Core-Cross-Linked Alginate Microcapsules for Cell Encapsulation

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Self-cross-linkable polyelectrolyte pairs comprised of poly(methacrylic acid, sodium salt-*co*-2-[methacryloyloxy]-ethyl acetoacetate) (70:30 mol ratio, A70) and poly-L-lysine are incorporated into CaAlg beads to form either a covalently cross-linked shell or a core-cross-linked bead. In both cases the reactive polyanion is added to a solution of sodium alginate that may contain live cells and dropped into a calcium chloride gelling bath. Subsequent exposure to poly-L-lysine (15−30 kDa) leads to formation of a cross-linked shell, while exposure to lower molecular weight poly-L-lysine (4−15 kDa) leads to formation of an interpenetrating matrix of covalently cross-linked synthetic polymer within the CaAlg template. The resulting spherical composites are resistant to chemical and mechanical stress yet remain cyto-compatible. This approach to cell-encapsulation may be useful for cell immuno-isolation in therapeutic cell transplants.

Introduction

Transplantation of encapsulated allogeneic or xenogeneic cells expressing therapeutic compounds or peptides is a promising approach for the treatment of diseases such as neurological disorders, dwarfism, hemophilia, lysosomal storage disorders, diabetes, and cancer. To avoid immune rejection by the host, the transplanted cells are typically protected by a semipermeable membrane, which allows the exchange of oxygen, nutrients and metabolites, while obscuring the encapsulated cells from the host's immune system.[1−5]

The most common cell encapsulation system involves the alginate/poly-L-lysine/alginate (APA) microcapsules derived from the original protocol of Lim and Sun.[5] These capsules are primarily composed of alginate, a naturally occurring polysaccharide composed of β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues. Calcium ions are used to cross-link G-rich regions of the alginate chains, and the resulting calcium alginate (CaAlg) hydrogel beads are coated with poly-L-lysine (PLL) to strengthen the bead surface and control permeability. A final coating with alginate is applied in order to hide the PLL from the host[4] and make the capsules biocompatible.

While APA capsules meet many of the requirements for immuno-isolation of cells when implanted into mice, they show insufficient strength when implanted into larger animals such as dogs.[6] This may be due to weakening of the hydrogel core by exchange of calcium with other physiological ions and the loss of the protective polyelectrolyte coatings.

A number of studies have attempted to address the challenge of long-term mechanical stability by varying the molecular weight (MW) or G/M ratio of the alginate,[4,7−9] the cross-linking ion,[9,10] and the polyelectrolytes[11−14] used to coat the capsule. Covalent cross-linking of the coating layer[15−19] or the alginate core[20−22] has also been investigated.

Another approach has been to examine the use of alternate hydrogel cores,[2,23] including those made of composite materials. Reinforcement of the alginate core through the formation of an interpenetrating network or composite may lead to improved mechanical properties while maintaining most of the desirable properties of alginate. In addition, it may be easier to adjust capsule properties such as strength and permeability independently of each other. One requirement would be to maintain the ability of the alginate to form calcium complexes through its extended G blocks.

A number of alginate composite materials have been explored for cell encapsulation.[24] Compounds added to the alginate forming the bead core were designed to be thermally (agarose[25]), ionically (carrageenan[26]), or photochemically gelled[27] or designed to modify viscosity or water content (carboxymethylcellulose[28]), act as wall-forming materials (cellulose sulfate,[29] heparin[28]), control permeability, or provide an improved environment for cell growth (chitlac, lactitol-functionalized chitosan[30]). For example, capsules designed for longer-term cell implantation have been prepared with alginate-cellulose sulfate composite cores where the cellulose sulfate acted as a viscosity modifier and was thought to be a better wall builder than alginate when forming polyelectrolyte complexes (PEC) with the polycations used to coat the capsules.[29] Still other composite cores have been formed by the in-diffusion of polymeric or monomeric species followed, in some cases, by cross-linking or polymerization. Gaserod et al. found that the in-diffusion of low MW chitosan into CaAlg beads resulted in the formation of an alginate-chitosan gel in the core of the capsule that was able to withstand the loss of calcium.[31] Childs and co-workers formed a composite capsule composed of alginate and poly(sodium acrylate-*co*-*N*-vinylpyrrolidone) formed by photopolymerization of monomers allowed to diffuse into the CaAlg beads.[32]

We recently described polyelectrolytes bearing complementary reactive groups that underwent a covalent cross-linking

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reaction to produce a cross-linked PEC coating on CaAlg capsules.[33] In contrast to photochemical cross-linking approaches, the described cross-linking by condensation reaction occurs spontaneously upon contact.

These materials and their cross-linking reaction might also be used to reinforce the core of the capsules. This paper describes the addition of the reactive polyanion, poly(methacrylic acid, sodium salt-*co*-2-[methacryloyloxy]ethyl acetoacetate), p(MAANa-*co*-MOEAA), 70:30 A70, to the CaAlg core followed by exposure to PLL. This could lead to two scenarios, depending on the relative ability of the two reactive polyelectrolytes to diffuse through the CaAlg matrix: (1) Larger PLL chains (MW ≥ 15−30 kDa) should penetrate no further than 30 μm into the composite bead, the norm for APA-type capsules,[33] covalently cross-linking with the A70 chains that they encounter. If the A70 present in the core has some mobility in the CaAlg, it should diffuse to the capsule surface to further reinforce the shell, which should have a thickness largely defined by the penetration depth of the PLL. (2) Smaller PLL chains (MW < 15 kDa) should be able to diffuse into the hydrogel bead, and react with A70 at deeper levels. Given appropriate concentrations and stoichiometry of the polyelectrolytes, it might be possible to form a covalently cross-linked network throughout the core.

The potential benefits of this counter-diffusion of the two reactive polyelectrolytes include a different control mechanism over wall thickness and location, compared to the usual sequential layer-by-layer deposition of polyelectrolytes. The first scenario described above would lead to covalently cross-linked analogs to the conventional APA capsules. The second scenario should lead to covalently core-cross-linked beads for which loss of alginate due to calcium−sodium exchange or oxidative breakdown[34] would be less critical. A third scenario, sequential exposure of the composite beads to both high and low MW PLL, may permit separate control over shell and core cross-linking. In all cases the beads would receive a final coat of alginate or other polyanions.

Interaction of the synthetic polyanion, A70, 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 investigated. The internal morphology, mechanical strength, permeability of the capsules, and the viability of encapsulated murine cells are described.

