Diels-Alder Crosslinked Hyaluronic Acid Hydrogels for Tissue Engineering

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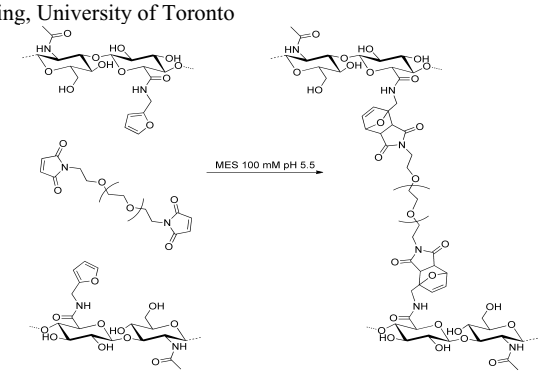
Statement of Purpose: Hyaluronic acid (HA) is a naturaly occuring nonsulfated glycosaminoglycan found ubiquitously throughout the extracellular matrix with reported roles in embryonic development, tissue organization, wound healing, and angiogenesis. HA is also biocompatabile, biodegradable and nonimmunogenic, and as such holds considerable promise within tissue engineering applications [1]. In order to translate these diserable biological properties to a mechanica lly robust hydrogel, HA must be covalently crosslinked. Current crosslinking methods often require a coupling agent, catalyst or photo -initiator, which may be cytotoxic, or involve a multi -step synthesis of a HA -derivatve suitable for crosslinking, increas ing the complexitity of the system. Accordingly, we sought to develop a novel procedure to prepare HA hydrogels via Diels -Alder chemistry. We have previously exploited this chemistry for conjugation of antibodies to nanoparticles for targeted drug delivery [2], and here we demonstrate its applicability to crosslink HA. In this simple, one -step strategy, stable hydrogels are formed without the use of a crosslinking agent. This highly selective "click" crosslinking reaction between furan -modified HA (HA -Furan) a nd bimaleimide-PEG ($(MI)_2$PEG) eliminates the formation of by -products, and is highly efficient under aqueous conditions – optimal for hydrogel formation.

Methods: HA-Furan derivates were synthesized in a simple one step reaction by conjugating furfuryla mine to HA using 4 -(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) coupling reagent. The degree of substitution (DS) was determined from ^{1}H NMR spectra. HA -Furan hydrogels were prepared by combining separate solutions of HA -Furan and $(MI)_2$PEG dissolved in MES buffer (Scheme 1). HA -Furan concentration was held constant while the concentration of (MI) $_2$PEG was varied to examine differences in hydrogel properties relative to the furan:maleimide ratio. Evidence of chemical crosslinking was verified using FTIR spectroscopy. Hydrogels were characterized by oscillatory rheology, equilibirum swelling, and degradation studies using hyaluronidase. Finally, MCF -10A cells were cultured on HA -Furan crosslinked hydrogels to examine possible cytotox ic effects.

Results: HA-Furan was synthesized with controlable DS, by varying the molar ratios of DMTMM:Furan:HA. Ratios of 4:2:1, 2:1:1, and 1:0.5:1 yielded HA -Furan with 75 ± 8% (n = 4), 61 ± 7% (n = 4), and 49 ± 6% (n = 9) DS, respectively. HA -Furan 49 % DS was employed, as lower substituted HA gels have been shown to display higher cellular bioactivity [3]. FTIR spectra displayed an increased absorption at 1459 cm^{-1} (C=C in Diels-Alder adduct), providing proof for chemical crosslinking.



Scheme 1. Schematic represe ntation of the formation of Diels-Alder crosslinked HA hydrogels.

Hydrogels were further analyzed according to the furan:maleimide (F:M) ratio. Rheological measurements showed that the shear elastic modulus (G') increased with maleimide concen tration. However, once an equal molar ratio of furan:maleimide had been reached, G' did not increase substationally with further increasing crosslinker concentration (see Table 1). This implies that the crosslinking efficiency is very high, and closely corresponds to the maximum number of theoretical crosslinks. A similar correlation was observed within *in vitro* degradation studies, where degradation rates depended on crosslinker concentration, with 1Furan:0.5MI hydrogels degrading the fastest, and 1Furan:2MI hydrogels degrading the slowest. Overall, enzymatic recognition was retained with the furan -modification on HA, suggesting HA -Furan crosslinked hydrogels would be a suitable biomaterical for *in vivo* implantation. There was no significant loss in mass observed in the negative -control where hydrogels were immersed in PBS only.

Table 1. Characterization of HA-Furan Hydrogels

Hydrogel Formulation	G' (Pa)	Degradation Rate (%/hour)
1Furan:0.5MI	275.59 ± 53.8 (n=4)	3.40 ± 0.19 (n = 3)
1Furan:1MI	556.9 ± 36.7 (n = 4)	2.23 ± 0.06 (n=3)
1Furan:2MI	679.17 ± 62.1 (n = 4)	2.06 ± 0.04 (n=3)

Prelimary cell studies with immortalized breast cancer cells (MCF-10A) showed normal cellular growth after 10 days under standard cell culture conditions, indica ting that HA-furan hydrogels are cytocompatible.

Conclusions: Diels-Adler click chemistry serves as a highly efficient crosslinking method for the formation of HA hydrogels, and is suitable for cell culture.

We acknowledge funding from NSERC and the CIH R Training Program in Regenerative Medicine (CMN).

References: 1. Allison DD et al. Tissue Engineering. 2006; 12: 2131-2140. 2. Shi M. et al. Angew. Chem. Int. Ed. 2007; 46: 6126-6131. 3. Eng D. et al. Acta. Biomaterialia. 2010; 6: 2407-2414.