Self-Crosslinkable Polymers for Cell Immuno-Isolation

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Introduction

Cell encapsulation within semipermeable polymer shells or beads has long been explored as a promising approach to the treatment of enzyme deficiency disorders.[1,2,3] Encapsulation of non-autologous cells can provide mechanical protection and immuno-isolation, permitting the use of standard cell lines that are genetically modified to express key enzymes or other actives missing in patients. Lysosomal Storage Disorders such as Tay-Sachs and Heuer's disease, as well as Diabetes form exciting targets for this approach.

The most common approach involves cell-encapsulation in alginate-poly-L-lysine-alginate (APA) microcapsules as first described by Lim and Sun.[4] Gel strength has emerged as a limiting factor in the life times of such devices. Recent approaches to strengthen these gel beads include *i.a.* the replacement of calcium with barium ions[5], the use of photochemical crosslinking,[6] and the use of high viscosity, high guluronic acid alginate.[7]

This paper describes the use of self-cross-linking polyelectrolytes to covalently reinforce the surface of calcium alginate gels. A matched set of acrylic polycations and polyanions, containing primary amino groups and electrophilic groups, respectively, were coated sequentially onto calcium alginate beads and found to self-crosslink into an outer shell that offers protection against mechanical and chemical challenges. Confocal laser fluorescent microscopy was used to measure the thickness of the resulting crosslinked polyelectrolyte complex layer. SEC studies show molecular weight cut-offs between 150 and 200 kDa, suitable for immune isolation. Results of the viability of C_2C_{12} mouse cells within these capsules will be presented.

Experimental

Materials. Sodium alginate (Keltone LV, M_n = 428 kDa) was a gift of the Nutrasweet Kelco Company. [2-(Methacryloyloxy)ethyl] trimethyl-ammonium chloride (MOETAC), 2-aminoethyl methacrylate hydrochloride (AEM.HCl), methacrylic acid (MAA), 2-(methacryloyloxy)ethyl acetoacetate (MOEAA), poly-L-lysine (PLL, M_n = 15-30 kDA), 2,2'-azobis(2-methyl propionamidine) dihydrochloride (97%), and fluorescein isothiocyanate (FITC), were purchased from Sigma-Aldrich, and used as received.

Synthesis of Poly(methacryloyloxyethyltrimethylammonium chloride - *co* - aminoethyl methacrylate) 70:30, (p(MOETAC-co-AEM.HCl, C70):

MOETAC (5.59 g), aminoethylmethacrylate hydrochloride (AEM.HCl, 2.13 g) and 2,2'-azobis(2-methyl propionamidine) dihydrochloride (0.209 g) dissolved in 75 mL DI water in a 125 mL HDPE bottle were reacted at 65C for 24 hrs. The polymer was purified by dialysis in cellulose tubing (12 kDa MW cut-off, Spectrum Laboratories) against DI water followed by freeze-drying. Yield: 6.88 g (90 %).

Synthesis of Poly(methacrylic acid, sodium salt-*co*-2-[methacryloyloxyethyl] acetoacetate) 70:30; (p(MAA-co-MOEAA), A70):

Poly(methacrylic acid, sodium salt-*co*-2-[methacryloyloxyethyl] acetoacetate was prepared by free radical polymerization as previously described.[8] MAA (4.84 g), 2-(methacryloyloxy)ethyl acetoacetate (MOEAA, 5.43 g) and AIBN (0.132 g) dissolved in 100 mL ethanol were reacted as described. The polymer was isolated by precipitation in ethyl ether and then dried to a constant weight in a vacuum oven at 50 ºC. Yield: 8.74 g (85%).

Instrumentation.

The composition of p(MAA-*co*-MOEAA) was determined by ^{1}H NMR spectroscopy in DMSO-d_6 using a Bruker AV 200 spectrometer. The composition of p(MOETAC-*co*-AEM.HCl) was determined by potentiometric titration with 0.1M NaOH in a PC Titrate automatic titrator (Man Tech Associates). The degree of labelling with FITC was measured on a Varian Cary 50 BIO UV-Vis Spectrophotometer. Optical microscope images of complexes, capsules and microspheres were taken using an Olympus BX51 optical microscope fitted with a Q-Imaging Retiga EXi digital camera and ImagePro software. Phase contrast microscope images were taken using a Wild M40 microscope. A Confocal laser scanning imaging system equipped with an Argon-ion laser and a Nikon microscope using EZ-C1 software, version 1.50, was used to study the distribution of FITC labeled polymers in the microcapsules.

Molecular Weight Determination

Molecular weights of p(MAANa-*co*-MOEAA) was determined using a Waters 515 GPC system described previously,[8] calibrated with narrow-dispersed PEG standards. Samples were eluted at a flow rate of 0.80 mL/min with a mobile phase consisting of 0.3 M sodium nitrate in phosphate buffer (pH 7) prepared by dissolving 27.6 g monosodium phosphate, 101.98 g sodium nitrate, and 4.66 g sodium hydroxide in 4.0 L of HPLC grade water. The pH was adjusted to 7 with 1M NaOH. A 1% p(MAANa-*co*-MOEAA) solution was prepared by adding a stoichiometric amount of 1 M NaOH to the p(MAA-co-MOEAA) copolymer in the mobile phase.

