Development of a Separable 3D Cell Co-Culture System for the Study of Stem Cell Microenvironments

A Thesis
Presented to
The Academic Faculty

By

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<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>2D</td>
<td>Two Dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>Three Dimensional</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
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<tr>
<td>PEG-DA</td>
<td>Poly(ethylene glycol) diacrylate</td>
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<td>OPF</td>
<td>Oligo(poly(ethylene glycol) fumarate)</td>
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<tr>
<td>CSMA</td>
<td>Chondroitin sulfate methacrylate</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>TEA</td>
<td>Triethyl amine</td>
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<tr>
<td>DCM</td>
<td>Dichloromethane</td>
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<tr>
<td>FuCl</td>
<td>Fumaryl chloride</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>DMMB</td>
<td>Dimethylmethylene blue</td>
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<tr>
<td>hMSC</td>
<td>Human mesenchymal stem cell</td>
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SUMMARY

Stem cells represent an attractive cell choice for regenerative medicine applications due to their inherent ability to differentiate down multiple cell lineages given the correct cues. However, little is known concerning how the interactions, specifically the paracrine signaling effects, of native cells will influence the growth, proliferation, and differentiation of stem cells after implantation in vivo. This lack of fundamental knowledge necessitates the development of an in vitro model. Hydrogels are a biomaterial uniquely suited to the task of providing a tunable, cytocompatible environment in which to study these interactions between cell populations. By utilizing the technique of photolithography in conjunction with microfluidics, we developed and optimized a system to sequentially construct spatially segregated 3D co-culture constructs that will provide a basis for better understanding paracrine signaling between stem cells and other cell types native to orthopedic tissues. Additionally, by incorporating a degradable hydrogel interface between components, we demonstrated the separability of this system and the potential to isolate and recover individual populations after culture.
INTRODUCTION

Necessity of an in vitro tissue model and important factors in its design

The primary goal of tissue engineering is to restore and maintain tissues and organs by applying external mechanisms to encourage or augment the body's natural recuperative processes. Stem cells in particular represent an attractive cell type for these regenerative medicine applications because they are able to differentiate across a wide variety of cell lineages. However, little is known concerning the interactions between stem cells and native tissues in vivo. As a result, models that mimic tissues ex vivo have become increasingly important in the understanding of healthy tissue growth and function\(^1\). The design of these tissue models begins in vitro due to the high degree of control an in vitro experimental setting provides. In these models, environmental factors are manipulated through a variety of means to coax the cells to differentiate and replicate, taking on the form and function of the desired tissue\(^3\).

Prominent among the numerous environmental influences affecting cell growth and function (both in vitro and in vivo) is the surrounding extra-cellular matrix (ECM), including its mechanical properties and incorporated biofactors, and soluble signaling factors from neighboring cells. Additionally, a three-dimensional (3D) environment for cell growth has proven to be critical for a proper understanding of cell function and for modeling the complexities inherent in tissue physiology.\(^1\) The 3D culture system mimics the natural tissue environment of the cell far more effectively than the traditional 2D culture system; as a consequence the cells behave more like their in vivo counterparts in attributes such as migration, proliferation and apoptosis, and differentiation.\(^4\)\(^6\). The development of a workable and accurate
tissue model will involve the implementation of several technologies in tandem to optimize these environmental factors.

In this work, a system was developed and optimized to co-culture segregated cell types in an environment that closely represents in vivo 3D physiology, and to allow the separation and recovery of viable cells after co-culture. To accomplish this, we first developed and optimized a set of parameters that allow for the sequential fabrication of a cell-laden construct with an adequate thickness to simulate a native tissue environment. Next, we synthesized and characterized a polymer to be incorporated as a degradable adhesive, holding multiple cell-laden constructs in close proximity and allowing for their eventual separation. Finally, we integrated each of the system components and provide proof-of-concept in our ability to create a construct with two segregated cell populations with a degradable adhesive between them allowing for their separation and recovery. The successful implementation of this system will allow future studies to discern the long-term effects on stem cell fate decisions in response to co-culture with native cell types.

