evidence for faster erosion of hypertrophic cartilage and remodeling of subchondral bone in the presence of MSCs. It should also be noted that surface regularity was significantly improved with transplanted MSCs [see Figure IV-4 (c)]. Previous reports suggested that cartilage fibrillation would proceed when immature surface cartilage lost sufficient mechanical support during the degradation of the scaffold, especially when experiencing vigorous repetitive loading at the weight bearing regions of articular cartilage [62, 163, 166]. Therefore, we hypothesize that the smoother articular surface observed in the OPF/MSC group was a result of faster subchondral bone formation in this group (as compared to the OPF group), which provided sufficient mechanical support for the articular surface. In addition to biomechanical considerations, implanted cells may also contribute to cartilaginous matrix secretion and remodeling, which result in better surface regularity and integration as other investigators reported [150, 164].

Histological scores of other neo-cartilage parameters did not reveal statistical differences for the implantation of MSCs. This is not an uncommon finding for the selected 12 week implantation time and the rabbit model used. For example, Solchaga et al. reported that the implantation of bone marrow in hyaluronan-based sponges accelerated the first stages of the osteochondral repair progress but did not significantly affect medium- (12 week) and long-term (24 week) outcomes of the repair process [138]. Several other studies, which involved cell implantation and cell tracking in osteochondral
defects also demonstrated that implanted cells can survive and participate in the initial repair process, but are finally replaced by host cells [163, 165, 167]. We hypothesize that a similar phenomenon occurred in our study, as we know from previous studies that the cells survive the crosslinking process [74, 75]. Additionally, cells from surrounding host tissues can migrate into the defect, thus explaining the dependence of cell and GAG content on position within the defect. Further research is necessary to elucidate the fate of encapsulated MSCs during implantation and the migration of host cells as well as their involvement in osteochondral tissue repair.

TGF-β1 has been reported to promote not only MSC chondrogenic differentiation, but also to induce osteogenic differentiation in vivo [168, 169]. Therefore, not surprisingly, the highest scores in bone filling and bone morphology occurred for the group including both TGF-β1 and MSCs [see Figure IV-4 (b)]. According to previous studies, TGF-β1 probably participates indirectly in vascularization, which controls the rate of bone formation [158]. Additionally, previous investigations suggested that TGF-β1 also affects chondro/osteoclasts and facilitates cartilage matrix resorption and bone remodeling during endochondral ossification [169]. This may explain the occurrence of more remodeled trabecular bone as seen in the group with TGF-β1 compared to the other two groups. Although TGF-β1 delivery to the bony tissue of osteochondral defects may elicit side effects, such as bone upgrowth into the cartilaginous region of the defects and inflammation [101, 158], such adverse effects were not observed in our study.

Surprisingly, in the current study cell morphology and GAG production of neo-cartilage were not improved by the addition of MSCs or TGF-β1. In fact, a thick
fibrous layer was frequently noticed at the articular surface in the presence of TGF-β1, as shown in Figure IV-3. Since many studies indicated that a high dosage of TGF-β1 is related to the occurrence of fibrosis and osteophyte formation in articular cartilage defects [11, 100], we hypothesize that the fibrous layer as seen in the MSC/OPF/TGF group may be linked to the high local concentration of TGF-β1. A previous in vitro study investigating the release of TGF-β1 from similar OPF/GMP composites has shown that TGF-β1 had a burst release within the first 3 days (65.5 ± 3.3% in PBS and 60.8 ± 3.4% in PBS containing collagenase), and a sustained release up to 28 days (84.6 ± 1.1% in PBS and 83.6 ± 1.5% in PBS containing collagenase) [81]. The TGF-β1 concentration was selected based on an in vitro study, where 600 ng TGF-β1/ml hydrogel achieved the best chondrogenic differentiation of MSCs in similar hydrogel composites [75]. However, the formation of fibrous tissue in vivo emphasizes the significant difference between the in vitro and in vivo environments. In addition to TGF-β1 loaded on GMPs, some other bioactive agents from both the synovial joint and the defect site of the wound may also be involved in the repair process, as evidenced by the cartilage repair for the OPF and OPF/MSC groups.

Comparing the results of this study to those from a previous study in our laboratory, where TGF-β1 alone (200 ng/g gel) was delivered from a similar OPF/GMP composite to an osteochondral defect [81], we found that the combined delivery of both MSCs and TGF-β1 did not exhibit significant improvement in neo-cartilage formation for the MSC seeding density and differentiation stage tested. Although MSCs are reported to be immunoprivileged and immunosuppressive [170-172], histoincompatibility may be a
factor that could affect neo-cartilage formation in the presence and absence of TGF-β1 in this case.

However, controversial results were found in some other studies, demonstrating that delivery of cells and growth factors enhanced cartilage repair as compared to the individual delivery of cells or growth factors. Sharma et al. injected PEG-DA and hyaluronic acid solution containing MSCs and 150 ng of TGF-β3 subcutaneously in nude mice, and they found that scaffolds with MSCs and TGF-β3 produced the highest quality cartilage [173]. Similarly, another study investigating the transplantation of periosteal cells in a fibrin gel to a full-thickness defect in rabbit knees showed that the addition of TGF-β1 (5 ng/ml gel) resulted in better osteochondral integration and improved zonal architecture [164].

The different results for cartilage repair from similar strategies may be due to the differences in cellular microenvironment of defects, scaffold properties as well as growth factor release patterns. The complexity of the in vivo healing environment requires further understanding of the roles of cells and growth factors and their interactions during tissue repair. While OPF/GMP composites supported osteochondral tissue growth, further in vivo assessment (with and without cells) is necessary to optimize scaffold properties, stem cell seeding density and differentiation stage, growth factor dosage and release kinetics for the best osteochondral tissue regeneration. Design of an osteochondral construct of a layered architecture, which allows the delivery of suitable growth factors and cells to both cartilage and bone regions, may also be necessary.
Conclusions

This study investigated the effect of MSC delivery with a biodegradable hydrogel composite in the presence or absence of TGF-β1 on osteochondral tissue repair in a rabbit model. The results revealed that the OPF-based hydrogel composites partially degraded at 12 weeks after implantation, which allowed for cartilaginous and bony tissue formation. The implantation of MSCs with the hydrogel composites did not elicit a persistent inflammation, and facilitated subchondral bone formation in the presence of TGF-β1. However, the delivery of MSCs either with or without TGF-β1 did not improve cartilage morphology for the MSC seeding density and differentiation stage tested.
Figure IV-1: Histological sections showing representative 12-week tissue repair at defect sites treated with OPF hydrogel composites alone. Sections stained with H&E (a), Safranin O (b), and Masson’s trichrome (c) at 2x display cartilaginous tissue growth into the subchondral region. Small arrows define the edges of the defects. Boxed regions are shown at 10x magnification to illustrate clustered, spherical chondrocyte-like cells in the neo-surface (d) and hypertrophic cartilage with calcified matrix in the subchondral region (e). Bar is 1 mm for (a-c) and 250 μm for (d, e).
Figure IV-2: Histological sections showing representative 12-week tissue repair at defect sites treated with OPF hydrogel composites containing MSCs. Sections stained with H&E (a), Safranin O (b), and Masson's trichrome (c) are demonstrated at 2× magnification. Small arrows define the edges of the defects. Boxed regions are shown at 10× magnification to illustrate a mixture of hyaline cartilage and fibrocartilage in the chondral region (d) and partially degraded composites remaining in the subchondral region (e). The big arrow indicates microparticles in a hydrogel composite remained in the defect. Bar is 1 mm for (a-c) and 250 μm for (d, e).
**Figure IV-3:** Histological sections showing representative 12-week tissue repair at defect sites treated with OPF hydrogel composites containing MSCs and TGF-β1. Sections stained with H&E (a), Safranin O (b), and Masson’s trichrome (c) at 2x display a thick fibrous layer at the articular surface and significant restoration in the subchondral region. Small arrows define the edges of the defects. Boxed regions are shown at 10x magnification to illustrate the excessive fibrous tissue growth into the joint surface (d) and remodeling tissue in the subchondral region (e). Bar is 1 mm for (a-c) and 250 μm for (d, e).
Figure IV-4: Histological scoring for overall defect (a), subchondral region (b) and cartilage region (c). Data are shown as average scores with error bars representing standard derivation for n=12. The symbol (*) indicates a statistical difference between groups (P<0.05).
Figure IV-5: Histological score distribution for subchondral morphology (a) and cartilage morphology (b).
### Table IV-1: Experimental groups tested in this study.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Scaffolds</th>
<th>Cell seeding density</th>
<th>Growth factor dose</th>
<th>Repetitions</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPF</td>
<td>OPF+GMPs</td>
<td>----</td>
<td>----</td>
<td>12</td>
</tr>
<tr>
<td>OPF/MSC</td>
<td>OPF+GMPs</td>
<td>MSCs 10 million cells/ml</td>
<td>----</td>
<td>12</td>
</tr>
<tr>
<td>OPF/MSC/TGF</td>
<td>OPF+GMPs</td>
<td>MSCs 10 million cells/ml</td>
<td>TGF-β1 600 ng/ml</td>
<td>12</td>
</tr>
</tbody>
</table>
Table IV-2: Histological scoring system for evaluation of overall tissue filling (a), subchondral bone repair (b), and cartilage repair (c) in rabbit osteochondral defects.

(a) Overall defect evaluation (throughout the entire defect depth)  

<table>
<thead>
<tr>
<th>Description</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Percent filling with neo-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>

(b) Subchondral bone evaluation (within the bottom 2 mm of defect)

<table>
<thead>
<tr>
<th>Description</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>3. Percent filling with neo-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 neo-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></td>
<td>Cartilage evaluation (within the upper 1 mm of defect)</td>
</tr>
<tr>
<td>---</td>
<td>------------------------------------------------------</td>
</tr>
<tr>
<td>6.</td>
<td>Morphology of neo-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 tissue (spherical morphology observed with &lt;75% of cells)</td>
</tr>
<tr>
<td></td>
<td>No tissue</td>
</tr>
<tr>
<td>7.</td>
<td>Thickness of neo-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>8.</td>
<td>Joint surface regularity</td>
</tr>
<tr>
<td></td>
<td>Smooth, intact surface</td>
</tr>
<tr>
<td></td>
<td>Surface fissures (&lt;25% neo-surface thickness)</td>
</tr>
<tr>
<td></td>
<td>Deep fissures (≥25% neo-surface thickness)</td>
</tr>
<tr>
<td></td>
<td>Complete disruption of the neo-surface</td>
</tr>
<tr>
<td>9.</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>10.</td>
<td>Chondrocyte and GAG content of neo-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>11.</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>
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CHAPTER V: IN VITRO GENERATION OF AN OSTEOCHONDRAL CONSTRUCT USING

INJECTABLE HYDROGEL COMPOSITES ENCAPSULATING RABBIT MARROW

MESenchYMAL STEM CELLS†

Abstract

Injectable, biodegradable hydrogel composites of crosslinked oligo(poly(ethylene glycol) fumarate) (OPF) and gelatin microparticles (MPs) were utilized to fabricate a bilayered osteochondral construct consisting of a chondrogenic layer and an osteogenic layer, and to investigate the differentiation of rabbit marrow mesenchymal stem cells (MSCs) encapsulated in both layers in vitro. The results showed that MSCs in the chondrogenic layer were able to undergo chondrogenic differentiation, especially in the presence of TGF-β1-loaded MPs. In the osteogenic layer, cells maintained their osteoblastic phenotype. Although calcium deposition in the osteogenic layer was limited, cells in the osteogenic layer significantly enhanced chondrogenic differentiation of MSCs in the chondrogenic layer. The greatest effect was observed when MSCs were encapsulated with TGF-β1-loaded MPs and cultured with osteogenic cells in the bilayered constructs. Overall, this study demonstrates the fabrication of bilayered hydrogel composites that mimic the structure and function of osteochondral tissue, along with the application of these composites as cell and growth factor carriers.

Abbreviations

ALP, alkaline phosphatase; APS, ammonium persulfate; BMP, bone morphogenetic protein; CM, chondrogenic medium; DMEM, Dulbecco’s modified Eagle’s medium; DMMB, dimethylmethylene blue; DMSO, dimethyl sulfoxide; ECM, extracellular matrix; FBS, fetal bovine serum; FGF, fibroblastic growth factor; GAG, glycosaminoglycan;

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GAPDH, glyceraldehyde-3-phosphatase dehydrogenase; GM, general medium; β-GP, β-glycerophosphate; GPC, gel permeation chromatography; IGF, insulin-like growth factor; MP, gelatin microparticle; MSC, mesenchymal stem cell; OM, osteogenic medium; OPF, oligo(poly(ethylene glycol) fumarate); PBS, phosphate buffered saline; PEG, poly(ethylene glycol); PEG-DA, poly(ethylene glycol)-diacrylate; RT-PCR, reverse transcriptase-polymerase chain reaction; TEMED, N,N,N',N'-tetramethylethylenediamine; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

**Introduction**

Articular cartilage and subchondral bone are two distinct tissues that contribute to the unique and important functions of the articular joint. Subchondral bone serves as a mechanical support for the articular surface [174], while articular cartilage protects bone from high stresses and allows for low-friction movements within the joint [174]. Due to its complex structure and poor access to progenitor cells, articular cartilage has limited ability to regenerate once injured [1, 2]. Thus, degenerative changes in cartilage and subchondral bone cause severe joint pain and progressive loss of joint function, which affects many people of different ages [2].

Recently, tissue engineering has become a promising strategy for tissue regeneration, by combining scaffolds, cells, and bioactive molecules [135]. In this approach, mesenchymal stem cells (MSCs) hold great promise because MSCs can be easily isolated from the bone marrow and expanded without losing their capacity to differentiate into cells of various mesenchymal lineages, including chondrocytes and osteoblasts [136]. Many studies have reported the successful generation of cartilage or bone tissue alone *in vitro* using MSCs [59, 95].