Experimental Section

Materials. Sodium alginate (Keltone LV) was a gift from the Nutrasweet Kelco Company (San Diego, CA). Methacrylic acid (MAA, 99%), 2-[methacryloyloxy]ethyl acetoacetate (MOEAA, 95%), fluorescein O-methacrylate (97%), poly-L-lysine hydrobromide (PLL, M_n = 15−30 kDa, 4−15 kDa and 1−4 kDa), fluorescein isothiocyanate (FITC, 90%), FITC-conjugated bovine serum albumin (BSA-FITC, M_n = 66 kDa), FITC-conjugated dextran (dextran-FITC, M_n = 10, 70, 150, 250, and 500 kDa), rhodamine B isothiocyanate (RITC, mixed isomers), 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES, 99%), and trypan blue stain (0.4 wt % in 0.85% saline) were purchased from Sigma-Aldrich, Oakville, ON, and were used as received. 2,2′-Azobis(isobutyronitrile) (AIBN, 99.95%) was purchased from Dupont (Mississauga, ON) and used as received. Sodium chloride (reagent), sodium nitrate (reagent), *N*,*N*-dimethylformamide (DMF, reagent), and anhydrous ethyl ether were obtained from Caledon Laboratories Ltd. (Caledon, ON). Calcium chloride (Fisher), trisodium citrate dihydrate (Analar, EMD Chemicals, Gibbstown, NJ), and sodium dihydrogen orthophosphate (BDH, ON) were used as received. Ethanol from Commercial Alcohols (Brampton, ON) and serum-free media (SFM) from Gibco (Mississauga, ON) were used as received. Sodium hydroxide and hydrochloric acid solutions were prepared from concentrates (Anachemia Chemical, Rouses Point, NY) by diluting to 0.100 or 1.00 M with deionized water. The preparation of A70, its FITC-labeled analog A70*f* and poly(methacrylic acid, sodium salt) (A100) were described previously.[33]

Synthesis of Fluorescent Version of PMAANa, A100f. MAA (1.91 g; 22 mmol), fluorescein O-methacrylate (89.7 mg; 0.22 mmol), and AIBN (73.6 mg, 0.447 mmol) were dissolved in 18 mL of ethanol and heated at 60 °C for 21 h. The polymer was isolated by precipitation in ethyl ether (200 mL), washed three times with 50 mL of ethyl ether to remove the unreacted fluorescein methacrylate, and dried to a constant weight under vacuum at 50 °C. Yield: 1.36 g (68%). The fluorescence labeling of A100*f* was determined to be 0.42 mol % based on total monomer groups by UV/vis spectroscopy.

Poly(methacrylic acid, sodium salt-*co*-2-[methacryloyloxy]ethyl acetoacetate) (p(MAA-*co*-MOEAA), 70:30 (A70) of Different Molecular Weights. A70 with different MWs were obtained by copolymerization of MAA and MOEAA in ethanol at 60 °C, with isolation by precipitation into ethyl ether, as described previously.[33] Reported reactivity ratios of MOEAA and methyl methacrylate (MMA) in toluene of about 1 and 1,[35] and of MAA to MMA (in *i*PrOH) of 0.33 and 0.78[36] suggest there should not be an unacceptable drift of copolymer composition from the initial comonomer feed ratio, especially given the low percentage of MOEAA of 30%. Monomer to initiator (AIBN) ratios of 20:1, 100:1, and 800:1 were used and resulted in A70 of 22, 42, and 149 kDa, respectively. Fluorescein-labeled versions of A70-22k, A70-42k, and A70-149k were prepared using FITC, as described previously,[33] and resulted in degrees of labeling of 0.22, 0.34, and 0.32 mol % of the total monomer units, respectively, determined by UV/vis spectroscopy. The polymers were neutralized with 1 M NaOH to form the sodium salt of A70.

Rhodamine-Labeled Poly-L-lysine (PLLr). PLL with MW of 1−4 kDa, 4−15 kDa, or 15−30 kDa (55.5 mg, 0.265 mmol of lysine·HBr units) was dissolved in 5 mL of 0.1 M $NaHCO_3$ buffer solution at pH 9 in a 20 mL glass vial. RITC (2.7 mg, 0.005 mmol) dissolved in 0.5 mL of DMF was added to the PLL solution and the mixture was stirred for 1 h at 20 °C. The resulting solution was dialyzed for at least 120 h against deionized water using cellulose tubing (Spectrum Laboratories, 3.5 kDa MW cutoff for 4−15 and 15−30 kDa PLL and 1 kDa MW cutoff for 1−4 kDa PLL), with daily water changes. The dialyzed polymer solutions were freeze-dried, and the polymers were dried further to constant weight in a vacuum oven at 50 °C. Final yields of isolated, labeled polymers were 10% for the 1−4 kDa, 56% for 4−15 kDa, and 40% for the 15−30 kDa PLL*r*, with degrees of labeling of 0.76, 0.77, and 0.62 mol %, respectively.

Characterization. Molecular weights of A100*f*, A70 and dextran-FITC samples were determined by gel permeation chromatography (GPC) with a system consisting of a Waters 515 HPLC pump, Waters 717 plus Autosampler, three Ultrahydrogel columns (0−3, 0−50, 2−300 kDa), and a Waters 2414 refractive index detector. Samples were eluted with a flow rate of 0.8 mL/min and the system was calibrated with commercially available narrow dispersed molecular weight polyethylene glycol (PEG) standards (Waters, Mississauga, ON).

Dextran-FITC samples were eluted with 0.1 M $NaNO_3$, while for A100*f* the mobile phase was 0.3 M $NaNO_3$ in 0.05 M phosphate buffer (pH 7). A100*f* was prepared for GPC analysis by the addition of a stoichiometric amount of 1 M NaOH to the MAA-containing precursor polymer followed by dilution with the mobile phase.

Polymer compositions were determined by ^{1}H NMR using a Bruker AV 200 spectrometer for samples dissolved in DMSO-d_6.

Preparation of Ca(A/A70) Composite Beads. The Ca(A/A70) beads were prepared following the procedure described by Ross et al.[37] Sodium alginate (0.045 g), and A70 or A70*f* (0.015 g) were dissolved in 3.0 g saline (0.9% NaCl) to form a solution containing 1.5 wt % sodium alginate and 0.5 wt % A70 or A70*f*. The pH was adjusted to 7 with 0.1 M NaOH. The solutions were filtered with sterile filters (0.45 μm, Acrodisc Syringe Filter, Pall Corporation, U.S.A.). A syringe pump

(Rassel Mechanical Inc. pump, model #A-99) was used to extrude this solution through a 27-gauge blunt needle (Popper & Sons, New York) at a rate of 30.1 mL/h. A concentric airflow (4 L/min) passing by the needle tip is used to induce droplet formation. The droplets were collected in 30 mL of a 1.1 wt % calcium chloride and 0.45 wt % sodium chloride gelling bath. A total of 20 min after bead formation was complete, the supernatant was removed, and the resulting concentrated Ca(A/A70) composite bead suspension (about 3 mL) was washed in sequence with 4-fold volumes of (a) 1.1 wt % $CaCl_2$, 0.45 wt % NaCl for 2 min; (b) 0.55 wt % $CaCl_2$, 0.68 wt % NaCl for 2 min; (c) 0.28 wt % $CaCl_2$, 0.78 wt % NaCl for 2 min; (d) 0.1 wt % CHES, 1.1 wt % $CaCl_2$, 0.45 wt % NaCl for 3 min; and then (e) 0.9 wt % NaCl for 2 min and stored in saline.