An Ubbelohde viscometer (viscometer constant: 0.00314 cSt/s), was used to determine molecular weights of MOETAC-based polycations dissolved in 1M NaCl at 20 ± 0.1 ºC.

Preparation of Ca-alginate beads:

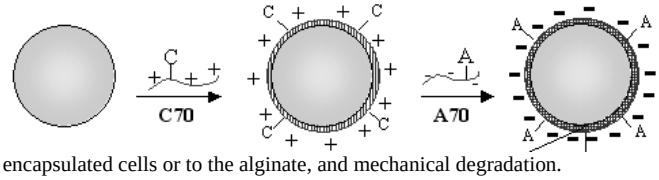
Calcium alginate beads were prepared as described by Ross et al.[9] A 1.5% aqueous solution of sodium alginate was filtered through sterile 0.45 µm Acrodisc syringe filters (Pall Corp., USA). A modified Sage syringe pump (Orion/Thermo Electron) was used to extrude the alginate solution at a rate of 99.9 mL/hr through a 27-gauge blunt needle (Popper and Sons), with a concentric airflow (4 L/min) used to induce droplet formation. The droplets were collected in a 1.1% calcium chloride bath (10× alginate volume) causing the formation of Ca-alginate beads. These beads were washed in sequence with four-fold volumes of a) 1.1% $CaCl_2$, 0.45% NaCl for 2 minutes; b) 0.55% $CaCl_2$, 0.68% NaCl for 2 minutes; c) 0.28% $CaCl_2$, 0.78% NaCl for 2 minutes; d) 0.1% CHES, 1.1% $CaCl_2$, 0.45% NaCl for 3 minutes; and then e) 0.9% NaCl for 2 minutes.

These beads (3 mL) were subsequently exposed to 0.05% PLL (10 mL) for 6 minutes and then washed with 15 ml of a) 0.1% CHES, 1.1% $CaCl_2$, 0.45% NaCl for 3 minutes; b) 1.1% $CaCl_2$, 0.45% NaCl for 2 minutes; and c) 0.9% saline for 2 minutes. Finally the beads were coated with 0.03% (w/v) sodium alginate (10 mL) in saline for 4 minutes followed by three washes with 0.9% saline. Coatings with the synthesized polyelectrolytes C70 and A70 were carried out in a similar fashion.

Osmotic stress caused by hypotonic solutions was used to compare the mechanical strength of different microcapsules, based on a published procedure.[10]

Results and Discussion

Calcium alginate beads as used for cell encapsulation consist of highly hydrated alginate networks that are ionically crosslinked through their guluronic acid residues. Failure modes in long-term implantation may include calcium loss to the host, immune or inflammatory response of the host to the



encapsulated cells or to the alginate, and mechanical degradation.

Figure 1. Sequential coating of calcium alginate beads with polyelectrolytes.

Exposure to aqueous solutions of poly-L-lysine or other polycations, followed by exposure to a biocompatible polyanion, usually alginate, has been a common approach to improve strength as well as permeability control of calcium alginate beads containing live cells (Fig. 1).

Introduction of matched crosslinkable groups (primary amine and acetoacetate) into a pair of polycations and polyanions, respectively should provide a route to *in-situ* covalent crosslinking of this outer polyelectrolyte

complex shell, without the use of low molecular weight crosslinking agents. Figure 2 shows an initial pair of polyelectrolytes designed for this purpose, called C70 and A70. Model experiments had shown that these two copolymers react instantaneously with each other in aqueous solutions, to form a crosslinked network that does not dissolve under conditions where purely electrostatic complexes dissociate

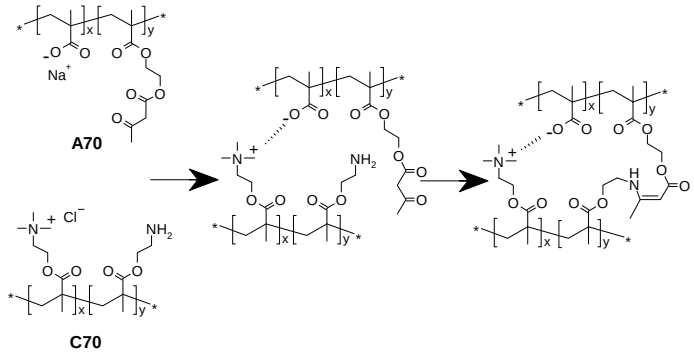


Figure 2. Reactive copolymers for *in-situ* crosslinking: A70: poly(methacrylic acid, sodium salt-*co*- 2-[methacryloyloxy]ethyl acetoacetate), p(MAANa-*co*-MOEAA) 70:30; C70: poly[2-(methacryloyloxy)ethyl]trimethylammonium chloride)-*co*-(2-aminoethyl methacrylate) methacrylate), p(MOETAC-*co*-AEM) 70:30