Hydrogels are an ideal candidate for an artificial extracellular matrix in 3D cell culture

Culturing cells in a 3D environment normally involves seeding or encapsulating cells on or in artificial ECM scaffolding. For the artificial ECM scaffold, crosslinked networks of synthetic or biological polymers, or hydrogels, are often used. A hydrogel's capacity to retain large volumes of aqueous solution makes it an attractive candidate for an ECM, as this attribute better facilitates the exchange of soluble factors between cells in culture. Additionally, the polymers comprising the hydrogel can be chemically modified before, during, or after the
gelation process to include numerous biofactors that will further influence cell growth, migration, adhesion, and proliferation within the ECM\textsuperscript{7-9}. The crosslinked polymers that comprise a hydrogel can be either synthetic or biological in nature; however, in order to be compatible with living systems they cannot induce cytotoxic effects on cells encapsulated within their network. Due to its non-cytotoxic and biocompatible nature, synthetic poly(ethylene)glycol (PEG) based hydrogels (such as poly(ethylene glycol)-diacrylate (PEG-DA) and oligo(polyethylene glycol) fumarate (OPF) ) have become popular choices for synthetic hydrogels in biological applications\textsuperscript{10-13}. Additionally, PEG-based hydrogels have been shown to be conducive to the integration of various biofactors in the hydrogel network\textsuperscript{14-15} allowing for the adjustment and fine-tuning of the ECM properties, an invaluable property when designing an \textit{in vitro} model that would closely approximate living tissues.

In addition to the synthetic PEG-based hydrogels, hydrogels have been successfully created from derivatives of biological polymers such as hyaluronic acid\textsuperscript{16}, heparin\textsuperscript{17}, and chondroitin sulfate\textsuperscript{18-21}. Chondroitin sulfate methacrylate (CSMA) hydrogels are of particular interest in the implementation of this co-culture system because they are readily degraded by the enzyme chondroitinase ABC lyase\textsuperscript{22}, making them an ideal candidate for a degradable adhesive to temporarily hold two or more cell populations in a close proximity.

\textit{Photolithographic methods allow for the controlled patterning of spatially defined hydrogel constructs}

In the creation of an ideal tissue model, the size and shape of the construct is very important, as the exchange of soluble factors and nutrients between cells is diffusion limited due
to the lack of a vasculature. Additionally, proper spatial relationships between cells are necessary to mimic in vivo conditions\textsuperscript{23-25}, making constructs on the millimeter to microscale ideal\textsuperscript{26}. Because milli- to micro-scale constructs are needed, a crosslinking method that would allow for microscale spatial control would be the most suitable for application in the 3D co-culture system. Numerous methods of crosslinking a synthetic or biological polymer into a hydrogel network exist, including heat molding (or thermal crosslinking), which uses heat to drive the crosslinking process; stereolithography or additive photopatterning, which uses a laser to crosslink the gel one layer at a time; and photolithography, which exposes the gel to ultra violet (UV) light to initiate polymerization\textsuperscript{27}. Of the crosslinking techniques available for use, photolithography possesses the necessary advantage because it allows hydrogels to be photopatterned on the microscale with relatively high throughput and spatial control. In photolithography, a photomask defines the pattern to be transferred to the photosensitive polymer\textsuperscript{28}. Transparent areas on the mask allow photons through to the polymer solution where they initiate the polymerization process, and the hydrogel forms beneath\textsuperscript{29}. Variations on this technique have been used in the past to pattern hydrogel scaffolds in which cells are encapsulated or seeded on the surface\textsuperscript{12, 30-32}.

Whereas photolithography allows for fairly precise control of hydrogel shape, the technology of microfluidics involves the use of flow cells to study the dynamics of fluids on the micro scale. When these technologies are used in conjunction with one another, the location and timing of the photopolymerizable process can be controlled, allowing for the fabrication of complex 3D hydrogel constructs\textsuperscript{33}. The temporal control imparted by the use of microfluidic flow cells also allows for the application of discrete photolithographic steps to produce multiple hydrogel components as part of a single laminated structure, as demonstrated previously by Cheung et al.\textsuperscript{34} and shown in Figure 1. This approach to hydrogel patterning can be extended to
the patterning of cells, where different cell types are patterned in series and laminated together to form a single construct, provided the inspiration for the project.

Figure 3: A hydrogel structure created through the sequential photopatterning of individual components (each shown as a different colored gel). Each gel color can be thought of as containing a different cell type, demonstrating the utility of this system in the creation of a 3D co-culture construct. Scale bar = 200 µm. Cheung et al. *Lab on a Chip* 2007.

**Summary of the development and optimization of the 3D co-culture system**

While the photopatterning of microscale hydrogel constructs for 3D cell culture has been achieved\(^{35-37}\), current techniques are limited in their ability to produce hydrogels with thicknesses greater than 500 µm and thus do not fully recreate the thicknesses inherent in many tissues.

Oxygen has been implicated previously as a potential culprit in the premature termination of the photo-polymerization process and may limit the thickness of the photopatterned hydrogels\(^{33, 38}\). The oxygen molecule's propensity to react with the free radicals that form during the polymerization reaction may cause the reaction itself to be quenched, resulting in the formation of a hydrogel with lower crosslinking density, lower patterning fidelity, and lower thickness.