Analogous cell-containing composite capsules were prepared by preparing a core solution containing 1.5 wt % sodium alginate, 0.5 wt % A70, and 2 million C_2C_{12} cells per mL of saline. This suspension was extruded to form capsules as described above, except for the use of an Orion sage pump, model #M362 located in a sterile laminar flow hood, and a liquid flow rate of 99.9 mL/hour. The cell containing capsules were washed as above and stored in serum-free media at 4 °C for further use.

Coating of Ca(A/A70) Composite Beads with Poly-L-lysine and Sodium Alginate To Form (A/A70)PA Capsules (Typical Procedure). A dense suspension of Ca(A/A70) composite beads (3 mL) was exposed to 10 mL of 0.05 or 0.5 wt % PLL (pH = 8, saline) for 6 min and washed once each with 12 mL of (a) 0.1 wt % CHES, 1.1 wt % $CaCl_2$, and 0.45 wt % NaCl for 3 min; (b) 1.1 wt % $CaCl_2$, 0.45 wt % NaCl for 2 min; and (c) 0.9 wt % saline for 2 min. The resulting (A/A70)P capsules were then coated with 10 mL of 0.03 wt % sodium alginate in saline for 4 min, followed by three washes with 12 mL of 0.9 wt % saline. The final (A/A70)PA composite capsules were stored in the last saline solution.

Capsule Characterization. Capsules and PECs were examined with an Olympus BX-51 optical microscope fitted with a Q-Imaging Retiga EXi digital camera and ImagePro software. The average diameters of the beads and capsules were determined by analyzing three batches of approximately 50−100 beads or capsules each.

Phase contrast microscope images were taken using a Wild M40 microscope, and confocal images were taken with a ZEISS LSM 510 confocal laser scanning microscope (CLSM) fitted with air-cooled Argon and HeNe lasers (LASOS; LGK 7628-1) and running LSM Image browser software (version 3.5).

Chemical and Mechanical Stress Test. Dense microcapsule suspensions in saline (100 μL) were placed in 15 mL of polypropylene conical tubes and exposed to 5 wt % (170 mM, 5 mL) sodium citrate for 5 min, followed by exposure to 5 mL of 2 M sodium chloride. The tubes were attached flat to a wheel tilted at 30° from horizontal and rotated at 30 rpm for 15 min at room temperature. The capsules were then washed with water, stained with trypan blue, and observed by optical microscopy.

Cell Culture. The cell line used was the C_2C_{12} cell line (American type Culture Collection [ATCC], Rockville, MD; catalog No. CRL-1772). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY) and 100 U/mL penicillin−100 μg/mL streptomycin (Gibco, Grand Island, NY) in the presence of 5% CO_2 with 100% humidity at 37 °C in a water-jacketed incubator.

Cell Viability. The number of viable cells per capsule was determined with an Alamar Blue assay.[38] A total of 100 μL of capsules were loaded in a 24-well plate with 500 μL media. A 50 μL aliquot of Alamar Blue reagent was added to each sample and the plate was incubated at 37 °C for 4 h. After incubation, 100 μL of supernatant was taken from each well and placed in a microtiter plate. The fluorescence of each sample was read with a Cytofluor II fluorimeter, with an excitation wavelength of 530 nm and an emission wavelength of 590 nm. The number of viable cells per capsule was determined by comparing the fluorescence intensity with a standard curve generated from a known number of cells.

The effect of the encapsulation procedure itself on cell viability has been measured in our laboratory a number of times for APA capsules by crushing freshly formed capsules, staining them with trypan blue, and counting both live and dead cells. On day 1, the percentage of live cells is consistently above 95%. The number of cells/capsule can, hence, be compared to that of APA, assuming similar capsule volumes, to get a measure of the viability in the new capsules.

Permeability Measurements. Capsule permeability was evaluated using dextran-FITC or BSA-FITC. The procedure with dextran-FITC was a modified version of a published procedure[39] and employed samples having nominal MWs of 10, 70, 150, 250, and 500 kDa. In the case of dextran-FITC, the capsules (0.2 g) were suspended in saline (0.2 mL) and exposed to 1.0 mL of 0.0015 or 0.05 wt % dextran-FITC in saline for 24 h at room temperature. When BSA-FITC was employed, the capsules (0.5 g) were suspended in a minimum amount of saline (~0.1 mL), exposed to 5 mL of 0.05 wt % BSA-FITC for 24 h at room temperature, and then washed 5 times with saline (5 mL) to remove free protein.

The microcapsules were then examined by CLSM. A microcapsule suspension (100 μL) was placed on a microscope slide within a Teflon ring (7 mm diameter, 3 mm depth) and images were obtained at the capsule equator. Intensity profiles were obtained from the CLSM images with a 25-pixel wide line using UTHSCSA Image Tool software (version 3.0).

Results and Discussion

The aim of this work is to explore covalent reinforcement of CaAlg beads with a cross-linked interpenetrating network formed by reaction between a polyanion present in the core, and a polyamine diffusing in from the outside. We had shown earlier that aqueous solutions of electrophile-containing polyanions such as A70 form cross-linked PECs upon contact with polyamines, such as PLL, due to the facile reaction between the acetoacetate groups on the polyanion and the primary amines on the polycations.[33,40,41] We also showed that this reaction can be used to form persistent, cross-linked shells around CaAlg beads.[33,40]

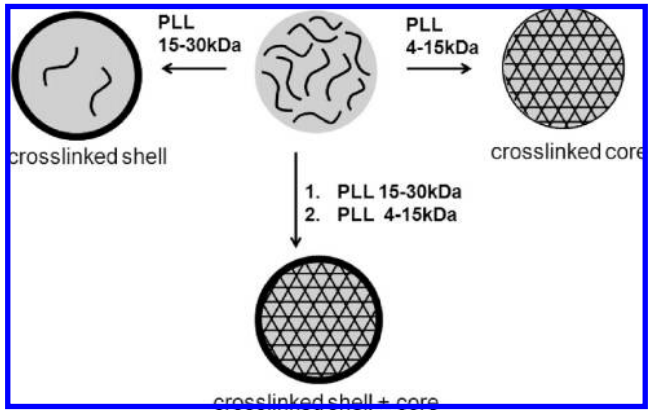
Here, we explore the ability of these polyelectrolytes to form cross-linked networks throughout the bead, leading to formation of a permanent three-dimensional support structure for cell transplantation and other uses.

