CA ALGINATE COMPOSITE MICROCAPSULES FOR CELL ENCAPSULATION

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Introduction

Encapsulation of cells within semi-permeable polymer shells or beads is a potentially powerful tool for the treatment of enzyme deficiency disorders such as LSD, hemophilia, dwarfism, and diabetes.[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. 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.e.* the replacement of calcium with barium ions[5], the use of photochemical cross linking,[6] the use of high viscosity, high guluronic acid alginate,[7] and the use of composite materials to modify viscosity, water content or wall formations.[8]

This presentation will describe the feasibility of using synthetic reactive polyanions based on methacrylic acid to augment the core material of Ca alginate microcapsules. The synthetic polyelectrolyte would be initially trapped in the CaAlg gel core by ionic or physical cross-links, and subsequently covalently cross-linked with a low MW polyamine allowed to diffuse into the core from the outside. Interaction of the synthetic polyanion(s) with calcium, diffusion of these polyanions within CaAlg hydrogel matrices and interaction with polycations at both the capsule surface and within the capsule core were studied. Confocal laser scanning microscopy (CLSM) was used to measure the MWCO of microcapsules, and to study the distribution of polyelectrolytes within these capsules. Confocal studies show molecular weight cut-offs between 150 and 200 kDa, suitable for immuno-isolation. The mechanical stability, the viability of encapsulated cells and biocompatibility of the capsules with a murine host will be presented.

Experimental

Materials. Sodium alginate (Keltone LV, M_n = 428 kDa) was a gift of the Nutrasweet Kelco Company. Methacrylic acid (MAA), 2-(methacryloyloxy)ethyl acetoacetate (MOEAA), poly (methacrylic acid, sodium salt) (PMAANa, M_n = 5400 Da), poly-L-lysine (PLL, M_n =1-4 and 15-30 kDa), fluorescein isothiocyanate (FITC), Rhodamine B isothiocyanate, fluorescein isothiocyanate (FITC)-conjugated BSA (M_n= 66 kDa), fluorescein isothiocyanate (FITC)-conjugated Dextran (M_n= 10, 70, 150, 250 and 500 kDa) and trypan blue were purchased from Sigma-Aldrich, and used as received. 2,2'-azobis(isobutyronitrile) (AIBN) from Dupont and serum free media (SFM) from Gibco were used as received.

Synthesis of Poly(methacrylic acid, sodium salt, A100-40k: MAA (5.00 g) and AIBN (0.095 g) dissolved in 45 mL ethanol in a 60 mL HDPE bottle were reacted at 60 °C for 24 hrs. The polymer was purified by precipitation in diethyl ether, and then dried to constant weight at 50 °C in a vacuum oven. Yield: 4.81 g (96 %).

Synthesis of Poly(methacrylic acid, sodium salt-*co*-2-[methacryloyloxyethyl] acetoacetate); (p(MAA-co-MOEAA), : 90:10 (A90), 80:20 (A80), 70:30 (A70), 60:40 (A60) and 50:40 (A50):
Poly(methacrylic acid, sodium salt-*co*-2-[methacryloyloxyethyl] acetoacetate was prepared by free radical polymerization as previously described.[9] MAA (7.84 g), MOEAA (2.28 g) and AIBN (0.166 g) dissolved in 100 mL ethanol were reacted at 70°C for 24 hours as described. The polymer was isolated by precipitation in diethyl ether and then dried to a constant weight in a vacuum oven at 50 ºC. Yield: 9.34 g (94%) p(MAA-*co*-MOEAA), A90. A80 (ethanol, yield: 85%), A70 (ethanol, yield: 85%), A60 (THF, yield: 71%) and A50 (1:1 THF/ethanol, yield: 85%) were prepared in a similar fashion. A70 polymers of several different MW were prepared by employing different monomer to initiator ratios during polymerization. The preparation of fluorescent analogs of A70 (A70*f*) was described previously[10].

