Optimization of Injectable Anisotropic Chitosan-based Hydrogels and *in vivo* Evaluation

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Statement of Purpose: Hydrogels offer an attractive approach for cartilage scaffolding because of the possibility of providing both liquid and solid phases similar to the natural environment. Further, injectable hydrogels offer a minimally invasive alternative to arthroscopic surgeries and ease of incorporation of cells and biologically active agents. Although many types of injectable hydrogels have been developed using various chemistries, in general they lack practical use due to the conditions necessary for gelation, difficulty in creating anisotropic matrix configuration mimicking native cartilage, and the mechanical strength. Chitosan-based temperature sensitive hydrogels using β-glycerophosphate have attracted significant attention due to their gelation near body temperature and possibility of blending other molecules without compromising gelation characteristics. However, they lack necessary mechanical strength in addition to the cellular signaling required for cartilage regeneration. The objectives of this study were to investigate the bio-mimicry of an anisotropic chitosan-based hydrogel and evaluate the developed injectable hydrogels for gelation in the body, stability, and immune response. We evaluated the effect of blending gelatin, hyaluronic acid (HA), and beta tri-calcium phosphate (β-TCP) on *in vitro* and *in vivo* gelation, linear and cyclical mechanical strength.

Methods: Initially, hydrogels were formulated isotropically to match three of the four layers of articular cartilage. Gelatin, HA, and β-TCP for the superficial, radial, and calcified zones, respectively were blended in different amounts and tested for formation of solution, gellability, and the quickness of gelation. The transitional zone was formed by altering the amounts of superficial and radial zones. Compressive, rheological, and physical characteristics were assessed at the physiological conditions. Hydrogel durability was assessed by cyclic compression at a frequency of 0.026Hz. Formation of anisotropic hydrogels was also tested.

Male BALB/c mice 8-10 weeks old (21-24 g) were used to test the *in vivo* gelation, stability, and overall systemic effect. Animal care was provided in accordance with the OSU IRB guidelines and all procedures were performed with the approval of the OSU Animal Care and Use Committee. Sterile solutions of chitosan-gelatin, chitosan-HA, and chitosan-HA-β-TCP were subcutaneously injected in the dorsal region. Sham animals went through the same process with the injection of saline. Three animals per condition were used. At day 5, animals were sacrificed hydrogels were harvested along with the adjoining skin and analyzed by histology. Also, liver and spleen were harvested. In tandem, hydrogels formed *in vitro* were analyzed for changes in matrix distribution.

Results: Multiple methods were attempted during the hydrogel formulations and unsuccessful due to chitosan precipitation during pH adjustment. Methods include the material addition time, heat and stirring, and addition of pre-heated water to solution. The compressive modulus was improved significantly by increasing chitosan concentration. Further, increasing the chitosan concentration of the hydrogels increased the rate of gelation as well as the structural integrity at physiological diameters. Cyclical tests demonstrated repeatable strength and durability with no sign of failure. Addition of HA to the hydrogels improved structural integrity relative to chitosan/gelatin hydrogels. Anisotropic gels were formed with different gradations of chitosan-based gels and no lamination was observed.

New concoctions were injectable into the subcutaneous region via 22G needle. Solutions gelled under physiological condition. At day five, all animals showed no gross changes in lymph node, liver, kidney and body weight relative to sham controls. Mice were stable and healthy through the duration of the study. Histological (H&E) analysis of the injected hydrogels showed gelation and attachment of the hydrogel to the surrounding tissue. Also, no inflammatory cell invasion was found in the gels with the lack of any tissue necrosis. Hydrogels formed *in vitro* and *in vivo* appeared similar in morphology.

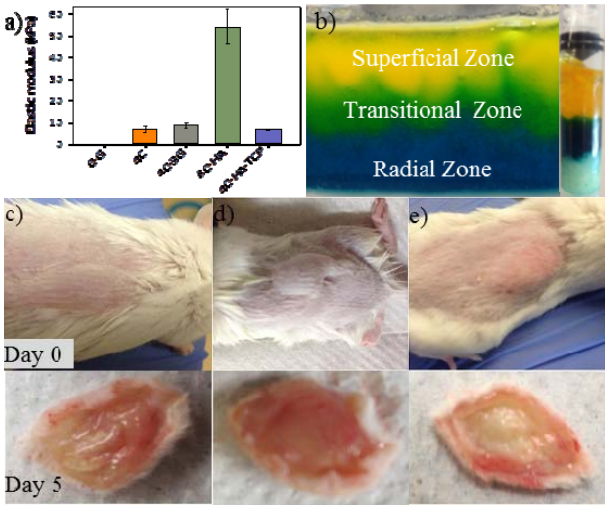

Figure: Properties of chitosan-based hydrogels *in vitro* and *in vivo*. (a) elastic modulus chart showing differences between isotropic hydrogels, (b) anisotropic formation of hydrogel. Gross images of injected hydrogels in respective zones (c) superficial, (d) radial, (e) calcified.

Conclusions: Increasing compressive strength, rate of gelation, and durability of the hydrogels is suggested to be resultant of increasing chitosan concentration. Structural integrity of the hydrogel is suggested to be the resultant of the addition of HA. Injectable hydrogels gelled and were stable inside the body with no immune response from the host after five days. Hydrogels were optimized to achieve higher mechanical strengths with the possibility of *in vivo* gelation.

Acknowledgments: Financial support from OCAST (HR12-023) and Edward Joullian Endowment. Authors thank Mr. Curtis Andrew for histological processing.