The first aspect of the 3D cell culture system optimized here is the photopatterning of both OPF/PEG-DA and CSMA hydrogels of sufficient thickness to model tissues by performing the crosslinking in a controlled, low oxygen environment. Additional aspects of system
development developed in this thesis are: (i) synthesizing and characterizing bulk-crosslinked CSMA hydrogel swelling and degradation properties; (ii) optimizing the sequential photopatterning process to ensure no cell types mix between crosslinking steps; (iii) combining the technologies and techniques developed in parts (i) and (ii) to create a sequentially photopatterned hydrogel structure that remains intact after swelling and that can be separated through the incorporation of a degradable CSMA interface; (iv) using techniques employed in part (iii) to create a cell-laden construct with two segregated cell populations, and separating these populations be degrading the CSMA interface between them. The final system as it would be implemented in the co-culture of multiple cell types is diagrammed in Figure 2.

Figure 4: Visual representation of the final implementation of the system in creating a separable 3D co-culture structure. The steps are as follows: (1) A hydrogel containing cell type 1 is photopatterned (though any shape is theoretically possible, a square is shown here for illustrative purposes); (2) Residual polymer solution containing cell type 1 is removed and the degradable CSMA hydrogel adhesive is photopatterned in contact with the first hydrogel; (3) Residual CSMA polymer solution is removed and a hydrogel containing cell type 2 is photopatterned in contact with the CSMA glue; (4) The construct is placed in an environment suitable for cell growth, and the cell populations exchange soluble signaling factors with one another, influencing the behaviors of their respective counterparts; (5) The CSMA hydrogel adhesive is enzymatically degraded, separating the hydrogels containing each cell type; (6) The cell populations, now isolated, are ready to be analyzed.
MATERIALS AND METHODS

Synthesis, purification, and characterization of the oligo(poly(ethylene glycol) fumarate) and poly(ethylene glycol) diacrylate polymers

The polymer OPF was synthesized according to methods described previously\textsuperscript{39}. A solution of Triethylamine (TEA) and fumaryl chloride (FuCl) was added dropwise to a solution of PEG dissolved in methylene chloride (MeCl; Thermo Fisher Scientific) (0.9:1 and 2:1 molar ratios of FuCl to PEG and TEA to FuCl, respectively) under Nitrogen. The addition was performed over the course of 5 hours at 4\textdegree{}C and 2 days further at room temperature. MeCl was then removed through rotovaporation (Buchi) and TEA hydrochloride through filtration, and the OPF recrystallized in ethyl acetate, washed in ethyl ether, and vacuum dried at 25\textdegree{}C. The final product was stored at -20\textdegree{}C.

The PEGNDA was also prepared as previously described\textsuperscript{40}. 0.1 mmol/mL dry PEG, 0.4 mmol/mL acryloyl chloride, and 0.2 mmol/mL TEA were combined in anhydrous dichloromethane (DCM) under nitrogen and continuous stirring and were left to react overnight. The resulting solution was washed with 2 M K\textsubscript{2}CO\textsubscript{3} and separated into aqueous and DCM phases to remove any residual hydrochloric acid. The DCM phase was subsequently dried with anhydrous MgSO\textsubscript{4}, and PEG-D A was precipitated in diethyl ether, filtered, and vacuum dried. The PEG-D A was stored in a sealed container at -20\textdegree{}C and protected from light. The molecular weight distribution of both the OPF and PEG-D A was determined with gel permeation chromatography (Shimadzu).
Chondroitin sulfate (15 g) (Sigma Aldrich) was dissolved in 60 mL of distilled/deionized H₂O (25% w/v) and heated to 60°C with continuous stirring. Once the temperature of the reaction vessel stabilized, 60 mL of methacrylic anhydride was added dropwise at an approximate rate of ~1 mL/min. After the addition of the acrylate, 135 mL of 5 N NaOH was added dropwise at an approximate rate of ~1 mL/min. The pH of the reaction was then recorded, and the reaction was allowed to proceed for 24 h. After the completion of the reaction the pH of the solution was again recorded. The product was then precipitated in a mixture of cold methanol and isopropanol, filtered, and vacuum dried. Samples for experiments were dialyzed for three days, after which the purified solution was subsequently frozen in liquid nitrogen and dried in a lyophilizer (Labconco) for an additional four days. The product was dissolved in D₂O and characterized with H¹NMR. The synthesis was repeated and the amount of NaOH added during each repetition was varied, resulting in CSMA hydrogels with differing physical properties (see below).