This approach involves adding the reactive polyanion, A70, to the sodium alginate solution, prior to gelling with calcium chloride. The resulting primary CaAlg beads thus contain A70 homogeneously distributed throughout. Subsequent exposure of these beads to aqueous solutions of PLL should lead to formation of cross-linked A70−PLL networks, with morphologies dependent on the MWs and mobilities of the two polyelectrolytes used.

The commonly used 15−30 kDa PLL should not be able to penetrate the CaAlg matrix more than a few tens of micron[33,40,42] and, hence, should result in formation of a thin cross-linked shell similar to those formed in the conventional layer-by-layer approach.[33,40] On the other hand, use of lower MW PLL should facilitate diffusion into the beads, resulting in thicker cross-linked layers or even core-cross-linked networks, depending on polyelectrolyte stoichiometry and concentrations.

The MW and mobility of the A70 is similarly expected to play an important role. The mobility of A70 inside the CaAlg should depend both on its MW and on its calcium binding strength. Accordingly, PLL and A70 with a range of MWs have been explored.

Scheme 1. Capsule Morphologies Formed when Embedding Reactive Polyanion within the CaAlg Capsule, Followed by Reaction with High MW PLL, Low MW PLL, and Sequentially with High and Low MW PLL



Scheme 2. Natural and Synthetic Polyelectrolytes Used in this Study

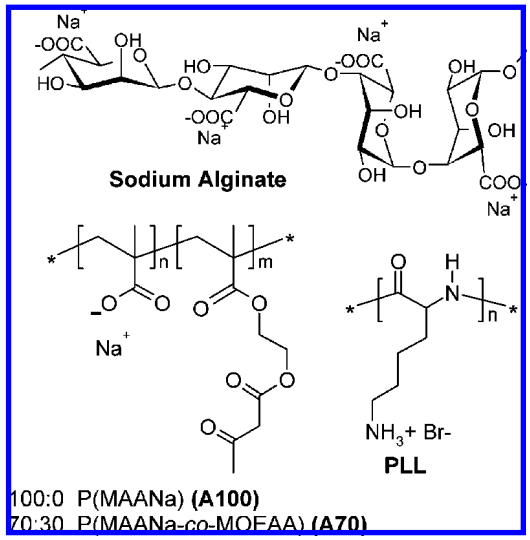


Table 1. Polymer Properties

polymer	M_n (kDa)/PDI[a]	MAA/MOEAA[b]
A100	38/2.6	
A100*f*	29/2.8	
A70-22k	22/3.1	70:30 (±3)
A70-42k	42/2.4	70:30 (±3)
A70-149k	149/1.7	70:30 (±5)

[a] M_n and polydispersity index (PDI) determined by GPC. [b] Copolymer composition in mol %, determined by ^{1}H NMR.

The synthetic polyanions are described in Scheme 2 and Table 1. Monomer to initiator ratios of 20:1, 100:1, and 800:1 were used to obtain A70 having number average MWs of 22, 42, and 149 kDa, respectively. Attempts to prepare higher MW polymer resulted in gelation, attributed to covalent cross-linking during polymerization. Fluorescently-labeled versions of the polymers were prepared by reaction with FITC (A70*f*) or RITC (PLL*r*) or via copolymerization with fluorescein O-methacrylate (A100*f*). Unless specifically noted, A70 or A70*f* with a MW of 42 kDa was employed.

(A/A70)PA Composite Capsules. As the initial step in the capsule formation would be the gelation of the alginate/polyanion mixture in $CaCl_2$, model experiments were carried out to understand the interaction between $CaCl_2$ and both A70 and A100, in comparison with CaAlg.

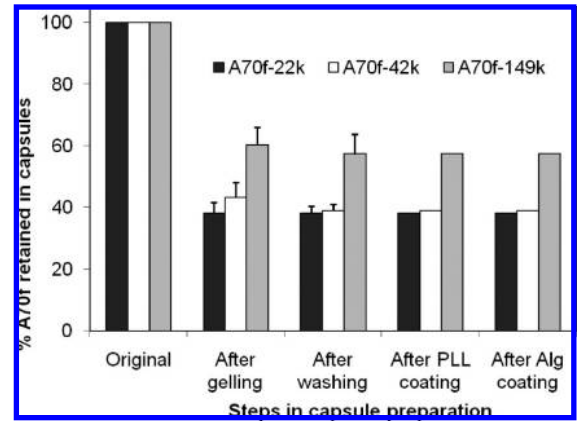


Figure 1. Percentage of A70*f* remaining in the composite microcapsules at different stages of the capsule preparation. Error bars show the standard deviation for select experiments performed in triplicate.

CaAlg is a solid gel that can resist moderate mechanical stresses, while A100 was found to form a liquid coacervate in presence of $CaCl_2$. A70 (22, 42, or 149 kDa) showed no macroscopic phase separation in the presence of $CaCl_2$, suggesting that these complexes may retain some mobility even within CaAlg beads.

CaAlg beads containing the synthetic polyanions were prepared by dripping saline solutions containing 1.5 wt % sodium alginate and 0.5 wt % polyanion, adjusted to pH 7, into a $CaCl_2$ bath. The resulting composite beads were exposed to 0.05 wt % PLL (15−30 kDa), washed with saline, and then coated with a 0.03 wt % sodium alginate solution. The final capsules had an average diameter of 650 μm and appeared identical to the initial Ca(A/A70) beads, but their surface was easily stained by trypan blue, indicating the presence of the polycation. In addition, the surface appeared pink when PLL*r* was used.

To determine the fraction of A70 trapped in the capsules, the supernatant solutions used during the preparation of capsules containing A70*f* were analyzed by UV−vis spectroscopy. This showed that 60 ± 5% of the original A70*f*-22k or A70*f*-42k, and 40 ± 5% of the A70*f*-149k were lost from the beads, predominantly to the gelling bath (Figure 1). No additional A70*f* loss was observed during the subsequent PLL coating process. Uncoated Ca(A/A70*f*) beads stored in a 6-fold excess of saline at 4 °C lost an additional 3% of the original A70*f* after 2 days and 16% after 3 months. In contrast, (A/A70*f*)PA capsules did not lose a significant amount of A70*f* to the supernatant over 8 months of storage.

Thus, A70*f* is lost principally in the gelation step during which the droplets were found to shrink to about 60% of their original volume, a value consistent with a number of other studies.[43,44] Core liquid is expelled from the gelling beads along with any polymer chains that are not physically entangled or ionically bound within the CaAlg gel. Use of higher MW A70 increases the retention of the polymer, possibly due to enhanced entanglement.

CLSM showed that A70*f* is initially homogeneously distributed within the Ca(A/A70*f*) beads (Figure 2a). Images obtained 1−2 h after coating these beads with PLL and sodium alginate (Figure 2b), show in addition a very thin outer shell, presumably formed by concentration of some of the A70*f* in the form of a PLL/A70*f* cross-linked shell.

