Characterizing Properties of Thiol-ene Crosslinked Hyaluronic Acid Hydrogels for Meniscus Tissue Engineering

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Introduction: Meniscal injuries and degeneration are common causes of osteoarthritis in the knee. Tears in the inner region of the meniscus, known as the white region, often fail to heal due to avascularity and often leads to further degeneration of the meniscal tissue. Research into the tunability of hydrogel properties including polymer chemistry, degree of substitution (DOS), cross-linking density, and swelling medias provides insight on how to design a potential tissue restoration hydrogel with the proper mechanical properties needed to promote repair.[1] In this study, the ability to tune hyaluronic acid-based hydrogel material properties by rationally controlling pentanoate percent substitution and resulting crosslink density with a thiol-ene reaction was investigated. Different molar ratios of pentenoic anhydride to hyaluronic acid were reacted and the resulting functionalized hyaluronic acid polymer was cross-linked using a thiol-ene click chemistry for hydrogel synthesis. The use of a rapid thiol-ene click chemistry and controlled available reactive groups provides potential for step growth polymerization to produce a more homogenous crosslinked network. The effect of degree of substitution on the material properties of pentanoate functionalized hyaluronic acid (PHA) hydrogels was determined through uniaxial compressive testing, swelling assessment, frequency sweep data, and low field nuclear magnetic resonance studies (LF-NMR) to determine relative mesh size. The hypothesis was that increasing the degree of substitution would increase the mechanical load capabilities, decrease swelling ratio, and decrease the average mesh size of the polymer network. Achieving a greater understanding of the tunability of this hydrogel system and properties in media used for *in vitro* systems enables future investigation of meniscal fibrochondrocyte mechanosensitive response to the mechanical environment established in PHA hydrogels.

Materials and Methods: PHA Polymer Synthesis: 60kDa hyaluronic acid (Lifecore Biomedical) was functionalized with reactive -ene groups using different molar ratios of hyaluronic acid to pentenoic anhydride. Proton Nuclear Magnetic Resonance (^{1}H NMR) was used to determine the DOS for the resulting pentanoate-modified hyaluronic acid (PHA) polymer. Cross-Linking Mechanism: The hydrogels were synthesized using a photoinitiated thiol-ene click chemistry reaction with a 1:1 molar ratio of thiol from DTT (dithiothreitol, Millipore Sigma) to ene from PHA. The photo initiator, lithium phenyl-2,4,6-trimethylbenzoylphosphinate (Millipore Sigma) at 0.1mM initiated crosslinking at a wavelength of 312nm. Sample preparation: Following synthesis, the hydrogels were swollen in 10 mM NaCl for 24 hours. The hydrogels were cut into 8mm disks using a biopsy punch and swollen in water, PBS, or DMEM with 10% FBS, 1% L- Glutamine, 1% Pen/Strep for 24 hours. Swelling degree was defined as the ratio of the mass of the wet disk to that the dry disk. The compressive secant modulus was determined as the slope of the elastic deformation region of stress/strain curve, varying for each substitution rate. The region was selected by identifying the slope at 5% strain prior to plastic deformation. Data was collected using a TA RSA3 Dynamic Mechanical Analysis at a strain rate of 0.005 mm/s for uniaxial testing. Frequency Sweeps: Frequency sweep data was collected at a frequency range of 99.95-.02 Hz and analyzed for viscoelastic properties at 1 Hz. LF NMR: Hydrogel samples were tested at 20 °C using a Bruker Minispec mq-20 operated at 0.47 T and 20 MHz. Transverse relaxation time (T_2) data was obtained via the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence. A total of 30,000 echoes were collected with a 90°-180° pulse separation (τ) of 0.2 ms and a recycle delay of 10 s, using 4 scans.

Results and Discussion: Swelling Degree: As hypothesized, increased DOS resulted in a trend indicating a decrease in the swelling degree. Disks swelled in water showed the predicted increase in swelling degree when compared to the disks swelled in different media with high ion concentrations. [Figure 1A]

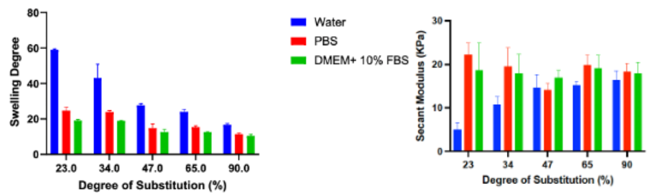

Figure 1. A. Swelling and B. secant modulus with increased degree of substitution swollen in H2O, PBS, and DMEM (+10% FBS).

Frequency Sweeps: The storage and loss moduli also trend with controlled DOS. Preliminary data trends indicate an increase in the storage modulus with increased DOS. LF-NMR: Preliminary results suggest reducing DOS leads to decreased mesh size as hypothesized.

Compressive secant modulus: The predicted trend of decreasing modulus with increasing substitution was distinguishable in the lower degrees of substitution for the disks in water, whereas at the 47% substitution the trend begins to plateau. For the disks equilibrated in media with high ionic strengths, the modulus increased when compared to water but there was a less distinguishable trend between the degrees of substitution [Figure 1B]. The data shows that the stress response to strain from the electrostatic repulsion interactions in media with increased ionic strength dominate over the thiol-ene crosslinks. Thus, the differences in the mechanical and physical data as a function of DOS are not as pronounced as was seen in water. The highest average modulus achieved was 24.5 kPa for the 23% DOS in PBS, which may not be significantly different from the other moduli in PBS or media.

Future Work: Results illustrate the ability to tune HA hydrogel network formation by modulating DOS using thiol-ene chemistry. Further, the differences of hydrogel properties in water compared to solutions with increased ionic strength provide critical insight into the behavior of the hydrogels in media relevant for *in vitro* studies. Current studies include investigation of the PHA hydrogels for mechanosensitive proteins such as Piezo 1,2 and YAP/Taz signalling, and analysis of cell spreading to determine meniscal fibrochondrocyte response to hydrogel network formation and resulting mechanical properties. **References:** (1) Kiyotake, E. A.; Douglas, A. W.; Thomas, E. E.; Nimmo, S. L.; Detamore (2) Townsend, J. M.; Andrews, B. T.; Feng, Y.; Wang, J.; Nudo, R. J.; Van Kampen, E.; Gehrke, S. H.; Berkland, C. J(3)Townsend, J. M.; Sali, G.; Homburg, H. B.; Cassidy, N. T.; Sanders, M. E.; Fung, K.-M.; Andrews, B. T.; Nudo, R. J.; Bohnstedt, B. N.; Detamore,