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Mechanical loading regulates human MSC differentiation in a multi-layer hydrogel for osteochondral tissue engineering

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ABSTRACT

A bioinspired multi-layer hydrogel was developed for the encapsulation of human mesenchymal stem cells (hMSCs) as a platform for osteochondral tissue engineering. The spatial presentation of biochemical cues, via incorporation of extracellular matrix analogs, and mechanical cues, via both hydrogel crosslink density and externally applied mechanical loads, were characterized in each layer. A simple sequential photopolymerization method was employed to form stable poly(ethylene glycol)-based hydrogels with a soft cartilage-like layer of chondroitin sulfate and low RGD concentrations, a stiff bone-like layer with high RGD concentrations, and an intermediate interfacial layer. Under a compressive load, the variation in hydrogel stiffness within each layer produced high strains in the soft cartilage-like layer, low strains in the stiff bone-like layer, and moderate strains in the interfacial layer. When hMSC-laden hydrogels were cultured statically in osteochondral differentiation media, the local biochemical and matrix stiffness cues were not sufficient to spatially guide hMSC differentiation after 21 days. However dynamic mechanical stimulation led to differentially high expression of collagens with collagen II in the cartilage-like layer, collagen X in the interfacial layer and collagen I in the bone-like layer and mineral deposits localized to the bone layer. Overall, these findings point to external mechanical stimulation as a potent regulator of hMSC differentiation toward osteochondral cellular phenotypes.

1. Introduction

Lesions to articular cartilage and the underlying subchondral bone in articulating joints can lead to joint pain, reduced joint function, and overtime osteoarthritis [1]. This type of lesion is particularly problematic because it extends across two distinctly different tissues, highly compliant hyaline cartilage and stiff subchondral bone. These two tissues are connected by a thin interface that is defined by the tidemark flanking cartilage and the cement line flanking subchondral bone [2]. This transitional region is critically important to the overall function of the joint as it enables efficient transfer of load between these two very different tissues, while minimizing stress concentration and reducing failure [3,4].

Tissue engineering is a promising strategy for regenerating osteochondral tissues, but the complexity of this type of tissue requires multi-layer scaffold designs and ultimately different cell types [5,6]. Bi-layer scaffolds are the simplest approach [7], whereby a stiff layer, typically inorganic mineralized matrix (e.g., hydroxyapatite and β-tricalcium phosphate) representing the bone region is topped with a soft polymeric layer (e.g., poly(lactic-co-glycolic acid), collagen, agarose, etc.) representing the cartilage region [8–12]. Other approaches have employed the same scaffold chemistry in both layers and instead varied the physical properties (e.g., pore structure [13]) and/or the biochemical cues (e.g., tissue-specific ECM-analogs [14–17], growth factors [18–20], or genes [21,22]). A few studies have developed multi-layer scaffolds to capture the interfacial layer [23–28]. These scaffold-based approaches have relied either on endogenous cells infiltrating into the scaffold or on exogenous cells delivered within the scaffold upon implantation [5]. Several studies have used mesenchymal stem cells (MSCs) in their undifferentiated state within both layers, but achieving the appropriate differentiation has been limited

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[29,30]. To overcome this shortcoming, MSCs pre-differentiated down the chondrogenic or osteogenic lineage have been used with some success [31]. Recent studies have shown that using osteogenically differentiated MSCs in the bone-like layer with undifferentiated MSCs in the cartilage-like layer leads to improved chondrogenic differentiation in this layer [30,32].

To date, multi-layer scaffold designs for osteochondral tissue engineering have primarily focused on physical cues from scaffold architecture or biochemical cues to create layers that are conducive to tissue engineering cartilage or bone. One key component that has been largely missing is mechanical loading. It is well-known that mechanical cues are important in chondrogenesis and osteogenesis [33–35] and that osteochondral tissues in articulating joints are continually subjected to mechanical forces. Therefore, a better understanding of how mechanical forces are translated through these complex multi-layer scaffolds and how they impact cells is needed. A few studies have utilized finite element modeling as a means to predict the local mechanical stresses and strains that are present in complex multi-layered osteochondral scaffolds [36,37]. However, the impact on cells remains under-studied.

The overall objective for this study was to develop a multi-layered biomimetic hydrogel for osteochondral tissue engineering and to characterize the hydrogel within a mechanically relevant environment, specifically under dynamic compressive loading. Human MSCs (hMSCs) were investigated for their clinical relevance and potential in regenerating cartilage and bone. The hydrogel was designed from the same base chemistry, but with varying mechanical properties from soft to stiff and tissue-specific biomolecules within each layer. Specifically, a stable poly(ethylene glycol) hydrogel was chosen as the base chemistry because biological moieties are readily incorporated in a controlled manner while the mechanical properties can be independently tuned and maintained over the course of the experiment [38–40]. Two extracellular matrix (ECM) molecules, chondroitin sulfate and RGD, were chosen. Chondroitin sulfate is the main glycosaminoglycan in cartilage and creates a unique environment that is hyperosmotic, which for cartilage cells enhances tissue synthesis [41] especially under dynamic compression [42,43]. RGD, a cell adhesion peptide, provides a mechanism for cells to sense substrate stiffness and may act as a mechanosensor to dynamic compression [44]. RGD has been shown to support chondrogenesis [45,46] with low concentrations improving differentiation [46]. RGD has also been shown to support osteogenesis over a range of concentrations [47,48].

