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Development of 3-D Hydrogel Culture Systems With On-Demand Cell Separation

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Abstract

Recently there has been an increased interest in the effects of paracrine signaling between groups of cells, particularly in the context of better understanding how stem cells contribute to tissue repair. Most current 3-D co-culture methods lack the ability to effectively separate 2 cell populations after the culture period, which is important for simultaneously analyzing the reciprocal effects of each cell type on the other. Here, we detail the development of a 3-D hydrogel co-culture system that allows us to culture different cell types for up to 7 days and subsequently separate and isolate the different cell populations using enzyme-sensitive glues. Separable 3-D co-culture laminates were prepared by laminating PEG-based hydrogels with enzyme-degradable hydrogel adhesives. Encapsulated cell populations exhibited good segregation with well-defined interfaces. Furthermore, constructs can be separated on-demand upon addition of the appropriate enzyme and cell viability remains high throughout the culture period, even after laminate separation. This platform offers great potential for a variety of basic cell signaling studies as the incorporation of an enzyme-sensitive adhesive interface allows the on-demand separation of individual cell populations for immediate analysis or further culture to examine persistence of co-culture effects and paracrine signaling on cell populations.

Keywords

Hydrogel; Mesenchymal Stem Cell; Co-Culture; Micropatterning; Poly(ethylene glycol)

1. Introduction

Stem cells represent an attractive option for regenerative medicine applications due to their ability to differentiate toward multiple cell lineages [1–3]. However, there is a lack of information on how stem cells' interactions with native cells may influence stem cell differentiation as well as the phenotype of the neighboring, differentiated cells. Therefore, the development of novel co-culture systems that allow the study of these complex interactions in a controlled, *in vitro* setting would be advantageous for optimizing a variety

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of regenerative medicine strategies. Such a system would enable fundamental studies on the effects of paracrine signaling between cells to better understand both how this communication is important in development as well as how it contributes to tissue repair [4–7].

Many co-culture studies performed to date have been carried out in 2-D culture plates that lack structural cues [5,8–10]. As model systems, 3-D tissue-engineered constructs can better mimic the organization and cell-cell interactions of tissues within the body [11,12], while avoiding the variability between samples that is observed in 3-D tissue explants [13,14]. For co-culture experiments, 2-D transwell systems have often been employed because they allow rapid separation of cell types and thus enable individual population analysis post-culture [5,15]. A variety of systems utilizing hydrogels with embedded cells have been used to achieve 3-D equivalents of the transwell system to examine the effects of soluble signaling in a more physiologically-relevant geometry [16–22]. For smaller-scale constructs (each layer <500 μm), microfluidic devices are generally employed to form multiple streams of cell-laden materials that are then gelled in a variety of geometries, ranging from parallel layers [17,18] to beads [22] to fiber-like structures or tube-like structures [20]. For larger constructs (each layer ~ 1 mm), hydrogels have been successfully layered in molds on top of porous scaffolds or other gels [19,21]. However, regardless of the fabrication method, most current 3-D co-culture methods lack the ability to effectively separate two cell populations after the culture period, which is important for simultaneously analyzing the reciprocal effects of each cell type on the other [16–22]. A more physiologically representative, 3-D *in vitro* co-culture system that allows post-culture separation of segregated cell populations would enhance the ability to study these effects and allow for the possible establishment of complex cell-cell interactions normally occurring between cells in native tissues.

Poly(ethylene glycol) (PEG), a biocompatible synthetic polymer, forms the basis for many hydrogel carriers utilized in 3-D culture systems that, when cross-linked, recreates a hydrophilic, biocompatible environment similar to native extracellular matrix (ECM) [11,23]. Additionally, PEG-based hydrogels such as oligo(PEG fumarate) (OPF) can be modified to present a variety of controlled, tailored environments representing different aspects of the native 3-D tissue [24–26]. Previously, we reported a photopatterned cell co-culture system based on PEG-diacrylate and OPF hydrogels with distinct patterning of two cell populations [27]. The current work investigates stimuli-sensitive polymers, including both polysaccharides and peptides that degrade on-demand when exposed to specific enzymes as a means to separate cell populations encapsulated in these PEG-based gels. It has been shown that proteoglycans like alginate, chondroitin sulfate, and heparin can be modified with acrylate groups that allow them to be cross-linked to form hydrogels [28–31]. Likewise, the inclusion of short matrix metalloproteinase (MMP)-sensitive peptides in PEG-based acrylated polymers allows for the formation of degradable hydrogels with improved cell migration, cell spreading, and tissue formation within the gels [32–34].