However, due to the different properties of articular cartilage and subchondral bone, a bilayered architecture with proper cell and extracellular matrix distribution is desirable because it mimics the complex structure of osteochondral tissue and contributes
to the function of the articular joint. Some attempts have been made to fabricate cartilaginous and bony layers independently before integrating the two components together by suturing or gluing [3, 84]. However, implantation of these bilayered constructs has typically revealed poor integration between layers, which may cause long-term device failure. Consequently, investigators are exploring methods to construct well-integrated bilayered scaffolds that can guide the chondrogenic and osteogenic differentiation of cells in different regions of the same scaffold during co-culture. The challenge lies in maintaining the appropriate chondrogenic and osteogenic phenotype under a single set of cell culture conditions [3, 5].

A degradable macromer, oligo(poly(ethylene glycol) fumarate) (OPF), has been developed in our laboratory and shown to be suitable as an injectable hydrogel carrier for cells and growth factors for both cartilage and bone tissue engineering [74, 78]. OPF can be synthesized from fumaryl chloride and poly(ethylene glycol) (PEG). The fumarate double bonds in the macromer chains allow this material to crosslink to form a hydrogel network, and the hydrolysis of ester linkages results in the degradation of the crosslinked hydrogel [73]. Previous work in our laboratory has demonstrated the cytocompatibility of OPF hydrogels with various cell types, including MSCs [74-76]. For example, rat MSCs encapsulated in OPF hydrogels and cultured in osteogenic medium have been shown to be viable and to differentiate down the osteogenic lineage, as evidenced by calcium deposition after 4 weeks [76]. Gelatin microparticles (MPs) can be incorporated into this hydrogel during the time of crosslinking for controlled release of growth factors [69, 70]. Previous studies have shown that MPs can be loaded with transforming growth factor-β1 (TGF-β1), an important molecule for in vitro and in vivo chondrogenesis, through
polyionic complexation and encapsulated in OPF hydrogels. The hydrogel composites containing TGF-β1-loaded MPs enable controlled release of the growth factor [69, 70]. Additionally, rabbit MSCs encapsulated with TGF-β1-loaded MPs in OPF hydrogels showed an increase in gene expression of type II collagen and aggrecan, indicating enhanced chondrogenic differentiation [75]. Further, the cellularity of the constructs and the distribution of the MPs in the OPF hydrogels were confirmed qualitatively via both light microscopy and histology [75]. It should also be noted that OPF allows the fabrication of a multi-layered structure with good integration between layers using a multi-step crosslinking procedure, as evidenced by swelling and mechanical studies [81, 90]. All these features suggested that OPF hydrogel composites could be applied for fabrication of osteochondral constructs.

The present study explored the fabrication of a bilayered OPF/MP composite consisting of a chondrogenic layer and an osteogenic layer; MSCs were encapsulated within these composites and MSC differentiation in both layers was investigated. Specifically, the study asked the following questions: (1) whether MSCs can undergo chondrogenic and osteogenic differentiation in the respective layers of a bilayered hydrogel composite; (2) how cells in the osteogenic layer affect MSC chondrogenesis in the chondrogenic layer; and (3) how cells in the osteogenic layer, in combination with TGF-β1-loaded MPs, influence MSC chondrogenesis in the chondrogenic layer.

Materials and Methods

Experimental Design

The overall experimental design is shown in Figure V-1. In order to develop a set of cell culture conditions that is suitable for both osteogenic and chondrogenic
differentiation of MSCs, an osteogenic culture study and a chondrogenic culture study were first performed with single-layer OPF hydrogel composites.

In the osteogenic construct culture study, MSCs were first cultured in a tissue culture flask with osteogenic medium for various periods of time (0, 3 and 6 days) to induce osteogenic differentiation (groups OS0, OS3, OS6 in Figure V-1). The precultured MSCs were then encapsulated in hydrogel composites with blank MPs and subsequently cultured in chondrogenic medium (CM) supplemented with (+) or without (-) β-glycerophosphate (β-GP); osteogenic differentiation was assessed. The purpose of this study was to examine whether the osteoblastic phenotype (induced by preculture) can be maintained within OPF hydrogel composites exposed to chondrogenic culture conditions with (or without) the osteogenic supplement β-glycerophosphate.

In the chondrogenic construct culture study, MSCs were encapsulated in OPF hydrogel composites containing either blank MPs (group BLK) or TGF-β1-loaded MPs (group TGF in Figure V-1) to examine their effect on chondrogenic differentiation of encapsulated MSCs. Cell constructs were cultured in chondrogenic medium supplemented with β-GP.

The next step was an osteochondral construct culture study, where bilayered hydrogel composites consisting of a chondrogenic layer at top and an osteogenic layer at bottom were fabricated. More specifically, in the top layer, MSCs were encapsulated in OPF hydrogels with either blank MPs (BLK) or TGF-β1 loaded MPs (TGF), while in the bottom layer, OPF hydrogel composites with blank MPs were utilized to encapsulate either MSCs (MSC) or 6-day osteogenically precultured cells (OS). Four formulations of bilayered constructs [groups BLK(MSC), TGF(MSC), BLK(OS), TGF(OS) in Figure V-1]
were cultured in CM supplemented with β-GP for 28 days. Samples from the top and bottom layers were analyzed to evaluate chondrogenic and osteogenic differentiation of the cells, respectively.

**OPF Synthesis and Characterization**

OPF was synthesized from fumaryl chloride and poly(ethylene glycol) with a nominal molecular weight of 10,000 g/mol (Sigma, St. Louis, MO) according to a previously established method [73]. Molecular weights of both the parent PEG and the resulting OPF were determined by gel permeation chromatography (GPC; Model 410; Waters, Milford, PA) using a refractive index detector (n=3). The purified macromer was stored at –20°C and sterilized prior to use by exposure to ethylene oxide for 14 h.

**Gelatin Microparticle Fabrication**

Gelatin microparticles were fabricated from acidic gelatin (Nitta Gelatin Inc., Osaka, Japan) and crosslinked with 10 mM glutaraldehyde (Sigma, St. Louis, MO) following established procedures [74]. After drying, MPs were sieved to obtain particles of 50-100 μm in diameter and sterilized with ethylene oxide for 14 h.

**Rabbit Marrow MSC Isolation and Preculture**

Rabbit marrow MSCs were isolated from the tibias of 4 month old New Zealand white rabbits as previously described [75]. Briefly, after anesthesia, the rabbit tibia was punctured with a 16-gauge needle and 4–5 ml of bone marrow were aspirated through a sterile tube into a 10 ml syringe containing 5000 U of heparin. The bone marrow was filtered through a cell strainer (40 μm) and cultured in general medium (GM) containing Dulbecco’s modified Eagle’s medium (DMEM), 10% v/v fetal bovine serum (FBS; Gemini, Calabasas, CA), 250 μg/l fungizone, 100 mg/l ampicillin, and 50 mg/l
gentamicin for 2 weeks. In order to reduce any interanimal variation, a pool of rabbit marrow MSCs from a total of six rabbits was mixed together, and then cryopreserved in medium containing 20% FBS and 10% dimethyl sulfoxide (DMSO) in liquid nitrogen. Prior to use, MSCs were thawed at 37°C and expanded in T-75 flasks with GM up to passage three. For osteogenic preculture, cells were expanded in GM up to passage two, and then, for the third passage, cells were cultured in osteogenic medium (OM), which is DMEM supplemented with 10% v/v FBS, 50 mg/l ascorbic acid, 10 mM β-glycerophosphate, 10^{-8} M dexamethasone, 250 µg/l fungizone, 100 mg/l ampicillin, and 50 mg/l gentamicin (all from Sigma), for 6 days (3 days in the OS3+ group in Figure V-1) before encapsulation. Cells from a single pool of rabbit marrow were used in all three studies and the cells used for each study were from each expansion of cryopreserved cells.

Fabrication of Single-layer Hydrogel Composites

Before encapsulation, sterile MPs were loaded with TGF-β1 (R&D Systems, Minneapolis, MN) by swelling in an aqueous solution of the growth factor at pH 7.4 for 15 h according to a previously reported method [79]. The volume of TGF-β1 loading solution needed for equilibrium swelling of the MPs (110 µl) was combined with 22 mg of MPs. The loading solution had a concentration of 3.6 µg TGF-β1/ml phosphate buffered saline (PBS; Gibco) to achieve a final loading of 600 ng TGF-β1/ml in the crosslinked scaffolds. This growth factor amount has been shown to promote the chondrogenic differentiation of rabbit MSCs encapsulated in hydrogel composites in vitro [75]. Blank MPs were loaded with PBS in a similar fashion.
For fabrication of a single-layer hydrogel composite, 0.1 g of sterile OPF and 0.05 g of sterile poly(ethylene glycol) diacrylate (PEG-DA; Nektar Therapeutics, Huntsville, AL) were first dissolved in 300 µl of PBS and mixed with swelled MP solution (either TGF-β1-loaded or blank). The mixture was then added to equal volumes (46.8 µl) of the thermal radical initiator solutions, 0.3 M ammonium persulfate (APS) and 0.3 M N,N,N',N'-tetramethylethylenediamine (TEMED) in PBS. A cell suspension (6.7 million cells in 168 µl of PBS) was subsequently added to the polymer solution to achieve a concentration of 10 million cells/ml final suspension. After gentle mixing, the suspension was quickly injected into Teflon molds (6 mm diameter, 1 mm thickness), followed by incubation at 37°C for 8 min. In the osteogenic construct culture study, all hydrogel composites contained blank MPs, while in the chondrogenic construct culture study, hydrogel composites contained either blank MPs or TGF-β1-loaded MPs, as shown in Figure V-1.

Fabrication of Bilayered Hydrogel Composites

Bilayered hydrogel composites were fabricated via a two-step crosslinking procedure. The desired composition for the osteogenic (bottom) layer (Figure V-1) was first injected into the bottom 1 mm of Teflon molds (6 mm diameter, 2 mm thickness) and incubated for 4 minutes, allowing for partial crosslinking. Meanwhile, another polymer-cell suspension was prepared, and then injected into the partially filled Teflon molds to form the chondrogenic layer. The resulting bilayered constructs were then incubated at 37°C for 8 minutes to achieve crosslinking.

All hydrogel constructs were transferred into 12-well tissue culture plates. Each construct was cultured with 2.5 ml chondrogenic medium, which was 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, Franklin Lakes, NJ), 1 mM sodium pyruvate (Sigma), 50 mg/l ascorbic acid, 10^{-7} M dexamethasone, 250 mg/l fungizone, 100 mg/l ampicillin and 50 mg/l gentamicin. In some of the groups, CM was supplemented with β-glycerophosphate, as indicated in Figure V-1. The medium was changed every 3 days. In the osteogenic and chondrogenic construct culture studies, the hydrogel composites were cultured up to 14 and 21 days, respectively, while in the osteochondral construct culture study, samples were cultured up to 28 days.

At the time points (days 0, 7, 14, 21 and 28), samples were removed from culture medium, rinsed in PBS and collected for quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) (n=4) and for biochemical assays (n=4). Bilayered samples were dissected with a blade to separate the chondrogenic layer and osteogenic layer; samples from each layer were stored for analysis.

Real-time PCR

Samples from the chondrogenic construct culture study and the top layer of the osteochondral construct culture study were subjected to RT-PCR analysis to quantify MSC chondrogenic differentiation. Total RNA was extracted and reverse-transcribed to cDNA as described previously [75]. The final cDNA was then subjected to real time PCR (7300 Real-Time PCR System, Applied Biosystems, Foster City, CA) to determine the expression of genes for collagen type II, aggrecan and collagen type I.

Gene expression data were analyzed using the 2^{ΔΔCt} method as described previously [137, 147]. Briefly, all gene expression data were normalized to the
expression of a house-keeping gene, glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) and expressed as the fold ratio compared to baseline gene expression of a control group at day 0. In the chondrogenic construct culture study, the control group contained a total of four samples from the BLK and TGF groups immediately after encapsulation. Similarly, in the osteochondral construct culture study, four top-layer hydrogels containing MSCs were collected from all the groups at day 0 and served as a control. The sequences of the primers for GAPDH, type II collagen, aggrecan, and type I collagen were as follows [75]: GAPDH: 5’-TCACCATCTTCCAGGAGCGA-3’, 5’-CACAATGCGAAGTGGTGCT-3’; type II collagen: 5’-AACACTGCCAACGTCCAGAT-3’, 5’-CTGCAGGCACGTATAGGTGA-3’; Aggrecan: 5’-GCTACGGAGACAAGGATGAGTTC-3’, 5’-CGTAAAAGACCTCACCCTCCAT-3’; type I collagen: 5’-ATGGATGAGGAACTGGCAACT-3’, 5’-GCCATCGACAAGAACAGTGTAAGT-3’.

**Biochemical Assays**

Samples from the osteogenic construct culture study and the bottom layer of the osteochondral construct culture study were analyzed for DNA, alkaline phosphatase (ALP) enzyme activity, and calcium content to characterize osteogenic differentiation of the cells. In the osteochondral construct culture study, a total of four bottom-layer samples from the BLK(MSC) and TGF(MSC) groups were collected after encapsulation and analyzed to represent day 0 values for both groups. Similarly, the BLK(OS) and TGF(OS) shared bottom-layer samples for biochemical assays at day 0.
At each time point, samples were homogenized with a pellet grinder (Fisher Scientific) and stored in 500 μl of ddH₂O. The homogenates were then stored at -20°C until analysis, when they were subjected to three freeze-thaw-sonication cycles (30 min at -80°C, 30 min at room temperature, 30 min of sonication) for complete extraction of DNA from the cell cytoplasm.