When the coated capsules were treated with excess 170 mM (5 wt %) sodium citrate for 18 h to liquefy the CaAlg core, the

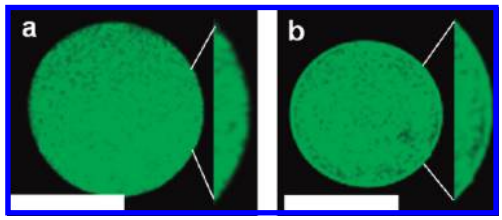


Figure 2. CLSM equatorial optical section of Ca(A/A70*f*) composite capsule: (a) uncoated and (b) coated with PLL (15−30 kDa; 0.05 wt %, 6 min) and then alginate (0.03 wt %, 4 min). Images were taken within 1−2 h of capsule preparation. The scale bar is 500 μm.

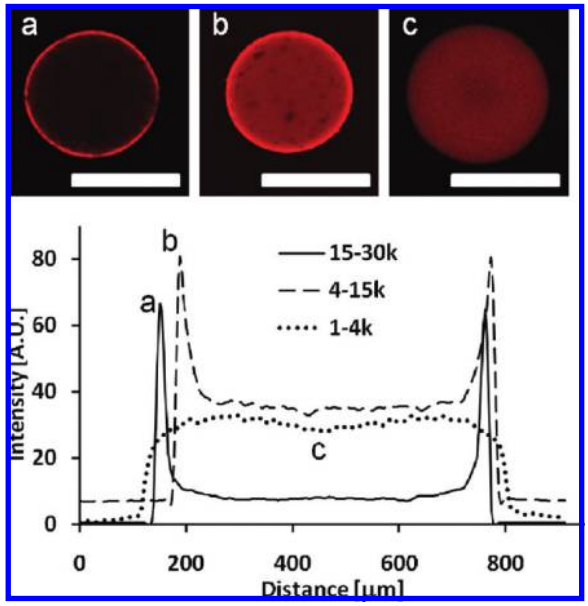


Figure 3. (Top) CLSM equatorial optical sections of (A/A70)PA capsules made with PLL*r* of (a) 15−30 kDa; (b) 4−15 kDa; and (c) 1−4 kDa. (Bottom) 25 pixel wide line profiles taken from the images. Coating conditions: PLL*r* (0.05 wt % in saline, 6 min); Alg (0.03 wt %, 4 min).

capsules retained the fluorescently labeled A70*f*, but swelled (40−50% diameter increase), indicating the absence of significant core cross-linking. Model studies subsequently suggested that formation of a cross-linked A70/PLL network requires equimolar or greater amounts of PLL relative to A70. Accordingly, fluorescently labeled PLL was used to track the in-diffusion of the polycation and determine the fraction that reaches the core of the beads.

CLSM images of capsules coated with 0.05% solutions of PLL*r* show that higher MW PLL*r* (15−30 kDa) is concentrated near the capsule surface, while the lowest MW PLL*r* (1−4k) is evenly distributed throughout the composite microcapsules (Figure 3a and c), in agreement with a number of earlier reports.[45−48] The intermediate MW PLL*r* (4−15 kDa) showed both formation of a distinct shell, and significant in-diffusion to the core of the bead (Figure 3b). The shell formed by 4−15 kDa PLL*r* was thicker (36 μm width at half-height of the line-out shown in Figure 3, bottom) than that formed by the 15−30 kDa PLL*r*, (23 μm), which is consistent with the deeper penetration expected of this intermediate MW polycation. Hence, reacting Ca(A/A70) beads with PLL of appropriate MW and concentrations, could lead to capsules that are reinforced both internally and externally.

The integrity of uncoated and PLL(15−30k)-coated Ca(A/A70) composite beads in the presence of sodium citrate and sodium chloride was examined by optical microscopy and

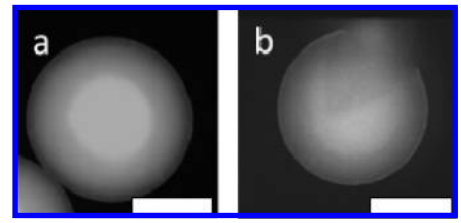


Figure 4. Fluorescence microscopy images (greyscale) of (A/A70*f*)P(4−15 kDa, 0.05 wt %) A(0.03 wt %) capsules: (a) as formed and (b) after being exposed to citrate (170 mM) and NaCl (2 M) and manually cut with a microknife. Scale bar: 300 μm.

compared with that of classical APA microcapsules. Uncoated beads composed of Ca(A/A70) or CaAlg dissolve when exposed to 170 mM (5 wt %) sodium citrate, a good calcium chelator. In contrast, addition of sodium citrate to PLL(15−30k)-coated capsules such as APA or Ca(A/A70)PA caused the core of the beads to dissolve, while the shells consisting of the PEC survived. One test for covalent cross-linking of the shell is to expose citrate-treated capsules to 2 M NaCl while vigorously agitating, which dissolves (in the case of 1−4 and 4−15k PLL) or noticeably weakens (in the case of 15−30k PLL) shells held together by just ionic interactions, compared to their cross-linked analogs. A better test, especially for the higher MW PLL, involves exposing the shells to 0.1 M sodium hydroxide, which neutralizes the ammonium ions of PLL leading to rapid and almost complete dissociation of all electrostatic PECs used here, as described earlier by Dusseault et al.[17]

Figure 4a shows a composite bead coated with 0.05 wt % PLL (4−15 kDa). After exposure to sodium citrate followed by 2 M sodium chloride, the capsule was manually cut (Figure 4b), revealing both a thin cross-linked shell and A70*f* diffusing out through the hole in the shell. The shell is self-supporting in saline, but it is clear that the core of the bead is not cross-linked, likely due to the presence of insufficient amounts of PLL. These capsules hence resemble the cross-linked-shell capsules prepared earlier using sequential exposure of CaAlg beads to polycation and A70,[33,40] except that the reactive A70 is here supplied from the interior of the capsule. The outer surface of the capsule, after coating with alginate, then resembles the conventional APA capsules.

Composite beads coated with low MW PLL (1−4 kDa, 0.05 wt %), which were shown in Figure 3c to have PLL*r* homogeneously distributed throughout the beads, dissolved within seconds upon exposure to sodium citrate (70 mM). This indicates that although this low MW PLL readily penetrates the interior of the beads, at the present concentration of 0.05% it is unable to cross-link the A70 to the extent necessary to give a cross-linked shell or core. The chains may be too short to effectively bridge between A70 chains. A sodium citrate concentration of 70 mM was found to be sufficient to extract calcium from both CaAlg and composite beads, and was used henceforth.