CSMA hydrogel characterization: fold swelling ratio and degradation time

CSMA gel solutions for photo-crosslinking were produced through the addition of a volume of phosphate buffered saline (PBS) and 0.05% w/v D2959 photoinitiator (Ciba) to an appropriate mass of purified CSMA in order to obtain gels with compositions of 90% w/w H₂O. The gel solution was then dispersed between two glass slides (Fisher) with a 1mm separation and
exposed to UV light for 10 minutes. Individual gels of uniform size were excised and placed into PBS where they were allowed to reach their equilibrium swelling mass overnight. For the swelling studies, CSMA hydrogels were crosslinked and their equilibrium swelling masses recorded. The hydrogels were subsequently lyophilized and their dry mass measured and recorded. The fold swelling ratio was calculated by dividing the equilibrium swelling mass by the dry mass \( W_s/W_d \). For the degradation studies, CSMA hydrogels (90% w/w H\textsubscript{2}O) were crosslinked in the same fashion and swollen overnight in PBS. After swelling, PBS was removed and replaced with 1.75 mL of a chondroitinase buffer solution containing Tris (50mM), sodium acetate (60 mM), and Bovine Serum Albumin (BSA, 0.02% w/v) (Sigma) at a pH of 8.0, to which 0.25 mL of the enzyme chondroitinase ABC (1 U/mL, 0.01% w/v BSA; Sigma) was added. The gels were then incubated with the enzyme at 37°C and checked every 30 minutes for complete degradation. Control gels were created as well, to which 2 mL of chondroitinase buffer solution was added. The data was analyzed using one-way ANOVA followed by a Tukey-Kramer multicomparison test \( (n = 5 \text{ for the swelling studies and } n = 3 \text{ for the degradation studies, with } \alpha = 0.01 \text{ for both post-hoc tests}) \).

Fabrication of microfluidic devices and photolithography masks

Photopatterning experiments were performed in a microfluidic device fabricated from polydimethylsiloxane (PDMS)\textsuperscript{41} (Sylgard 184, Corning). Each microfluidic device was comprised of a 2 mm thick rectangular chamber in the center with three inlet and three outlet channels positioned symmetrically on either side. Prior to molding, a poly(urethane) master mold was fabricated through techniques described previously.\textsuperscript{42} PDMS (10:1 base:curing agent ratio)
was poured over the master and allowed to cure at 70 °C for 2 h. Afterwards, the PDMS was removed from the mold, and individual devices were excised and subsequently bonded to cover glass using oxygen plasma treatment\textsuperscript{43}. Medical grade platinum-cured silicone micro tubing was used for fluidic connections. In the inlet and outlet channels, holes for fluidic connections were punched to a size determined by the outer diameter of the tubing. The tubing was connected to the device via type 304 90°-angled stainless steel 21 gauge tubes. 21 gauge Luer lock dispensing needles were attached to the opposite ends of the tubing to allow for connection to syringes containing polymer solution. A contact-bonded, overlying PDMS enclosure was fabricated using a different poly(urethane) mold to isolate the device in a nitrogen atmosphere. Masks used in the photolithographic patterning were designed in AutoCad and printed at 10,000 dpi (CAD Art Services).

**Hydrogel photopatterning optimization: OPF/PEG-DA and CSMA**

Photopatterning experiments were performed in a microfluidic device fabricated from PDMS, detailed previously. The photomasks used consisted of a series of 800 µm to 3000 µm squares. Devices were equilibrated with N\textsubscript{2} or room air prior to loading the polymer. The photomask was aligned and the polymer solution injected and allowed to crosslink under 365 nm light for 12 min (see Figure 3). Using a dissecting microscope and ImageJ software (NIH), the dimensions of the hydrogels were measured immediately after crosslinking and after reaching equilibrium swelling. Measurements were compared using ANOVA and Tukey's post-hoc test \((n = 3, p \leq 0.05)\). Linear regression was performed to determine the correlation between mask size and the size of the resulting hydrogel.
Photopatterning of the CSMA polymer was attempted, and effects of crosslinking time, UV lamp intensity, and the presence or absence of oxygen on the photopatterned hydrogels investigated. The CSMA polymer solution, 90% w/w H\textsubscript{2}O and 0.05% w/v D2959 photoinitiator, was prepared and injected into a microfluidic flow cell, which was either equilibrated with a nitrogen atmosphere 30 minutes prior or left in room air. CSMA hydrogels were photopatterned in a series of square blocks, with sizes ranging from 550 µm to 3000 µm. Crosslinking time was varied from 8 minutes to 12 minutes, and UV lamp intensity varied from 10.5 mW/cm\textsuperscript{2} to 11.5 mW/cm\textsuperscript{2}.

