Crosslinked Hyaluronic Acid Hydrogel Networks Designed as Mechano-stimulators

Pushkar S. Varde[1,2], Julie M. Hasenwinkel[1,2].
[1]Department of Biomedical and Chemical Engineering, [2]Syracuse Biomaterials Institute, Syracuse University, Syracuse, NY 13244

Statement of Purpose: The purpose of this study is to design and characterize a unique crosslinked hyaluronic acid (HA) based hydrogel that is able to perform work through the process of releasing the residual stress trapped in the network during fabrication. The unperturbed conformation of high molecular weight HA is a random coil. We intend to disentangle these coils and align the molecular chains by flowing them through a syringe needle and rapidly crosslinking them to form a network. Through this approach we are able to retain molecular chain alignment and trap residual stress in the network. This stress can then be released via manipulating the crosslinked network, by either degrading the HA molecules or cleaving the crosslinks. As the residual stress is released, the hydrogel network will shrink and exert a force on any body attached to it, in essence performing work on that body. We present here results for the shrinkage characterization and force generated by the shrinking hydrogel when the backbone HA molecules are degraded. Availability of binding sites on HA for cell surface receptors would enable the use of this unique biomaterial as a mechano-stimulator for applications such as peripheral nerve repair and wound closures.

Materials: 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. (1). Proton NMR was used to determine degree of methacrylation. Following rheological characterization, a composition consisting of 60mg/ml solution of HAGMa with a 4:1 ratio of crosslinker poly(ethylene glycol) diacrylate (PEGDa), and 1% wt/vol of photoinitiator Irgacure 2959 was chosen for making samples. The pre-crosslinking solution was injected into a rectangular mold at a flow rate of 0.5 ml/min and crosslinked by exposing the solution to a 100W, 365nm UV lamp for 5 mins. The clear pre-crosslinking solution turns opaque within 40 seconds of exposure to UV thus forming crosslinked hydrogels. The hydrogels were swollen in PBS for a day before any characterization experiments were performed.

Shrinkage characterization: Hydrogels were subjected to enzymatic degradation with hyaluronidase (Hyase), a naturally occurring enzyme that cleaves the principal disaccharide unit in the HA molecule (2). Along with the rapidly crosslinked samples, another set of samples were made by introducing a delay between injecting pre-crosslinking solution into the mold and crosslinking. This allowed for relaxation of the polymer molecules, thereby leading to the loss of any molecular orientation. Such samples will be referred to as negative controls. Three rapidly crosslinked samples, with average length of 12 ± 0.2mm, were exposed to 5µg/ml and 10µg/ml of hyaluronidase for a period of 48hrs. Samples exposed to PBS served as controls. Negative controls were also exposed to hyaluronidase. The enzyme was dissolved in PBS and temperature was maintained at 37°C in a temperature controlled shaker. The enzyme had to be replenished every 4 hours due to reduction of its activity (3,4). Images of the samples were captured before introducing the enzyme, at every 4hr timepoint, and at the end of 48hrs alongside a reference scale. The dimensions of the samples were measured using Image J software and plotted as percent normalized length change over the course of the experiment.

Force characterization: A device consisting of an aluminum cantilever-differential variable reluctance transducer (DVRT) pair was custom built for measuring the force generated by HA hydrogels during the process of shrinking. 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 non-contact DVRT will record this deflection and provide a way to quantify the force. The cantilever was calibrated for various known loads and known displacements/deflections. A PBS reservoir housed the gel and enzyme during the experiment. The gel was glued onto a fixed stage on one end and to the calibrated cantilever on the other end using epoxy glue. Hyaluronidase enzyme was replenished every 4 hours similar to the shrinkage experiments. The entire setup was maintained at 37°C during the experiment by placing it in a temperature controlled water bath.

Results: The rapidly crosslinked samples exposed to hyaluronidase demonstrated a decrease in sample length over 48 hours. As the concentration of hyaluronidase was increased, the magnitude and rate of the shrinkage also increased. All samples stayed intact throughout the experiment. The change in the width of the samples was minimal (~1.5%). Samples exposed to 10µg/ml of hyaluronidase had the most shrinkage in length with an average of 1.4 ± 0.22mm or 11%. This translates to an average shrinkage rate of 27.5µm/hr. Control samples had very minimal change in their lengths (1.6%). Compared to the samples that were rapidly crosslinked to conserve some chain alignment, the extent and rate of shrinkage for negative controls exposed to hyaluronidase was significantly lower ($p<<0.05$). Their behavior was similar to control samples where the network was not degraded. The rate of shrinkage of the gels measured thus far; correspond well with the rates at which stepper motors have been used to successfully stretch neuronal bundles (5). So these gels could potentially perform the same function as the stepper motors when attached to neuronal bundles. Figure 1 shows representative results comparing the force measured by the custom built device from a control sample and a shrinking sample that were exposed to 10µg/ml of hyaluronidase. The extent of shrinkage was comparable to that seen in the shrinkage experiments performed on samples of the same composition for the same duration. The device recorded a gradual increase in the force generated as a result of the shrinking, and a net force of 3mN over the 48hr duration from the shrinking sample demonstrating that the gel is capable of performing work on the attached cantilever.

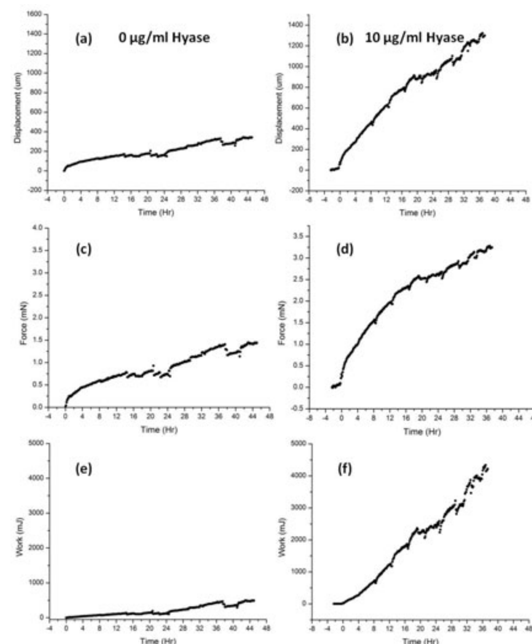

Fig.1 Representative data showing cantilever displacement indicative of sample length shrinkage (a,b), measured force (c,d), and work performed (e.f) versus time for a control sample (left), and a crosslinked sample (right) exposed to 10µg/ml of hyaluronidase over a period of 48 hours.

Conclusions: Crosslinked HA hydrogels were successfully fabricated under conditions that should lead to storage of residual stress in the network. This stress was released by degrading the network with hyaluronidase enzyme, leading to shrinkage of the gel and force generation as a result of a change in molecular orientation within the gels. We recorded an average shrinkage of 11% in the rapidly crosslinked samples exposed to 10µg/ml of hyaluronidase which resulted in a net force of 3mN generated by the sample. We believe this shrinkage is a consequence of releasing the stress that was trapped in the network by the crosslinking process, which restricted the recoiling of the HA molecular chains. Future work involves testing different compositions to tailor the shrinkage rate for specific applications and to better understand and further characterize the mechanism of shrinkage. Hydrolysable crosslinker molecules will also be studied in order to release the residual stress in the network by cleaving only the crosslinks in the HA networks.

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