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Characterization of slow-gelling alginate hydrogels for intervertebral disc tissue-engineering applications

Emily A. Growney Kalaf, Reynaldo Flores, J. Gary Bledsoe, Scott A. Sell *

Parks College of Engineering, Aviation & Technology, Department of Biomedical Engineering, Saint Louis University, 3507 Lindell Boulevard, St. Louis, MO 63103, USA

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ABSTRACT

Reversal of intervertebral disc degeneration can have a potentially monumental effect on spinal health. As such, the goal of this research is to create an injectable, cellularized alginate-based nucleus pulposus that will restore disc function; with the primary goal of creating an alginate gel with tailorable rates of gelation to improve functionality over standard $CaCl_2$ crosslinking techniques. Gelation characteristics of 1% sodium alginate were analyzed over various molar concentrations of a 1:2 ratio of $CaCO_3$:glucono-δ-lactone (GDL), with 10% $CaCl_2$ as the control crosslinker. Alginate construct characterization for all concentrations was performed *via* ultimate and cyclic compressive testing over a 28 day degradation period in PBS. Dehydration, swell testing, and albumin release kinetics were determined, and cytotoxicity and cell homogeneity tests showed promise for cellularization strategies. Overall, the 30 and 60 mM GDL alginate concentrations presented the most viable option for use in further studies, with a gelation time between 10 and 30 min, low hysteresis over control, low percent change in thickness and weight under both PBS degradation and swelling conditions, and stable mechanical properties over 28 days *in vitro*.

1. Introduction

Alternatives to highly-invasive surgical procedures for lower back pain caused by severe intervertebral disc (IVD) degeneration include the constantly advancing field of minimally-invasive injections into the nucleus pulposus (NP). Of high importance is the maintenance of mechanical strength of the NP to allow for proper dampening of loads from the upper body. Hydraulic dampening occurs naturally in an intermittent cyclic nature, with high interstitial fluid and osmotic pressure; any injectable NP replacement strategy must be able to withstand these constant loads [1,2]. Chemonucleolysis therapy is also highly prevalent in IVD treatments, and has been shown to induce further degeneration and disc shrinkage, and treatment is typically used in conjunction with separate injections of mechanically stable polymers [3,4]. Alginate has been considered a promising hydrogel for use in injectable NP replacement strategies, with researchers indicating its use in both acellular and cellular strategies [5–9].

Alginate ($(C_6H_8O_6)_n$) a biocompatible, naturally occurring polysaccharide found in brown algae, has become a useful biomaterial for transporting cells without harm, serving as a vehicle for biological cues and providing structure [10–12]. With alginate polymerizing due to the presence of easily dissociable crosslinkers (*i.e.* divalent cations), the creation of uniform alginate macroblock geometries is quite difficult as the boundary of the alginate is tightly and rapidly bound upon contact with the crosslinking solution. The gelation is also diffusion-limited due to the tight crosslinking around the surface; this aberrant crosslinking causes variations in mechanical strength, gelation time, geometry, and diffusivity, particularly toward the centroid [13]. With controlled gelation, many more opportunities arise for the creation of alginate geometries including more tightly controlled mechanical properties, cell encapsulation transporting the cells into a site, and modification of structure and template for cell growth.

Calcium carbonate ($CaCO_3$) is a highly accessible calcium ion substitute for calcium chloride ($CaCl_2$) that dissociates when exposed to an excess of hydrogen atoms. Glucono-δ-lactone (GDL, $C_6H_{10}O_6$) is an optimum dissociating agent for the $CaCO_3$ in alginate; it hydrolyzes into gluconic acid at a rate dependent on pH and temperature, allowing the alginate to be poured into molds before gelation occurs [14]. However, timing is an important aspect of gelation with regard to homogenous cell-encapsulation. There must be a balance of time where the alginate crosslinks to a certain density in a timely manner to prevent cells settling to the bottom of the mold.