After crosslinking using a predefined set of the specified parameters, any hydrogels formed were excised from the flow cell and imaged under a dissecting scope (Leica), both immediately after crosslinking and after immersion for 24 hours in a solution of phosphate buffered saline. See Figure 4 for a summary of both OPF/PEG-DA and CSMA hydrogel chemistries.
**System optimization: flow cell washing**

During the serial photopatterning of different cell types, it would be necessary to completely remove cell populations in between crosslinking steps to ensure that no mixing between the populations occurred. In order to ensure that existing photopatterned gels would not obstruct removal of residual cell/polymer solution, polystyrene beads 10 µm in diameter (used in lieu of cells) were homogeneously dispersed at a concentration of $10 \times 10^6$ beads/mL in a polymer solution comprised OPF ($M_n = 10,000$ kDa) and PEG-DA ($M_n = 3,400$ kDa) (50% w/w OPF/PEG-DA, 75% w/w H$_2$O, 0.05% w/v D2959 photoinitiator). The crosslinking was performed in a controlled, oxygen-free environment to mitigate the inhibitive effects of oxygen on the crosslinking process. A prefabricated microfluidic flow cell was allowed to equilibrate with a nitrogen atmosphere, and after 30 minutes the polymer solution was injected into the flow cell. Hydrogels were photopatterned for 12 minutes under UV light (375 nm wavelength) and a nitrogen atmosphere. The hydrogels were photopatterned in an array of alternating square blocks, with block spacing varying from 100 µm to 750 µm. After photopatterning, the remaining bead/polymer solution was washed from the flow cell using an isotonic PEG solution, and the process was recorded using dark field microscopy. This process is illustrated in Figure 5.

**Figure 5:** Simulated washing of cells from a microfluidic flow cell after crosslinking one set of OPF/PEG-DA hydrogels. (Left) Photograph and computer rendering of the new flow cell design. (Right) A summary of the optimization process: (1) Inject macromer solution (with polystyrene beads) into device (dark blue); (2) Photopattern hydrogel blocks (light blue); (3) Wash out residual macomer/bead solution with bead-free macromer solution of poly(ethylene glycol) (red).
System optimization: hydrogel lamination and demonstration interface stability and of interface separability through the incorporation of a degradable CSMA adhesive

A hydrogel tri-laminate consisting of two OPF/PEG-D A hydrogels with a CSMA hydrogel at the interface was created in order to demonstrate the inherent stability of the structure after swelling, and to show that the OPF/PEG-D A portions may be separated upon degradation of the CSMA interface. OPF/PEG-D A hydrogels (75% w/w H₂O, 0.05% w/v D2959) were photo-patterned for 15 minutes in a prefabricated PDMS flow cell. CSMA (90% w/w H₂O, 0.05% w/v D2959) was then injected into the flow cell and allowed to crosslink for 10 minutes. The laminate was then excised, the CSMA stained with dimethylmethylen blue (DMMB), and immersed in 1.75 mL of a chondroitinase buffer solution (pH of 8.0) to which 0.25 mL of a solution containing the enzyme chondroitin sulfate ABC lyase was added. CSMA control gels (90% w/w H₂O, 0.05% w/v D2959) were also created, stained with DMMB, placed in 2 mL of chondroitinase buffer solution, and incubated at 37°C. The laminates and control gels were periodically monitored to check for complete degradation. Pre- and post-degradation images of the laminates were taken using an inverted Nikon TE2000U microscope and a digital camera.

To qualitatively study the interface of a laminate consisting of two differentially swollen hydrogels (similar to an OPF/PEG-D A/CSMA laminate), the methacrylated fluorophores rhodamine B and fluorescein were added to two OPF/PEG-D A gel solutions. The first solution consisted of OPF (molecular weight of 10,000 kDa) (OPF 10k) and PEG-D A to which a methacrylated fluorescein was added with a final concentration of 2.5 mM. A second gel
solution was likewise prepared, but with OPF 3.4k in place of OPF 10k and to which methacrylated rhodamine B was added to achieve a final concentration of 0.25 mM. A PDMS flow cell was placed into a larger PDMS chamber and flushed for 30 minutes with nitrogen gas. The OPF 10k gel solution was then injected into the flow cell and photo-patterned under a UV lamp. Afterwards, an isotonic solution of PEG 10k and PEG 20k (50% by mass, 75% w/w H₂O) was injected into the flow cell to wash out the remaining OPF 10k solution. The OPF 3.4k solution was subsequently injected and photo-patterned. Both gels were crosslinked for 12 minutes. The laminate was then excised and fluorescent images were obtained using a Leica MZ16F dissecting microscope.

