Injectable Alginate Hydrogels with Embedded Chitosan Microspheres for Controlled, Localized Protein Release

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Statement of Purpose:
Alginate hydrogels provide an ideal protein release scaffold for tissue engineering due to having high biocompatibility and the ability to gel *in situ* with simple ionic crosslinking mechanisms. Chitosan is another attractive biocompatible polymer that can be formed into solid structures via phase inversion techniques. Here, monodisperse protein-loaded chitosan microspheres are incorporated into calcium-crosslinked alginate hydrogels and the release of protein is measured. We show how the fabrication of chitosan microspheres can be controlled with respect to size and composition to tune protein release. Also, the gelation of alginate by a glucono-δ-lactone (GDL) and calcium phosphate mechanism is characterized for injectable applications.

Methods:
Alginate was purified by dialysis and activated charcoal as previously described [1]. A 2.66 wt% solution of alginate was made and into it 1.5 wt% of hydroxyapatite powder (Sigma Aldrich) was homogenized. Chitosan (MMW, Sigma Aldrich) was dissolved at 2 wt% into 2% acetic acid. 2 wt% polyvinyl alcohol (PVA, 13-23 kDa, Sigma Aldrich) was added (crystal or soluble) as a porogen to the chitosan solution and removed after sphere solidification by heating in 80°C water for 6 hrs. Microspheres were formed by inserting a stainless steel blunt tip needle into PVC tubing to form a coaxial droplet generator. The continuous phase consisted of a solution of mineral oil, hexane, triethylamine, and Span® 80 (Croda, Edison, NJ). The dispersed phase consisted of the chitosan and PVA solution. After microsphere formation in the tube, spheres were solidified in a bath of 50% 2M sodium hydroxide and 50% ethanol for 1 hr and washed twice with water and hexane. For external crosslinking of chitosan spheres, a 24 hr soak in 5 mM genipin solution (Wako Chemicals) was performed. Protein was loaded by soaking in a 5 wt% bovine serum albumin (BSA, Fisher) solution for 48 hrs. Spheres were dried either by lyophilization or at room temperature. Characterization of microsphere surface morphology and porosity was performed with an ESEM (FEI Quanta 600i, Hillsboro, OR). Dried microspheres were added to the alginate solution at various concentrations and mixed well. To activate solidification of the gels, a freshly prepared 0.2 mg/μl glucono-delta-lactone (Sigma Aldrich) solution was mixed with the alginate solution for 10 secs prior to gelation. Rheological measurements were performed with an AR-G2 Rheometer at 37°C (1 Hz, 1% Strain, TA Instruments, Newcastle, DE). Protein release was measured at 37°C in phosphate buffered saline (PBS, HyClone) using a BCA Protein Assay (Pierce).

Results:
Monodisperse chitosan microspheres were successfully prepared and their size tuned by varying the flow rate of the continuous phase (Figure 1). These spheres can be incorporated into alginate hydrogels for protein release.

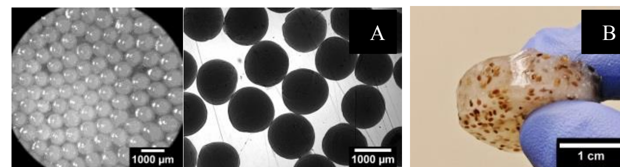

Figure 1: (A) Chitosan microspheres with low polydispersity can be prepared by coaxial flow at various sizes. Scale bars 1000 μm. (B) Chitosan microspheres were successfully incorporated into an alginate disk. Scale bar 1 cm.

Gelation time, defined as the time where G'>G'', was determined for mixtures with varying amounts of GDL but constant calcium phosphate content, and could be tuned between 130 and 570 seconds (Figure 2).

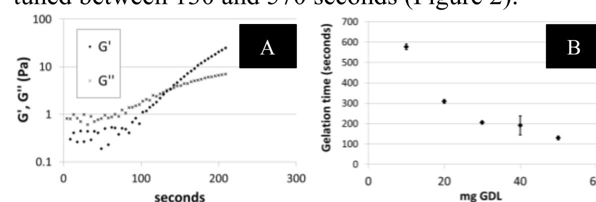

Figure 2: (A) Sample gelation measurement for alginate hydrogel crosslinked with calcium phosphate and GDL. (B) Gelation time can be tuned by varying GDL amount and holding calcium phosphate constant (2 wt%).

Protein release can be tuned by the addition of PVA porogen into the chitosan microspheres (Figure 3). Soluble PVA porogen allowed the greatest protein release.

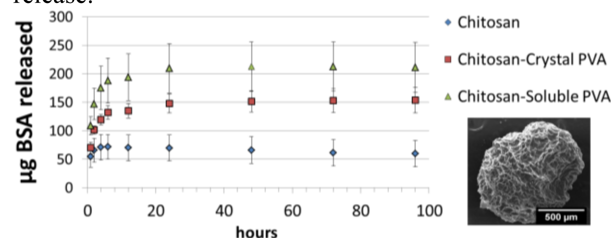

Figure 3: Bovine serum albumin release curves from microspheres with various PVA porogen. Shown is an SEM image of a chitosan microsphere prepared by lyophilization. Scale bar 500 μm.

Conclusions:
An alginate hydrogel embedded with chitosan microspheres has been constructed, with the ability to tune microsphere size and rate of alginate gelation. Addition of a PVA porogen to the microspheres has been shown to increase protein release. Future studies will determine the bioactivity of the released protein and its ability to influence cells in a localized and controlled manner for tissue regeneration applications.

References:
[1] Jeong, S. I., Krebs, M. D., Bonino, C. A., Samorezov, J. E., Khan, S. A., & Alsberg, E. (2010). Electrospun chitosan–alginate nanofibers with in situ polyelectrolyte complexation for use as tissue engineering scaffolds. Tissue Engineering Part A, 17(1-2), 59-70.

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