In this project, the authors modified, synthesized, and characterized a slow-gelling alginate for potential cellular or acellular use in injectable NP replacement. The characterization within these methods is a continuation of previous studies, with greater depth into the mechanical and cytologic properties of the alginates [15]. It is hypothesized that the use of *in situ* crosslinking alginate will create an analogue to a healthy NP, and subsequent gelation would occur within a surgically relevant time to prevent liquid extrusion. Modification of the gelation characteristics of the alginate is hypothesized to change the mechanical and

* Corresponding author.
E-mail address: ssell@slu.edu (S.A. Sell).

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physical properties of the gels, while maintaining appropriate gelation time. As alginates are also capable of maintaining NP phenotype *in vitro* and *in vivo* [16,17], the slow-gelling alginates are hypothesized to have the potential to maintain cell viability and phenotype while allowing a homogenous cell suspension by preventing cell clumping that occurs in instantaneously cross-linked alginate [18]. Injectable strategies have successfully restored height and function to the IVD, but without the regenerative potential that a cellularized approach would offer [19]. As such, a tissue engineered cellularized injectable scaffold of bioresorbable and bioactive materials may provide sufficient mechanical stability to the IVD while enhancing the reparative capacity of the body through incorporation of appropriate cells. This work is the first to provide a full mechanical characterization of controlled gelling alginates, while also evaluating protein release kinetics and cell viability. The unique, cellularized injectable strategy proposed in this article can also be applied to other applications such as the central nervous system and bone marrow transplants [20–22].

2. Experimental section

2.1. Physical characterization of slow gelling alginate hydrogels

2.1.1. Hydrogel synthesis

Stock solutions of 1.0% w/v alginate were made by using sodium alginate powder ($C_6H_9NaO_7$, Acros Organics, CAS: 9005-38-3, New Jersey, USA) and Millipore 18.2 MΩ DI water. After complete dissolution, the aqueous alginate was stored at 4 °C and used within one week of mixing [12]. Calcium carbonate ($CaCO_3$) (Fisher Scientific, CAS: 471-34-1, Nazareth, PA, USA) and D-(+)-gluconic acid δ-lactone (GDL) ($C_6H_{10}O_6$, Sigma-Aldrich, CAS: 90-80-2, St. Louis, MO, USA) were added in a 1:2 M ratio with varying molar concentration (provided in 2.1.2) to the alginate solution; first with addition of $CaCO_3$, followed by the addition of GDL after vortexing. A calcium ion to carboxyl molar ratio of 0.18 is used initially, and is adjusted with a multiplication factor while keeping the molar ratio of CaCO3:GDL constant at 1:2 to preserve pH [15]. Instantaneously gelling alginate was prepared as 1% w/v in DI water with no addition of $CaCO_3$, and crosslinked by either submerging the alginate in 10% $CaCl_2$ (Fisher Scientific, CAS: 10,035-04-8) solution or by addition of $CaCl_2$ to poured alginate.

2.1.2. Gelation time

The inverted test tube method was used to quantify alginate hydrogel gelation time [23]. $CaCO_3$ powder was added in molar volumes of 15 mM, 30 mM, 45 mM, 60 mM, and 75 mM, labeled respectively as 1×, 2×, 3×, 4×, and 5× [15]. GDL in molar concentrations of 30 mM, 60 mM, 90 mM, 120 mM, and 150 mM respectively, was added to the corresponding alginate:$CaCO_3$ mixture and vortexed. The tubes were inverted every 30 s at room temperature; gelation time was determined to be at the point where the alginate did not flow upon inversion. General pH testing was performed with pH test strips 4.5–10, which were later followed by measurement with a pH meter. Control alginates were created at a 10:1 ratio of $CaCl_2$ to alginate using 10% $CaCl_2$. Subsequent testing of the alginates continued at 1×, 2×, and 3× concentrations based on the gelation time, with $CaCl_2$ as control.

2.1.3. Alginate disc and macroblock preparation

Flat alginate discs of uniform 1 mm thickness were prepared for rheology, swelling, SEM, diffusivity, and cell distribution measurements by placing the solution of alginate and crosslinkers between two glass platens with 1 mm silicone spacers. Control discs were created by pipetting $CaCl_2$ between glass platens along the circumference of the flattened alginate disc to crosslink. Discs were punched from the alginates using a circular punch 20 mm in diameter, creating a uniform thin slab geometry which allowed for reproducibility and uniformity throughout testing. Larger volume alginate hydrogels were prepared for porosity, degradation, cytotoxicity, and mechanical testing. These hydrogels were created by pipetting the alginate into well plates of desired sizes after addition of crosslinkers. Gelation took place at room temperature or 37 °C. Control alginates were crosslinked by submerging in $CaCl_2$ for one hour.

