Patterning Biochemical Signals into Nanofibrous Hydrogels to Spatially Control Cell Behavior

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Statement of Purpose: Spatially defined biochemical and biophysical signals at tissue interfaces *in vivo* motivate the creation of novel materials to mimic the natural spatial heterogeneity of extracellular matrix (ECM). In particular, recent studies have demonstrated that spatial tethering of biomolecules onto flat surfaces and non-fibrous hydrogels may direct various cell behaviors including morphology, differentiation, and proliferation *in vitro*[1,2]. While these material systems have had success in spatial biochemical patterning, each system is non-fibrous and therefore lacks the structural biophysical features present in the native cellular microenvironment. To permit spatially defined biochemical signals in a biophysically relevant scaffold, we introduce electrospun norbornene modified hyaluronic acid (NorHA) and demonstrate its utility to pattern multiple ligands and spatially control cell behavior in a nanofibrous hydrogel.

Methods: NorHA (Fig.1A) was synthesized by modifying HA-TBA (tetrabutylammonium) with norbornene carboxylic acid in anhydrous dimethyl sulfoxide (DMSO) using 4-dimethylaminopyridine and di-tertbutyl dicarbonate. Norbornene groups permit covalent attachment of thiolated molecules in the presence of a radical initiator for spatially defined crosslinking and/or patterning. After synthesis, solutions of 3.25 wt% NorHA, 2.5% polyethylene oxide (carrier polymer), 1% bovine serum albumin, dithiothreitol (DTT), and 0.05% I2959 (photoinitiator) in PBS were electrospun and imaged by scanning electron microscopy (SEM) (Fig.1B). Scaffolds were crosslinked by exposure to UV light (10 mW/cm^2, 15min), and by limiting the amount of DTT within the network, only a fraction (0.4) of the available norbornene groups are theoretically consumed during crosslinking. Next, scaffolds were hydrated in a solution containing thiolated peptides (0.5mM HS-RGD [for cell adhesion], 0.25mM GCEEE-FITC, GCDD-Rho B, or GCDDD-Methoxycoumarin), and then photopatterned by a second exposure to UV light (10 mW/cm^2, 90sec) through a photomask so that RGD and fluorophore attachment occurred only at exposed regions (Fig.1C,D). After washes with PBS over 72 hours to remove any unattached RGD and fluorescent peptides, this procedure was repeated to demonstrate patterning of multiple ligands (Fig.1E), or scaffolds were seeded with NIH 3T3 fibroblasts (5,000-10,000 cells/cm^2), cultured for 3 days in growth media, and stained for actin (FITC-phalloidin) and nuclei (DAPI). Cell density (nuclei/mm^2) and cell area were quantified in ImageJ.

Results: SEM imaging indicated smooth fiber morphology and submicron diameter fibers (220±50nm), while confocal imaging of fibers in the dry state (510 ±97nm) and hydrated state (740 ±140nm) demonstrated hydrogel swelling behavior. Patterning of scaffolds with thiolated peptide fluorophores showed pattern fidelity to 50μm within the nanofibrous network (Fig.1D). This patterning process is repeatable, enabling independent pattering of three separate ligands onto the same scaffold (Fig.1E). By including thiolated RGD during patterning (with GCDD-Rho to indicate pattern), cellular behavior is also spatially altered. Fibroblasts populate the patterned regions with covalently attached RGD with high fidelity (Fig.1F,1G) such that cell density is altered between areas with or without RGD present (Fig.1H). For cells that are adherent, cell area is altered such that cells on regions with RGD have larger areas (more spreading) compared to the limited number of cells contained in regions without RGD (Fig.1I). Although not shown, this general behavior is also observed in culture of human umbilical vein endothelial cells (HUVECs), pointing to the broader applicability of the material system.

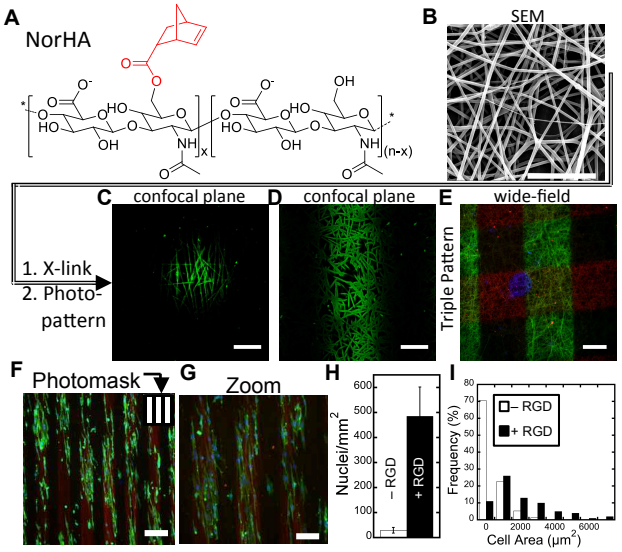

Figure 1: (A) Norbornene hyaluronic acid (red indicates norbornene group). (B) SEM of unhydrated NorHA scaffold. (C) Confocal plane of a patterned 50 μm circle, or (D) line containing GCEEE-FITC. (E) Three different molecules patterned onto the same scaffold. (F,G) Photomask and corresponding patterned fibroblast response to 100 μm patterned lines of RGD (Green:F-actin, Blue:DAPI). (H) Fibroblast density and (I) area on nanofibrous scaffolds with (black) or without (white) RGD. Scale bars: (B) 5 μm (C,D) 25μm, (E) 100μm, (F) 200μm, (G) 100 μm.

Conclusions: Importantly, this work introduces spatial control of biochemical signaling in a scaffold with more biophysically relevant features (hydrogel mechanics, nanofibrous topography), thus enabling tissue-engineering strategies that better mimic natural ECM architecture. Ongoing and future work is underway observing cell response to spatial signaling in scaffolds with nanofiber alignment (attained through electrospinning mandrel collector speed) to further probe the roles biochemical and biophysical signals play in altering cell responses.

References: 1. McBeath, R., *et al. Dev Cell* (2004).
2. DeForest, C.A. *et al. Nature Materials* (2009).