DNA content was determined using the PicoGreen assay (Molecular Probes, Eugene, OR) according to the manufacturer’s instructions. ALP enzyme activity of each homogenized sample was measured by using an Alkaline Phosphatase Assay from Sigma (St. Louis, MO) as previously described [76]. After the DNA and ALP assays, each homogenized sample was mixed with an equal volume of 1 N acetic acid. Calcium content of the hydrogels was then determined using a calcium quantification assay (Diagnostic Chemicals; Charlottetown, PEI, Canada) as described previously [76].

Samples from the chondrogenic construct culture study and the top layer of the osteochondral construct culture study were subjected to DNA, glycosaminoglycan (GAG), and calcium assays to evaluate chondrogenic differentiation. In the chondrogenic construct culture study, a total of four samples from the BLK and TGF groups were collected after encapsulation and analyzed to represent day 0 values for both groups. Similarly, in the osteochondral construct culture study, the BLK(MSC) and BLK(OS) groups shared top-layer samples for biochemical assays at day 0, and the TGF(MSC) and TGF(OS) groups shared samples at day 0.

The analysis of GAG content required samples to be homogenized and digested in 500 μl of a 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); all from Sigma-Aldrich) at 56°C for 16 h. The homogenates were then frozen down and subjected to the same free-thaw-sonication cycles prior to analysis. DNA and calcium assays were performed on the lysate as described above. GAG content was determined using a biochemical assay, the dimethylmethylene blue dye (DMMB) assay (Sigma-Aldrich), as previously described [146].

Microplate readers (BIO-TEK Instrument, Winooski, VT) were utilized for all the absorbance/fluorescence measurements. Cell-free constructs were also fabricated, cultured, and analyzed with the samples. For all assays, fluorescence or absorbance of the cell-free hydrogels was subtracted from the values of experimental groups to account for fluorescence/absorbance caused by the scaffold material alone.

Statistical Analysis

DNA, GAG, calcium contents, ALP activity and gene expression data are reported as means ± standard deviation. Repetitive ANOVA and Tukey’s multiple comparison tests were used to determine possible significant differences (p < 0.05) between groups.

Results

Osteogenic Construct Culture Study

In the osteogenic construct culture study, we examined whether osteoblastic phenotype (induced by preculture) can be maintained within OPF hydrogel composites in the subsequent chondrogenic culture conditions, with an osteogenic supplement. DNA, ALP and calcium results are presented in Figure V-2 (a-c). For all the treatments, DNA content significantly decreased from day 0 to day 7, but the values remained similar after
day 7. At day 7, hydrogels with osteogenically precultured cells were found to have a higher DNA content than those with MSCs, and a significant difference was observed between OS0+ and OS6+ groups. However, by day 14, this difference among groups was no longer present.

ALP results revealed a significantly higher ALP enzyme activity at days 7 and 14 than at day 0 for all the treatments. However, there was no significant change in ALP activity between treatment groups.

At day 14, there was higher calcium deposition in the hydrogels containing cells that were precultured in osteogenic medium for three or six days (and subsequently cultured in CM with β-GP), compared to hydrogels containing cells expanded in general medium. Specifically, 8.29 ± 3.62 μg of calcium were deposited in the OS6+ samples (and 7.55 ± 7.84 μg in the OS3+ samples), compared to 0.38 ± 0.41 μg of calcium deposited in the OS0+ samples by day 14. The difference between the OS6+ and OS0+ groups was significant. Additionally, for MSCs precultured in osteogenic medium for 6 days, a significant difference in calcium content was observed at day 14; constructs cultured in chondrogenic medium with β-GP (OS6+) had much higher calcium deposition than those cultured without β-GP (OS6-).

*Chondrogenic Construct Culture Study*

In the second study, we investigated whether MSCs will undergo chondrogenic differentiation when encapsulated in OPF hydrogel composites with blank MPs or TGF-β1-loaded MPs, and subsequently cultured in chondrogenic medium containing β-GP. The combined influence of β-glycerophosphate and TGF-β1 on scaffold mineralization was also evaluated.
Quantitative gene expression data for collagen type II, aggrecan, and collagen type I at each time point are depicted in Figure V-3 (a-c). The results revealed an increase in collagen type II and aggrecan gene expression over time for both BLK and TGF groups. Specifically, a significantly higher collagen type II gene expression compared to day 0 was seen in the group containing blank MPs at day 28, while in the group with TGF-β1 loaded MPs, significantly increased expression was observed at both day 14 and day 28 (and in the case of aggrecan gene expression, also at day 7). The presence of TGF-β1 significantly affected the gene expression of chondrogenic markers, as evidenced by significant differences between the BLK and TGF groups in collagen type II gene expression at day 28 and in aggrecan gene expression at day 14 and day 28.

For collagen type I gene expression, although the levels for TGF-β1 treated samples increased at day 7 and day 14 compared to day 0, gene expression subsequently decreased; the values on day 21 were found to be statistically equivalent to day 0. For the samples with blank MPs (no TGF-β1 treatment), there was no significant change in collagen type I gene expression over time.

Results for the biochemical assays, which quantified DNA, GAG and calcium content, are presented in Figure V-4 (a-c). For both BLK and TGF groups, there was a significant decrease in DNA content between day 0 and day 7, but the values on days 7, 14 and 21 did not show any significant difference. At day 7, BLK samples had a significantly higher DNA amount than TGF samples, but there was no difference in DNA content between the two treatments at days 14 and 21.

There was a trend of increasing GAG/DNA content for both BLK and TGF groups over the 21-day culture period; TGF samples had higher GAG/DNA values than
BLK samples at each time point. However, statistical analysis revealed no significant difference in GAG/DNA content between groups at each time point during the culture period.

Little calcium deposition was observed for either BLK or TGF samples when cultured in the chondrogenic medium with β-GP. The values for each treatment were found to be below 1 μg at each time point and statistically equivalent to those at day 0.

Osteochondral Construct Culture Study

After testing the effect of our set of culture conditions (specifically, CM + β-GP) on both MSC chondrogenic and osteogenic differentiation in single-layer composites, we developed a bilayered hydrogel composite consisting of a chondrogenic layer at top and an osteogenic layer at bottom. Chondrogenic and osteogenic differentiation of the cells in each respective layer, as well as the signaling between layers, were examined in this study.

RT-PCR analysis for gene expression of collagen type II, aggrecan, and collagen type I was performed on the top layer of the bilayered hydrogels to evaluate chondrogenic differentiation, as shown in Figure V-5 (a-c). For all the treatments, cells showed an increase in collagen type II and aggrecan gene expression over time. Significantly higher expression levels of both genes were found at days 14 and 28 (compared to day 0) in the TGF(OS) treatment group. Samples with osteogenic precultured cells in the bottom layer (indicated by (OS) in Figure V-5) exhibited higher gene expression in both markers at every time point than those with MSCs in the bottom layer (indicated by (MSC) in Figure V-5); this trend was observed both in the presence and absence of TGF-β1. Interestingly, although there was a significant difference in gene
expression between BLK and TGF groups in the single-layer chondrogenic construct culture study, when the same formulations were co-cultured with MSCs encapsulated in the bottom layer, the difference in gene expression between BLK(MSC) and TGF(MSC) treatments at any time point was not significant (compare Figure V-5 (a-b) to Figure V-3 (a-b)). However, when osteogenic precultured cells were seeded in the bottom layer, they seemed to have a synergistic effect with TGF-β1 on chondrogenic differentiation of the cells in top layer, as evidenced by a significant increase in collagen type II gene expression at day 28 and in aggrecan gene expression at days 14 to 28 compared to all the other groups. Additionally, it should also be noted that samples without TGF-β1 loading on the top layer but with osteogenic cells in the bottom layer (group BLK(OS) in Figure V-5) demonstrated a higher gene expression in the chondrogenic markers than the samples with TGF-β1 and MSCs at bottom (group TGF(MSC) in Figure V-5).

Although there was no significant difference in collagen type I gene expression among formulations at each time point, the trend over time was different between MSC and OS treatments. The samples with MSCs at bottom showed an increase in collagen type I gene expression at later time points as compared to day 0, whereas when the bottom layer contained osteogenic cells, collagen type I gene expression decreased or remained unchanged over time.

DNA content of the top and bottom layers at each time point is shown in Figure V-6 (a) and Figure V-7 (a), respectively. For both layers, the results exhibited a decreasing trend over time with significant difference between day 0 and later time points. In the top layer, more DNA was seen in samples with OS cells at bottom than those with just MSCs at bottom. In particular, DNA content in the top layer of the BLK(OS) group
was found to be significantly higher than that for the BLK (MSC) or TGF(MSC) group at day 28. Similarly, a higher DNA content was seen in the bottom layer for OS treatment groups compared to MSC treatment groups at each time point, either with or without TGF-β1. There was significantly more DNA in the bottom layer of BLK(OS) or TGF(OS) samples than BLK(MSC) constructs at day 7. No significant difference in DNA content of the bottom layer was observed at days 14 and 28 among formulations.

GAG content, indicative of chondrocytic phenotype, was measured for top layer hydrogels and is shown in Figure V-6 (b); GAG content was measured to further investigate chondrogenic differentiation of the encapsulated MSCs. For all four formulations, significantly higher GAG/DNA levels were observed at later time points (days 7, 14 and 28) compared to day 0. Significant differences in GAG/DNA values among groups were only found between the BLK(OS) group and both the BLK(MSC) and TGF(MSC) groups. However, this could be due to the artifact of higher DNA content in the BLK(MSC) group compared to the other two groups at this time point. The results of calcium content in top-layer hydrogels are depicted in Figure V-6 (c). All the formulations had little calcium deposition (less than 1 μg) in the top layer and the results showed no differences among the groups.

For the bottom layer, ALP enzyme activity and calcium amount were measured to evaluate osteogenic differentiation of the cells, as shown in Figure V-7 (b) and (c). There was a general trend of increasing ALP activity over time for all the treatments. However, no significant differences were observed among the groups at any time point. A significant increase in ALP amount compared to day 0 was only observed for MSC treatment groups at day 28, likely because OS cells had a higher ALP level at day 0 than
MSCs. Compared to the results obtained from the single-layer study [Figure V-2 (b)], the ALP values in this study were in a similar range.

Calcium content measurements indicated a generally low calcium deposition in the bottom layer of the bilayered scaffolds. At day 14, cells in BLK(OS) group produced $2.04 \pm 2.74 \, \mu g$ calcium, which was a little higher than the other treatments. However, due to the large standard deviation, no statistical difference was found among groups or over time.

**Discussion**

Guiding both osteogenic and chondrogenic differentiation of MSCs in different regions of the same composite scaffold is challenging. We hypothesized that osteogenically precultured MSCs can maintain their phenotype in OPF hydrogel composites that are subsequently cultured with CM containing $\beta$-GP. Additionally, we hypothesized that this medium would support chondrogenic differentiation of MSCs encapsulated in the hydrogel composites. These hypotheses were first examined with an osteogenic construct culture study and a chondrogenic culture study using single-layer OPF hydrogel composites.

Bilayered hydrogel composites consisting of a chondrogenic layer and an osteogenic layer were then fabricated and cultured in CM containing $\beta$-GP. We specifically investigated: (1) the differentiation of MSCs in both layers of the bilayered hydrogel composites; (2) the effect of cells in the osteogenic layer on MSC chondrogenesis in the chondrogenic layer; (3) the effect of cells in the osteogenic layer combined with TGF-\(\beta\)1-loaded-MPs on MSC chondrogenesis in the chondrogenic layer.
In the osteogenic construct culture study, we developed a protocol for osteogenic differentiation, which involved preculture of MSCs in osteogenic medium in a tissue culture plate, encapsulation of the MSCs in OPF/MP hydrogel composites and subsequent culture of cell constructs in a chondrogenic environment with β-glycerophosphate.

Our results revealed a decrease in DNA content from day 0 to day 7, which was consistent with previous studies using the same scaffold for chondrogenic and osteogenic differentiation [75, 76]. As previously reported, although the radical initiators were biocompatible at the concentration used in the present study, the encapsulation process may still result in cell death at early time points; cell number remains the same at later time points [75, 76]. Alternatively, the decrease in DNA content could be due to the culture in the chemically defined, serum-free, chondrogenic medium. It has been shown that the serum-free condition is beneficial for MSC chondrogenic differentiation but not for cell proliferation [41]. During the process of chondrogenic differentiation, encapsulated MSCs may stop proliferating, resulting in a decrease in cell number. In the present study, after switching the cells to chondrogenic medium for 7 days, we found more DNA in the constructs with osteogenically precultured cells than in those with just MSCs, suggesting a more active role of the osteogenically precultured cells in the chondrogenic environment. Additionally, during the preculture in the tissue culture plate before encapsulation, we also observed a faster proliferation rate of the cells in the osteogenic medium than that of cells in general medium (data not shown). Based on these observations, we speculate that, during osteogenic preculture, cells may produce growth
factors that support cell survival and enhance proliferation.

It has been shown that osteogenic differentiation of MSCs includes three periods: proliferation, extracellular matrix maturation, and mineralization [49]. ALP is considered as an early marker for osteogenic differentiation; it is expressed after cell proliferation and is involved in the onset of extracellular matrix mineralization [49]. The significant increase in ALP activity at days 7 and 14 compared to day 0 suggested that cells continued osteogenic differentiation in the chondrogenic environment, after preculture. This could be attributed to the chemical compounds common to both chondrogenic medium and osteogenic medium, including dexamethasone and ascorbic acid. However, little difference in ALP activity among the groups was observed. In a previous study investigating rabbit MSC osteogenic differentiation in osteoblast-conditioned medium, the authors found similar levels of ALP produced by cells in a control medium compared to cells in conditioned or chemically supplemented medium. They inferred that MSCs have a natural predisposition to differentiate into the osteogenic lineage in the absence of any growth factors and cytokines [175]. Alternatively, the lack of difference in ALP activity among groups could be due to the difficulty in extracting ALP, a cell membrane-associated molecule, from hydrogels, as reported in a similar study involving rat MSC osteogenic differentiation in OPF hydrogels [76].

