Tuning of Stiffness Anisotropy in Collagen Sheets by Planar Stretch

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Introduction: Mechanical properties of the extracellular matrix plays an important role in regulating cell fate such as differentiation of mesenchymal stem cells (MSCs) based on stiffness [1], or guided cell migration based on stiffness gradients (a.k.a durotaxis) [2]. What is less understood is the effect of *matrix stiffness anisotropy* (i.e. different stiffness values in different material directions) on cell response. Most natural tissues are substantially stronger along the load bearing direction than the direction transverse to the longer axis (such as tendons, muscle etc.). Notably, the cells of such tissues are elongated along the stiffer direction. There are not many biomaterials to emulate and study the effects of stiffness anisotropy on cellular response. It has been shown that aligned collagen threads induce tenogenic differentiation of MSCs [4]. Therefore, a controlled method of generating a biomaterial with tunable stiffness anisotropy is needed to better understand the cellular fate in anisotropic tissues. This study reports the effects of a controlled unidirectional stretch process on the stiffness/strength anisotropy of collagen sheets.

Methods: *Electrocompaction:* Type-I collagen solution (Advanced Biomatrix, CA) was electrocompacted as rectangular sheets of 25x30x0.2 mm between two planar electrodes. Electric current electrophoretically mobilizes collagen molecules and compacts them under the effects of mechanisms published before [3-5]. Collagen molecules are randomly (i.e. isotropically) oriented in such sheets as evidenced by compensated polarized imaging (CPI, magenta color indicates randomness in Fig. 1, 0% stretch). *Mechanical Stretch:* A customized mechanical device stretched the randomly-oriented collagen sheets at 35μ/s to 25%, 50% and 75 % of their initial length. The degree of alignment of collagen molecules was assessed by CPI (Olympus BX51, blue color indicates molecular alignment in the SW-NE direction). Samples were tested either in uncrosslinked form or after crosslinking in genipin. *Mechanical Properties*: Samples were tested in tension (Rheometrics Inc., NJ) along the stretch direction (**L**ongitudinal) and transverse to the stretch direction (**T**ransverse). Cross sectional area of sheet samples were measured with Leica TCS SP2 multi-photon confocal microscope in hydrated state. Stress-strain curves were established using sample geometry and load-displacement data. Moduli in the longitudinal (E_L) and transverse (E_T) directions were calculated as the slope of the linear region. Stiffness anisotropy was expressed as SA = (E_L/E_T). *Cell Morphology*: MSCs (Lonza) were seeded on random, intermediate and fully stretched sheets to determine the effects of stiffness anisotropy on cell morphology. One-way analysis of variance (ANOVA) was performed within crosslinked and uncrosslinked groups separately with significance set at $p<0.05$.

Results: Molecular alignment increased gradually with stretch as indicated by the emergence of blue color in the polarized images (Fig. 1). E_L increased about two-fold (Fig. 2a) and four-fold (Fig. 2b) with stretch whereas there was a significant decline in E_T (Fig. 2a, 2b). Crosslinking increased the stiffness by about 100-fold (Fig 2a. vs. 2b). The stiffness anisotropy increased by about 8-fold with stretch (Fig. 3). The failure stress increased 3-fold from 4 MPa to 12 MPa with stretch for crosslinked samples. The MSCs became elongated gradually with increasing stiffness anisotropy (Fig 4.)

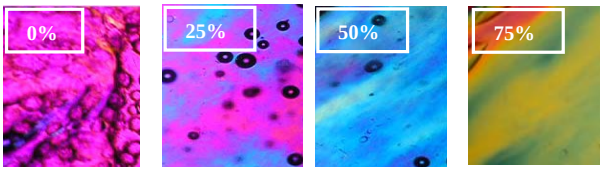

Fig. 1: 0% stretch shows no alignment where as 75 % extrusion gives the maximum alignment. 25% and 50% stretch provides intermediate levels of alignment.

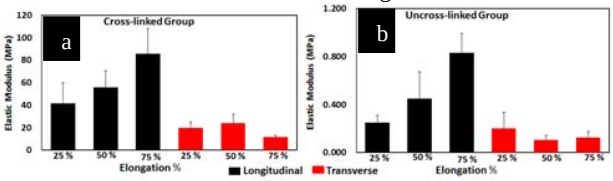

Fig. 2: a) Transverse and longitudinal stiffness of cross-linked and b) Uncross-linked collagen sheets at different stretch levels

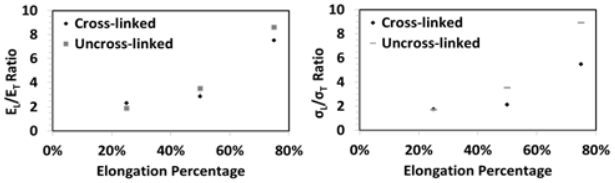

Fig. 3: Stiffness (left) and failure stress (right) anisotropy in collagen sheets increased with stretch.

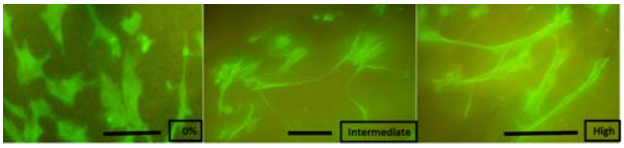

Fig. 4: MSCs cystoskeletal morphology at 0%, intermediate and high stretch levels (Scale bar 100μm).

Conclusions: We were able to tune the stiffness anisotropy by 10 fold (1 to 10) and the stiffness value by 100 fold by using stretching and crosslinking processes. This amounts to creation of a material profile where the modulus can be changed from several hundred kPa, to single digit MPa, to hundred MPa. Importantly, the cell elongation had a dramatic and direct response to this anisotropy. Therefore the presented collagen model holds promise for systematic investigation of the effects of matrix anisotropy on cellular morphology and fate.

References: 1.Engler et al.,Cell 2006;126(4):677-89 2. Sergey et al. Cell 2012; 151, 1513–1527, December 21, 2012 3. Cheng et al., Biomaterials, 2008 4.Kishore et al., Biomaterials, 2012, 33(7), 2137-44; 5.Uquillas et al. Ann Biomed Eng, 2012, 40(8), 1641-53.