Creation and degradation of a hydrogel tri-laminate with a degradable CSMA interface, and assessment of cell population segregation and viability before and after degradation

To determine the length of time required to separate hydrogel tri-laminate components, molding devices were constructed by placing a 1 mm-thick spacer of PDMS between two glass slides that contained a cavity to accommodate the polymer solution. The tri-laminate was created by successively crosslinking and subsequently masking a layer of PEG-DA, CSMA, and PEG-DA (90% w/w H₂O, 0.05% w/v D2959) under 365 nm light at 10.5 mW/cm² for 12 min, as shown in Figure 6. The hydrogels were divided into 1.5 mm wide sections and allowed to reach equilibrium swelling, whereupon they were added to a chondroitinase buffer solution (200 mM tris-HCl, 240 mM Na-acetate) containing the enzyme chondroitinase ABC.
at 0.25, 0.5, and 1 U/mL to assess degradation time.

To establish if cells retained viability after separation of the tri-culture laminate, the same experiment was repeated with encapsulated human mesenchymal stem cells (hMSC, passage 6; Texas A&M) inside the PEG-DA hydrogels at a density of $10 \times 10^6$ cells/mL. One tri-laminate included a group of cells in the first layer stained with Orange CMRA and in the second layer a group of cells stained with Green CMFDA (Invitrogen); these were visualized using fluorescence microscopy to ensure population segregation. After encapsulation, the sectioned laminates were allowed to culture for 1 day in growth media (alpha-MEM (Mediatech), 16.5% fetal bovine serum, 1% L-glutamine, and 1% glutamicin/amphotericin B) after which they were added to the chondroitinase buffer solution containing the enzyme chondroitinase ABC at 0.25, 0.5, and 1 U/mL. After separation of the PEG-DA layers, the gels were cultured for an additional day in growth media and their viability assessed with a LIVE/DEAD assay (Invitrogen) in conjunction with confocal microscopy.

RESULTS

CSMA hydrogel characterization: fold swelling ratio and degradation time

After synthesizing multiple batches of the CSMA polymer, bulk crosslinking yielded hydrogels with fold swelling ratios ranging from 18 to 38 minutes and degradation times ranging from 80 to 210 minutes, depending on the polymer batch used. The degradation time of the hydrogel correlated reasonably well with the fold swelling ratio ($r^2 = 0.6103$); hydrogels with
higher fold swelling ratios generally exhibited shorter degradation times and hydrogels with lower fold swelling ratios took longer to degrade, as expected (Figure 7).

**Hydrogel photopatterning optimization: OPF/PEG-DA and CSMA**

A cross-sectional diagram of the PDMS microfluidic flow cell with and without the respective nitrogen chamber is shown in Figure 5. The dimensions of the OPF/PEG-DA hydrogels photopatterned were found to correlate strongly with the dimensions of the photomask to produce those hydrogels ($r^2 = 0.9987$ and $r^2 = 0.9927$ for hydrogels photopatterned with and without nitrogen, respectively) (Figure 8). The geometry of the hydrogel was easily adjusted by changing the photomask. Additionally, the feature fidelity and thickness was found to be significantly increased in the OPF/PEG-DA hydrogels photopatterned under nitrogen.

Thicknesses of greater than 1 mm were obtained for hydrogels as small as 900 μm in width, and these thicknesses were maintained for all hydrogels photopatterned with nitrogen. Hydrogels photopatterned under nitrogen were also found to swell significantly less than those photopatterned in room air, suggesting a more highly crosslinked network.

The same set of experiments was repeated with CSMA hydrogels. Similarly to the results obtained with OPF/PEG-DA hydrogels, the dimensions of the photopatterned CSMA hydrogels...
once again correlated strongly with the dimensions of the applied photomask (Figure 9).

However, CSMA hydrogels could not be photopatterned at all without the use of the nitrogen chamber. The CSMA hydrogels that were photopatterned exceeded 1 mm in thickness, and gels as small as 650 μm across could be successfully photopatterned using these techniques.

Additionally, detailed shapes and geometries could be obtained by altering the shape of the photomask used.

Figure 8: The effects of photopatterning under nitrogen on OPF/PEG-DA hydrogel thickness, patterning fidelity, and percent increase in size after swelling. (a) Hydrogel dimensions correlated well to the dimensions of the photomask used to pattern the gel, both with and without nitrogen. (b) Different hydrogel shapes are obtained by altering the geometries of the photomask (shown in the bottom left-hand corner of each image). Also, patterning OPF/PEG-DA hydrogels under nitrogen (bottom set of images) eliminated the presence of rounded corners and edges present in hydrogels photopatterned in room air (top set of images, noted by black arrows) and enhanced hydrogel features (scale bar = 1 mm). (c) Hydrogel thickness was significantly enhanced when patterned with nitrogen, with hydrogels as small as 900 μm exceeding thickness of 1 mm or more. (d) Hydrogels patterned with nitrogen increased significantly less in thickness than those patterned in room air, suggesting a more highly crosslinked network. n=3, error bars indicate ± standard deviation.
System optimization: flow cell washing