The specific aims of this study were twofold. First, the aim was to develop and characterize a multi-layered hydrogel with controlled presentation of biochemical and mechanical cues for capturing the three main regions of osteochondral tissues: cartilage, bone, and the interface comprised of calcified cartilage. Specifically, a multi-layered hydrogel was designed based on (a) a compliant cartilage-like layer containing 1% chondroitin sulfate [42] and 0.1 mM RGD [44], (b) a stiffer bone-like layer consisting of 10 mM RGD, and (c) an interfacial region that combines the soft and stiff layers. The second aim was to employ undifferentiated hMSCs and investigate whether the local cues presented by a multi-layer hydrogel under the application of intermittent dynamic compression are sufficient to impact the fate of hMSCs when presented with a mixed osteochondral media.

2. Materials and methods

2.1. Macromer synthesis

Poly(ethylene glycol) dimethacrylate (PEGDM) macromer was synthesized via microwave methacrylation [49]. Briefly, 4600 g mol^{-1} poly(ethylene glycol) (PEG) (Fluka, Sigma–Aldrich) was melted and reacted with methacrylic anhydride in the presence of hydroquinone (Sigma–Aldrich). The reaction mixture was dissolved in methylene chloride and purified by multiple precipitations with ethyl ether, filtration, and drying under vacuum. The degree of methacrylate substitution on each end of the PEG molecules was determined to be 93% by ^{1}HNMR (Varian VYR-500). Specifically, the area under the curve for the vinyl resonance peaks (δ = 5.7 ppm, δ = 6.1 ppm) was compared to the area under the curve for the methylene peaks associated with the PEG backbone (δ = 4.3 ppm).

YRGDS (Genscript) was reacted in a 1:1.1 molar ratio with excess acryloyl-PEG-N-hydroxysuccinimide (3400 Da; Laysan Bio, Inc.) in 50 mM sodium bicarbonate buffer (pH 8.4) overnight at room temperature. The degree of attachment was determined to be 94% using the spectroscopic Fluoraldehyde™ o-Phthalaldehyde (Pierce) method of detection. In addition, fluorescently labeled YRGDS was synthesized as follows. In brief, acryloyl-PEG-RGD in 0.1 M sodium bicarbonate (pH 8.5) was reacted with Alexa Fluor 488 cadaverine in a 1:1 molar ratio using 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) as a coupling agent over 24 h while stirring. Acryloyl-PEG-RGD and acryloyl-PEG-fRGD were purified by dialysis against deionized water (dI-H_2O), recovered by lyophilization and stored at 4 °C.

Methacrylated chondroitin sulfate (ChSMA) was synthesized as previously described [42,50]. Briefly, chondroitin sulfate A (Sigma), containing ~30% chondroitin-6-sulfate and ~70% chondroitin-4 sulfate was dissolved at 25% (w/v) in dI-H_2O and reacted in a 1:8 ratio with methacrylic anhydride. The reaction temperature was held at 4 °C for 24 h and the reaction pH was maintained at 8. The reaction product was precipitated in chilled methanol and dialyzed against dI-H_2O. The purified product was recovered via lyophilization and the degree of methacrylation was determined to be 23% via ^{1}HNMR (Varian VYR-500), indicating that, on average, there were 23 methacrylate groups present on each ChSMA molecule. Specifically, the area under the curve for the vinyl resonance peaks (δ = 5.5–6.2 ppm) was compared to the area for the acetyl groups (δ = 1.7–2.0 ppm). Multiple methacrylate substitutions are possible due to the free hydroxyl groups present in each repeat unit of the ChS [51].

2.2. Acellular multi-layered hydrogel fabrication

Two macromer solutions were used to create the multi-layered hydrogels for fabrication characterization: 10% (g/g) PEGDM, top, and 30% (g/g) PEGDM, bottom. Each macromer solution was combined with 0.05% (g/g) photoinitiator Irgacure 2959 (BASF). To visualize each layer in acellular hydrogels, 0.1% (g/g) fluorescein-o-methacrylate (Sigma) was added to the 10% PEGDM macromer solution, and 0.1% (g/g) rhodamine methacrylate (Sigma) was added to the bulk 30% PEGDM macromer solution. The macromer solutions were exposed to 365 nm light with an intensity of ~5 mW cm^{-2} for a range of times, referred to as polymerization time (0–10 min: bottom, plus 10 min: top). Cubic (5 × 5 × 5 mm) hydrogels were fabricated and imaged using confocal laser scanning microscopy. ImageJ software was used to characterize the resultant interface thicknesses. Multi-layered hydrogels were also fabricated with biochemical cues where the two macromer solutions were prepared: 10% (g/g) comprised of 90:10 (PEGDM:ChSMA) by weight with 0.1 mM RGD (top layer) or 30% (g/g) PEGDM with 10 mM RGD (bottom). Hydrogels were formed as described above. For hydrogels with the fluorescently labeled RGD, hydrogels were formed as described above and at room temperature, but with a redox initiating system consisting of ammonium persulfate (0.025M APS) and tetramethylethylenediamine (0.0125M TEMED). The concentration of the redox initiators was chosen to achieve similar polymerization behavior of the PEG

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