In these studies, enzyme-sensitive hydrogels were incorporated in particular locations in 3-D patterned constructs to promote the separation of cell-laden PEG-based gels. The on-demand separation of cell populations via enzyme exposure afforded viable cells that could be cultured post-separation. Furthermore, layered constructs containing multiple stimuli-sensitive gels were prepared and could release cell populations in a specific order based upon exposure to enzymes. This novel separable co-culture technology was also integrated into more complex, interlocking 3-D constructs to demonstrate the versatility of our technique to enable separation of a variety of co-culture geometries that may be tailored for a wide range of tissue types.

2. Materials and Methods

Polymer Synthesis and Characterization

Oligo(poly(ethylene glycol)fumarate) (OPF) (PEG, $M_n = 18,300 \pm 100$ Da, polydispersity index [PDI] 4.8 ± 0.2), poly(ethylene glycol)-diacrylate (PEGDA, $M_n = 3,730 \pm 20$ Da, PDI 1.1 ± 0.1), chondroitin sulfate methacrylate (CSMA), and Acrl-PEG-GRGDS (RGD) were synthesized according to established methods [24,26,30,35].

Alginate MAm (alginate methacrylamide) was prepared by dissolving alginate (1.0 g, $M_w = 29$ kDa, NovaMatrix) in a buffer solution (1% w/v, pH 6.5) of 50 mM 2-morpholinoethanesulfonic acid (Sigma) containing 0.5 M NaCl.[28] *N*-hydroxysulfosuccinimide (2.3 mmol, 500 mg) and *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (4.6 mmol, 872 mg) were added to the solution and allowed to react for 5 min. *N*-(3-aminopropyl) methacrylamide (MAm) (2.3 mmol, 411 mg) was then added and the reaction stirred at room temperature for 24 h. The polymer was precipitated with the addition of excess of acetone, dried under reduced pressure, and rehydrated to a 1% w/v solution in deionized water (dH₂O). The alginate MAm solution was then dialyzed against dH₂O (MWCO 1,000 Da, Spectrum Laboratories Inc.; Rancho Dominguez, CA) for 3 days and lyophilized.

PEG-acrylate derivatized with MMP-cleavable peptide GGGLGPAGGK (Acrl-PEG-LGPA-PEG-Acrl) was prepared by reacting the peptide GGGLGPAGGK (129.9 μ mol, 100 mg) with Acrl-PEG-SVA (313.4 μ mol, 971.6 g, $M_n = 3,400$ Da, LaysanBio) in 50 mM sodium bicarbonate buffer (pH = 8.5) for 3 h [32]. Subsequently, the Acryl-PEG-LGPA-PEG-Acrl was purified via dialysis against dH₂O (MWCO 3500–5000 Da, Spectrum Laboratories Inc.) for 2 days and the purified product isolated by lyophilization.

OPF and PEGDA were characterized via gel permeation chromatography to determine molecular weight and polydispersity. CSMA and alginate MAm were characterized via NMR to determine the extent of methacrylate modification for each enzyme-sensitive molecule.

PEG ($M_n = 10,000$ and $3,400$ Da), chondroitin sulfate A ($M_w = 78,000$ Da), fumaryl chloride, triethylamine, acryloyl chloride, *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride, *N*-hydroxysulfosuccinimide were purchased from Sigma–Aldrich (St Louis, MO). Dichloromethane, ethyl acetate, anhydrous potassium carbonate and ethyl ether were obtained from Fisher Scientific (Waltham, MA). Alginate ($M_w = 29,000$ DA) was purchased from NovaMatrix (Sandvika, Norway). Acrl-PEG-SVA ($M_n = 3,400$ Da) was purchased from LaysanBio (Arab, AL). GRGDS adhesion peptide was purchased from PeproTech (Rocky Hill, NJ) and GGGLGPAGGK matrix-metalloproteinase sensitive peptide was purchased from AAPPTec (Louisville, KY).

