Synthesis of norbornene-modified hyaluronic acid hydrogels via water-based DMTMM coupling

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Statement of Purpose: Synthesis of chemically modified hyaluronic acid (HA) polymers has enabled the fabrication of hydrogels, such as norbornene-modified HA (NorHA) hydrogels, that can provide tunable mechanical properties. However, current protocols for the synthesis of NorHA polymers relies on the use of dimethyl sulfoxide (DMSO)[1], a solvent that is cost-intensive and requires extensive dialysis to be removed. To address this, we developed a NorHA synthesis protocol free of organic solvents based on the triazine derivative 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM)[2] and tested the degree of norbornene substitution, hydrogel mechanical properties, and embedded cell viability to assess their use in tissue engineering and regenerative medicine applications.

Methods: HA was modified with 5-norbornene-2-methylamine using DMTMM, (Fig. 1A), precipitated with ethanol, and the degree of substitution (DS) was determined using proton nuclear magnetic resonance (^{1}H NMR). NorHA hydrogels (2 wt%) were prepared via a ultra-violet (UV) light (3 mW/cm^2) activated thiol-ene reaction using dithiothreitol (DTT) and lithium phenyl(2,4,6-trimethylbenzoyl)phosphinate (LAP)[1] (Fig. 1B). Mechanical analysis was performed using oscillatory photorheology (1 Hz, 1% strain, 3 mW/cm^2), and viability of encapsulated human mesenchymal stromal cells (hMSCs, 4x10^6/mL) in 2 wt% NorHA gels was investigated through Live/Dead staining (24 h, calcein A [live cells] and propidium iodide [dead cells]).

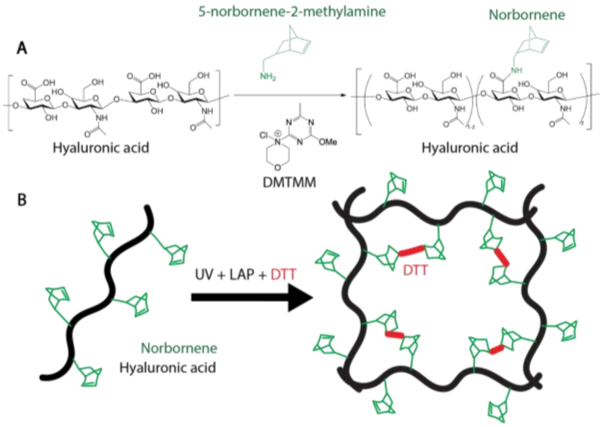

Figure 1. Schematics of NorHA **A** synthesis using 5-norbornene-2-methylamine and DMTMM, and **B** crosslinking via light-induced thiol-ene reaction[1].

Results: NorHA polymers were synthesized through the activation of HA carboxylic acids with DMTMM and amide-coupling, and both the reaction time and temperature of the synthesis were altered to tailor the degree of substituted norbornenes (DS). Increasing the temperature from 20 ºC to 37ºC yielded a two-fold increase in DS (Fig. 2A), and increased reaction times were coincident with higher DS that increased over 48 hours (Fig. 2B). While the crosslinker concentration (DTT) had little influence on the storage modulus (G') for low substituted NorHA polymers (DS 10.5%), high substitutions (DS 38.5%) resulted in stiffer hydrogels as indicated by the increase in G' for increasing DTT (Fig. 2C). G' values for medium substitutions (DS 25.5%) decreased for high DTT concentrations, probably due to DTT auto-crosslinking (Fig. 2C). High viability of encapsulated hMSCs was observed for NorHA hydrogels synthesized from DMTMM and DMSO at 24 h of culture (DMSO 75.5% ± 11.6%, DMTMM 88.0% ± 6.6%, Fig. 2D).

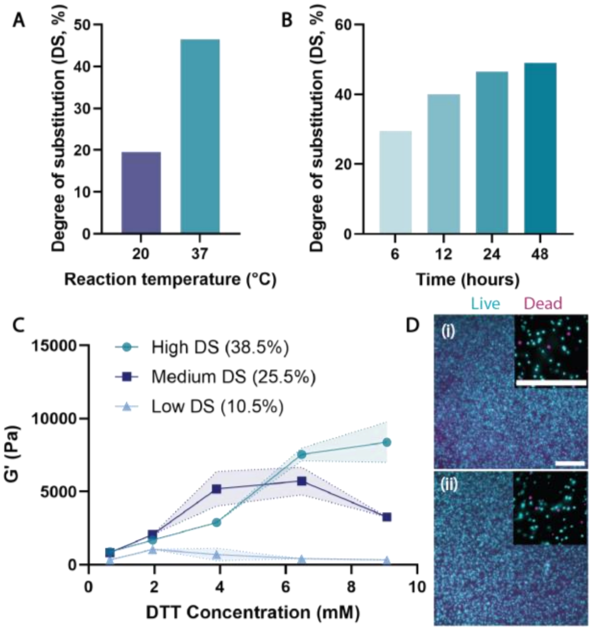

Figure 2. Degree of substitution (DS, %) as a function of **A** reaction temperature (24 h), and **B** reaction time at 37ºC. **C** Storage modulus (G') of NorHA hydrogels as a function of DS and DTT. **D** Representative images of live-dead staining of hMSCs cultured for 24 h in NorHA synthesized in DMSO (i) and DMTMM (ii, scale bars = 500 µm).

Conclusions: DMTMM coupling of norbornenes to HA enabled the synthesis of NorHA polymers free of organic solvents with tunable degree of substitution that can be purified through ethanol precipitation. The use of water-soluble DMTMM eliminates the need for extensive dialysis while maintaining the tunability and cytocompatibility of crosslinked hydrogels. Moreover, reducing the use of solvents provides promise as means towards the development of green and sustainable syntheses.

References: [1](Gramlich, W.M. et al. Biomaterials 2013), [2](Loebel, C. et al. Carbohydrate Polymers 2015).