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Systematic study of alginate-based microcapsules by micropipette aspiration and confocal fluorescence microscopy

Rachelle M. Kleinberger [a], Nicholas A.D. Burke [a], Kari Dalnoki-Veress [b], Harald D.H. Stöver [a,*]

[a] Department of Chemistry and Chemical Biology, McMaster University, 1280 Main Street West, Hamilton, Ontario L8S 4M1,Canada
[b] Department of Physics and Astronomy, McMaster University, 1280 Main Street West, Hamilton, Ontario L8S 4M1,Canada

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ABSTRACT

Micropipette aspiration and confocal fluorescence microscopy were used to study the structure and mechanical properties of calcium alginate hydrogel beads (**A** beads), as well as **A** beads that were additionally coated with poly-L-lysine (**P**) and sodium alginate (**A**) to form, respectively, **AP** and **APA** hydrogels. **A** beads were found to continue curing for up to 500 h during storage in saline, due to residual calcium chloride carried over from the gelling bath. In subsequent saline washes, micropipette aspiration proved to be a sensitive indicator of gel weakening and calcium loss. Aspiration tests were used to compare capsule stiffness before and after citrate extraction of calcium. They showed that the initial gel strength is largely due to the calcium alginate gel cores, while the long term strength is solely due to the poly-L-lysine–alginate polyelectrolyte complex (PEC) shells. Confocal fluorescence microscopy showed that calcium chloride exposure after PLL deposition led to PLL redistribution into the hydrogel bead, resulting in thicker but more diffuse and weaker PEC shells. Adding a final alginate coating to form **APA** capsules did not significantly change the PEC membrane thickness and stiffness, but did speed the loss of calcium from the bead core.

1. Introduction

Encapsulation of genetically engineered therapeutic cells has been proposed for treatment of several enzyme and hormone deficiency disorders, including diabetes [1], Parkinson's [2], and lysosomal storage disorders [3]. Encapsulation of allogeneic cells in a semi-permeable membrane can provide physical immune protection, and may enable long-term delivery of therapeutic peptides. The most common approach to cell encapsulation involves embedding cells in a calcium alginate bead that is then coated with a polycation, commonly poly-L-lysine (PLL), to form a stable polyelectrolyte complex (PEC) shell to increase the stability, and tune the permeability, of the membrane [4].

These beads are usually further coated with alginate in order to hide the PLL from the host's immune system, resulting in capsules known as alginate/PLL/alginate or **APA** capsules [4]. A recent study by Tam et al. suggests that the final layer of alginate does not significantly alter the outer composition of the membrane, casting doubt on the need for this final alginate coating [5].

The properties of **APA** capsules depend significantly on the type and concentration of alginate, PLL and gelling ion (typically calcium) used, as well as on the protocols used for their preparation and storage. Capsules can fail in their immune-protective role in a number of ways, which may include mechanical rupture, loss of the outer alginate coating followed by fibrotic overgrowth [6], and even degradation of alginate by redox processes or hydrolysis [7].

A key issue is the loss of calcium through exchange with, *e.g.*, sodium [8–10], leading to bead swelling [10], decreased gel modulus [11], and even rupture of the **APA** membrane [4].

Improved understanding and control of capsule properties are key challenges in the field, and have led to calls for greater standardization between different labs [12]. Many novel approaches to create covalently crosslinked capsules still use calcium alginate and polycations, such as PLL [13–16].

Tissue-like capsule stiffness and long-term mechanical integrity [17] are critical for successful transplantation of encapsulated therapeutic cells, as well as for related use of these gels in stem cell differentiation and regenerative medicine [18–21].

The mechanical stability of **APA**-type capsules has often been studied using ensemble pass/fail screening for the fraction of intact capsules following exposure to hypotonic media [15,22], or shaking with glass beads [23]. The thickness of the membrane formed by the PLL–alginate PEC has been used to indicate capsule strength in some studies [4,24] though at times the strongest capsules were reported to be those having a thin shell formed using higher molecular weight (MW) PLL [24]. In other studies, swelling ratios were used as an indicator of strength [4,25].