Device Fabrication

Layered Constructs—Molding devices for hydrogels containing multiple (straight) layers (see Fig. 1) were constructed by placing a 1-mm-thick spacer of polydimethylsiloxane (PDMS, Dow Corning Sylgard 184; Essex-Brownwell, Inc.) between two glass slides that contained a cavity to accommodate the polymer solution.

Tri-laminates—In the latter part of these studies, hydrogel laminates with complex geometries (referred to as tri-laminates) were formed via a series of replica moldings with PDMS molds [36]. Devices consisted of a 1-mm-thick chamber with two cavities for macromer solutions (see Fig 2A). Briefly, we fabricated PDMS devices by curing 1-mm-thick and 2-mm-thick PDMS (10:1 base:curing agent) in a dish at 37 °C for 12 h, cutting the

PDMS using printed AutoCAD (Autodesk, San Rafael, CA) drawings as templates, and bonding a 1-mm-thick PDMS design on top of a 2-mm-thick slab of PDMS using oxygen plasma treatment [37]. The bonded devices were coated with a layer of parylene-C using a Labcoater PDS 2010 (Specialty Coating Systems; Indianapolis, IN) [38,39]. Coated devices were loaded with macromer solution, covered with a glass slide, and clamped to allow for hydrogel formation. An additional 1-mm-thick PDMS rectangular frame was contact-bonded to a glass slide to contain the hydrogels during lamination (see below) (Fig. 2A).

Hydrogel Construct Characterization and Separability

Layered Constructs—A 3-layered laminate was created by cross-linking a layer of PEGDA under 365 nm light at 10.5 mW/cm² for 12 min and subsequently masking it to prevent excess cross-linking during the rest of the procedure. These steps were repeated to add successive layers of CSMA, and PEGDA to the construct [each layer was 90% w/w dH₂O, 0.05% w/v D2959 photoinitiator (Ciba)]. The tri-layer samples were then added to a buffer solution (200 mM tris-HCl, 240 mM Na-acetate) containing the chondroitinase ABC at 0.125, 0.25, or 0.5 U/mL to assess degradation time ($n=3$). To create a 5-layer laminate, layers of AcrI-PEG-LGPA-PEG-AcrI (LGPA) and PEGDA were added to the 3-layered construct using the same methods (Fig. 1). Five-layer laminates were first added to a solution of collagenase (2 mL, 2,200 U/mL, 3 mM CaCl₂ in PBS) followed by chondroitinase ABC (2 mL, 0.5 U/mL) ($n=3$); the process was then repeated in reverse order to demonstrate the capability to sequentially separate hydrogel components.

Tri-laminates—Macromer solutions of OPF/PEGDA 1:1 [90% w/w dH₂O, 0.018 M ammonium persulfate (APS) and 0.018 M N,N,N',N'-tetramethylethylenediamine (TEMED)] were prepared. The macromer solution was pipetted into the cavities in the coated PDMS mold and the solutions cross-linked at 37 °C for 20 min. The resulting gels were extracted from the mold and arranged in a separate 1-mm-thick rectangular PDMS frame on a glass slide (see Fig. 2A). The gels were placed against the inner walls of the PDMS frame, which allowed the spacing between the interlocking gels to be fixed at ~400 μ m. A macromer solution of alginate MAm (98% w/w dH₂O, 0.018 M APS/TEMED) was pipetted between the two gels and the solution cross-linked at 37 °C for 20 min to form a tri-laminate. Constructs were removed from the PDMS frame and allowed to reach equilibrium swelling in PBS.

Laminate dimensions prior to swelling were measured using an epifluorescent microscope (Nikon Eclipse TE2000-U, Nikon Instruments Inc.; Melville, NY) and compared to the laminate design created with AutoCAD software ($n = 4$) (Fig. 2B, Table 1). Additionally, tri-laminates were added to alginate lyase solutions (2 mL, 10 U/mL in PBS, pH = 7.4) and observed visually every 15 min until the OPF/PEGDA components separated ($n=11$) [40].