Core-Cross-linked (A/A70)PA Capsules. For Ca(A/A70) beads that retain roughly 40% of their original A70 loading, exposure to 0.05 wt % PLL corresponds to a ratio of cross-linking groups (amine/acetoacetate) of about 2:1. UV/vis analysis of a supernatant PLL*r* (15−30 kDa) solution after coating showed that only half of this PLL was actually absorbed by the capsules and, thus, the PLL-coated beads have an overall amine/acetoacetate ratio of approximately 1:1. However, much of this bound PLL is involved in electrostatic complexation and is concentrated in the dense shell at the surface as shown in Figure 3a. This indicates that the effective amine/acetoacetate

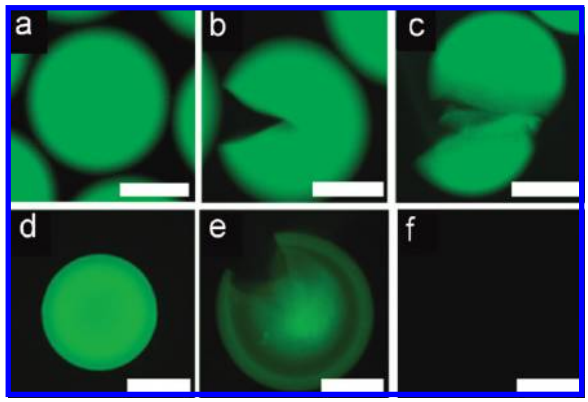


Figure 5. Fluorescence microscopy images of (a) (A/A70*f*)P(4−15k, 0.5 wt %)A(0.03 wt %) and (d) (A/A100*f*)P(4−15k, 0.5 wt %)A(0.03 wt %); (b,e) the beads were manually cut, and then exposed to excess 70 mM sodium citrate; (c,f) the beads after further treatment with 2 M NaCl. The scale bar is 300 μm.

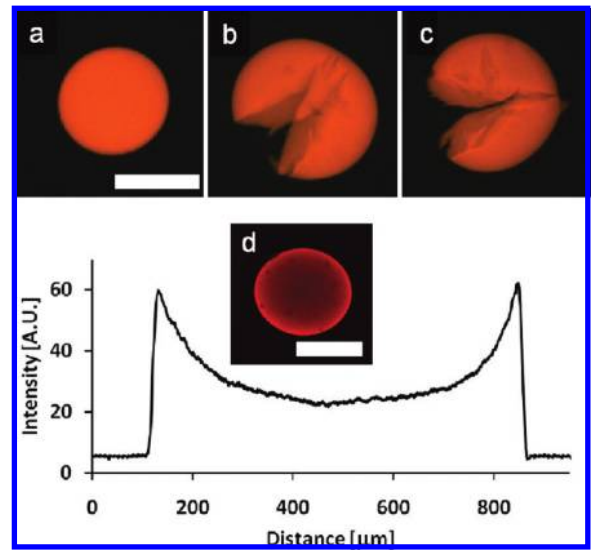


Figure 6. Fluorescence microscopy images of core-cross-linked (A/A70)PLL*r* (4−15k, 0.5 wt %)A (0.03 wt %) composite capsules to show the location of PLL*r* (4−15 kDa). (a) As formed; (b) after addition of excess 70 mM sodium citrate and crushing; and (c) following addition of excess 2 M NaCl. The scale bar is 500 μm. (d) CLSM middle optical section and intensity line profile of a capsule such as in (a).

ratio in the core is much lower and explains the absence of core-cross-linking in the resulting capsules.

Analysis of the in-diffusion patterns (Figure 3) indicated that the intermediate MW PLL (4−15 kDa) might represent a good compromise between ease of in-diffusion and a MW high enough to cross-link the A70 in the core, provided it is available in sufficiently high concentration to compensate for incomplete capture and preferential binding to the shell.

Accordingly, we explored increasing the PLL (4−15k) concentration from 0.05 to 0.5 and 1 wt %. Coating using 1% PLL solution resulted in wrinkling of the bead surface, while 0.5% PLL (4−15 kDa) resulted in smooth bead surfaces. Figure 5a shows (A/A70*f*)PA capsules coated with 0.5 wt % PLL (4−15k), followed by alginate (0.03 wt %). The resulting capsules were manually cut and exposed to 70 mM citrate (Figure 5b) and then 2 M sodium chloride (Figure 5c). The capsules undergo little swelling and there is minimal loss of A70*f*, demonstrating that sufficient PLL has diffused into the core to cross-link the bead throughout. The cross-linked beads also survived subsequent treatment with 0.1 M NaOH (not shown).

When the A70*f* (42 kDa) was replaced with A100*f* (35 kDa), rendering covalent cross-linking impossible, the resulting (A/A100*f*)P(4−15k, 0.5 wt %)A(0.03 wt %) capsules (Figure 5d) swelled considerably when exposed to 70 mM citrate for about 5 min. The outer layer, consisting of an electrostatic complex of PLL with alginate and A100*f*, appears to swell more than the inner core, revealed when the shell is cut (Figure 5e). Subsequent exposure to 2 M sodium chloride completely dissolved both shell and core within three minutes (Figure 5f), confirming again that the permanent structure shown in Figure 5a−c for the A70-containing capsule is indeed based on covalent cross-linking.

Interesting is the location of the PLL because it will be important for both cross-linking and biocompatibility. Accordingly, Ca(A/A70) beads were coated with 0.5 wt % PLL*r* (4−15 kDa) and then examined by fluorescence microscopy and CLSM (Figure 6). As observed in Figure 5, capsules exposed to citrate and manually crushed, followed by the addition of 2 M NaCl underwent only some swelling and showed minor loss of PLL*r*, confirming the role of PLL in the covalent cross-linking of both shell and core. The presence of a distinct PLL*r* shell in addition to core cross-linking suggests that the higher MW fraction of PLL*r* (4−15 kDa) is limited to forming a surface network, while the lower MW fraction can diffuse into the core to cross-link with A70. The presence of the distinct shell in Figure 6c, after exposure to 2 M NaCl, indicates that it does not involve electrostatic binding of excess PLL to alginate, but rather covalent bonding, to A70.

CLSM analysis of a (A/A70)PLL*r* (4−15k, 0.5 wt %)A (0.03 wt %) capsule such as that shown in Figure 6a shows the concentration of PLL falling more gradually moving from shell to core (Figure 6d), compared to the analogous beads coated with 0.05 wt % PLL*r* (4−15 kDa) shown in Figure 3b. The width at half-height is now close to 100 μm. The confocal image in Figure 6d was taken at reduced gain settings compared to Figure 3b to avoid saturating the CCD detector.

Gaserod et al.[47] had reported that presence of a competing cation such as calcium can enhance in-diffusion of chitosan, but not of PLL, into CaAlg beads. We similarly found that exposure of the Ca(A/A70) beads to PLL*r* (4−15 kDa, 0.5 wt %) in 1.1 wt % $CaCl_2$ and 0.45 wt % NaCl instead of the standard 0.9% saline, did not lead to significantly increased in-diffusion of PLL (images not shown).