In contrast to the ALP results, calcium deposition, which is a late marker of osteogenic differentiation, was observed to have higher values in OS3+ and OS6+ samples than in either OS0+ samples or OS6- samples at day 14. This underscores the importance of both osteogenic preculture, and the addition of β-GP to the subsequent culture medium, on cell osteogenic differentiation. A previous review on sequential gene
expression during osteoblast differentiation indicated that the onset of mineralization is associated with many factors besides ALP expression, such as co-expression of osteopontin and osteocalcin as well as the maturation of the extracellular matrix (ECM) [49]. Without osteogenic preculture, it is possible that the signals in chondrogenic medium with added β-glycerophosphate were not enough for MSCs to undergo osteogenic differentiation, or that these conditions led to a delay in matrix mineralization, resulting in significantly lower calcium deposition in OS0+ samples compared to OS6+ samples. Similarly, the significant difference in calcium amount between OS6+ and OS6- groups indicates that β-GP could have played an important role in providing further signals for maintaining osteoblastic phenotype and enhancing calcium deposition in chondrogenic medium.

Both ALP and calcium results confirmed that the osteoblastic phenotype was maintained in a chondrogenic environment with the osteogenic supplement β-GP. This protocol provides an exciting new method for using the same culture medium for both chondrogenic and osteogenic differentiation of MSCs in different portions of a scaffold material.

**Chondrogenic Construct Culture Study**

It has been shown that when MSCs differentiate to chondrocytes, they start to express chondrocyte-specific marker genes such as collagen type II and aggrecan, along with downregulating the gene expression for collagen type I, which is produced by undifferentiated MSCs [137]. RT-PCR results from the chondrogenic construct culture study demonstrated general trends of increasing gene expression of collagen type II and aggrecan, and decreasing gene expression of collagen type I over time, which confirmed
the chondrogenic differentiation of MSCs encapsulated in OPF composites and cultured in chondrogenic medium supplemented with β-glycerophosphate in the absence or presence of TGF-β1. Additionally, the effect of TGF-β1 on MSC chondrogenic differentiation was found to be significant, as evidenced by the earlier appearance of a marked increase in gene expression of chondrogenic markers, as shown in Figure V-3 (a-b).

DNA results showed a trend of decreasing DNA content over time in all studies, as reported, for instance, in the chondrogenic construct culture study [Figure V-4 (a)]. Significantly lower DNA content was found in the TGF group compared to the BLK group at day 7, indicating a possible inhibitory effect of TGF-β1 on cell proliferation during the early portion of the culture period of the OPF hydrogel composites. Similar results were also seen in an osteogenic study by Roostaeian et al., in which the addition of TGF-β1 resulted in a 44% decrease in cellular growth of rabbit MSCs when compared to medium alone [176].

GAG production, associated with chondrocytic phenotype of the cultured cells, exhibited higher values for both groups at later time points than at day 0, which together with gene expression results confirmed chondrogenic differentiation, particularly in the presence of TGF-β1 [Figure V-4 (b), Figure V-3]. However, GAG production was comparatively low and there was no statistical difference between groups; this may be because of the tight polymer network surrounding cells, which may have limited the deposition of matrix.

It should be noted that all the cell proliferation, gene expression and matrix production data in this study were comparable to those from a previous study where the
same formulations were cultured in chondrogenic medium [177]. This study indicated that the addition of β-GP to chondrogenic medium did not significantly affect the chondrogenic potential of the medium.

Little calcium deposition was observed in the present study, where constructs were cultured in chondrogenic medium supplemented with β-GP, either in the presence or absence of TGF-β1. Although β-GP has been reported to have a stimulatory effect on osteogenesis and calcification, its function with respect to mineralization seems to require the presence of other signals, such as those present in hypertrophic chondrocytes. Coe et al. reported that the addition of 10 mM β-GP to hypertrophic chondrocytes resulted in stimulation of type X collagen synthesis and the onset of mineralization. In contrast, the addition of β-GP to non-hypertrophic chondrocytes failed to induce expression of type X collagen or to induce calcium production [178]. Alternatively, serum could also be an important factor that synergizes with β-GP to induce calcification. Calcium deposition was noted when MSCs were encapsulated in OPF hydrogels and cultured in osteogenic medium, which has similar components to the medium used in the present studies, but also has serum [76]. Although an osteogenic supplement, β-GP, was utilized as one of the medium components in the present study, the results indicated that the chemically defined, serum-free medium seemed to provide a favorable environment for chondrogenic differentiation, without eliciting calcium deposition.

Osteochondral Construct Culture Study

Combining the results from the osteogenic and chondrogenic construct culture studies, we hypothesized that chondrogenic medium with β-GP would support both chondrogenic differentiation and osteogenic differentiation of MSCs in OPF/MP
composites in vitro. In the osteochondral construct culture study, we fabricated bilayered composites consisting of a chondrogenic layer and an osteogenic layer.

Chondrogenic differentiation of the MSCs in the top (chondrogenic) layer was evaluated via gene expression of chondrocytic markers, collagen type II and aggrecan, as well as an MSC-associated marker, collagen type I. As expected, the results showed upregulation of chondrogenic markers and downregulation of collagen type I during the culture period for all the formulations, which further confirmed the chondrogenic potential of the culture medium.

By comparing gene expression data from the MSC and OS treatment groups, it was apparent that the addition of osteogenic cells in the bottom layer of the composites enhanced the chondrogenic differentiation of the MSCs in the top layer. Samples without TGF-β1 in the top layer, but with osteogenic cells in the bottom layer [BLK(OS)], demonstrated either similar or higher gene expression of chondrogenic markers and a lower gene expression of collagen type I, compared to the samples with TGF-β1 loaded MPs in the top layer and MSCs in the bottom layer. Thus, it is likely that the osteogenic cells in the bottom layer of the BLK(OS) group produced chondro-inductive signals and had a positive effect on the differentiation of the cells in the top layer. In fact, studies in our laboratory have shown that rat MSCs cultured in osteogenic medium either express genes for or secrete many molecules of bioactive factors related to cartilage and bone formation, including TGF-β1, fibroblastic growth factor-2 (FGF-2), vascular endothelial growth factor (VEGF), bone morphogenetic protein-2 (BMP-2), and insulin-like growth factor-1 (IGF-1) [93, 96]. These paracrine signals could have contributed to the chondrogenic differentiation of the cells in the top layer.
Similarly, bioactive molecules secreted by osteogenic cells in bilayered constructs may also explain the synergistic effect of OS cells and TGF-β1 on chondrogenic differentiation of MSCs in the top layer. Numerous studies have reported that the combination of TGF-β1 with other growth factors such as IGF-1, FGF-1, or BMP-2, has a synergistic effect on MSC chondrogenic differentiation in vitro, probably due to regulatory cross-talk between the signaling cascade of each growth factor [43, 44, 115, 116].

In contrast, when MSCs were encapsulated in the bottom layer of the bilayered hydrogel, the effect of TGF-β1 on cell differentiation in the top layer was not as obvious as in the chondrogenic construct culture study. In that single-layer study, where the same formulations as the top layers of the osteochondral construct culture study were cultured, the TGF group showed significantly higher collagen type II and aggrecan gene expression than the MSC group. Since the same amount of TGF-β1 was loaded in the single-layer and bilayered constructs, the addition of a bottom layer with more MSCs (in the bilayered construct) could have resulted in each cell experiencing a lower TGF-β1 concentration as compared to the conditions in the single-layer hydrogel. A previous study has shown that with a lower dosage of TGF-β1, gene expression of collagen type II and aggrecan by MSCs declined in OPF/MP composites [75].

The incorporation of OS cells in the bottom layer affected not only MSC chondrogenic differentiation in the top layer, but also influenced cell number for both the top and bottom layers. As shown in Figure V-7 (a), more cells were present in the bottom layer of the hydrogels for OS groups than MSC groups at day 7, which was in accordance with the results obtained from the osteogenic construct culture study [Figure V-2 (a)].
The results of both studies indicated that after in vitro osteogenic preculture, the cells were more resilient than plain MSCs, and were able to survive the crosslinking procedure better and differentiate in OPF hydrogels. Additionally, the precultured cells in the bottom layer could have produced bioactive molecules that supported cell proliferation in the top layer through a paracrine effect, as speculated before. This was evidenced by higher DNA content in top layer hydrogels for OS treatment groups than MSC treatment groups at each time point [Figure V-6 (a)]. Similar results were also found in a study by Nakaoka et al., in which they demonstrated that proliferation and differentiation of chondrocytes were enhanced by soluble factors produced from osteoblasts in a co-culture system with both direct and indirect contact [130].

In fact, when comparing DNA results from the top and bottom layers to their corresponding single-layer culture results (chondrogenic and osteogenic construct culture studies, respectively), we found the values to be comparable. The results suggested that the increase in thickness of bilayered hydrogels did not affect cell growth, which further underscored the advantages of an OPF/MP crosslinked network for nutrient transport and cell growth.

GAG production in the top-layer hydrogels also exhibited similar values to those obtained in the single-layer study for the same formulations [Figure V-4 (b) and Figure V6 (b)]. The increased GAG production compared to day 0 provided additional evidence of MSC chondrogenic differentiation. It is also important to see that no calcium was deposited in this layer under the influence of the osteogenic cells in the bottom layer [Figure V-6 (c)].

The continued osteogenic differentiation of the bottom-layer cells was confirmed
by the increased amount of ALP over time and the presence of some calcium deposits in the constructs [Figure V-7 (b-c)]. Although numerous studies have reported that chondrocytes have a positive effect on the osteogenic differentiation of MSCs in co-culture systems [50, 179], similar values for ALP secretion and calcium deposition were observed in our bilayered study and single-layer study for either MSCs or precultured cells (Figure V-2 and V-7), suggesting a limited effect of the top-layer cells on the osteogenic differentiation of the bottom-layer cells. Since the MSCs in the top layer were undergoing chondrogenic differentiation into chondrocyte-like cells, the bioactive factors produced by these cells could be limited in amount and thus explain the limited effect of these cells on osteogenic differentiation. Additionally, TGF-β1 released from MPs in the top layer might have inhibited calcium deposition, explaining the difference between the calcium amount in the BLK(OS) group and the TGF(OS) group. Previous studies have also reported a similar inhibitory effect of TGF-β on osteogenic differentiation of both MSCs and osteoblasts differentiated from MSCs, as evidenced by reduced ALP activity and mineral deposition [108].

Conclusions

In the current work, we developed a set of culture conditions, which not only promote chondrogenic differentiation of MSCs encapsulated in OPF hydrogel composites, but which also maintain the osteoblastic phenotype of precultured MSCs encapsulated in the same hydrogel composites. A bilayered osteochondral construct containing a chondrogenic layer and an osteogenic layer was fabricated using OPF hydrogel composites containing gelatin MPs and MSCs. The culture of the bilayered constructs showed that MSCs were able to undergo chondrogenic differentiation in the
chondrogenic layer. Although calcium deposition in the osteogenic layer was limited, the osteogenic cells in this layer enhanced MSC chondrogenic differentiation in the chondrogenic layer. Most importantly, osteogenic cells in the bone layer exhibited a synergistic effect with TGF-β1 loaded MPs, promoting chondrogenesis in the chondrogenic layer, as evidenced by enhanced levels of gene expression for collagen type II and aggregan. The present study demonstrated the fabrication of bilayered osteochondral constructs using biodegradable hydrogel composites for the co-delivery of growth factors and MSCs for cartilage tissue engineering.
Figures

◆ Osteogenic Construct Culture Study

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◆ Chondrogenic Construct Culture Study

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Plate preculture Hydrogel culture