To determine if the devices were reusable for multiple cell encapsulations, OPF/PEGDA gels were prepared from three separate devices and the resulting hydrogels' dimensions were analyzed via epifluorescence ($n = 5$). The devices were subsequently autoclaved and used to prepare additional gels. This process was repeated for a total of five autoclave cycles. Multiple measurements (e.g. block height) were made of the dimensions of the gels from each device. The measurements were normalized to the original measurement of that dimension in that device prior to autoclaving (Fig. 2C).

Cell Patterning and Co-Culture

Before encapsulation, human mesenchymal stem cells (hMSCs, p4, Texas A&M) were thawed and plated at 4×10^6 cells/flask in medium containing alpha-Minimum Essential Medium (alpha-MEM; Invitrogen), 16.3% fetal bovine serum (FBS; Hyclone), 1%

antibiotic/antimycotic solution (A/A; Mediatech), and 2% L-glutamine (Mediatech). Cells were expanded to passage 5 with medium changes every 3 days, lifted using 0.05% trypsin/0.53 mM ethylenediaminetetraacetic acid (EDTA) and used at passage 6 for encapsulation experiments. A PEG modified adhesive peptide sequence (Acrl-PEG-GRGDS) was incorporated into hydrogels encapsulating MSCs to promote viability of the embedded cells [41,42]. Cell populations were distinguished from each other during co-culture experiments by differentially staining aliquots of hMSCs with 10 μ M CellTracker Orange CMRA or CellTracker Green CMFDA reagents (Invitrogen) per manufacturer's recommendations 1 day before encapsulation.

Tri-laminates—Cells were patterned into interlocking hydrogel laminates using the previously described replica molding method. Devices were sterilized via autoclaving prior to use. Macromer solutions of OPF/PEGDA (1:1 wt ratio of polymers; 90% wt/total wt dH₂O, 0.018 M APS and 0.018 M TEMED, 10 $\times$ 10⁶ cells/mL (initial seeding density), 1 μ mol/g Acrl-PEG-GRGDS) containing green- or orange-stained hMSCs were cross-linked in the cavities of a coated PDMS mold at 37 °C for 20 min. The resulting gels were extracted from the mold using a spatula and laminated using the previously described method. Laminated constructs were placed in tissue culture plates with alpha-MEM growth medium.

Image analysis of patterning—The gels containing stained cells were allowed to reach equilibrium swelling overnight. They were then rinsed for 1 h in PBS and subsequently imaged at 4X on an epifluorescent microscope (Nikon Eclipse TE2000-U). A total of 11 overlapping images were acquired for each gel. The green and red channels were merged for each image and then stitched together to provide a single image with an overall view of the entire construct to demonstrate spatial patterning of the two cell populations (Fig. 3).

Cell Viability

Layered Constructs—Three-layer hydrogels containing encapsulated hMSC populations with CSMA, alginate MAM and LGPA adhesive layers were created using the previously described methods. Cells were cultured in the 3-layered constructs for 1 day after which cell viability was assessed for a subset of samples using LIVE/DEAD staining (see below for details). Adhesive layers on remaining tri-laminates were removed by exposure to the appropriate enzyme (CSMA to 2 mL chondroitinase ABC in buffer at 0.5 U/mL for 4 hours, alginate MAM to 2 mL alginate lyase in media at 10 U/mL for 30 min, and LGPA to 2 mL collagenase in media at 2200 U/mL for 1.5 h). The separated gel components containing hMSCs were cultured for an additional 1 or 3 days (2 or 4 days total culture time), at which point their cell viability was quantified and compared with viability levels at day 1 (before enzyme exposure) (Fig. 4A).

Tri-laminates—Complex tri-laminates containing homogeneous populations of hMSCs were fabricated using the previously described methodology. Constructs were cultured for 1, 7, and 14 days, with medium changes every 3 days. After each time point, cell viability was evaluated using LIVE/DEAD staining (see below). At day 7, a set of gels were placed in alginate lyase solutions (2 mL, 10 U/mL in growth medium) until the cell-laden OPF/PEGDA gels separated (about 30 min, Fig. 5A). The separated components were then cultured for an additional 7 days at which point cell viability was assessed (14 days total culture time).

Note that for all above experiments, cell viability was assessed several days – 1 week after enzyme exposure. This assessment period was chosen because it is of similar duration to what is required by ASTM standards for cytotoxicity testing of biomaterials (ASTM

Standard F813, 2007 (2012), “Standard Practice for Direct Contact Cell Culture Evaluation of Materials for Medical Devices”).

