A Viscoelastic Tissue-Mimetic Hydrogel for Modelling Chondrogenesis

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Statement of Purpose: Cartilage-related injuries and conditions are very common, yet current treatments fail to address cartilage regeneration. Creating 3D *in vitro* environments which capture the complex biochemical cues and viscoelastic properties necessary for cartilage is vital for the development of effective *in vitro* models and robust tissue engineering approaches to cartilage regeneration. In this study, a cartilage-mimetic PEG hydrogel prepared from hydrazone crosslinks, a covalent adaptable network, was designed and used to tune growth factor release rate and study the effect of viscoelasticity on chondrogenesis.

Methods: *Materials:*8-arm 20 kDa PEG-Hydrazide (PEG-Hz) and PEG-Benzaldehyde (PEG-Bz), and 8-arm 20kDa PEG-Aldehyde (PEG-Ald) were synthesized in house and verified by ^{1}H-NMR. Chondroitin Sulfate A (10-30 kDa) was modified with aldehydes (ChS-Ald) through oxidation with sodium periodate and aldehyde substitution was determined by hydroxylamine hydrochloride titration. Recombinant Human TGF-β3 was thiolated with Traut's Reagent, then reacted with 1 kDa Maleimide-PEG-Hydrazide linker. *Tethered fluorescent probe:* Acellular PEG hydrogels were fabricated with PEG-Hz crosslinked with either 100% PEG-Bz or 100% PEG-Ald. Dansyl Hydrazide was pre-tethered to either PEG-Bz or PEG-Ald at a ratio of 1:200 fluorescent probe to reactive group; release was measured by fluorescence. *Cartilage-mimetic hydrogel fabrication:* Hydrogel conditions are as follows: PEG-Hz crosslinked with A) 100% PEG-Bz; B) 100% PEG-Bz, TGF- β tethered to PEG-Bz; C) 25% PEG-Bz, 75% PEG-Ald, TGF-β3 tethered to PEG-Bz; D) 25% PEG-Bz, 75% PEG-Ald, TGF-β3 tethered to PEG-Ald. Conditions B, C, and D were formulated with 1% CS-A and 50 nM TGF-β3. RGD (0.1mM) was tethered to PEG-Bz in all conditions. Gels were made at 8% total PEG w/w. Murine bone marrow-derived MSCs expanded to P3, encapsulated at 50M cells/mL, and cultured in defined chondrogenic media (DMEM with 1% ITS+, 100 nM Dexamethasone, 50 ug/mL L-ascorbic acid, 20 ug/mL Gentimicin, and 1% Pen/Strep. *Histology:* Hydrogels were fixed (4% paraformaldehyde), dehydrated, and paraffinized. Sections were stained for glycosaminoglycans (GAGs) with Safranin O and nuclei were stained with Weigert's Hematoxylin. Immunohistochemistry was assessed for Collagen I, Collagen II, and Collagen X.

Results: PEG-hydrazone cartilage-mimetic hydrogels were designed with different viscoelastic responses to study the role of dynamic networks on TGF-β3 release rate on chondrogenesis of murine MSCs. To study the effect of bond reversibility on the release rate of tethered molecules, dansyl hydrazide was tethered into acellular PEG hydrogels fabricated with either 100% benzyl (slow reversing) or 100% alkyl hydrazone (faster reversing) bonds. Release of the fluorescent probe measured in the supernatant was significantly greater in the 100% alkyl hydrazone condition compared to the 100% benzyl hydrazone condition, with the majority of probe release occurring within the first week, indicating that reversibility rate of hydrazone bonds affects the rate and degree of release of tethered molecules (Figure 1).

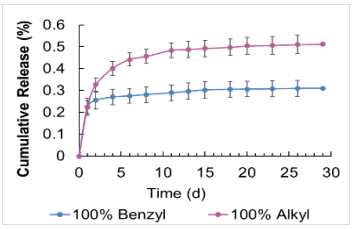

Figure 1. Release of dansyl hydrazide.

When MSCs were cultured the viscoelastic cartilage-mimetic hydrogels, collagen II was increased in the more viscoelastic conditions, C and D, compared to the less viscoelastic condition B and control condition A (Figure 2). Minimal Collagen I was present in all conditions. Preliminary quantification indicates increased Collagen X in condition D with the releasable TGF-β3. Control condition A did not stain positively for GAGs, while conditions B, C, D stained strongly for GAGs throughout, likely due to retention of Aldehyde-modified Chondroitin Sulfate in those hydrogel formulations. Cells in conditions C and D are majority round in shape, indicative of a chondrogenic phenotype. Median diameters of cells was quantified as a measure of hypertrophy, which were higher in both conditions C and D than conditions A and B. Cells in conditions C and D appeared to form columnar structures, indicating potential proliferation in these conditions.

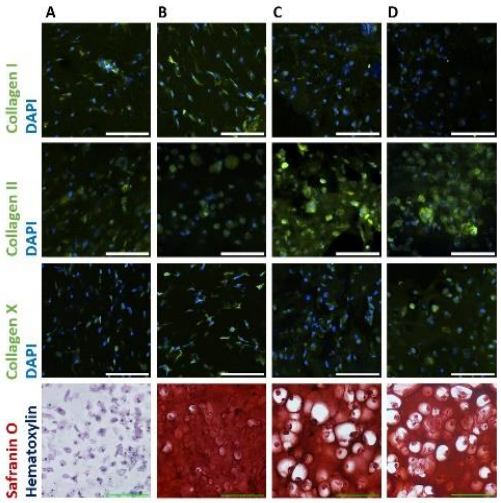

Figure 2. Immunohisto-chemical staining for Collagen I, II, and X and histological staining for GAGs at day 7. Scalebars represent 100 μm.

Conclusions: Findings show improved signs of chondrogenesis in conditions C and D compared to conditions A and B. Comparing conditions C and D indicates that viscoelasticity, controlled for initial modulus and biochemical cues, does affect chondrogenesis. Further studies are underway to understand the significance of these differences.

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