◆ Osteochondral Construct Culture Study

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Figure V-1: A schematic representation of the overall experimental design. Four groups were included in the osteogenic construct culture study, namely OS0+, OS3+, OS6+ and OS6- groups. Two groups were included in the chondrogenic construct culture study, namely BLK and TGF groups. In the osteochondral construct culture study, four groups were included, namely BLK(MSC), TGF(MSC), BLK(OS), and TGF(OS) groups. OS cells in this study were precultured with osteogenic medium for 6 days. CM: chondrogenic medium; GM: general medium; β-GP: β-glycerophosphate; MPs: gelatin microparticles; OM: osteogenic medium; OS: osteogenic; TGF-β1: transforming growth factor-β1.
Figure V-2: Biochemical assay results for single-layer OPF/MP hydrogel composites encapsulating rabbit marrow MSCs (OS0), 3-day osteogenically precultured MSCs (OS3) or 6-day osteogenically precultured MSCs (OS6); all composites were cultured in chondrogenic medium supplemented with (+) or without (-) β-glycerophosphate. DNA content (a), ALP enzyme activity (b), and calcium content (c) of the samples are presented. Within a given group, a significant difference (p<0.05) compared to day 0 value (day 7 value for calcium assay) is noted with (*). Samples indicated with (#) had a significant difference from the other groups at the same time point (p<0.05). Error bars represent means ± standard deviation for n = 4.
Figure V-3: Quantitative gene expression of collagen type II (a), aggrecan (b), and collagen type I (c) for single-layer hydrogel composites encapsulating rabbit marrow MSCs and blank MPs (BLK) or rabbit marrow MSCs and TGF-β1-loaded MPs (TGF). Data are presented as a fold ratio after being normalized to GAPDH expression. The expression level of controls (Day 0, BLK group) is represented as one. Within a given group, a significant difference in gene expression ($p<0.05$) compared to day 0 value (control) is noted with (*). Samples indicated with (#) had a significantly higher gene expression than the other groups at the same time point ($p<0.05$). Error bars represent means ± standard deviation for $n=4$. 
Figure V-4: Biochemical assay results for single-layer OPF hydrogel composites encapsulating rabbit marrow MSCs and blank MPs (BLK) or rabbit marrow MSCs and TGF-β1-loaded MPs (TGF). DNA content (a), GAG content normalized to µg DNA (b), and calcium content (c) of the samples are presented. Within a given group, a significant difference ($p<0.05$) compared to day 0 value is noted with (*). Samples indicated with (#) had a significant difference from the other groups at the same time point ($p<0.05$). Error bars represent means ± standard deviation for $n=4$. The BLK and TGF groups shared samples at day 0.
Figure V-5: Quantitative gene expression of collagen type II (a), aggrecan (b), and collagen type I (c) for the top layer of the bilayered OPF hydrogel composites. The top layer of each composite contained encapsulated rabbit marrow MSCs and blank MPs (BLK) or rabbit marrow MSCs and TGF-β1-loaded MPs (TGF), which were co-cultured with a bottom layer consisting of an OPF hydrogel/blank MPs composite encapsulating MSCs (MSC) or osteogenic cells (OS). Data are presented as a fold ratio after being normalized to GAPDH values. The expression level of controls [Day 0, BLK(MSC) group] is represented as one. Within a given group, a significant difference in gene expression (p<0.05) compared to day 0 value (control) is noted with (*). Samples indicated with (#) had a significantly higher gene expression than the other groups at the same time point (p<0.05). Error bars represent means ± standard deviation for n = 4.
Figure V-6: Biochemical assay results for top layer of the bilayered hydrogels. The top layer of each composite contained encapsulated rabbit marrow MSCs and blank MPs (BLK) or rabbit marrow MSCs and TGF-β1-loaded MPs (TGF), which were co-cultured with a bottom layer consisting of an OPF hydrogel/blank MPs composite encapsulating MSCs (MSC) or osteogenic cells (OS). DNA content (a), GAG content normalized to μg DNA (b) and calcium content (c) of the samples are presented. Within a given group, a significant difference ($p<0.05$) compared to the day 0 value is noted with (*). Samples indicated with (#) had a significant difference from the other groups at the same time point ($p<0.05$). Error bars represent means ± standard deviation for $n = 4$. At day 0, the BLK(MSC) and BLK(OS) groups shared top-layer samples for all biochemical assays; and similarly the TGF(MSC) and TGF(OS) groups shared samples.
Figure V-7: Biochemical results for bottom layer of the bilayered hydrogels. The bottom layer of each composite consisted of an OPF hydrogel/blank MPs composite encapsulating MSCs (MSC) or osteogenic cells (OS); these were co-cultured with a top layer containing encapsulated rabbit marrow MSCs and blank MPs (BLK) or rabbit marrow MSCs and TGF-β1-loaded MPs (TGF). DNA content (a), ALP enzyme activity (b) and calcium content (c) of the samples are presented. Within a given group, a significant difference ($p<0.05$) compared to the day 0 value is noted with (*). Samples indicated with (#) had a significant difference from the other groups at the same time point ($p<0.05$). Error bars represent means ± standard deviation for $n = 4$. At day 0, the BLK(MSC) and TGF(MSC) groups shared bottom-layer samples for all biochemical assays; and similarly the BLK(OS) and TGF(OS) groups shared samples.
CHAPTER VI: THE EFFECTS OF TRANSFORMING GROWTH FACTOR-B3 AND PRECULTURE PERIOD OF OSTEOCGENIC CELLS ON THE CHONDROGENIC DIFFERENTIATION OF RABBIT MARROW MESCNYMAL STEM CELLS ENCAPSULATED IN A BILAYERED HYDROGEL COMPOSITE

Abstract

In this work, injectable, biodegradable hydrogel composites of crosslinked oligo(poly(ethylene glycol) fumarate) (OPF) and gelatin microparticles (MPs) were utilized to fabricate a bilayered osteochondral construct. Rabbit marrow mesenchymal stem cells (MSCs) were encapsulated with transforming growth factor-β3 (TGF-β3)-loaded MPs in the chondrogenic layer and cocultured with cells of different periods of osteogenic preculture (0, 3, 6 and 12 days) in the osteogenic layer to investigate the effects of TGF-β3 delivery and coculture on the proliferation and differentiation of cells in both layers. The results showed that, in the chondrogenic layer, TGF-β3 significantly stimulated chondrogenic differentiation of MSCs. Additionally, cells of various osteogenic preculture periods in the osteogenic layer, along with TGF-β3, enhanced gene expression for MSC chondrogenic markers to different extents. In the osteogenic layer, cells maintained their alkaline phosphatase activity during the coculture; however, mineralization was delayed by the presence of TGF-β3. Overall, this study demonstrated the fabrication of bilayered hydrogel composites that mimic the structure and function of osteochondral tissue, along with the application of these composites as cell and growth factor carriers, while illustrating that encapsulated cells of different

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degrees of osteogenic differentiation can significantly influence the chondrogenic differentiation of cocultured progenitor cells in both the presence and absence of chondrogenic growth factors.

**Abbreviations**

ALP, alkaline phosphatase; APS, ammonium persulfate; BMP, bone morphogenetic protein; calcein AM, calcein acetoxymethyl ester; CM, chondrogenic medium; DMEM, Dulbecco's modified Eagle's medium; EthD-1, ethidium homodimer-1; FBS, fetal bovine serum; ES, embryonic stem cell; FGF, fibroblastic growth factor; GAG, glycosaminoglycan; GAPDH, glyceraldehyde-3-phosphatase dehydrogenase; GM, general medium; IGF, insulin-like growth factor; MP, gelatin microparticle; MSC, mesenchymal stem cell; OM, osteogenic medium; OPF, oligo(poly(ethylene glycol) fumarate); OS, osteogenic; PBS, phosphate buffered saline; PEG, poly(ethylene glycol); PEG-DA, poly(ethylene glycol)-diacrylate; RT-PCR, reverse transcriptase-polymerase chain reaction; TEMED, N,N,N',N'-tetramethylethylenediamine; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

**Introduction**

Articular cartilage is an avascular tissue with limited ability to self-repair once injured. Injured articular cartilage can lead to degeneration of subchondral bone, resulting in severe pain and loss of mobility of the affected joint [1, 2]. Therefore, for long-term repair of articular cartilage, it is necessary to develop a construct consisting of both articular cartilage and subchondral bone to mimic the structure and fulfill the functions of native osteochondral tissue [3, 26, 82].

Mesenchymal stem cells (MSCs) are progenitor cells that reside in many tissues, including bone marrow [39, 136]. MSCs can be easily isolated and expanded without losing their ability to differentiate into different connective tissue cell types, such as chondrocytes and osteoblasts. Therefore, for the generation of an osteochondral construct, MSCs are an ideal candidate cell population, which circumvents the need for invasive isolation of chondrocytes and osteoblasts from limited sources [26]. However,
investigation of the chondrogenic and osteogenic differentiation of MSCs, especially in a coculture environment, stands as a fundamental aspect of the development of a cellular construct for osteochondral tissue repair.

A three-dimensional network is known to be important for MSC proliferation and differentiation, since it mimics the in vivo environment [67]. Among the numerous scaffold materials available for tissue engineering approaches for osteochondral repair, hydrogels possess the key ability to allow for ample intake of nutrients and removal of wastes to support the viability of encapsulated cells [55, 141]. Recently, a biodegradable and injectable oligomer, oligo(poly(ethylene glycol) fumarate) (OPF), has been developed in our laboratory [73]. The double bonds in the OPF backbone allow it to crosslink to form a hydrogel network under physiological conditions; and the crosslinked material may degrade via ester hydrolysis. Additionally, gelatin microparticles (MPs) have been incorporated in OPF hydrogels as a digestable porogen and a drug delivery vehicle [70, 81]. The resulting hydrogel composites containing growth-factor-loaded MPs have been shown to support both chondrogenic and osteogenic differentiation of encapsulated MSCs [75, 76, 177].

In addition to a three-dimensional scaffold, chemical supplements and growth factors are also found to be crucial for guiding cell differentiation in vitro [41, 50]. Osteogenic differentiation of MSCs in vitro usually occurs with osteogenic supplements in a culture medium, including dexamethasone, ascorbic acid, and β-glycerophosphate [50]; whereas chondrogenic differentiation requires not only a chemically-defined, serum-free medium but also the presence of chondrogenic growth factors, especially
members of the transforming growth factor-β (TGF-β) family [41], such as TGF-βs and
bone morphogenetic proteins (BMPs).

Another strategy to enhance chondrogenesis is via coculture. Several studies have
investigated the coculture of chondrocytes and osteoblasts in vitro, and they found that
the proliferation and differentiation of chondrocytes were regulated by cell-cell contact
and by soluble factors secreted by the osteoblasts [130, 131]. Similarly, the coculture of
MSCs or embryonic stem cells (ES) with other cells types, such as chondrocytes and
hepatic cells, also demonstrated enhanced levels of chondrogenic differentiation of the
MSCs [39, 134]. Recently, a bilayered hydrogel coculture system has been developed in
our laboratory for osteochondral tissue engineering applications [180]. The study found
that TGF-β1-loaded MPs in the chondrogenic layer of the hydrogel composites and
MSC-derived osteogenic cells in the osteogenic layer synergistically enhanced MSC
chondrogenesis in the chondrogenic layer.

In the present work, we fabricated bilayered OPF hydrogel composites
encapsulating MPs and MSCs in each layer; and we specifically asked the questions: (1)
whether MSCs can undergo chondrogenic and osteogenic differentiation in the respective
layers of a bilayered hydrogel composite; (2) whether TGF-β3-loaded MPs affect MSC
chondrogenic differentiation in the chondrogenic layer; (3) whether TGF-β3-loaded MPs
and cells of different periods of osteogenicpreculture encapsulated in the osteogenic
layer have a combined effect on MSC chondrogenic differentiation in the chondrogenic
layer.
Materials and Methods

Experimental Design

In this study, bilayered hydrogel composites consisting of a chondrogenic layer at top and an osteogenic layer at bottom were fabricated, as shown in Figure VI-1. More specifically, in the top layer, MSCs were encapsulated in OPF hydrogels with either blank MPs (noted with −) or TGF-β3-loaded MPs (noted with +), while in the bottom layer, OPF hydrogel composites with blank MPs were utilized to encapsulate MSCs, 3-day osteogenically precultured cells (OS3 cells), 6-day osteogenically precultured cells (OS6 cells), or 12-day osteogenically precultured cells (OS12 cells). Eight formulations of bilayered constructs (groups OS0±, OS3±, OS6±, and OS12± in Figure VI-1) were cultured in chondrogenic medium (CM) supplemented with 10 mM β-glycerophosphate for 28 days. Samples from the top and bottom layers were analyzed to evaluate chondrogenic and osteogenic differentiation of the cells, respectively. Similar hydrogel composites containing TGF-β1-loaded MPs in the top layer were also fabricated for comparison (data shown in Appendix).

OPF Synthesis and Characterization

OPF was synthesized from fumaryl chloride and poly(ethylene glycol) (PEG) with a nominal molecular weight of 10,000 g/mol (Sigma, St. Louis, MO) according to a previously established method [73], and sterilized prior to use by exposure to ethylene oxide.

Gelatin Microparticle Fabrication and Growth Factor Loading

Gelatin microparticles were fabricated from acidic gelatin (Nitta Gelatin Inc., Osaka, Japan) and crosslinked with 10 mM glutaraldehyde (Sigma, St. Louis, MO)
following established procedures [74]. After drying, MPs were sieved to obtain particles of 50-100 μm in diameter and sterilized with ethylene oxide.

Before encapsulation, sterile MPs were loaded with TGF-β3 (R&D Systems, Minneapolis, MN) by swelling in an aqueous solution of the growth factor at pH 7.4 for 15 h according to a previously reported method [79]. The volume of TGF-β3 loading solution needed for equilibrium swelling of the MPs (110 μl) was combined with 22 mg of MPs. The loading solution had a concentration of 3.6 μg TGF-β3/ml phosphate buffered saline (PBS; Gibco) to achieve a final loading of 600 ng TGF-β3/ml in the crosslinked scaffolds. This growth factor amount has been shown to promote the chondrogenic differentiation of rabbit MSCs encapsulated in hydrogel composites in vitro [75]. TGF-β1-loaded MPs were prepared in a similar fashion using a solution of the same growth factor concentration. Blank MPs were loaded with PBS alone.

Rabbit Marrow MSC Isolation and Preculture

Rabbit marrow MSCs were isolated from the tibiae of six 4-month-old New Zealand white rabbits as previously described [75]. The bone marrow was cultured in general medium (GM) containing Dulbecco’s modified Eagle’s medium (DMEM), 10% v/v fetal bovine serum (FBS; Gemini, Calabasas, CA), 250 μg/l fungizone, 100 mg/l ampicillin, and 50 mg/l gentamicin for 2 weeks, and then cryopreserved as described previously [180]. For MSC expansion, the cryopreserved cells were thawed at 37°C and cultured in T-75 flasks with GM up to passage three (12 days), as shown in Figure VI-1. Osteogenic cells were cultured from the same batch of cryopreserved MSCs for 12 days, as shown in Figure VI-1, with both GM and osteogenic medium (OM), which was DMEM supplemented with 10% v/v FBS, 50 mg/l ascorbic acid, 10 mM
β-glycerophosphate, $10^{-8}$ M dexamethasone, 250 μg/l fungizone, 100 mg/l ampicillin, and 50 mg/l gentamicin (all from Sigma). Specifically, osteogenic cells for the bottom layer of OS3±, OS6± and OS12± groups were exposed to OM for 3, 6 and 12 days immediately prior to encapsulation, respectively.