Narrow-disperse calcium alginate beads were first prepared by extruding a 1.5w% sodium alginate solution, optionally containing C2C12 mouse cells, into a 1.1 calcium chloride bath. Following a standard washing procedure, these calcium alginate beads ("A") were exposed, in sequence, to dilute solutions of the polycation ("C70") and the polyanion ("A70"). When the resulting "AC70A70" capsules were exposed to 1 M sodium citrate solutions to extract the calcium, the crosslinked polyelectrolyte shells remained as intact spherical shells. Unlike comparable non-crosslinked polyelectrolyte complexes, these crosslinked shells also survived exposure to 2M saline (Figure 3a). AC70fA70f capsules, prepared using FITC-labelled C70 and A70, show shell thicknesses of up to 35 micron in confocal laser scanning microscopy (CLSM) images (Figure 3b). Shell thicknesses vary with MW and exposure time, in agreement with recent, analogous studies, of poly-L-lysine shells.[11] Layers of similar thickness are observed irrespective of which polyelectrolyte is labelled, indicating the presence of a homogeneous mixture of the two polyelectrolytes C70f and A70f at the surface of the alginate bead.

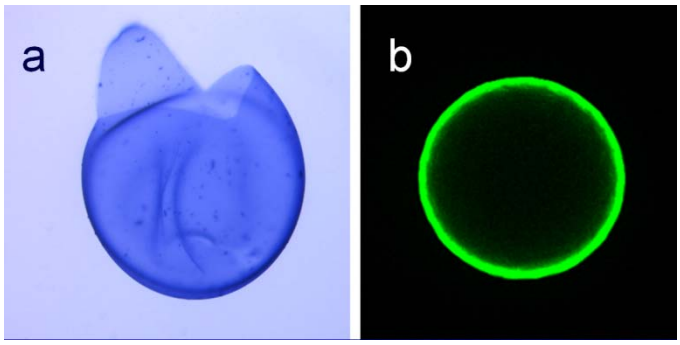


Figure 3. a) OM image of a Trypan Blue stained shell remaining after exposure to 1M citrate and 2M saline (cut open to reveal capsular nature). b) CLSM image from the equatorial region of a Ca Alg bead exposed to 0.5% solutions of C70f and A70f for 10 min each (AC70fA70f bead).

Calcium alginate beads bearing the cross-linked shell demonstrated greater resistance to osmotic pressure changes compared to conventional beads coated with poly-L-lysine and alginate (APA beads). Permeability of the crosslinked shells were studied using CLSM studies of fluorescently labeled dextrans with molecular weights up to 500,000Da. Both APA and AC70A70 capsules were found to have molecular weight cut-offs between 150,000Da and 200,000Da, suitable for exclusion of immune-related proteins. Figure 4 shows a phase contrast microscope image of C2C12 cells encapsulated in AC70A70 capsules, after incubation for one week.

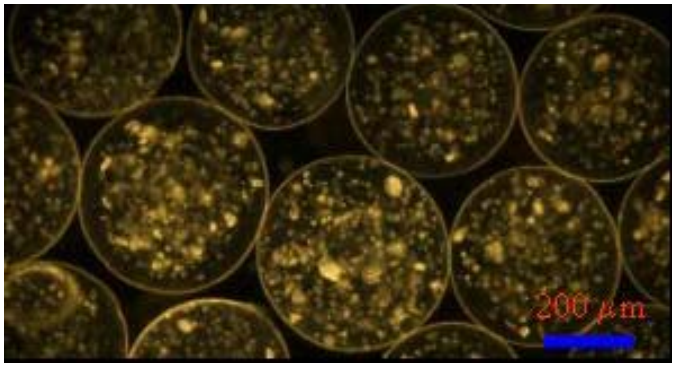


Figure 4. Phase contrast microscope image of C_2C_{12} cells encapsulated in AC70A70 after incubation for 1 week. Polyelectrolyte coatings prepared with 0.5% solutions and 10 min exposure time.

Cell viability in *in-vitro* studies of AC70A70 beads was found to be slightly lower than in the control APA capsules, but cells were replicating. Experiments are in progress to explore cell viability and *in vivo* performance of AC70A70 and analogous capsules.

Conclusions

We have shown that covalently cross-linked shells can be formed around Ca-Alg capsules by coating with oppositely charged polyelectrolytes bearing complementary amine and acetoacetate reactive groups. Use of fluorescently labelled analogs of these polyelectrolytes indicates that they are concentrated near the surface of the calcium alginate beads. The cross-linked nature of this outer shell was demonstrated by its resistance to citrate and sodium chloride. These cross-linked shell capsules have greater resistance to osmotic pressure changes, compared to APA control capsules. The cross-linked polyelectrolyte coating have suitable molecular weight cut-offs for cell encapsulation. C_2C_{12} cells were viable within calcium alginate capsules coated with the new polyelectrolytes.

Such covalent reinforcement through self-crosslinking polyelectrolytes may permit long-term strengthening of calcium alginate beads. This approach combines using inherently low-toxicity polymer-bound reactive groups with the ability to modify coating thickness and permeability. Studies of the *in vivo* use of these and related capsules are being conducted and will be reported shortly.

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