Three-dimensional Co-Culture in Micropatternable Hydrogels with Cell-Release Capabilities

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Introduction: Local delivery of marrow stromal (stem) cells (MSCs) offers a potentially viable alternative to auto-/allograft transplants for repair of injured tendons/ligaments. Knowledge regarding MSC interactions with native cells and resulting effects on cell proliferation, matrix production, and tissue repair would enhance development of these strategies. Here, we detail methods for 1) controlled cell patterning in three dimensions (3D) using a photolithographically prepared co-culture system based on oligo(polyethylene glycol)-fumarate (OPF) hydrogels, and 2) facile separation of modules of cells post-culture for further analyses using a photocrosslinkable, enzyme-digestible "glue" based on chondroitin sulfate (CS).

Methods: OPF was synthesized from a poly(ethylene glycol) precursor (PEG, M_n=10kDa).[1] PEG-diacrylate (PEG-DA) crosslinker was synthesized from PEG precursor (M_n=3.4kDa).[2] Photocrosslinkable CS was synthesized using two different schemes. First, N-(3-aminopropyl) methacrylamide was conjugated to CS using *N*-(3-dimethylpropyl)-*N*-ethylcarbodiimide hydrochloride and *N*-hydroxysulfo-succinimide in dH_2O to produce CS-methacrylamide (CSMAm).[3] Alternatively, methacrylic anhydride was added dropwise to a solution of CS in dH_2O to produce CS-methacrylate (CSMA). CSMAm and CSMA hydrogels (90% initial water content) were photocrosslinked using 365nm light with 0.05% D2959 photoinitiator (Ciba) and assessed for fold swelling. Degradability of conjugated CS in the presence of chondroitinase ABC (Sigma) was assessed using an enzyme activity assay and swollen hydrogel disks (3mm-diameter, 1mm-thick, *n*=3) in 0.25U/mL enzyme.

Serial photolithographic patterning of cell-containing OPF:PEG-DA hydrogels was performed within a 2mm-thick, PDMS-based microfluidic flow cell using 10,000dpi photomasks (CAD Art Svcs). Macromer solutions (75% initial water content) were prepared with a 1:1 ratio of OPF:PEG-DA and 0.05% w/v D2959 and containing 10×10^6 cells/mL. Cells were either primary bovine tendon/ligament fibroblasts (P2) or primary bovine marrow stromal cells (bMSCs, P2). Devices were enclosed within an N_2 chamber and purged for 30min with N_2 gas to inhibit oxygen quenching of the free radical polymerization. A 3×5 square array of homogeneous or alternating cell populations was sequentially patterned by loading the 1st gel solution/cell type, crosslinking through a photomask, washing, reloading with the 2nd gel solution/cell type, and crosslinking again without re-exposing the 1st set of cells. At 1, 7, and 14d, hydrogels were stained with LIVE/DEAD dye (Invitrogen) for 1h and imaged using an LSM 510 confocal microscope (Zeiss). Other samples were assayed for DNA content using the PicoGreen assay (Invitrogen) (*n*=3). Data were analyzed using ANOVA and Tukey's post-hoc test or a Student's *t*-test ($p \le 0.05$).

Results and Discussion: Enzyme-mediated degradation of CSMAm does not occur, likely due to steric inhibition of the enzyme resulting from the high degree of conjugation (Fig. 1A). Figure 1B reveals that bulk hydrogels of CSMA may be synthesized with multiple degrees of methacrylation that affect gel swelling ratio and degradation, making it a more viable alternative. Gel degradation time correlates with swelling ratio, suggesting the network mesh size affects penetration and/or activity of chondroitinase ABC.

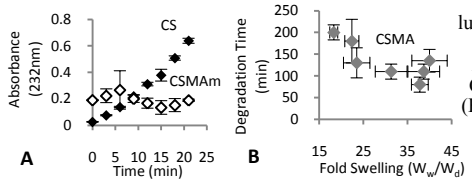

Figure 1. (A) Evolution of degradation products (Abs. 232nm) for CS and CSMAm polymers. (B) CSMA hydrogel degradation time (min) vs. fold swelling ratio.

In the second part of this study, we laminated OPF:PEG-DA hydrogels in complex patterns and evaluated viability of cells in long term culture. Figures 2A (fibroblasts) and 2B (bMSCs) demonstrate that remaining patterned cells are viable after 14d in culture. DNA content (Fig. 2C), indicative of cell number, shows little decrease over time, which supports the use of this system for long-term culture. We are able to pattern a complex array of cells (Fig. 2D) with controlled location of each cell population (Fig. 2E) that is consistent throughout the 1.5mm-thick blocks. Patterning CSMA gels between blocks enables separation after long term culture via enzymatic degradation (Fig. 2F), demonstrating our ability to separate cell populations after co-culture for further gene/protein expression analyses.

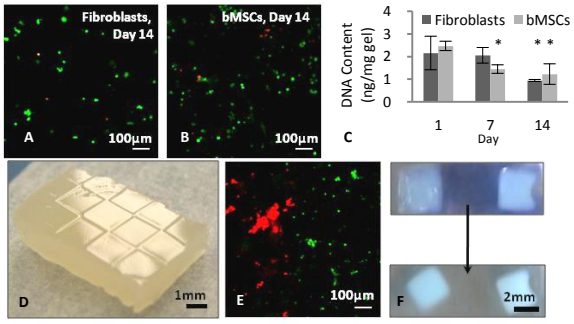

Figure 2. LIVE/DEAD stain of (A) fibroblasts and (B) bMSCs at 14d. (C) PicoGreen assay for DNA content (*p<0.05 vs. day 1). (D) 3×5 array patterning of hydrogels containing cells. (E) Patterning of two distinct cell populations stained with either CellTracker Green or Orange. (F) Separation of patterned OPF gels containing CSMA (stained with DMMB) between OPF blocks via enzymatic cleavage.

In these studies, we have developed a spatially controlled 3D environment for culturing multiple cell types over weeks, followed by separation of blocks to analyze each cell type separately. Such a system has tremendous potential to enable better understanding of paracrine effects on a range of stem cell functions.

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