LIVE/DEAD staining—For all viability studies, hydrogel constructs ($n = 3$ for all experiments) were analyzed using LIVE/DEAD staining. Constructs were rinsed in phosphate buffered saline (PBS) and subsequently incubated with staining solution (1 μ M calcein AM, 1 μ M ethidiumhomodimer-1) for 2 h. The stained gels were imaged through the z -axis of the gel (stack depth = 0–1000 μ m; 10 μ m intervals) via confocal microscopy (Zeiss LSM 510, Carl Zeiss MicroImaging LLC, Thornwood, NY). For quantification of % viable cells, images 50 μ m apart were analyzed through the entire thickness of three individual gels for each condition (~12–18 images each), and the percentage of live cells were calculated. The measurements were normalized to the Day 1 cell viability. The normalized data were then combined, averaged, and reported in Fig. 4B and Fig. 5B. Representative z -stacks from each gel were selected and a single image >200 μ m into the gel is presented from each time point in Fig. 4C and Fig. 5C.

Statistics—All measurements were compared using analysis of variance and Tukey's *post hoc* test ($p < 0.05$) performed by SYSTAT (version 12.00.08) software packages. Results are reported as mean $\pm$ standard deviation (SD).

3. Results

Hydrogel Construct Characterization and Separability

Layered Constructs—Layered constructs were prepared via serial photopolymerization of chondroitin sulfate methacrylate (CSMA) between two PEGDA layers as described in the Methods section. This procedure generated 1 mm $\times$ 3 mm tri-laminates that remained together after reaching equilibrium swelling (<24 h). To determine the length of time required to separate the PEGDA layers, the constructs were exposed to either a 0.125, 0.25, or 0.5 U/mL chondroitinase ABC solution in tris(hydroxymethyl)aminomethane (Tris) buffer. The CSMA adhesive layer dissolved completely in 4 h when exposed to 0.5 U/mL chondroitinase ABC solution, allowing for facile separation of the non-degradable PEGDA components. To demonstrate the ability to sequentially separate hydrogel blocks, 5-layer laminates incorporating several adhesive interfaces with different modes of degradation were prepared (Fig. 1). In Figure 1A, PEGDA gels (shown by arrows) were selectively and sequentially separated from the laminates upon exposure to a 2,200 U/mL collagenase solution in PBS for 30 min to degrade the collagenase-sensitive component (middle panel; grey “pac-man” denotes absence of collagenase-sensitive layer), followed by placement in a 0.5 U/mL chondroitinase solution in Tris buffer for 4 h to degrade the CSMA (bottom panel; white “pac-man” denotes absence of chondroitinase-sensitive layer). The separation order of the PEGDA components depended on the sequence in which the adhesive interfaces were degraded. It was found that the PEGDA blocks could easily be released in the reverse order by exposing an identical construct to chondroitinase first (Fig. 1B middle panel), followed by collagenase (Fig. 1B bottom panel).

Tri-laminates—We also investigated the potential of the separable co-culture design to be used in a layered construct with more complex geometry. To do so, interlocking laminated hydrogels were prepared via a series of replica moldings with parylene-coated PDMS molds as described in the Methods section (Fig. 2A). This process facilitated the creation of 3-D ~1 mm $\times$ 4.4 mm $\times$ 11.6 mm OPF/PEGDA-alginate MAM- OPF/PEGDA tri-laminates with tissue-scale thickness (~1 mm) that remained together after reaching equilibrium swelling.

Construct dimensions prior to swelling were measured and compared to the hypothetical laminate design created with AutoCAD software. These measurements corresponded to the original AutoCAD drawing of the device as illustrated in Fig. 2B. To determine the time required to separate interlocking hydrogel components, after fabrication, swelled interlocking laminates were exposed to 10 U/mL alginate lyase solutions and the OPF/PEGDA components were separated within 30 min (observation of $n=12$). This separation time scale was observed in both PBS- and media-based enzyme solutions.

In addition, in order to ascertain if the parylene-coated devices were reusable for multiple cell encapsulations, it was necessary to examine the extent to which the coated devices produced consistent constructs over multiple uses and sterilization cycles. There were no significant changes in gel dimensions for any of the devices after five autoclave cycles (Fig. 2C).