In order to ensure that different cell populations would not mix in between crosslinking steps, it was necessary to devise a new microfluidic flow cell design that would facilitate the complete removal of residual cells. The final flow cell design was then implemented in all further experiments, and is shown in Figure 5. After varying the distance between photopatterned hydrogels, it was found that the large majority of beads could be cleared from the flow cell after patterning with a minimum horizontal spacing between 100 μm and 250 μm, as shown in Figure 10.
System optimization: hydrogel lamination and demonstration interface stability and of interface separability through the incorporation of a degradable CSMA adhesive

After photopatterning and visualization with the acrylated fluorescent dye, the interface between the two differentially swollen photopatterned OPF hydrogels remained stable (Figure 11a). CSMA was successfully laminated between two PEG-DA hydrogels as well, and after swelling the hydrogel construct remained stable. Upon addition of chondroitinase, the CSMA interface dissolved, leaving the two PEG-DA hydrogels intact (Figure 11b).
Creation and degradation of a hydrogel tri-laminate with a degradable CSMA interface, and assessment of cell population segregation and viability before and after degradation

CSMA hydrogels were found to degrade readily upon exposure to chondroitinase ABC lyase, and lamination to PEG-DA gels did not appreciably affect CSMA degradative properties. PEG-DA gels remained laminated after swelling and were separated upon addition of chondroitinase. The time required to separate the two PEG-DA gels varied from less than 1 hour for 1 U/mL chondroitinase to over 4 hours at 0.25 U/mL. After visualization with fluorescence microscopy, the two hMSC populations (distinguished with Cell Tracker Green CMFDA and Orange CMRA) remained separated after the encapsulation process (Figure 12). Additionally, as shown in Figure 13, cell viability was maintained throughout the process of encapsulation and after separation, with no observable decrease in viability regardless of the concentration of enzyme used to degrade the CSMA interface of the tri-laminate.

Figure 12: Creation of hydrogel tri-laminate with two segregated labeled cell populations separated with a degradable CSMA interface. (A) Visual representation of the sequential patterning of the multi-layer hydrogel laminate. Red and green layers correspond to the two segregated cell populations, while the purple layer represents the hydrogel adhesive. (B) Fluorescent image of a trilaminate with encapsulated hMSC stained with cell tracker orange and green (shown in red and green, respectively) (scale bar = 0.5 mm).

Figure 13: LIVE/DEAD confocal microscopy images of cells (A) before degradation of the CSMA interface and after using (B) 0.25 U/mL, (C) 0.5 U/mL, and (D) 1 U/mL of chondroitinase. Green indicates viable cells, and red indicates dead. Scale bar = 100 µm.
DISCUSSION

The utilization of artificial biomimetic scaffolds such as hydrogels in the formation of 3D tissue-engineered constructs has been accomplished in a variety of ways and is useful for the creation of in vitro tissue models enabling the study of 3D cellular physiology\textsuperscript{27}. A better understanding of this physiology will result in more effective tissue-replacement therapies and techniques, such as the engineering of stem cells outside of the body before implantation to ensure successful engraftment\textsuperscript{1}. Additionally, by combining 3D cell encapsulation techniques with co-culture, the manner in which different cell populations interact and influence one another can be determined. Current transwell co-culture techniques are limited in their inability to reproduce 3D tissue physiology. While several techniques exist that allow for the co-culture of multiple cell types in 3D\textsuperscript{11, 44-47}, they are generally very specialized in their implementation and do not allow for the recovery of viable segregated cell populations for in vitro post-culture analysis. The system developed here overcomes both of these obstacles, while maintaining the ability to pattern cells in a tunable 3D microenvironment similar to native tissues and tissue components.

The photopatterning technologies and system developed here represent the very first working prototype of its kind. The photolithographic methods of patterning hydrogel structures yield architectures whose dimensions correlate strongly with those of the applied photomask. This suggests that the system can be easily calibrated, depending on the size and type of geometry desired. Additionally, by altering the shape of the photomask, any number of hydrogel shapes is obtainable. This consequential versatility and ease of use is a product of the photopatterning technique\textsuperscript{24, 28} and thus, by extension, an inherent property of our system.
Alteration of hydrogel shape could be employed to distinguish between different cell populations housed in the microstructure; this would be especially useful if the system is expanded to include the culturing of three or more different cell populations.

