Sulfated Alginate to Sequester Endogenous Growth Factors Secreted by Mesenchymal Stem Cell Spheroids

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Statement of Purpose: Cell-based therapies represent a promising solution to a myriad of diseases and conditions. However, the rapid and dramatic loss of cells upon injection into the target site remains a key bottleneck, severely stunting the promise of this approach. Mesenchymal stem cells (MSCs) are broadly studied for use in cell therapies due to their potential for autologous use and secretion of bioactive factors that stimulate tissue regeneration. The therapeutic potential and viability of MSCs can be enhanced by aggregating the cells into spheroids and transplantation using engineered materials such as alginate. Compared to monodisperse cells, MSC spheroids secrete increased quantities of potent growth factors. Unfortunately, even as spheroids, the bioactive nature of the MSC secretome is diminished by cell death or differentiation once implanted. We propose that the inclusion of sulfate groups within an alginate hydrogel would sequester endogenous biomolecules secreted by entrapped MSC spheroids, thereby prolonging the therapeutic effect of MSCs by presenting these factors to neighboring responsive cells. In this way, implanted MSCs can migrate out of the spheroid, differentiate, or even die, and their therapeutic secretome will remain to augment the healing microenvironment. In these studies, we hypothesized that the biophysical properties of alginate gels would be influenced by the degree of sulfation and method of crosslinking.

Methods: Alginate was reacted in formamide with chlorosulfonic acid at 60°C for 2.5 hours to couple sulfate groups to the polymer backbone. Degree of sulfation was controlled through the percent chlorosulfonic acid added to the reaction, which was studied at 0, 1 or 2 percent. Sulfate groups were detected *via* dimethylmethylene blue assay (DMMB), used to detect the glycosaminoglycan (GAG) component, and nuclear magnetic resonance (NMR). Alginate was then crosslinked using covalent or ionic methods. Sulfated alginate hydrogels were covalently crosslinked using adipic acid dihydrazide (AAD), 1-hydroxybenzotriazole hydrate (1-HOBT), and 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), at a ratio of 1:10 sulfated to non-sulfated alginate. Alginate gels were ionically crosslinked by pipetting $CaCl_2$ on a 3.5 kD dialysis membrane on top of the gels to allow for ion diffusion for 10 minutes. The mechanical properties were evaluated *via* compressive testing. 40,000-cell MSC spheroids were entrapped in covalently crosslinked sulfated alginate at a concentration of 200,000 MSCs/gel. Viability of the cells was assessed *via* live/dead stain.

Results: Increasing the percentage of chlorosulfonic acid from 0, 1, to 2 percent in the alginate reaction increased the number sulfate groups on the backbone, as confirmed by the DMMB assay for GAG (**Fig. 1A**). NMR peaks confirmed the increased sulfate degree of substitution achieved using higher percent chlorosulfonic acid reactions. Mechanical properties of ionically crosslinked alginate gels were dependent upon the degree of sulfation, with higher moduli correlating with more sulfate groups (**Fig. 1B**). Additionally, unlike covalently crosslinked gels which did not depolymerize, ionically crosslinked gels disintegrated within 24 hrs. Regardless of the degree of sulfation, the elastic modulus of covalently crosslinked hydrogels remained unchanged (**Fig. 1C**). MSC spheroids remained viable in covalently crosslinked gels at all degrees of sulfation examined (**Fig. 1D**).

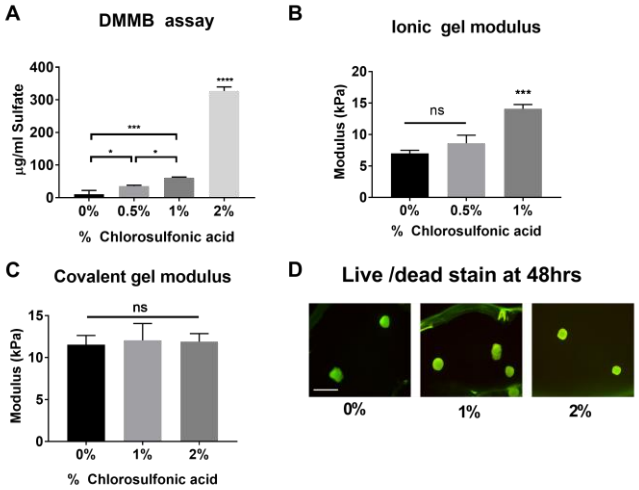

Figure 1. (A) Quantification of GAG content, an indicator of the extent of sulfation, revealed that increased sulfation was attained when incubating in increased concentrations of chlorosulfonic acid. **(B)** Ionically crosslinked hydrogels exhibit increasing modulus with increasing sulfation. **(C)** The degree of sulfation does not affect the compressive modulus of covalently crosslinked hydrogels. **(D)** Live/dead stain of spheroids in covalently crosslinked sulfated gels. Scale bar = 1000 µm, n=3-4; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Conclusions: We demonstrated that the degree of sulfation can be readily controlled through the amount of chlorosulfonic acid added to the reaction. Viability of spheroids within covalently crosslinked hydrogels was also high, indicating it is an effective method for entrapping cells. The mechanical properties of ionically crosslinked gels were enhanced due to increasing sulfation, while covalently crosslinked gels were unaffected. This finding allows us to decouple the relationship between mechanical properties and sulfation to probe the role of each factor in future studies. Compared to previous studies that load only one growth factor at a time, this strategy facilitates the sequestration and presentation of a complex mixture of factors from the sulfated hydrogel that exhibit angiogenic and anti-inflammatory properties.