Cell Patterning

After establishing that enzyme-separable constructs could be prepared, the encapsulation and segregation of cell populations in these laminates was investigated. Replica-molding of hydrogels with PDMS devices generated interlocking laminated gels, which contained segregated cell populations. Differential staining of hMSCs encapsulated in opposing laminated OPF/PEGDA gels revealed a pattern of cells congruent to device design and segregation of cell populations across the entire construct after one day of culture (as demonstrated through stitched epifluorescent microscopy images). Well-defined interfaces including corners and straight edges existed between the two encapsulated cell populations and the non-fluorescent alginate MAm adhesive (Fig. 3A). The uniformity of this pattern throughout the entire laminate depth was verified by transversely sectioning the construct and imaging the cross sections via epifluorescent microscopy. The images showed a consistently straight interface between the two populations (Fig. 3B).

Cell Viability

To examine the effect of both the encapsulation and separation techniques, viability was quantitatively assessed for the layered hydrogel constructs containing hMSCs after culture in medium for up to 4 days. LIVE/DEAD assays of laminates 3 days after enzymatic separation of the cellularized PEGDA components revealed predominately viable cells throughout the thickness of the gels when imaged via confocal microscopy (Fig. 4C). 80–90% of the cell populations remained viable 1 and 3 days after dissolution of the enzyme-sensitive adhesive (2 and 4 days total culture time), with no significant changes in viability between before and after degradation of the adhesive interface (Fig. 4B).

Similarly, quantitative assessment of cell viability for interlocking laminate constructs revealed live cells throughout the entire gel thickness when imaged with confocal microscopy after 1, 7, and 14 days in culture. A set of laminates were degraded at day 7 in alginate lyase solution and the separated gels cultured for an additional week. LIVE/DEAD staining of the OPF/PEGDA components 1 week post-separation demonstrated predominately live cells throughout the thickness of the gels, and the cell viability was comparable to that of intact laminates cultured for the same total time (Fig. 5C). In these samples, 75–90% of the cell populations remained viable throughout the culture period, with no significant changes in viability even after laminate separation (Fig. 5B).

4. Discussion

These studies demonstrate the development of a novel patterned, 3-D co-culture system with on-demand separation capabilities. First, robust tri-layered constructs were formed using

enzyme-sensitive adhesives and PEGDA. The facile separation of the PEGDA components was achieved through exposing the constructs to an enzyme solution specific to the adhesive. Additionally, multi-layered constructs containing two different enzyme-sensitive adhesives can be selectively separated upon sequential exposure to specific enzyme solutions (Fig. 1). These results illustrate the potential this system has for use in the time-dependent culture of 3 or more cell types. With multiple adhesive layers, cell types can be selectively removed from the construct through addition of the appropriate enzyme, allowing for the response of the remaining cells to the removal of one of the co-culture populations to be observed. Notably, previous studies from our laboratory with a non-degradable version of this layered culture system (PEG/OPF blocks only) indicate that the presence of differing types of neighboring cells affect the phenotype of mesenchymal stem cells, adipocytes, and osteoblasts, indicating that this system does not provide a barrier to diffusion of soluble factors between cell types [43], further supporting the suitability of using these materials as part of a 3D co-culture platform.

In additional studies, we transferred this ability to separate non-degradable components to interlocking tri-laminate constructs designed to maximize the exchange of soluble signaling factors between cell types. A series of replica moldings with parylene-coated PDMS molds yielded 1-mm thick interlocking laminated hydrogels (Fig. 2A). The replica molding technique was chosen for its ability to easily produce gels in the shape of a device with high fidelity between the device and resulting constructs [44,45]. The parylene coating reduces the gas permeability of the PDMS and inhibits oxygen quenching of the cross-linking reaction, allowing for the formation of full-thickness gels in the devices [38]. Furthermore, it was determined that these parylene-coated devices were reusable for multiple cell encapsulations. Compared to previous methods employed in our laboratory and others to prevent oxygen quenching during polymerization, such as nitrogen gas purging, increasing photoinitiator concentration, and increasing UV light intensity [27,46], parylene coating has the advantage that it is much less time consuming and easier to apply than nitrogen purging and offers a much less cytotoxic alternative than substituting or adding excess free radical initiators or short-length crosslinkers [24,47–49]. Parylene coating is therefore amenable to both redox- and photo-initiated polymerization while limiting the presence of cytotoxic oxygen free-radicals [50]. The coated devices were determined to be reusable for up to 5 autoclaving cycles and consistently produced constructs of similar dimensions throughout the cycle periods (Fig. 2C).