Rhodamine-labeled Poly-L-lysine (PLL*r*): PLL (15-30k) (55.5 mg) was dissolved in 0.1M $NaHCO_3$ buffer solution (5 mL) at pH 9 in a 20 mL glass vial. Rhodamine isothiocyanate (2.7 mg) dissolved in 0.5 ml DMF was added to the PLL solution and stirred for 1 hour at 20 °C. The rhodamine-labelled PLL was purified by dialysis in cellulose tubing (3.5 kDa MW cut-off, Spectrum Laboratories) against DI water followed by freeze-drying. Rhodamine-labelled PLL (1-4k) was prepared in a similar fashion, and purified using 1 kDa MW cut-off dialysis tubing to minimize loss of low MW fractions.

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 degree of labelling with FITC and rhodamine were 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 Argon and HeNe lasers and a ZEISS microscope using LSM Image browser software, was used to study the distribution of FITC and rhodamine-labeled polymers in the microcapsules.

Molecular Weight Determination
Molecular weights of PMAANa (100-40k) and p(MAANa-*co*-MOEAA) were determined using a Waters 515 GPC system described previously,[9] 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). 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.

Preparation of Ca (Alginate-A70) composite microcapsules:
Ca(Alg-A70) microbeads were prepared using an approach described by Ross et al.[11] Aqueous solutions of 1.5 w% Na Alginate and 0.5 w% A70 or A70*f* were 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 alg-A70 mixture 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 (alg-A70) microbeads. 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% NaCl for 2 minutes. Finally the beads were coated with 0.03% (w/v) Na Alg (10 mL) in saline for 4 minutes followed by three washes with 0.9% saline.

Results and Discussion

Calcium alginate beads as used for cell encapsulation consist of highly hydrated alginate networks that are ionically cross-linked mainly through their guluronic acid residues. Exposure to aqueous solutions of PLL 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. 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, loss of polyelectrolyte coating, and/or mechanical degradation.

We have recently described the coating of alginate –poly-L-lysine cores with the reactive polyanions (A70) to form cross linked outer shells capable of sustaining the bead shape even after exposure of the bead to citrate and concentrated saline.[10] Here we describe the inclusion of 0.5% w/v reactive polyanion A70 with the sodium alginate used for the core material. We are exploring two hypotheses with this approach:
1. The A70 in the core would diffuse to the surface during coating with poly-L-lysine, forming a cross linked polyelectrolyte complex analogous to

the ones described earlier,[10] but with more control over shell thickness and location due to the counter-diffusion from core and outside.
2. Exposure of these composite beads to low MW polyamine might lead to in diffusion and cross linking throughout the cores, forming calcium alginate beads permanently reinforced throughout by an interpenetrating network of covalently cross-linked A70/PLL (Figure 1).
Model polyelectrolyte complexation studies show that calcium forms a liquid coacervate with A100, A90 and A80, while A70, A60 and A50 did not show any phase separation. These latter polyanions in particular would thus be quite mobile within calcium alginate beads, depending on their MW. These reactive polyelectrolytes have also been shown to react instantaneously with PLL and other polyamines, to form cross-linked networks that do not dissolve under conditions where purely electrostatic complexes dissociate.

Figure 1. Schematic representation of the formation of a cross-linked network by trapping a reactive polyanion within the CaAlg capsule followed by reaction with a low MW polycation bearing complementary reactive groups.

Narrow-disperse Ca (Alg-A70) beads were first prepared by extruding a aqueous solution of 1.5 w% sodium alginate and 0.5 w% A70, optionally containing C_2C_{12} mouse cells, into a 1.1% (w/v) $CaCl_2$ bath. Following a standard washing procedure, these Ca (Alg-A70) microbeads ("comp") were exposed, in sequence, to dilute solutions of PLL polycation ("P") and the alginate polyanion ("A"). When the resulting "comp-PA" capsules were exposed to 1 M sodium citrate to extract the calcium, the cross-linked polyelectrolyte layers remained as intact spherical shells. Unlike comparable non-cross linked polyelectrolyte complexes, these cross linked shells also survived exposure to 2M saline (Figure 2a), which shows that some of the A70 initially located in the core of the capsule has diffused to the capsule surface and cross linked with the PLL coating. The presence of A70*f* in the composite microbeads was analyzed by UV-visible spectroscopy, and found that ~ 40% of the original A70*f* (M_n= 22 or 42k) is retained in the gellation step during which the droplet shrinks. However, using high MW of A70*f* (M_n =149k), somewhat more A70*f* (~ 60%) is retained entrap in the Ca alg gel.