The introduction of a nitrogen chamber and photopatterning under a nitrogen atmosphere resulted in hydrogels with thicknesses greater than 1 mm and very high aspect ratios. These results are consistent with previous studies detailing the inhibitory effects of oxygen on the photopolymerization process\textsuperscript{38}. The thicknesses obtained using these photopatterning procedures are very similar to those found in native tissue environments, an important attribute for an \textit{in vitro} model capable of accurately representing an \textit{in vivo} environment. The ability to obtain relatively high (>1 mm) thicknesses with micropatterned hydrogels makes this system unique from previous, similar photopatterning techniques\textsuperscript{11, 35-37, 40}. Additionally, the fact that the system allows for the photopatterning of hydrogels on the microscale makes it ideal for studying tissue microenvironments\textsuperscript{27} and diffusion-limited paracrine signaling effects, as the effects of these endogenous signals lose their potency with distance from the secreting cell due to their dilution in the surrounding media.

The new microfluidic flow cell design allows for the successful removal of residual polymer solution containing cell-like components. After visualization a thin, uniform coating of polystyrene beads was noticed along the bottom of the flow cell. However, this coating was later found to be due to the settling of the beads along the surface of the coverglass – it is anticipated that when actual cells are used in place of these polystyrene beads, this settling effect will be far less significant, as the cells' density differs little from that of the aqueous polymer solution. The successful removal of residual cells from the flow cell after each crosslinking step, as well as the volume of washing solution necessary to remove them, depends on both the design of the flow
cell as well as the obstruction to flow caused by the hydrogel structure occupying the central chamber. The fact that different hydrogel shapes may alter the flow in different ways highlights an important limitation in the types and shapes of structures that can be created using this system. Some geometries may impede the removal of residual cells causing inhomogeneity in the encapsulation of subsequent cell populations. This problem could be circumvented by changing the design of the flow cell; this task is made easier by the fact that the flow cells can be easily fabricated with a relatively high throughput, as they are simple PDMS castings bonded to a piece of coverglass.

Differentially swollen hydrogels, once laminated together by sequential photopatterning, retained a stable interface that did not rupture after swelling. These results suggest that the system could be used to sequentially pattern hydrogel structures that contain not only different cell types but also different types of crosslinked hydrogels, as long as the necessary vinyl moiety is present in the constituent polymer. This would impart additional versatility to the system, as it would allow for the study of differing extracellular matrix components on cell growth and behavior. The CSMA hydrogel synthesis yielded a material that was successfully laminated with an existing OPF/PEG-DA hydrogel through photocrosslinking and further integrated into a hydrogel structure with multiple components. Upon addition of the enzyme chondroitinase ABC the CSMA interface dissolved, leaving the other hydrogel components intact and completely separate. After repeating these experiments with encapsulated cells, the CSMA degradation process appeared to have no immediate detrimental effects on cell viability after separation, and cell populations remained separated and distinct throughout the encapsulation process. This is especially important, as the successful recovery of viable cells after culture will allow for further experimentation on those populations.
One commonly employed method of non-contact, separable cell co-culture is the transwell system. Transwell works by providing two separated surfaces for cell growth in the same well, with one cell population plated on a removable membrane permeable to soluble factor signaling molecules. This versatile system has been used in the past to elucidate the effects of paracrine signaling and cross-talk between various cell populations with notable success\textsuperscript{48-50}. However, transwell culture is restricted to two dimensions, and thus is limited in its ability to accurately simulate native tissue physiology. More advanced 3D cell co-culture systems are often uniquely specialized in their applications and unable to allow for the facile separation and post-culture analysis of segregated cell types\textsuperscript{44-47}. The system developed here represents a significant advantage over both traditional transwell co-culture methods and current 3D co-culture systems in that it provides the physiologically representative 3D environment for cell growth, is versatile enough to answer a variety of important biological questions concerning stem cell growth and development, and allows for the sequential separation and recovery of viable cell populations through the incorporation of one or more stimulus-responsive adhesives. Additionally, the system can be potentially further tailored to more accurately represent tissue physiology by incorporating a multitude of biofactors, peptides, and other moieties into the hydrogel network\textsuperscript{14-15}.
CONCLUSION

The completion of this project represents the development of a novel hydrogel fabrication system that (a) allows for the sequential crosslinking of a hydrogel structure with various and easily alterable geometries; (b) produces hydrogel structures that are microns in width while still retaining thicknesses of greater than 1 mm, which would allow for the diffusion-limited exchange of soluble factors between cell populations while closely representing the dimensions of native tissue; (c) allows for the incorporation of different types of hydrogels into one cohesive architecture that retains its structural integrity over time. A direct consequence of (c) is the incorporation of the CSMA hydrogel as a temporary adhesive holding multiple hydrogels in close proximity. Taken together, the aspects of this system make it well suited for implementation in the production of a separable 3D co-culture system for the study of paracrine signaling effects between cell populations. Its inherent utility and versatility make it an ideal tissue model, and the information it will assist in obtaining from future co-culture studies will help to elucidate the biological mechanisms of stem cell development that could allow for the successful implementation of regenerative medicine therapies.
REFERENCES