Our capacity to use the PDMS molds to generate hydrogels with intended dimensions is highlighted by comparing the dimensions of interlocking constructs to the Auto CAD design dimensions (Fig 2B, Table 1). Furthermore, results indicate that the molding technique can produce a thin adhesive layer in the laminates with dimensions that are currently unobtainable through photopatterning techniques [27,32,51]. The thickness of the adhesive is controlled by the mold used to prepare the laminates. For these studies, the molds were hand-cut with finite dimensions, which limited the thickness of the adhesive layer to ~400 μm or greater; however, machined molds could be designed to create laminates with thinner adhesive interfaces. Thinner interfaces could facilitate faster diffusion of signaling molecules across the adhesive [52] and subsequently may result in a shorter culture time for observable intercellular responses to occur. Notably, the molding and laminating technique presented here, which results in well-defined, patterned populations of cells (Fig. 3), can be applied to a variety of geometries resulting in a plethora of possible co-culture designs to recapitulate the structure of a wide range of tissue types.

For these tri-laminate constructs, the OPF/PEGDA gels can be separated in 30 min when the construct is exposed to either a PBS- or media-based alginate lyase solution (Fig. 5A). The ability to degrade the laminates in a media-based enzyme solution suggests that cellularized

laminates could be degraded under benign conditions to maintain the viability of encapsulated cells. LIVE/DEAD staining revealed that viability for hMSCs in layered constructs was largely preserved for at least 3 days after exposure to enzyme, regardless of degradation time or enzyme (Fig. 4B and C). Additionally, cell viability was also maintained for at least 2 weeks of total culture and at least 1 week of culture after tri-laminate separation as is illustrated in Fig. 5B and C. This phenomenon occurs despite the presence of free radicals during cross-linking, as well as exposure to enzymes throughout laminate separation, both of which could have been potentially harmful to non-immortalized cell lines [47,53]. These results suggest that the methods of patterned cell encapsulation and subsequent laminate degradation described in this paper can be used for both short- and long-term co-culture experiments as may be needed to examine the role of paracrine interactions on cell phenotype. While, in these studies, enzymes that target molecules produced by mammalian cells (such as chondroitin sulfate or collagen) were employed as proof-of-concept experiments to show the flexibility of the type of adhesive that can be included in the system, removing these biomolecules deposited pericellularly through exposure to enzymes during the delamination step could affect the phenotype and/or differentiation of the embedded cells. Therefore, in the future, we envision the use of glues that respond to non-mammalian enzymes, such as the alginate employed in this study, or other biomolecules that do not alter the extracellular matrix surrounding the embedded cells.

In summary, we have successfully designed and implemented a hydrogel co-culture system that allows for the culture of two or more cell types in a highly tailorable environment representative of 3-D tissue physiology. The inclusion of enzymatically degradable adhesives in the system permits the recovery of viable cell populations after culture, regardless of the type of adhesive employed. The incorporation of multiple adhesive interfaces with different enzymatic susceptibilities enables the selective temporal separation of encapsulated cell populations contingent upon the order of enzyme exposure. This flexible platform offers great potential for a variety of basic cell signaling studies because the inclusion of an enzyme-sensitive adhesive interface allows the on-demand separation of individual cell populations for immediate analysis (such as immunostaining, real-time polymerase chain reaction (RT-PCR) etc.) or further culture to examine persistence of co-culture effects and paracrine signaling on specific cell populations. Therefore, in the future, this versatile construct could be used to better understand fundamental mechanisms and signals underlying stem cell fate decisions that could not have been elucidated in traditional culture systems.