Mechanical tests of single alginate capsules have been carried out using atomic force microscopy (AFM) [26], compression testing

Abbreviations: PEC, polyelectrolyte complex; PLL, poly-L-lysine; **A**, alginate; **AP**, alginate/poly-L-lysine; **APA**, alginate/poly-L-lysine/alginate; MW, molecular weight; G, guluronic acid; M, mannuronic acid.

* Corresponding author. Tel.: +1 9055259140x24983.
E-mail address: stoverh@mcmaster.ca (H.D.H. Stöver).

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[11,17,27–32], rheology [11], and deformation by centrifugal forces [33,34]. AFM measurements mainly provide information about local surface properties [26], while compression tests provide information about the whole bead from the analysis of Young's modulus [30], viscoelastic properties [35,36], and bursting forces [17,36,37].

Micropipette aspiration is a common technique for studying mechanical properties of cells [38–40] and small semi-permeable capsules [41–44]. The application of this method to larger, alginate-based capsules is rare, despite the simplicity of the technique and the quantitative information that can be extracted. Hunkeler and co-workers made limited use of aspiration to measure the membrane tension of capsules composed of an alginate/cellulose sulfate core coated with polymethylene-co-guanidine [45].

This paper describes a simple yet sensitive aspiration-based test of capsule stiffness, its validation and use to study the properties of model capsules as a function of preparation and storage conditions. Confocal microscopy was used to determine capsule morphology, in particular shell thickness, through the use of fluorescein labeled PLL.

2. Experimental

2.1. Materials

Sodium alginate (Pronova UP MVG, batch: BP-0908-01) was purchased from Novamatrix (Sandvika, Norway). Poly-L-lysine hydrobromide (PLL, M_n 15–30 kDa), fluorescein isothiocyanate (FITC) and HEPES sodium salt from Sigma-Aldrich (Oakville, ON Canada), and sodium chloride and calcium chloride from Caledon Laboratories (reagent grade, Georgetown, ON), were used as received. Trisodium citrate dihydrate (AnalaR) was purchased from EMD Chemicals (Gibbstown, NJ, USA) and was used as received. Sodium hydroxide and hydrochloric acid stock solutions (0.1 or 1.0 M) were purchased from LabChem (Pittsburgh, PA, USA).

2.2. PLL fluorescent labeling

FITC-labeled PLL, PLL*f*, was prepared as described earlier [16]. Briefly, PLL (HBr form, 99.5 mg, 0.48 mmol lysine) was dissolved in 0.2 M $NaHCO_3$ buffer (pH 9) and 1.0 mg (0.0026 mmol) of FITC dissolved in N,N-dimethylformamide was added. The PLL*f* was purified by dialysis and isolated by freeze-drying (64.5 mg, 82% HCl form) with a labeling degree of 0.61%, determined from the maximum absorbance at 495 nm, using the absorption coefficient of free FITC of 77,000 $M^{-1}\,cm^{-1}$ [46]. The extinction coefficient of PLL*f*, was 2.67 $mL\cdot cm^{-1}\cdot mg^{-1}$, measured at 495 nm.

2.3. Preparation of calcium alginate beads (**A** beads)

A solution of sodium alginate (5–10 mL, 1 wt.%) in saline was filtered (0.2 μm) and then extruded into 60 mL of gelling bath consisting of de-ionized water containing 1.1 wt.% $CaCl_2$ (100 mM) and 0.45 wt.% NaCl (77 mM), reflecting a 12–24 molar excess of Ca^{2+} over carboxylates. Extrusion was done at a rate of 0.5 mL/min, using a syringe pump and a flat-tipped 27 G needle fitted inside a 1 mm diameter tube that provided an annular airflow of 3–4 L/min. This airflow was adjusted to generate narrow disperse calcium alginate beads (**A** beads) with mean diameters of 500 to 600 μm, and standard deviations of about 50 μm. After extrusion was complete, the beads were kept in the gelling bath for another 10 min before being transferred into fresh gelling bath solution (using a 3:10 volume ratio of settled bead suspension to wash solution) for an additional 10 min, and before moving on to coating or aging experiments as described below. All solutions were pre-cooled to 4 °C and the gelling bath was placed in an ice bath during bead formation. The beads have smooth surfaces, and equatorial/axial aspect ratios not exceeding 1.1.

