Hybrid Bio/Synthetic Biomimetic Aggrecan: An Enzymatically Resistant Design Strategy

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Statement of Purpose. Aggrecan is a large proteoglycan (PG) found in load bearing tissues. It is composed of a protein core to which chondroitin sulfate (CS) is covalently bound in a very dense array (2-4nm spacing). Charged anionic groups on the CS chains draw water into the disc and electrostatic repulsions generated between closely packed GAG chains resist deformation thereby allowing the tissue to distribute mechanical forces[1]. Aggrecan degeneration is mediated enzymatically through cleavage of the core protein and leads to numerous degenerative conditions such as arthritis and intervertebral disc degeneration[2]. *The goal of this project is to develop an enzymatically resistant biomimetic replacement for the large PG aggrecan by replacing the core protein with a synthetic polymer*. To achieve close spacing of CS chains in our biomimetic aggrecan and mimic the bottle brush structure of native aggrecan, we have utilized a "grafting-through" step-growth approach to synthesis where CS spacing can be controlled by the length of the synthetic monomer.

Methods: We have identified a terminal primary amine on CS and investigated its ability to react with synthetic polymers and monomers[3]. ***Synthesis.*** Biomimetic aggrecan was synthesized via the linear step-growth polymerization of di-epoxide monomers with amine terminated CS. Biomimetic aggrecan was synthesized by reacting CS with the di-epoxide polyethylene glycol diglycidyl ether (PEG-DGE, MW ~526) or ethylene glycol diglycidyl ether (EG-DGE, MW 174.2) at pH 9.4 (0.1M sodium borate buffer (SBB), CS 25mg/mL). The effects of time (24-96hrs), temperature (21-45℃), and DGE concentration (10-100mM) were investigated. Conjugation was monitored using the fluorescamine assay, which detects free primary amines on un-reacted CS. Reactions were purified by dialysis against DI water (6-8K MWCO membrane). ***Characterization.*** To characterize the chemical structure and purity of the synthesized biomimetic aggrecan, samples were investigated *via* ^{1}H-NMR (D_2O solvent). The structure of the biomimetic aggrecans was investigated via transmission electron microscopy (TEM, staining with uranyl acetate). Viscosity for biomimetic aggrecan solutions (25mg/mL in PBS) was determined using an AR 2000ex Rheomoter (25°C, shear rate 10-200/s). Live/Dead assay was performed on NIH 3T3 Fibroblasts (10,000cells/cm^2 on tissue culture plastic, 48hr attachment, RPMI media, 5% fetal bovine serum, L-glutamine and 1% pen/strep) dosed with DGE monomer, biomimetic aggrecan or CS (UV sterilized).

Results: PEG-based and EG-based biomimetic aggrecan were synthesized via the reaction of CS with PEG-DGE and EG-DGE respectively. PEG-based and EG-based biomimetic aggrecan have similar chemistries but differ in the molecular spacing between CS bristles. The reactions progressed with time and were modulated by both temperature and di-epoxide concentration with

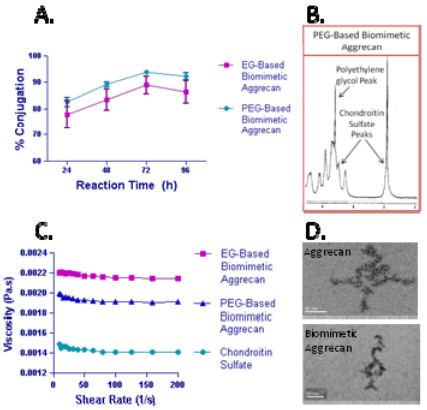

Figure 1:
(A) Reaction Kinetics (45℃, 20mM DGE)

(B) ^{1}H-NMR of PEG-based biomimetic aggrecan

(C) Viscosity (25mg/mL in PBS, 25℃)

(D) TEM images of aggrecan and PEG-based biomimetic aggrecan

reactions at 45°C achieving the highest % conjugation (PEG-DGE 92% and EG-DGE 86%) (Fig 1A). ^{1}H-NMR spectra (Fig 1B) of purified biomimetic aggrecan showed peaks corresponding to ethylene glycol/poly(ethylene glycol) at 3.6ppm while peaks corresponding to unbound epoxide groups (~2.6 and 2.8ppm) were not detected, indicating removal of un-reacted di-epoxide monomer. All peaks corresponding to CS remained similar to CS standard indicating no degradation of CS during polymerization. In TEM imaging of aggrecan (Fig 1C), CS can be seen arranged in a chain like structure on the protein core. The biomimetic aggrecan also takes on a beaded-chain like structure similar to that of native aggrecan. Viscosities of the biomimetic aggrecan solutions were observed to be higher than that of CS (2.2mPa.s for EG-based and 1.9mPa.s for PEG-based *vs*. 1.4mPa.s for CS) (Fig 1D). Cytotoxicity testing of the biomimetic aggrecans indicated high cell viability after exposure to 2mg/mL biomimetic aggrecan for 24hrs (>95% viability) while the monomers PEG-DGE and EG-DGE were acutely toxic to cells at concentrations of 1mM or greater (<15% viability).

Conclusions: Hybrid bio/synthetic biomimetic aggrecan macromolecules were synthesized using the di-epoxide linear step-growth polymerization strategy. These biomimetic aggrecan molecules may be utilized to treat degenerative disorders by restoring mechanical properties of degenerated tissues while avoiding enzymatic degradation due to the potentially enzymatically resistant nature of the synthetic core.

References: [1]Seog, J. et al., *Macromolecules*, 35, pgs 5601-5615, 2002. [2]Urban, J PG et al, A*rthritis Research and Therapy*, 5(3), 2003. [3]Sarkar S et al. *SFB Annual Meeting 2010* Seattle, Washington, USA. **We would like to acknowledge C. Winkler (Drexel University) for TEM imaging and The Coulter Foundation for funding.**

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