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Abbreviations

3-D	Three-Dimensional
PEG	Poly(ethylene glycol)
2-D	Two-Dimensional
ECM	Extracellular matrix
OPG	Oligo(PEG fumurate)

MMP	Matrix metalloproteinase
PDI	Polydispersity
PEGDA	PEG-diacrylate
CSMA	Chondroitin sulfate methacrylate
MAm	Methacrylamide
PDMS	Polydimethylsiloxane
APS	Ammonium persulfate
TEMED	N,N,N',N'-tetramethylethylenediamine
hMSCs	Human mesenchymal stem cells
Alpha-MEM	Alpha-Minimum Essential Medium
A/A	Antibiotic/antimycotic
EDTA	Ethylenediaminetetraacetic acid
SD	Standard deviation
Tris	Tris(hydroxymethyl)aminomethane
PBS	Phosphate buffered saline
RT-PCR	Real-time polymerase chain reaction

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Figure 1.

Incorporation of adhesive interface in hydrogel trilaminate allows for selective on-demand separation of gel components. A 5-layer hydrogel laminate can be selectively degraded using enzymes specific to the adhesive layers. A) Selectively exposing the laminate to first collagenase and then chondroitinase allows for the sequential separation of PEG-DA components from the primary hydrogel structure. B) Reversing the order of enzyme addition illustrates the ability to selectively remove individual components (scale bars = 1 mm).

Figure 2.

Comparison of dimensions of laminates formed from devices to design parameters and measured dimensions after multiple autoclaving cycles. A) Diagram illustrating fabrication of interlocking laminates: 1) Parylene coat PDMS device and sterilize the device, 2) Cross-link PEGDA/OPF solution (interlocking pieces) in coated device, 3) Laminate PEGDA/OPF hydrogels (interlocking pieces) with alginate MAM adhesive (between gels) in PDMS frame. B) The spacing, block height and width of complex laminates were measured as indicated on gel images were measured via epifluorescence and compared to designed AutoCAD dimensions ($n = 4 \pm \text{SD}$ for each measurement). C) Gel dimensions were measured after fabrication in devices subjected to at least five autoclaving cycles for three different molds ($n = 5 \pm \text{SD}$ for each autoclave cycle for each mold, $p < 0.05$); average measurements from three different molds are represented and values for each mold are normalized to measurements before autoclaving.

Figure 3. Two different cell populations remain separated in a complex trilaminar. Two distinct populations of labeled human MSCs were encapsulated in a complex interlocking trilaminar and remain spatially segregated after construct formation. Top view of the entire laminate (A) and transverse cross section of laminate (B). Scale bars are 1 mm.

Figure 4.

Cell viability after degradation of multiple adhesive interfaces. A) Illustration of a multi-layered hydrogel construct with a degradable adhesive interface. The effects of adhesive degradation on cell viability were examined for all three polymers: Alginate Mam, CSMA, and LGPA. B) Fraction of viable cells after dissolution of different adhesive interfaces at 1 and 3 days after dissolution (2 and 4 days total culture time). Cell viability was quantified from confocal images ($n=3\pm SD$, $p<0.05$; all values normalized to viability before degradation of the adhesive). C) Representative LIVE/DEAD stained confocal images of cells 3 days after dissolution of the corresponding interface (4 days total culture time) (scale bar = 100 μm).

Figure 5.

hMSC viability during laminate formation and separation. A) Enzymatic separation of an interlocking co-culture laminate by alginate lyase. B) Fraction of viable cells throughout the culture period (up to 14 days total culture time). Cell viability was quantified from confocal images ($n=3\pm SD$, $p<0.05$; all values normalized to Day 1 viability). C) Representative LIVE/DEAD stained confocal images of cells 1, 7, and 14 days after laminate formation and 7 days post-laminate degradation (14 days total culture time) (scale bars = 100 μm).

Table 1

Comparison of laminate measurements vs. original design.

Dimension of Interest	AutoCAD Design Measurement (mm)	Average Gel Measurement (mm)	% error from AutoCad
Block Height (a)	2	1.97 ± 0.13	1.5
Block Width (b)	2	1.95 ± 0.17	2.5
Block-to-Block Spacing (c)	0.4	0.39 ± 0.06	2.5
Block-to-Spine Spacing (d)	0.4	0.35 ± 0.10	12.5

The spacing, block height and width of complex laminates (as indicated on gel images, Fig 2B.) were measured via epifluorescence and compared to designed AutoCAD dimensions ($n = 4 \pm \text{SD}$ for each measurement).