The capsule shell plays important roles in permeation and biocompatibility and, thus, it may be advantageous to be able to carry out shell formation independent of the core-cross-linking process. To test the scope for separately controlling core-cross-linking and shell formation, CaAlg beads were sequentially coated with two PLL solutions of different MWs, a variation of the approach described by Prokop et al.[24] for coating simultaneously with a blend of low and high MW polycations. The expectation was that sequential coating would ensure that the higher MW PLL could coat the outside of the bead, without competition from the more mobile low MW PLL. Subsequently, the lower MW PLL would be able to diffuse through the outer PEC shell to cross-link inner regions not accessible to the high MW PLL. Hence, Ca(A/A70*f*) beads were first exposed to 0.05 wt % PLL (15−30k) for 1 min, followed without washing by another exposure to 0.5 wt % PLL (4−15k) for 6 min, and after a saline wash step, by the usual final coat with 0.03 wt % Alg for 4 min. The resulting capsules, after manual cutting and exposure to citrate and 2 M NaCl, show both the presence of a

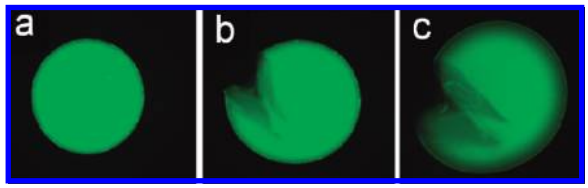


Figure 7. Fluorescence microscopy images of (A/A70*f*)PA capsule prepared by exposure to both high and medium MW PLL. Ca(A/A70*f*) composite beads were coated with 0.05 wt % PLL (15−30k; 1 min), and then with 0.5 wt % PLL (4−15k; 6 min), followed by 0.03 wt % Alg (4 min). (a) As formed; (b) after challenge with citrate and manual cutting; (c) after exposure to excess 2 M NaCl. The scale bar is 300 μm.

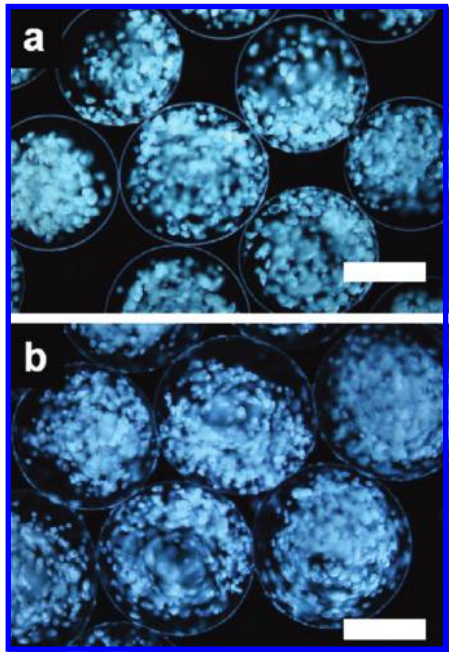


Figure 8. Phase contrast microscope image of C_2C_{12} mouse cells 7 days after being encapsulated in (a) APA and (b) (A/A70)PA microcapsules (coating conditions: PLL (15−30 kDa, 0.05 wt %)/6 min; Na-Alg (0.03 wt %)/4 min). Scale bar: 500 μm.

distinct outer shell formed by reaction of the higher MW PLL with A70*f* near the surface, and core-cross-linking between the lower MW PLL and A70*f* in the core (Figure 7a−c). In contrast, the capsules prepared using only 0.5 wt % PLL (4−15k) did not show a distinct outer shell (Figure 5c). This demonstrates the ability of the two-stage approach to give some independent control over shell and core cross-linking and may enable tuning of the MW cutoff as required for specific cell immuno-isolations.

In Vitro Cell Viability. C_2C_{12} mouse cells were encapsulated in APA capsules, the shell-cross-linked (A/A70)P(15−30k, 0.05 wt %)A capsules and the core-cross-linked (A/A70)P(4−15k, 0.5 wt %)A capsules. Cell-containing APA and shell-cross-linked (A/A70)P(15−30k, 0.05 wt %)A capsules are shown in Figure 8. The capsules were cultured in vitro for 1 week and the numbers of living cells per capsule were determined with the Alamar Blue assay. Note that the cell viability tests on the two new types of capsules were performed at different times each with an APA control, to take into account variables affecting cell growth that are unrelated to the presence of the new materials. Figure 9a shows that the average live cell numbers in these shell-cross-linked capsules are similar to those in APA capsules over the week long incubation, indicating that the A70 in the core of the (A/A70)PA capsules is not detrimental to cell viability.

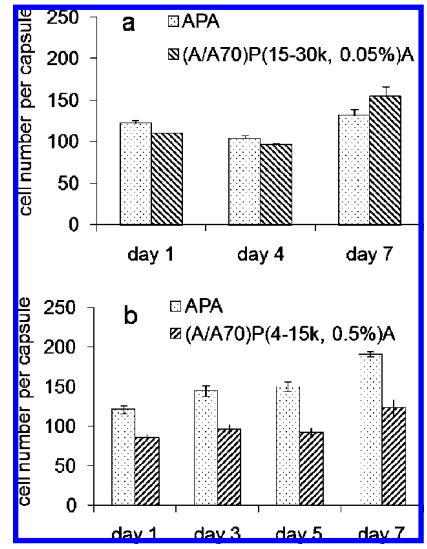


Figure 9. In vitro cell viability of C_2C_{12} cells encapsulated in APA and (A/A70)PA capsules over time. (a) APA and shell-cross-linked (A/A70)P(15−30k, 0.05 wt %)A capsules and (b) APA and core-cross-linked (A/A70)P(4−15k, 0.5 wt %)A capsules. Error bars show the standard deviation. Coating conditions: APA, PLL(15−30k, 0.05 wt %, 6 min), Alg(0.03 wt %, 4 min); shell-cross-linked (A/A70)PA, PLL(15−30k, 0.05 wt %, 6 min), Alg(0.03 wt %, 4 min); core-cross-linked (A/A70)PA, PLL(4−15k, 0.5 wt %, 6 min), Alg(0.03 wt %, 4 min).

The cell viability results for the core-cross-linked (A/A70)PA capsules are shown in Figure 9b. The APA capsules show higher cell numbers throughout the incubation although similar relative increases in cell numbers (50−60%) are seen for the two types of capsule. Comparison with the higher cell numbers observed in the case of analogous shell-cross-linked capsules prepared using only 0.05 wt % PLL(15−30 kDa) (Figure 9a) suggests that the lower initial cell viability in the present capsules is due to the larger amount of lower MW PLL used.