2.1.4. Rheology

Frequency and time sweeps were conducted on the AR 2000ex rheometer (flat plate geometry, 0° half angle) at 37 °C to obtain storage and loss moduli (G′ and G″) for accurate gelation characteristics. To analyze moduli over a varying frequency, thin slab alginate discs were loaded onto the rheometer platform. A preload of 0.2 N was applied to the gels followed by a frequency sweep from 1 to 10 Hz at 2% strain. Storage and loss modulus were calculated from the data and compared with $CaCl_2$ as control. To determine gelation time *via* time sweep, the gel solution was mixed with $CaCO_3$ and GDL and poured onto the platform, followed by consistent 10 rad/s frequency at 0.02% strain. Storage modulus crossover time at 37 °C was obtained and compared to the inverted tube method results. $CaCl_2$ control time sweep was completed by lowering the rheometer geometry on the uncrosslinked alginate followed by pipetting $CaCl_2$ around the cone at the moment the time sweep started.

2.1.5. Pore analysis

To view microstructure, gelled alginate discs prepared as previously described were flash frozen between liquid nitrogen-cooled aluminum platens and lyophilized (VirTis Sentry 2.0 Lyophilizer). Sample cross-sections were sputter coated with a gold anode and placed under high vacuum. The samples were imaged using a Zeiss EVO LS 15 SEM (Zeiss) at an accelerating voltage of 10 kV and a spot size of 6 pA. The resulting images were imported into ImageJ, and mean pore areas ($n > 30$ pores measured) were calculated and compared to the $CaCl_2$ control.

To analyze the porosity and pore volume of the varying gels, 1×, 2×, 3× and control $CaCl_2$ gels of 1 mL volumes were created in uniform cylinders by crosslinking in wells of a 24-well plate. Control gels were created by laying a sized Kimwipe over the top of the alginate and pipetting 2 mL of 10% CaCl2 over the top of the Kimwipe; after 20 min the Kimwipe was removed and the gel was agitated to allow for crosslinking around the entire surface. The gels were removed from the wells, measured for accuracy of volume using thickness and diameter measurements ($V_{cylinder} = 2\pi r^2 * \text{Thickness}$). The samples ($n = 3$) were frozen for 20 min at −80 °C and lyophilized for 12 h. The xerogels were weighed and dimensions measured to ensure little dimension change, followed by a 12 hour soak in absolute EtOH. The weight and volume of the ethanol-soaked gels were measured and the porosity of the gels were calculated by $\%P = (W_f - W_i)/(V_h * \rho_e)$, where W_f is the final weight of the ethanol-soaked xerogel, W_i is the weight of the xerogel, V_h is the measured volume of the xerogel, and ρ_e is the density of absolute ethanol at room temperature, known to be 0.789 g/cm^3 [24]. Pore volume was measured by $V_p = V_t - (m_s/\rho_s)$, where V_t is the volume of the dried hydrogel, m_s is the mass of the dried hydrogel, and ρ_s is the density of the alginate, known to be 1.6 g/cm^3 [25].

2.1.6. Water retention analysis

Alginate discs of predetermined sizes were weighed, measured, and air dried at room temperature for 48 h. After complete dehydration, the gels were reweighed and their dimensions measured, and placed in PBS to view the properties of the rehydration of the gels. Mass swelling ratio was determined using $Q_m = (W_s - W_d)/W_d$; where W_s is the weight of the swollen hydrogel, and W_d is the weight of the dry hydrogel [26].

2.1.7. Diffusivity & elution

2.1.7.1. Albumin diffusivity. Alginate (1% w/v) was dissolved into a solution of 2% Bovine Serum Albumin (BSA, MW: 67,000 Da) in DI water. After gelation, the discs ($n = 3$ per condition) were weighed and placed in a known volume of PBS under constant movement on an orbital