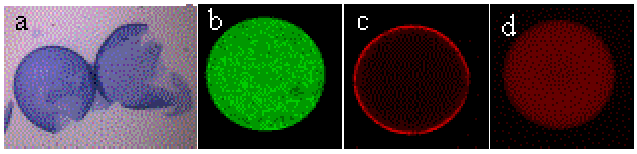
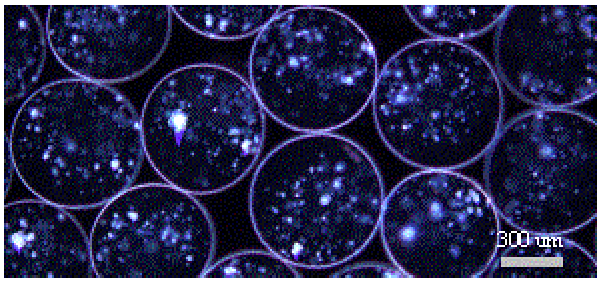


Figure 2. a) OM image of a Trypan Blue stained shell remaining after exposure to 1M citrate and 2M saline (cut open to reveal capsular nature). CLSM image from the equatorial region of a b) Ca (Alg-A70f) bead exposed to 0.05% solutions of PLL (15-30k, 6 min) and 0.03% NaAlg (4 min), c). Ca (Alg-A70)-PLL*r* 0.05% (15-30k, 6 min)-0.03% Alg (4 min). d) Ca (Alg-A70)-0.05% PLL*r* (1-4k, 6 min)-0.03% Alg (4 min).

Confocal Laser Scanning Microscopy (CLSM) was used to demonstrate the inclusion of the synthetic polyanion and to probe the distribution of A70*f* and PLL*r* within the capsules. After gelling in the $CaCl_2$ bath, A70*f* was initially found to be distributed throughout the beads. Similar images were obtained immediately after coating with PLL and alginate (Figure 2b). CLSM images of capsules prepared with PLL*r* (15-30k) (Figure 2c) show that the polycation is concentrated mainly at or near the capsule surface. However, PLL*r* (1-4k) (Figure 2d) is evenly distributed throughout the composite microcapsules, which is consistent with a number of analogous studies, of poly-L-lysine shells.[12] This could provide a unique opportunity to make capsules that are reinforced both internally and externally, which relies on: a) formation of a PLL/Alg wall of limited permeability, b) diffusion of low MW PLL into core, c) weak calcium binding of (low MW) A70 that allows diffusion of A70 towards the capsule surface and d) covalent cross-linking between complementary reactive groups.
MWCO's of the comp-PA microcapsules were studied using CLSM of dextran-FITC with molecular weights up to 500 kDa. Both APA and Comp-PA microcapsules were found to have molecular weight cut-offs between



150 kDa and 200 kDa, suitable for cell encapsulation.

Figure 3. Phase contrast microscope image of C_2C_{12} cells encapsulated in comp-PA after incubation for 1 week.
Figure 3 shows a phase contrast microscope image of C_2C_{12} cells encapsulated in comp-PA microcapsules, after incubation for one week. Cell viability in *in-vitro* studies of comp-PA beads was found to be slightly lower than in the control APA capsules, but cells were replicating, which indicates that A70 is not detrimental to cell viability. Experiments are in progress to explore cell viability and *in vivo* performance of comp-PA and analogous capsules.

Conclusions

We have shown that internal and external cross-linked network can be formed in Ca-Alg capsules by inclusion of A70 in conventional APA microcapsule cores. The distribution of these polyelectrolytes in the microcapsules was studied using fluorescent-labeled analogs. Its resistance to citrate and sodium chloride demonstrated the cross-linked nature of the shell. The cross-linked polyelectrolyte coating has suitable molecular weight cut-offs for cell encapsulation. C_2C_{12} cells were viable within calcium alginate composite capsules.
This study describes a promising approach to cell encapsulation and may ultimately be useful for clinical immunosuppressive therapies. Studies of the *in vivo* use of these and related capsules are being conducted and will be reported shortly.

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