Fabrication of Bilayered Hydrogel Composites

Bilayered hydrogel composites were fabricated via a two-step crosslinking procedure as described previously [180]. The desired composition for the osteogenic (bottom) layer was first prepared. Specifically, 0.1 g of sterile OPF and 0.05 g of sterile poly(ethylene glycol) diacrylate (PEG-DA; 4,000 Da nominal molecular weight, Monomer-Polymer & Dajac Labs, Feasterville, PA) were first dissolved in 300 μl of PBS and mixed with 110 μl of swelled MP solution (blank MPs). The mixture was then added to equal volumes (46.8 μl) of the thermal radical initiator solutions, 0.3 M ammonium persulfate (APS) and 0.3 M N,N,N’,N’-tetramethylethylenediamine (TEMED) in PBS. A proper cell suspension (6.7 million cells in 168 μl of PBS) was subsequently added to the polymer solution to achieve a concentration of 10 million cells/ml final suspension. After gentle mixing, the suspension for the osteogenic layer was quickly injected into the bottom 1 mm of Teflon molds (6 mm diameter, 2 mm thickness) and incubated for 4 min, allowing for partial crosslinking. Meanwhile, another polymer-cell suspension was prepared, and then injected into the partially filled Teflon molds to form the chondrogenic layer. The resulting bilayered constructs were then incubated at 37°C for 8 min to achieve crosslinking.

Each hydrogel construct was then cultured with 2.5 ml chondrogenic medium, which was 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, Franklin Lakes, NJ), 1 mM sodium pyruvate (Sigma), 50 mg/l ascorbic acid, 10^{-7} M dexamethasone, 10 mM β-glycerophosphate, 250 mg/l fungizone, 100 mg/l ampicillin and 50 mg/l gentamicin. The medium was changed every 3 days during a 28-day culture period.

At various time points (days 0, 7, 14, and 28), samples were collected for quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) (n=4), biochemical assays (n=4), live/dead assay (n=1) and histological analysis (n=1). Bilayered samples for RT-PCR and biochemical assays were dissected with a blade to separate the chondrogenic layer and osteogenic layer [180]; samples from each layer were stored for analysis. Samples loaded with TGF-β1 were analyzed only for RT-PCR (n=4) at day 28.

Real-time RT-PCR

Samples from the chondrogenic layer of the osteochondral constructs were subjected to RT-PCR analysis to quantify MSC chondrogenic differentiation as described previously [75]. Gene expression for collagen type II, aggrecan and collagen type I was determined, and data were analyzed using the $2^{-\Delta\Delta Ct}$ method as reported previously [137, 147]. All gene expression data were normalized to the expression of a house-keeping gene, glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) and expressed as the fold ratio compared to baseline gene expression of a control group at day 0. In this study, the control group contained four top-layer hydrogels encapsulating MSCs that were collected immediately after encapsulation.
Biochemical Assays

Samples from the osteogenic layer of the osteochondral constructs were analyzed for DNA, alkaline phosphatase (ALP) enzyme activity, and calcium content using microplate readers (BIO-TEK Instrument, Winooski, VT) to characterize osteogenic differentiation of the cells as described previously [180]. Bottom-layer samples containing MSCs, 3-day, 6-day and 12-day osteogenically precultured cells (n=4) were collected after encapsulation to represent day 0 values for the OS0±, OS3±, OS6± and OS12± groups respectively.

Similarly, samples from the chondrogenic layer of the constructs were digested in a proteinase K solution and subjected to DNA, glycosaminoglycan (GAG), and calcium assays to evaluate chondrogenic differentiation as previously described [146, 180]. Four samples from top layer hydrogels containing MSCs and blank MPs were collected immediately after encapsulation and analyzed to represent day 0 values for all the groups.

Confocal Fluorescence Microscopy

A live/dead assay was performed at days 0 and 28 after encapsulation as reported previously [114]. After rinsing with PBS, each layer of the construct was then placed in dye solutions containing 4 μM ethidium homodimer-1 (EthD-1) and 2 μM calcein acetoxyethyl ester (Calcein AM) (Invitrogen) for 30 min. Cells encapsulated in the hydrogel composites were examined by confocal fluorescence microscopy (Zeiss LSM 510, Thornwood, NY). Using argon laser excitation at 488 nm, the cells were imaged under a 10x objective, and the emitted light was collected using 505-526 nm and 612-644 nm filters.
**Histology**

At the various time points, bilayered hydrogel composites were fixed, dehydrated, and embedded in paraffin. Cross sections of 20 μm were prepared for Von Kossa staining using a method reported previously [76]. Briefly, the sections were exposed to 5% silver nitrate solution, exposed to UV light for 30 minutes to visualize calcium deposition, and counterstained with 0.1% Safranin O solution.

**Statistical Analysis**

DNA, GAG, calcium contents, ALP activity and gene expression data of the eight treatments (in Figure VI-1) at different time points are reported as means ± standard deviation. Repetitive ANOVA and Tukey’s multiple comparison tests were used to determine possible significant differences (p < 0.05) between groups. Additionally, gene expression data of hydrogels containing TGF-β3- and TGF-β1-loaded MPs at day 28 were compared similarly with repetitive ANOVA and Tukey’s multiple comparison tests (p < 0.05). Statistical significance symbols for Figure VI-2, 3 and 4 are defined in Table VI-1.

**Results**

**Cellularity in Both Chondrogenic and Osteogenic Layers**

DNA contents of the chondrogenic and osteogenic layers of the hydrogel composites are depicted in Figure VI-2 (A) and Figure VI-3 (A), respectively. For both layers of all the treatments, the DNA amount decreased from day 0 to day 7.

In the osteogenic layer [Figure VI-3 (A)], samples containing osteogenically precultured cells (the OS3±, OS6±, and OS12± groups) generally showed higher DNA values compared to those containing plain MSCs (the OS0± groups), either in the
presence or absence of TGF-β3. Specifically, with blank MPs in the chondrogenic layer, a significantly higher DNA value was observed in the osteogenic layer of the hydrogels containing 6-day osteogenically precultured cells (the OS6− group) than in those encapsulating MSCs (the OS0− group) at day 28. Similarly, with TGF-β3-loaded MPs in the chondrogenic layer, significantly more DNA was found in the osteogenic layer of samples of the OS3+ and OS6+ groups than in those of the OS0+ group at day 28.

As in the osteogenic layer, TGF-β3 did not show an effect on cell number in the chondrogenic layer [Figure VI-2 (A)]. However, cells in the osteogenic layer significantly influenced DNA content in the chondrogenic layer of the hydrogels. For example, MSCs in the chondrogenic layer that were cocultured with 3-day osteogenically precultured cells (the OS3± groups) demonstrated a significantly higher DNA amount than those cocultured with MSCs (the OS0± groups) at day 28, either with or without TGF-β3-loaded MPs. MSCs that were cocultured with 6-day osteogenically precultured cells (the OS6± groups) exhibited the highest DNA content compared to all the other groups (the OS0±, OS3±, and OS12± groups) during the culture with or without TGF-β3. However, the coculture with 12-day osteogenically precultured cells (the OS12± groups) did not result in a significant increase in DNA content in the chondrogenic layer when compared to the OS0± groups.

*Chondrogenic Differentiation of MSCs in the Chondrogenic Layer*

The chondrogenic differentiation of MSCs in the chondrogenic layer of the bilayered hydrogels was determined via gene expression of two chondrogenic markers, collagen type II and aggrecan, as well as the gene expression of an MSC-associated marker, collagen type I. Gene expression data are shown in Figure VI-4 (A-C).
For all the treatments, collagen type II gene expression was upregulated over time [Figure VI-4 (A)]. The presence of TGF-β3-loaded MPs enhanced collagen type II gene expression of MSCs encapsulated in the chondrogenic layer. At day 28, significantly higher levels of gene expression were observed in the groups with TGF-β3 (the OS0+, OS3+, OS6+ and OS12+ groups) than in the corresponding groups without TGF-β3 (the OS0−, OS3−, OS6− and OS12− groups, respectively). Additionally, 12-day osteogenically precultured cells had a combined effect with TGF-β3 (the OS12+ group), resulting in the highest level of collagen type II gene expression among all the treatments at day 28. In similar hydrogel composites, where the same amount of TGF-β1 was loaded in the chondrogenic layer, a combined effect of osteogenic cells and the growth factor was also observed. Collagen type II gene expression of MSCs exposed to TGF-β1 increased with the duration of osteogenic preculture of the cocultured osteogenic cells. However, with plain MSCs in the osteogenic layer, TGF-β1 alone only resulted in a 357 ± 211 fold increase in collagen type II gene expression at day 28, which was significantly lower than a 927 ± 148 fold increase caused by TGF-β3 alone.

Aggrecan gene expression levels also increased over time for all the treatments [Figure VI-4 (B)]. For the groups that were cultured with either osteogenic cells alone (the OS3−, OS6− groups) or with TGF-β3 alone (the OS0+ group), significant increases compared to a day 0 baseline were observed no earlier than day 28. However, when MSCs were cultured with both osteogenic cells and TGF-β3, significant increases relative to the day 0 baseline were observed as early as day 7 or 14 (day 14 for the OS3+ group, day 7 for the OS6+ group, and day 14 for the OS12+ group). Cells of 3-day and 6-day osteogenic preculture durations (the OS3± and OS6± groups) seemed to result in more
overall aggrecan gene expression of the MSCs in the chondrogenic layer than plain
MSCs (the OS0± groups) or 12-day osteogenically precultured cells (the OS12± groups),
with either TGF-β3-loaded or blank MPs in the top layer.

Collagen type I gene expression was found to decrease significantly at day 28
relative to day 0, when MSCs in the chondrogenic layer were cultured with either
TGF-β3, or with osteogenic cells, or with both. It should be noted that samples treated
with TGF-β1 alone exhibited a significantly higher collagen type I gene expression than
those treated with TGF-β3 alone.

GAG content, indicative of the chondrogenic phenotype, was measured for the
chondrogenic layer of the bilayered hydrogels to further investigate chondrogenic
differentiation of the encapsulated MSCs, as shown in Figure VI-2 (B). For all the
formulations, significantly higher GAG/DNA levels were observed at days 14 and 28
compared to day 0. At day 28, significant differences in GAG/DNA values among groups
were found between the OS0– group and the OS6– group; as well as between the OS0+
group and the OS3+, OS6+ and OS12+ groups.

Calcium deposition was determined for the chondrogenic layer of the hydrogels to
ensure that no bone upgrowth to the chondrogenic layer occurred with the combined
effects of TGF-β3 and β-glycerophosphate in the coculture medium. As illustrated in
Figure VI-2 (C), all the formulations had a calcium content of less than 1.1 μg during the
culture. No statistically significant difference was observed over time or among groups.

Osteogenic Differentiation of Cells in the Osteogenic Layer

ALP enzyme activity and calcium content were measured for the osteogenic layer
of the hydrogels to evaluate the osteogenic differentiation of the encapsulated cells. ALP
results over time are shown in Figure VI-3 (B). Immediately after encapsulation, ALP activity of cells in the osteogenic layer of the hydrogels was found to be higher in samples containing cells of a longer osteogenic preculture period (not significantly). During the subsequent hydrogel culture, ALP values of the osteogenic cells (the OS3±, OS6± and OS12± groups) slightly increased or remained unchanged, either with or without TGF-β3 in the top layer. In contrast, the OS0± groups containing MSCs in the osteogenic layer both exhibited significant increases in ALP activity at later time points compared to day 0. In particular, in the OS0− group, ALP activity of the cells significantly increased from day 0 to day 7, and then significantly decreased by day 28 relative to day 7. The OS0+ group had a significantly higher ALP activity at days 7, 14 and 28 compared to day 0, with a peak at day 14. Additionally, the ALP values of the OS0+ group were found to be significantly higher than those of all the other treatments at days 7, 14 and 28.

Calcium deposition in the osteogenic layer of the hydrogels for all the treatments is shown in Figure VI-3 (C). Although statistical analysis did not reveal any significant differences among the groups at each time point, significant increases in calcium deposition compared to day 0 values were found in some groups containing osteogenically precultured cells, for example, in the OS3− group at days 14 and 28, in the OS12− group at days 7, 14 and 28, and in the OS12+ group at days 7 and 14.

Confocal Fluorescence Microscopy

Confocal fluorescence microscopy images of cells encapsulated in both layers of the hydrogels at day 0 and day 28 are presented in Figure VI-5. The cells were stained with a live/dead assay kit, where calcein AM stained the live cells with a green
fluorescence and ethidium homodimer-1 (EthD-1) stained dead cells with a red fluorescence. Immediately after encapsulation, most of the cells encapsulated in each layer were alive, and they were well distributed within the hydrogel composites [Figure VI-5 (A)]. After 28 days of culture, cells in each layer of the hydrogel composites retained their viability in all the treatments [Figure VI-5 (B-C)]. However, cells from different groups showed a different distribution in the top (chondrogenic) layer of the hydrogels. As shown in Figure VI-5 (B), cells of the OS0- group were sparsely distributed in the top layer; whereas in the groups treated with TGF-β3, more cell aggregation was observed in the top layer of the hydrogels [noted with small arrows in Figure VI-5 (C)]. No difference in cell distribution was observed for cells encapsulated in the bottom (osteogenic) layer of the hydrogels [Figure VI-5 (B-C)].

