Enzyme-Sensitive Glues Allow for Sequential Separation & Recovery of Viable Cell Populations after 3D Co-Culture

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Introduction: Stem cells represent an attractive cell choice for regenerative medicine applications due to their ability to differentiate toward multiple lineages. However, there is a dearth of information on how interactions with native cells, specifically paracrine signaling effects, may influence stem cell fate. A more physiologically representative, 3D *in vitro* co-culture system that allows post-culture separation of segregated cell populations would enhance the ability to study these effects. Here, we fabricate multi-layer hydrogels with alternating non-degradable, cell-laden gels and enzymatically degradable interfaces using photopatterning techniques, and show that the non-degradable gels may be sequentially isolated through the separate digestion of each interface. Additionally, we demonstrate that different patterned cell populations remain segregated from each other, and that they remain viable after separation via enzymatic degradation of the adhesive interface.

Methods: Chondroitin sulfate methacrylate (CSMA) was synthesized through the dropwise addition of methacrylic anhydride and NaOH to a solution of chondroitin sulfate A (Sigma) dissolved in dH_2O. Poly(ethylene glycol) diacrylate (PEGDA) was synthesized from PEG precursor (M_n=3.4 kDa) using established techniques.[1] PEG-acrylate derivatized with the matrix metalloproteinase-cleavable peptide sequence GGGLGPAGGK was synthesized by reacting with Acrl-PEG-SVA (M_n=3.4kDa, LaysanBio; 1:2.2 molar ratio) in 50 mM $NaHCO_3$ (pH 8.5) for 3 h and purified by dialysis.

Molding devices for gel patterning were constructed by placing a 1 mm-thick spacer of polydimethylsiloxane between two glass slides that contained a cavity to accommodate the polymer solution. To determine the length of time required to separate hydrogel components, a tri-laminate was created by successively crosslinking and subsequently masking a layer of PEGDA, CSMA, and PEGDA (90% w/w dH_2O, 0.05% w/v D2959 photoinitiator (Ciba)) under 365 nm light at 10.5 mW/cm^2 for 12 min. The tri-layer laminate sections were added to a 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 (*n*=3). To demonstrate the ability to sequentially separate hydrogel components by incorporating interfaces with different modes of degradation, additional layers of Ac-PEG-GGGLGPAGGK and PEGDA were added using the same methods to create a 5-layer laminate (Figure 2). One 5-layer laminate was first added to a solution of collagenase (2,200 U/mL, 3 mM $CaCl_2$ in PBS) followed by chondroitinase ABC (1 U/mL) (*n*=2), while a second laminate was added to each enzyme solution in reverse order.

Cell viability after separation of the tri-layer laminate was examined by encapsulating human mesenchymal stem cells (hMSC, passage 6; Texas A&M) inside the PEGDA layers (10×10^6 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 (*n*=7). After encapsulation, another set of laminates were allowed to culture for 1 day in growth media (alpha-MEM, 16.5% fetal bovine serum, 2mM L-glutamine, and 1% gentamicin/amphotericin B) after which they were added to buffer solution containing chondroitinase ABC at 0.25, 0.5, and 1 U/mL. After separation of the PEGDA 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 (*n*=2; Fig. 1).

Results: Swollen hydrogels remained laminated. Epifluo--rescence microscopy verified separation of the two hMSC populations in the tri-layer laminate (distinguished with Cell Tracker Green CMFDA and Orange CMRA) after the encapsulation process (not shown). Additionally, cell viability was maintained after encapsulation and after enzymatic separation, with no observable decrease in viability regardless of the enzyme concentration used to degrade the CSMA interface (Fig. 1).

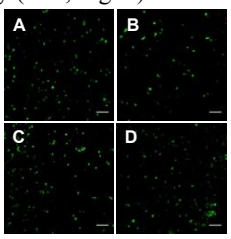

Figure 1: LIVE/DEAD confocal microscopy images of cells before degradation of the CSMA interface (a) and after using 0.25 U/mL (b), 0.5 U/mL (c), and 1 U/mL (d) of chondroitinase. Green indicates viable cells, and red indicates dead. Scale bar = 100 μm.

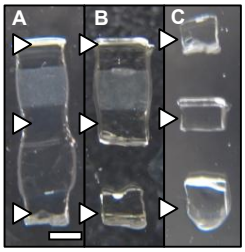

Figure 2:5-layer laminate (A) before the addition of enzyme; (B) after the addition of collagenase; and (C) chondroitinase. PEGDA gels are indicated by arrows. Scale bar = 1.5 mm

The time required to separate the two PEGDA gels in the tri-layer constructs varied from less than 1 hour for 1 U/mL chondroitinase to over 4 hours at 0.25 U/mL. Similarly, PEGDA gels in the 5-layer laminate were sequentially separated after the addition of collagenase, followed by chondroitinase at 4 U/mL (Fig. 2). The order of separation depended on the order in which the interfaces were degraded.

Conclusion: We have successfully designed and implemented a hydrogel system for the long-term 3D co-culture of segregated cell populations that remain viable after separation, with the potential to selectively separate cell types by incorporating adhesive interfaces with different enzymatic susceptibilities. This versatile platform can be used to selectively isolate specific cell populations for immediate analysis or further culture to examine persistence of co-culture effects on a variety of stem and native cell types.

Acknowledgements: Petit URS, NIH P40RR017447, NIH R21EB009153 **References:** [1]Hahn et al. *Biomaterials*. 2006;27:2519-24.