It was initially surprising that the diffusion of PLL into the capsule core did not have a more negative effect on cell viability. The preferred location of PLL*r* (4−15 kDa) in the shell, compared to the homogeneous distribution of A70*f* (Figure 5) obviously reflects the fact that PLL is applied from the outside, while A70 is found throughout the core. PLL in the bead core may exhibit reduced toxicity toward the encapsulated cells, as verified by the data in Figure 9b, because most of the PLL diffusing in from the outside should rapidly react with the A70 present throughout the core. Any residual unreacted PLL would likely be complexed by alginate, and thus rendered much less cytotoxic, as reported previously for the complexes formed between polystyrenesulfonate and polycations.[49]

On the other hand, the presence of excess PLL near the surface may require more than the standard final coating with 0.03 wt % alginate to prevent undesirable interactions with the host. One option will be to apply a coat of A70 to covalently bind this surficial PLL.

MW Cutoff of APA and Shell-Cross-Linked Microcapsules. It is important that capsules containing cells allow the diffusion of nutrients and metabolites, while preventing the ingress of components of the host immune system. The good cell viabilities observed for both shell- and core-cross-linked capsules demonstrate reasonable permeability of nutrients into the capsules. The ideal MW cutoff of the microcapsule membrane is one that provides immunoprotection of transplanted cells while allowing release of therapeutic protein. This will

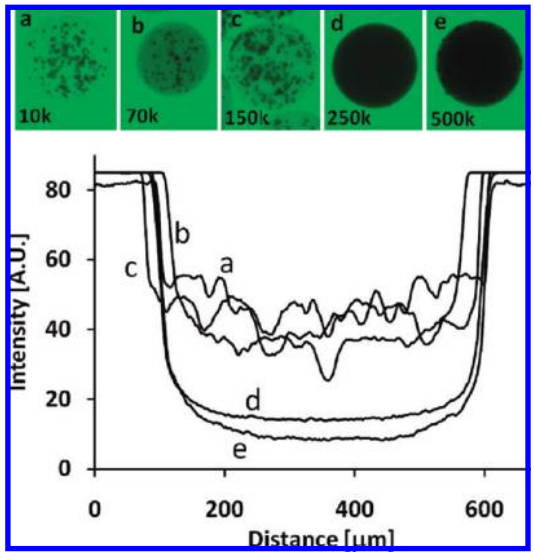


Figure 10. (Top) CLSM middle sections of cell containing (A/A70)P(4−15k, 0.5 wt %)A(0.03 wt %) capsules exposed for 24 h at room temperature to 0.05 wt % dextran-FITC with nominal MWs of (a) 10k, (b) 70k, (c) 150k, (d) 250k, and (e) 500k. Coating conditions: PLL (0.5 wt %, 6 min); Alg (0.03 wt %, 4 min). (Bottom) Line profile from images as above.

depend on the transplant type, with values of about 100−200 kDa commonly considered to be suitable for allografts.[50,51]

The MW cutoff of these new shell- and core-cross-linked capsules was estimated using a series of commercial dextran-FITC samples with nominal MWs of 10, 70, 150, 250, and 500 kDa.[39,52] GPC analysis showed that the samples had broad MW distributions, with polydispersity indices of 4.7 for the 150 kDa sample and approximately 2 for the other samples, and thus in-diffusion of the low MW fraction may occur with each of the samples. In addition, dextran may behave differently than globular proteins in solution, and as such, the use of dextran-FITC provides only a rough indication of the MW cutoff.

Shell-cross-linked (A/A70)P(15−30k, 0.05 wt %)A capsules exposed to 0.0015 wt % dextran-FITC solutions for 24 h and then examined by CLSM showed increasing in-diffusion with decreasing MW (data not shown). Similarly, core-cross-linked (A/A70)P(4−15k, 0.5 wt %)A capsules containing C_2C_{12} mouse myoblast cells exposed to dextran-FITC (0.05 wt %) for 24 h (Figure 10) showed that dextrans of 500 and 250 kDa are almost completely excluded, while dextrans having MW's of 10 and 70 kDa can diffuse in freely. At least some fraction of the polydisperse 150 kDa dextran also diffuses into the capsules (Figure 10c). Both the shell- and core-cross-linked capsules have MW cut-offs that are roughly 100−200 kDa, similar to APA capsules (data not shown), and in line with data reported for other capsules.[31,53−56] Work is in progress to find additional means of determining the MW cutoff and to explore decreasing this MW cutoff by varying the A70/PLL ratio, and adding additional layers of reactive polymers.

The permeability of APA and shell-cross-linked (A/A70)P-(15−30k)A microcapsules containing cells was also assessed by looking for the uptake of BSA-FITC (MW 66 kDa) (Figure 11). Both types of microcapsules were permeable to BSA-FITC, indicating a MW cutoff greater than 70 kDa, consistent with the dextran-FITC results.

Close inspection of Figure 11 reveals that some cells protrude from the surfaces of both APA and shell-cross-linked composite capsules, making them liable to recognition and attack by the host's immune system. This concern has been recognized and

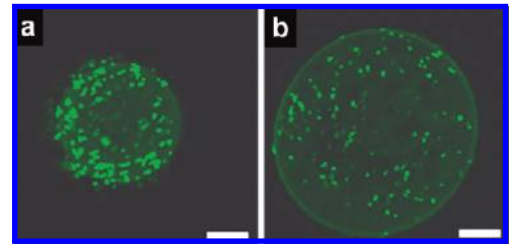


Figure 11. CLSM equatorial optical sections of (a) APA and (b) (A/A70)P(15−30k, 0.05 wt %)A microcapsules exposed to BSA-FITC. The scale bar is 200 μm.

can be addressed by using a double coaxial encapsulation approach that deposits a second, cell-free shell of CaAlg over the cell-containing capsules. In the case of core-cross-linked beads, the inner matrix could provide continued immuno-isolation in capsules whose outer shell has been breached by such surficial cells.

Research is in progress to optimize the amounts of A70 and PLL in such capsules and to explore their strength and host compatibility.

Conclusion

We have shown that internally and externally cross-linked networks can be formed in CaAlg beads by inclusion of A70 in conventional APA microcapsule cores. The distribution of the polyelectrolytes in the microcapsules was studied using fluorescently labeled analogs. While a portion of the A70 was lost during gelling, the remainder was distributed evenly throughout the beads. The depth of PLL penetration was controlled by varying the PLL MW, which allowed the formation of capsules in which just the shell or both the shell and core were covalently cross-linked. The resistance of these capsules to citrate and high ionic strength demonstrated the cross-linked nature of the core as well as the shell. The use of two MWs of PLL can give independent cross-linking of core and shell, which should give access to core-cross-linked capsules with shells exhibiting MW cut-offs suitable for cell encapsulation. Murine C_2C_{12} cells were viable within CaAlg composite capsules.

This study describes a promising approach to cell encapsulation and may ultimately be useful for immuno-isolation in cell-based therapies. Research is in progress to optimize the amounts of A70 and PLL in these capsules, and to explore their strength and host compatibility. Studies of the in vivo use of these and related capsules are being conducted and will be reported shortly.

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