*Light Microscopy and Histology*

A light microscopy image of a cross section of a bilayered hydrogel composite containing MSCs and MPs in both layers immediately after encapsulation is shown in Figure VI-6 (B). In the sample, cells (shown as small dots) and MPs (noted with a big arrow) were well distributed. No apparent border can be recognized between the layers, indicative of a good integration between layers.

Figure VI-6 (A, C-J) depicts histological cross sections of the hydrogel composites after 0 and 28 days of culture. The sections were prepared with Von Kossa staining and counter stained with Safranin O, which collectively stain mineralized matrix with a black color and GAG, a cartilaginous matrix component, with a red color. MPs (noted with big arrows) and cells (aggregation noted with small arrows) in the samples appear in dark red in the images. Similar to the results from confocal fluorescence images
[Figure VI-5 (A)] and the light microscopy image [Figure VI-6 (B)], MPs and cells were found to distribute evenly in both layers of the hydrogel composites after encapsulation [Figure VI-6 (A)]. At day 28, most of the MPs were degraded, while cells maintained their rounded phenotype. There was a trend of increasing cell aggregation [noted with small arrows in Figure VI-6 (D, F, H)] in the TGF-β3-treated groups, especially with osteogenic cells cocultured in the bottom (osteogenic) layer of the hydrogels. Although no significant difference was observed for GAG staining among the samples, a more intense staining for mineralized matrix was noticed in the blank MP treatments [Figure VI-6 (C, E, G, I)] than in the TGF-β3-loaded MP treatments [Figure VI-6 (D, F, H, J)] at day 28. The hydrogel composites also seemed to have more calcium deposition in the center than in the top or bottom surfaces [Figure VI-6 (E, G, I)].

**Discussion**

In the current work, bilayered composites consisting of a chondrogenic layer and an osteogenic layer were fabricated using OPF hydrogel networks containing gelatin MPs and MSCs. Specifically, we investigated: (1) the proliferation and differentiation of cells in both layers of the bilayered hydrogel composites; (2) the effect of TGF-β3-loaded MPs on the chondrogenic differentiation of MSCs in the chondrogenic layer; and (3) the combined effects of the duration of the osteogenic preculture of the cells in the osteogenic layer and TGF-β3-loaded MPs on MSC chondrogenesis in the chondrogenic layer.

Previously, our laboratory has fabricated a similar bilayered OPF construct containing TGF-β1-loaded MPs and MSCs in the chondrogenic layer, and containing 6-day osteogenically precultured cells in the osteogenic layer. The culture of the
bilayered construct showed that TGF-β1-loaded MPs in the chondrogenic layer and osteogenic cells in the osteogenic layer synergistically promoted chondrogenesis of MSCs encapsulated in the chondrogenic layer [180].

TGF-β3 was chosen for the current study because a previous study has shown that TGF-β3 was more effective than TGF-β1 in terms of promoting the chondrogenesis of human MSCs in pellet culture, which was evidenced by an earlier and greater deposition of GAG and type II collagen [103]. Additionally, TGF-β3 was also reported to have a biphasic stimulatory effect on DNA synthesis of osteoblasts, and to inhibit osteogenic differentiation of MSCs or MSC-derived osteoblasts [104, 108].

In vitro osteogenic culture period of MSCs has been shown to significantly affect their osteogenic gene expression and extracellular matrix production levels [49, 51], and even to influence their osteogenic potential when implanted in vivo [51-53]. Based on these findings, we hypothesized that cells of different durations of osteogenic preculture can provide different signaling to the cocultured MSCs in a bilayered OPF hydrogel composite. Therefore, this study investigated how the osteogenic preculture duration of cells in the osteogenic layer in combination with TGF-β3-loaded MPs in the chondrogenic layer would affect cell proliferation and differentiation in both layers of a bilayered hydrogel composite.

Cellularity in Both Chondrogenic and Osteogenic Layers

DNA content of both the chondrogenic layer and the osteogenic layer was examined at different time points, as shown in Figure VI-2 (A) and Figure VI-3 (A), respectively. In both layers, there was a significant decrease in DNA content from day 0 to day 7 for all the treatments. Previous studies using a similar single-layer hydrogel
composite for MSC chondrogenic and osteogenic differentiation have indicated that the encapsulation process may lead to some cell loss, especially at early time points [75, 76]. However, images of the live/dead assay in Figure VI-5 demonstrated that the majority of cells in both layers were alive after encapsulation [Figure VI-5 (A)] and that they maintained their viability during the subsequent culture [Figure VI-5 (B-C)], demonstrating the cytocompatibility of the macromer, crosslinker and initiators under the conditions explored in this study.

Statistical analysis revealed that TGF-β3 did not influence the cellularity of either layer. However, the cells encapsulated in the osteogenic layer significantly affected the DNA content of both layers. In particular, more DNA was seen in both layers in the treatments with osteogenically precultured cells (the OS3±, OS6±, and OS12± groups) than the treatments with plain MSCs (the OS0± groups). This was in accordance with our previous findings that osteogenic cells (6-days of osteogenic preculture) were more resilient than plain MSCs when encapsulated in the osteogenic layer of an OPF hydrogel composite and that they supported the proliferation of the cells in the chondrogenic layer, possibly through a paracrine effect [180]. Furthermore, in this study, we found that osteogenic cells of various osteogenic preculture periods in the osteogenic layer supported the proliferation of MSCs in the chondrogenic layer to different extents. As shown in Figure VI-2 (A), MSCs in the chondrogenic layer that were cocultured with 3-day osteogenically precultured cells (the OS3± groups) demonstrated a significantly higher DNA content compared to those cultured with plain MSCs (the OS0± groups) in the osteogenic layer at day 28; while MSCs that were cocultured with 6-day osteogenically precultured cells (the OS6+ groups) demonstrated the highest levels of
DNA, which were significantly higher than all the other treatments (the OS0+, OS3+ and OS12+ groups) at days 14 and 28 in the presence of TGF-β3. In contrast to the OS3± and OS6± groups, MSCs cocultured with 12-day osteogenically precultured cells (the OS12± groups) did not show a significant increase in DNA content compared to the control groups (OS0±) at any time point.

The difference in the ability of osteogenic cells to promote the proliferation of the cocultured MSCs may be related to their osteogenic differentiation levels. It is know that during the three stages of osteogenic differentiation (proliferation, matrix maturation, and mineralization), there is a reciprocal relationship between cell proliferation and the subsequent matrix maturation and mineralization [49, 51]. In this study, 3-day and 6-day osteogenically precultured cells can be considered as immature osteoblasts, which possess a great ability to proliferate. A previous review on osteogenic differentiation of osteoblasts indicated that immature osteoblasts actively express cell-cycle and cell-growth- regulated genes, which encode proteins that support proliferation [49], whereas 12-day osteogenically precultured cells represented more extensively differentiated osteoblasts, which express genes or extracellular matrix proteins associated with extracellular matrix maturation or mineralization and downregulate genes for proliferation [49]. Compared to 12-day osteogenically precultured cells, 3-day and 6-day osteogenically precultured cells may secrete more bioactive factors to support cell proliferation, thus resulting in a higher DNA amount of both layers due to autocrine and paracrine signaling.
Chondrogenic Differentiation of MSCs in the Chondrogenic Layer

It has been shown that when MSCs undergo chondrogenic differentiation, they start to express chondrocyte-specific marker genes such as collagen type II and aggrecan, along with downregulating expression of the gene for collagen type I, which is produced by undifferentiated MSCs [137]. Therefore, chondrogenic differentiation of MSCs encapsulated in the top (chondrogenic) layer of the constructs was determined by gene expression for collagen type II, aggrecan, and collagen type I. Gene expression data shown in Figure VI-4 (A-C) demonstrated upregulation of collagen type II and aggrecan gene expression and downregulation of collagen type I gene expression over time for all the treatments, indicating the chondrogenic differentiation of the MSCs encapsulated in the chondrogenic layer of the hydrogel constructs in the coculture environment.

The stimulatory effect of TGF-β3 on MSC chondrogenic differentiation was apparent, as higher levels of collagen type II and aggrecan gene expression were observed in the TGF-β3-treated groups (the OS0+, OS3+, OS6+ and OS12+ groups) than in the corresponding groups without TGF-β3 (the OS0−, OS3−, OS6− and OS12− groups, respectively). Similar results have been reported in the literature, showing the important roles of TGF-β3 for MSC chondrogenic differentiation [103, 105]. Comparing the results of TGF-β3 and TGF-β1 treatments at day 28 [Figure VI-4 (A-C)], where the same amount of TGF-β3 or TGF-β1 was loaded on MPs and encapsulated with MSCs in the chondrogenic layer of the bilayered hydrogel composites, we found that TGF-β3 was more efficient than TGF-β1 in enhancing MSC chondrogenic differentiation, when no osteogenic cells were involved in the bottom (osteogenic) layer. TGF-β3 resulted in higher levels of gene expression for collagen type II and aggrecan, as well as a lower
level of gene expression for collagen type I, than TGF-β1 at day 28. The results agreed with the findings from some previous studies. For example, Barry et al. have compared the chondrogenic potential of three TGF-β isoforms on human bone marrow derived MSCs [103]. They found that TGF-β2 and TGF-β3 are more effective than TGF-β1 in promoting chondrogenesis, as evidenced by an earlier and greater deposition of GAG and type II collagen.

The incorporation of osteogenic cells of three different osteogenic preculture periods in the osteogenic layer of the hydrogels all resulted in an increase in chondrocytic gene expression of the chondrogenic-layer cells (not significant) [Figure VI-4 (A-B)], suggesting their stimulatory effect on MSC chondrogenesis. The results were expected, since studies in our laboratory have shown that MSCs cultured in osteogenic medium either express genes for or secrete many bioactive factors related to cartilage and bone formation, including TGF-β1, BMP-2, fibroblastic growth factor 2 (FGF-2), vascular endothelial growth factor (VEGF), and insulin-like growth factor 1 (IGF-1) [93, 96]. Many studies including ours have suggested these paracrine signals could have contributed to the chondrogenic differentiation of the cocultured MSCs or chondrocytes [130, 134, 180].

Osteogenic cells demonstrated a combined effect with both TGF-β1 and with TGF-β3 on enhancing MSC chondrogenic differentiation in this study. It should be noted that the combined effects were more apparent in the TGF-β1 treatments than the TGF-β3 treatments at day 28, however, it was probably due to a more efficient potential of TGF-β3 alone for MSC chondrogenesis. Osteogenic cells of various osteogenic preculture periods, in combination with TGF-β3- (or TGF-β1-) loaded MPs, enhanced
collagen type II and aggrecan gene expression of the MSCs at different levels. For collagen type II gene expression, there was a trend that longer osteogenic preculture periods of cells in the osteogenic layer resulted in a higher collagen type II gene expression of the cocultured MSCs in the chondrogenic layer in the presence of TGF-β3-loaded MPs. A more apparent trend was seen in similar hydrogel composites containing TGF-β1. In contrast, for aggrecan gene expression, 3-day and 6-day osteogenically precultured cells in the bottom (osteogenic) layer resulted in a more rapid and greater upregulation in gene expression of the top-layer MSCs compared to plain MSCs or 12-day osteogenically precultured cells in the bottom layer. This was true in all the cases, with blank MPs, TGF-β3-loaded MPs, or TGF-β1-loaded MPs in the chondrogenic layer. For collagen type I gene expression, although no difference was observed among the osteogenic treatments, all the values decreased at day 28 compared to day 0. Our previous study indicated that the synergistic effect of TGF-β1 and osteogenic cells on MSC chondrogenic differentiation could be attributed to the cross-talk between the growth factor released from MPs and other growth factors secreted by osteogenic cells [180]. Therefore, the results of this study suggest possible differences in the soluble growth factors secreted by cells of various osteogenic preculture periods.

In another study, Nakaoka et al. cocultured chondrocytes and osteoblasts in a culture medium with or without osteogenic supplements [130]. Their results also indicated that the differentiation level of osteoblasts (caused by the presence of osteogenic supplements) influenced collagen type II and aggrecan gene expression levels of the chondrocytes. Additionally the chondrocytes showed a significant increase in collagen type X gene expression, indicative of differentiation into a hypertrophic
phenotype, under the combined effects of osteoblasts and osteogenic supplements in the culture medium. Although the results are not directly comparable with the present study due to the differences in the cells types, culture medium, and the coculture system selected, both studies underscore the importance of the investigation of the cell differentiation stages on the cell signaling in osteochondral tissue.

GAG production, associated with the chondrogenic phenotype of the cultured cells, exhibited higher values for all the groups at later time points than at day 0 [Figure VI-2 (B)], which provided additional evidence of MSC chondrogenic differentiation. However, GAG production was comparatively low, and no apparent difference was observed in the cross sections stained with Safranin O [Figure VI-6 (top layer of C-J)]. As previously reported, the tight polymer network may have limited the deposition of matrix [75]. Calcium content was low in the chondrogenic layer of bilayered hydrogels for all the treatments at all the time points [Figure VI-2 (C)], confirming that MSCs in the chondrogenic layer did not undergo osteogenic differentiation under the influence of both TGF-β3 and β-glycerophosphate in the medium.

Confocal microscopy images [Figure VI-5 (B-C)] and histological images [Figure VI-6 (C-J)] each showed differences in cell distribution in the top (chondrogenic) layer of hydrogels at day 28. Compared to the OS0-- (control) group [Figure VI-5 (B) and Figure VI-6 (C)], where the cells were sparsely distributed, more cell aggregates were observed in the TGF-β3 treatments [noted with small arrows in Figure VI-5 (C) and Figure VI-6 (D, F, H)] at day 28. Previous studies have shown that MSC condensation is important for inducing chondrogenesis during joint development [26]. Thus, a higher extent of
chondrogenic differentiation of MSCs in the presence of TGF-β3 in the current study may be related to a better cell-cell contact.