2.4. Preparation of **AP** and **APA** capsules

AP beads were prepared by adding 10 mL of 0.05 wt.% PLL or PLL*f* in saline (pH 7.0–7.5) to 3.0 mL of settled, freshly prepared **A** beads. After 6 min with occasional swirling, the supernatant was removed, and the as-formed beads washed a) once with fresh gelling bath and once with saline or b) twice with saline. Each wash took 2 min unless stated otherwise. Supernatant from coating and washing steps were analyzed for residual PLL*f* content using UV/Vis.

APA capsules were prepared by adding 1.67 mL of 0.03% sodium alginate to 0.5 mL of settled **AP** beads for 4 min, with occasional swirling. The resulting **APA** capsules were washed twice with saline for 2 min. All washing steps involved a 3:10 volume ratio of settled beads to coating/washing solution.

2.5. Monitoring the effects of storage/washing conditions on **A** and **AP** beads

Freshly prepared **A** beads and **AP** capsules were stored in saline (154 mM NaCl) at 4, 20 and 40 °C. All washing steps used a volume ratio of settled beads to washing solution of 3:10. Beads were monitored by microscopy and aspiration at room temperature, in a small amount of their own supernatant. Supernatants were monitored for Ca^{2+} content using ICP-MS.

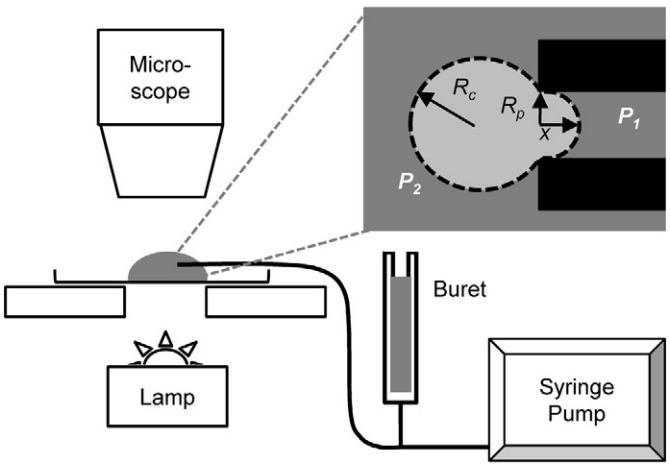
2.6. Citrate treatment

Sodium citrate (5 mL, 70 mM) and settled **AP** or **APA** capsules (0.5 mL) were mixed at room temperature for 5 min before the supernatant was removed and the settled, liquid filled capsules were washed once with saline (1.67 mL).

2.7. Micropipette aspiration (*Scheme* **1**)

A borosilicate disposable micropipette with an inner diameter of 290 μm and an outer diameter of 1120 μm (Fisher Scientific) was attached to a 10 mL glass buret (0.5 cm inner diameter, 65 cm length) using flexible Tygon tubing, forming a U-tube filled with water. A number of capillary tips from one batch were examined by optical or scanning electron microscopy (TESCAN VP SEM) and were found to have smooth, circular openings and near identical inner diameters of 291 ± 2 μm.

A syringe pump (NE-1600, New Era Pump Systems™) connected to the Tygon tubing *via* a Y-shaped connector allowed automated control of the height of the water column and hence the pressure differential. About 0.25–0.50 mL of a suspension of capsules in their own storage supernatant (unless indicated otherwise) was placed on a



Scheme 1. Setup for micropipette aspiration.