Development of an Injectable Hyaluronic Acid Based Hydrogel to Facilitate Stretch Growth of Axons

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Statement of Purpose: The goal of this work is to develop and characterize a unique polymeric biomaterial that can be used to enhance axonal regrowth and repair by providing mechanical stimulation. The growth cone is considered as a key player in axonal growth (1, 2). There are two main mechanisms of axonal elongation. The first is via growth cone extension, where the growth cone is searching for a target to bind to. The second occurs after the growth cone has synapsed with its desired target and the neuron elongates as the target relocates and pulls the neuron with it (3-5). The latter mechanical stimulation mechanism is called "stretch growth" or "towed growth". The neuronal network in the body grows in length with the growth of the body. *In vitro* studies have used calibrated micro-needles and mechanical micromotors to simulate and manipulate this phenomenon. The goal of such studies was to understand this mechanism and determine the limits of stretch growth. We aim to develop a hydrogel system that can perform the same function as a motor and "tug" on the neurons it is attached to, thereby providing mechanical stimulation to enhance their growth. The shear thinning property of hyaluronic acid (HA) enabled us to test if we could store a retractive stress in a rapidly crosslinked network under shear flow and then controllably release this stress and achieve shrinkage of the network scaffold along one desired axis. We investigated two strategies to achieve this goal. The retractive stress trapped in the crosslinked network was released either by manipulating the main backbone HA chains or by selectively breaking the crosslinks. For the former we crosslinked the network with a non-degradable crosslinker thereby we could target the HA chains, while for the latter crosslinking was performed using a hydrolysable crosslinker. HA also provides the benefit of preexisting sites that bind to cell surface receptors making it an ideal candidate.

Methods: Sodium salt of HA derived from *Streptococcus equi*, MW 1.6MDa, was modified to attach a pendant methacrylate group for photo-crosslinking. HA was derivatized with glycidyl methacrylate to obtain methacrylated HA (HAGMa) by adapting a method described by Bader et al. (6). Proton NMR was used to determine degree of methacrylation. Following rheological characterization, we tested the following compositions for their shrinkage properties. 60mg/ml HAGMa crosslinked with 3:1 and 4:1 ratio of poly(ethylene glycol) diacrylate (PEGDa), and 70mg/ml HAGMa crosslinked with 1:1 and 1:1.5 ratio of poly(ethylene glycol) Polylactic acid diacrylate (PEGPLADa). The photoinitiator used was Irgacure 2959. The pre-crosslinking solution was injected into a rectangular mold made out of glass slides at a flow rate of 0.5 ml/min and rapidly crosslinked by exposing the solution to a 100W, 365nm UV lamp for 5 mins. The hydrogels were swollen in PBS to equilibrium before any characterization experiments were performed.

Hydrogel characterization: PEGDa crosslinked hydrogels were subjected to enzymatic degradation with hyaluronidase (Hyase), a naturally occurring enzyme that cleaves the principal disaccharide unit in the HA molecule (2). PEGPLADa crosslinked hydrogels were subjected to hydrolytic degradation by immersing in PBS. For both types of gels suitable controls were tested to verify that shrinkage was on account of the experimental conditions. Images of the samples were captured at several time points and sample dimensions were measured using Image J to produce a profile of the shrinkage. The shrinking hydrogels are capable of performing work, by applying a force on any attached body, as a consequence of the shrinking. We characterized this using a custom built device that consisted of a cantilever and a non-contact differential variable reluctance transducer (DVRT) pair. The principle concept behind the design is that a shrinking hydrogel attached between a flexible calibrated cantilever and a fixed stage will deflect the cantilever. The DVRT will record this deflection and provide a way to quantify the force. The cantilever was calibrated for various known loads and known displacements and deflections.

Results: Rapidly crosslinked samples exposed to hyaluronidase or hydrolytic degradation demonstrated a decrease in sample length over a period of 48 hours as shown in figure 1. As the concentration of hyaluronidase was increased, the magnitude and rate of the shrinkage of the PEGDa crosslinked hydrogels also increased. Increasing the crosslinker concentrations slowed the rate and extent of shrinkage. The hydrolytically degraded PEGPLADa crosslinked samples demonstrated a higher shrinkage rate for the first 12 hours. All samples stayed intact throughout the experiment. The change in the width of the samples was minimal (~1.5%). 1:1 PEGPLADa crosslinked samples had the most shrinkage in length with an average of 2.01 ± 0.25mm or 15%. This translates to an average shrinkage rate of 41.94µm/hr. Control samples had very minimal change in their lengths. The 4:1 PEGDa crosslinked samples exposed to 10µg/ml Hyase generated an average force of 3.6mN as compared to 2.4mN for 3:1 PEGDa samples exposed to similar conditions. Experiments to characterize the force generated by PEGPLADa crosslinked samples are currently ongoing.

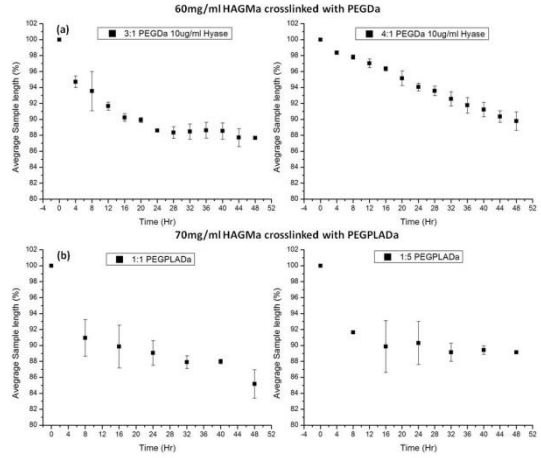

Fig.1. Scatter plots showing the sample shrinkage profile for (a) 60mg/ml HAGMa crosslinked with PEGDa and (b) 70mg/ml HAGMa crosslinked with PEGPLADa. The former was exposed to 10µglml Hyase while the latter were hydrolytically degraded to recover the retractive stress stored in the crosslinked networks. The 4:1 PEGDa crosslinked samples present a favorable linear shrinkage profile over the course of 48 hours, while all other compositions seem to demonstrate an exponentially decaying profile of the shrinkage. (n=3)

Conclusions: The experimental results demonstrate that a retractive stress can be stored in a HA network by rapidly crosslinking solutions under flow and then recovered via network manipulation. Two independent strategies were investigated using two different types of crosslinkers (PEGDa and PEGPLADa); and in both cases shrinkage as a result of experimental conditions was observed. The 4:1 PEGDa crosslinked samples exposed to 10µg/ml Hyase showed the most linear shrinkage profile for the 48hr duration and a 3.6mN average force generation as a consequence of the shrinkage, while 1:1 PEGPLADa crosslinked samples demonstrated the highest magnitude of shrinkage. Experiments are currently underway to characterize samples via optical birefringence to determine the degree of molecular chain orientation. We also aim to determine the strength of bonding between native cell adhesion sites on HA and primary neurons using atomic force microscopy (AFM).

References: **1)** Price R.D. et al., J. Plas, Reconstr Aes., 2007 10; 60(10):1110-9.; **2)** Larsen NE et al., Adv Drug Deliv Rev. 1991 10; 7(2):279-93.; **3)** Laurent TC et al., ARD. 1995 May 01; 54(5):429-32.; **4)** Luo Y, et al., J Control. Release. 2000 10/3; 69(1):169-84.; **5)** Borland et al., Immunol. 1998; 93(2):139-48.; **6)** Bader, R. A. et al., J. Biomed. Mater. Res, 2008,86A: 494–501