**Osteogenic Differentiation of Cells in the Osteogenic Layer**

The osteogenic differentiation of the cells encapsulated in the bottom (osteogenic) layer was assessed by their ALP enzyme activity and calcium deposition. ALP activity has been regarded as an early marker for the osteogenic differentiation of MSCs or osteoblasts [49, 51, 76]. Many previous studies investigating osteogenic differentiation of cells have reported that ALP activity of the cells increased during their proliferation period, peaked before mineralization, and then decreased [49, 51]. ALP results of this study in Figure VI-3 (B) showed that MSCs (in the OS0± groups) encapsulated in the osteogenic layer followed the characteristic trend for ALP activity, indicative of their osteogenic differentiation. Although the OS0+ group (with TGF-β3 in the top layer) had higher ALP levels than the OS0− group (without TGF-β3 in the top layer) at days 7, 14 and 28, the ALP peak in the OS0+ group was seen to shift to a later time point (day 14) compared to the ALP peak of the OS0− group at day 7. The results suggest that TGF-β3 may prolong the immature stage of MSCs and delay the osteogenic process.

ALP activity of osteogenic cells was higher than that of plain MSCs after the preculture at day 0. Additionally, there was a trend that longer exposure to the osteogenic medium resulted in higher ALP values (although the differences were not significant). During the subsequent culture in the hydrogel composites, ALP levels of the osteogenic cells slight increased or remained unchanged. This indicates that the peaks of their ALP values may have occurred during the osteogenic preculture in tissue culture flasks. However, the cells were able to maintain their osteoblastic phenotype in the hydrogels.
Calcium deposition is a marker for late-stage osteogenic differentiation [49, 51]. The results from the calcium assay on the osteogenic layer of the hydrogels did not reveal significant differences in calcium content among the groups, however significant increases in calcium content compared to the day 0 values were observed in some samples containing osteogenically precultured cells at later time points [Figure VI-3 (C)]. Von Kossa staining of the cross sections of the hydrogel composites at day 28 confirmed the deposition of mineralized matrix in the samples containing precultured osteogenic cells in the bottom (osteogenic) layer and blank MPs in the top (chondrogenic) layer [the OS3–, OS6– and OS12– groups in Figure VI-6 (E, G, I)]. In those samples, a darker staining of calcium deposition was observed in the middle area of the hydrogel composites, some even spread to the top layer, as shown in Figure VI-6 (G, I). More calcium deposition in the middle area of the hydrogels could be due to a faster degradation rate in the center of the hydrogels (seen from cross sections of the hydrogel composites without cells, data not shown), giving the cells more space to lay down matrix. In contrast to the blank MP treatments, no staining of calcium was observed in either layer of the TGF-β3 treatments [Figure VI-6 (D, F, H, J)]. This finding, along with the results from the ALP assay showing the delay of peak ALP activity in the presence of TGF-β3, suggest that TGF-β3, or chondrocyte-like cells induced by TGF-β3 had an inhibitory effect on the maturation and mineralization of osteoblastic cells encapsulated in the bottom layer. In fact, other studies investigating the effect of TGF-β3 on osteogenic differentiation of MSCs, MSC-derived osteoblasts and osteoblast-enriched bone cells, have also shown that TGF-β3 suppressed osteogenic differentiation and matrix synthesis of the cells [104, 108].
Conclusions

We fabricated a bilayered osteochondral construct with good integration between layers, using OPF hydrogel composites containing gelatin MPs and MSCs. A live/dead assay revealed that cell viability was maintained in both layers during the coculture, and the cellularity of each layer was influenced by the osteogenic preculture period of the cells encapsulated in the osteogenic layer. TGF-β3-loaded MPs significantly stimulated chondrogenic differentiation of MSCs in the chondrogenic layer. Additionally, cells of various osteogenic preculture periods in the osteogenic layer, along with TGF-β3, enhanced chondrogenic gene expression of the MSCs in the chondrogenic layer to different extents. In the osteogenic layer, osteogenically precultured cells maintained their osteoblastic phenotype, as evidenced by a sustained ALP activity and some calcium deposition. However, TGF-β3 showed an inhibitory effect on cell mineralization by Von Kossa staining. The results demonstrated the fabrication of bilayered osteochondral constructs using biodegradable hydrogel composites and suggested their application for the co-delivery of growth factors and MSCs for cartilage tissue engineering, while illustrating that encapsulated cells of different degrees of osteogenic differentiation can significantly influence the chondrogenic differentiation of co-cultured progenitor cells in both the presence and absence of chondrogenic growth factors.
Figure VI-1: A schematic representation of the overall experimental design. During the plate preculture, rabbit marrow mesenchymal stem cells (MSCs) were cultured with either general medium (GM) or osteogenic medium (OM) or both for a total of 12 days. However, cells were exposed to OM for different time periods: 0, 3, 6 and 12 days, and were named MSCs, OS3 cells, OS6 cells, OS12 cells, respectively. After cell encapsulation at day 0, bilayered hydrogel composites were cultured in chondrogenic medium (CM). Eight groups were included, namely the OS0±, OS3±, OS6± and OS12± groups. The top layer of the hydrogels encapsulated MSCs with blank microparticles (MPs) (indicated with −) or with transforming growth factor-β3 (TGF-β3)-loaded-MPs (indicated with +). The bottom layer of the hydrogel composites contained blank MPs and cells of different preculture periods: MSCs in the OS0± groups, OS3 cells in the OS3± groups, OS6 cells in the OS6± groups, and OS12 cells in the OS12± groups, respectively.
Figure VI-2: DNA content (A), GAG content normalized to µg DNA (B) and calcium content (C) for the top layer of the bilayered hydrogel composites. Error bars represent means ± standard deviation for n = 4. At day 0, all groups shared top-layer samples for the biochemical assays. Statistical significance symbols are defined in Table VI-1.
Figure VI-3: DNA content (A), ALP enzyme activity (B) and calcium content (C) for the bottom layer of the bilayered hydrogel composites. Error bars represent means ± standard deviation for n = 4. At day 0, the OS0± groups shared bottom-layer samples for all biochemical assays; and similarly the OS3± groups, OS6± groups, and OS12± groups shared samples respectively. Statistical significance symbols are defined in Table VI-1.
Figure VI-4: Quantitative gene expression of collagen type II (A), aggrecan (B), and collagen type I (C) for the top layer of the bilayered OPF hydrogel composites. Data are presented as a fold ratio after being normalized to GAPDH values. The expression level of controls (Day 0, OS0− group) is represented as one. At day 28, gene expression levels were compared between top layer hydrogels encapsulating TGF-β3 and those encapsulating TGF-β1. Error bars represent means ± standard deviation for n = 4. Statistical significance symbols are defined in Table VI-1.
Figure VI-5: Confocal fluorescence microscopy images of cells encapsulated in top and bottom layers of the bilayered composites after encapsulation (A) and after 28 days of culture (B-C) with live/dead reagents. A green fluorescence (calcein AM) designates live cells, whereas a red fluorescence (EthD-1) indicates dead cells. Arrows indicate aggregated rabbit marrow MSCs in OPF hydrogel composites. Scale bar represents 100 μm.
Figure VI-6: Histological sections stained with Von Kossa and Safranin O stainings illustrating cells encapsulated in top and bottom layers of the bilayered composites after encapsulation (A) and after 28 days of culture (C-J). (B) shows a light microscopy image of a cross section of a bilayered hydrogel encapsulating MSCs and blank MPs in each layer at day 0. Small arrows indicate aggregated cells in OPF hydrogel composites. Big arrows indicate MPs. Scale bar represents 1 mm in (B), and 250 μm in the histological images (A, C-J).
**Tables**

_Table VI-1:_ Definition of statistical significance symbols in Figures VI-2, 3, 4 and Figures A-2, 3, 4.

<table>
<thead>
<tr>
<th>Symbols</th>
<th>Statistical Significance ($p&lt;0.05$)</th>
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<tr>
<td>*</td>
<td>A significant difference between the values at a later time point and day 0 within a given group</td>
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<tr>
<td>**</td>
<td>A significant decrease compared to a previous time point within a given group</td>
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<tr>
<td>†</td>
<td>A significant difference between a TGF-β3-loaded MP treatment and its blank MP control at the same time point</td>
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<td>#</td>
<td>A significant difference compared to another group within the TGF-β3-treated groups or within blank MP groups at the same time point</td>
</tr>
<tr>
<td>§</td>
<td>A significant difference between the TGF-β3 and TGF-β1 groups, of the same bottom layer formulation at the same time point</td>
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CHAPTER VII: SUMMARY

Articular cartilage has limited ability to repair itself. Therefore, the overall objective of this research was to develop a biodegradable hydrogel composite as a cell and growth factor carrier for the repair and regeneration of articular cartilage in an osteochondral defect.

An initial study demonstrated that OPF hydrogel composites containing TGF-β1-loaded MPs supported MSC chondrogenic differentiation in vitro. Additionally, hydrogel composites of higher swelling ratios (larger mesh sizes) resulted in enhanced upregulation of chondrocyte-specific genes of the encapsulated MSCs, such as collagen type II and aggrecan.

Next, these hydrogel composites containing MSCs were implanted in an osteochondral defect in rabbits. After 12 weeks, OPF hydrogel composites partially degraded. Histological analysis revealed that the implantation of MSCs with the hydrogel composites facilitated subchondral bone formation in the presence of TGF-β1. However, the delivery of MSCs either with or without TGF-β1 did not improve cartilage morphology.

Accordingly, further investigation explored the fabrication of a bilayered construct to mimic native osteochondral tissue. An osteochondral construct was successfully fabricated using OPF/MP hydrogel composites and MSCs, consisting of a chondrogenic layer and an osteogenic layer. In vitro culture of the bilayered constructs showed good integration between layers. MSCs were able to undergo chondrogenic differentiation in the presence of TGF-β1 in the chondrogenic layer, while osteogenic cells maintained osteoblastic phenotype in the osteogenic layer. Most importantly, the
osteogenic cells exhibited a synergistic effect with TGF-β1-loaded MPs, promoting chondrogenic differentiation of MSCs in the chondrogenic layer.

A following study further investigated the combined effects of TGF-β3 and osteogenic cells of different preculture periods on the chondrogenic differentiation of co-cultured MSCs in a similar bilayered hydrogel composite. Compared to TGF-β1, TGF-β3 loaded on the MPs was more effective in stimulating chondrogenic differentiation of MSCs in the chondrogenic layer. Additionally, cells of various osteogenic preculture periods in the osteogenic layer, along with TGF-β3, enhanced chondrogenic gene expression of the MSCs in the chondrogenic layer to different extents.

Overall, these studies demonstrated the applications of OPF hydrogel composites as an injectable scaffold for MSC differentiation, suggesting their great potential for the co-delivery of growth factors and cells for osteochondral tissue engineering.

Future studies may involve investigation of the release kinetics of different bioactive molecules released from multilayered cell-laden hydrogels on the structure and function of osteochondral tissue. Additional investigation may focus on the effects of the mechanical properties of multilayered cell-laden hydrogels on their capacity to regenerate osteochondral tissue. Further investigation may emphasize the effects of the differentiation stage of cell populations encapsulated in different layers of multilayered cell-laden structure on osteochondral tissue formation and regeneration.
CHAPTER VIII: LITERATURE CITED


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Figure A-1: A schematic representation of the overall experimental design for a supplemental study. This study was performed using the same conditions as those in Chapter VI, except that TGF-β1 instead of TGF-β3 was used in the top layer of the hydrogel composites. In particular, during the plate preculture, rabbit marrow mesenchymal stem cells (MSCs) were cultured with either general medium (GM) or osteogenic medium (OM) or both for a total of 12 days. However, cells were exposed to OM for different time periods: 0, 3, 6 and 12 days, and were named MSCs, OS3 cells, OS6 cells, OS12 cells, respectively. After cell encapsulation at day 0, bilayered hydrogel composites were cultured in chondrogenic medium (CM). Eight groups were included, namely the OS0±, OS3±, OS6± and OS12± groups. The top layer of the hydrogels encapsulated MSCs with blank microparticles (MPs) (indicated with −) or with transforming growth factor-β1 (TGF-β1)-loaded-MPs (indicated with +). The bottom layer of the hydrogel composites contained blank MPs and cells of different preculture periods: MSCs in the OS0± groups, OS3 cells in the OS3± groups, OS6 cells in the OS6± groups, and OS12 cells in the OS12± groups, respectively.
Figure A-2: DNA content (A), GAG content normalized to μg DNA (B) and calcium content (C) for the top layer of the bilayered hydrogel composites containing blank MPs (−) or TGF-β1-loaded MPs (+). Error bars represent means ± standard deviation for n = 4. At day 0, all groups shared top-layer samples for the biochemical assays. Statistical significance symbols are defined in Table VI-1.
Figure A-3: DNA content (A), ALP enzyme activity (B) and calcium content (C) for the bottom layer of the bilayered hydrogel composites containing blank MPs (−) or TGF-β1-loaded MPs (+). Error bars represent means ± standard deviation for n = 4. At day 0, the OS0± groups shared bottom-layer samples for all biochemical assays; and similarly the OS3± groups, OS6± groups, and OS12± groups shared samples respectively. Statistical significance symbols are defined in Table VI-1.
Figure A-4: Quantitative gene expression of collagen type II (A), aggrecan (B), and collagen type I (C) for the top layer of the bilayered OPF hydrogel composites containing blank MPs (−) or TGF-β1-loaded MPs (+). Data are presented as a fold ratio after being normalized to GAPDH values. The expression level of controls (Day 0, OS0− group) is represented as one. Error bars represent means ± standard deviation for n = 4. Statistical significance symbols are defined in Table VI-1.