# Self-Cross-Linking Polyelectrolyte Complexes for Therapeutic Cell Encapsulation

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Self-cross-linking polyelectrolytes are used to strengthen the surface of calcium alginate beads for cell encapsulation. Poly([2-(methacryloyloxy)ethyl]trimethylammonium chloride), containing 30 mol % 2-aminoethyl methacrylate, and poly(sodium methacrylate), containing 30 mol % 2-(methacryloyloxy)ethyl acetoacetate, were prepared by radical polymerization. Sequential deposition of these polyelectrolytes on calcium alginate films or beads led to a shell consisting of a covalently cross-linked polyelectrolyte complex that resisted osmotic pressure changes as well as challenges with citrate and high ionic strength. Confocal laser fluorescence microscopy revealed that both polyelectrolytes were concentrated in the outer 7−25 $\mu$m of the calcium alginate beads. The thickness of this cross-linked shell increased with exposure time. GPC studies of solutions permeating through analogous flat model membranes showed molecular weight cut-offs between 150 and 200 kg/mol for poly(ethylene glycol), suitable for cell encapsulation. $C_2C_{12}$ mouse cells were shown to be viable within calcium alginate capsules coated with the new polyelectrolytes, even though some of the capsules showed fibroid overcoats when implanted in mice due to an immune response.

## Introduction

Cell encapsulation is a process by which live cells are entrapped within semipermeable matrices or membranes for biomedical applications.[1–6] Cells to be encapsulated can be designed to express therapeutic proteins.[7,8] As their use involves implantation into the host's body, often the peritoneal cavity, nonautologous cells require encapsulation that isolates them from the host's immune system, while permitting metabolic exchange and release of therapeutic proteins.[9] These capsules need to be compatible with both host and implanted cells and should not degrade in vivo. This requires capsule walls with molecular weight (MW) cut-offs of about 150 kDa, and ideally some form of cross-linking to strengthen the shell against biochemical and mechanical degradation.

The most common approach to such cell-encapsulation for immuno-isolation involves alginate-poly(L-lysine)-alginate (APA) microcapsules, as first described by Lim and Sun.[9] Alginate is a natural polysaccharide consisting of $\beta$-D-mannuronic acid (M) and α-L-guluronic acid (G) residues (Scheme 1) that can be ionically cross-linked by calcium ions through the G-rich regions of the alginate chains. These beads are coated with poly(L-lysine) (PLL) to strengthen the outer bead surface and control permeability,[10] followed by coating with a final layer of alginate to hide the cationic PLL from the host[4] and, thus, make the capsules biocompatible. While these APA capsules meet many of the requirements for immuno-isolation of cells implanted into mice,[11] they have shown insufficient strength when implanted into larger animals such as dogs and, presumably, humans.[12] This may be due to the slow loss of calcium or the polyelectrolyte overcoats.

Approaches to strengthen these microcapsules include the use of barium ions, which result in stronger microcapsules,[13] though at the possible cost of some neurotoxicity.[14] A range of other alginate-based systems have been explored, including ultrahigh viscosity alginate,[15] (Alg-cellulose sulfate)-poly(methylene-*co*-guanidine),[16] Alg-chitosan,[17] Alg-PLL-poly(acrylic acid),[18] Alg-poly(L-ornithine)-Alg,[19] Alg-PLL-poly(ethylene glycol), Alg-chitosan-poly(ethylene glycol), and Alg-PLL-poly(ethylene glycol)-Alg.[20]

Fully synthetic systems, such as poly(hydroxyethyl methacrylate-*co*-methyl methacrylate) (p(HEMA-*co*-MMA)[21] and polyphosphazene,[2] have been used as wall formers as well, though there are no in vivo results available for p(HEMA-*co*-MMA) capsules, and polyphosphazenes have been shown to elicit an immune response.[22]

Cross-linking of Alg-chitosan capsules with glutaraldehyde or carbodiimide was shown to improve their mechanical strength,[23] though these small-molecule cross-linkers raise serious toxicity issues. Photopolymerization of monomer-functionalized alginate,[24–28] or of monomers within the alginate core,[29,30] was used to strengthen the capsules, though irradiation and the presence of photoinitiator and monomers again posed challenges to the encapsulated cells. Recently, Hallé and co-workers[31,32] studied APA microcapsules in which a photoactivated cross-linker was used to covalently link the PLL chains with other PLL chains and with adjacent alginate chains in both the surface of the core and the outer coating. These capsules displayed greatly improved mechanical stability while maintaining the cell viability and permeability of standard APA capsules.

In this paper, formation of a self-cross-linked polyelectrolyte skin on flat calcium alginate (Ca-Alg) model gels and around Ca-Alg beads is examined. This approach is based on cross-linking through complementary reactive groups attached to two oppositely charged polyelectrolytes. Being polymer-bound, these

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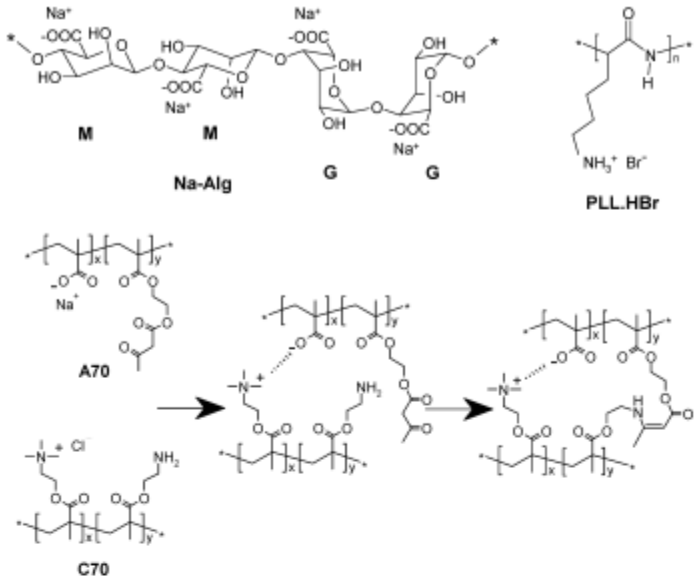
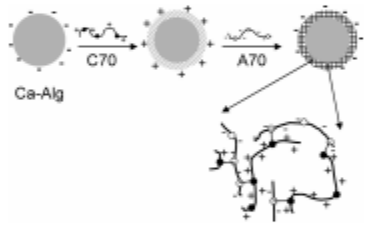
**Scheme 1.** Natural and Synthetic Polyelectrolytes Used To Prepare Coated Capsules




**Scheme 2.** Formation of Cross-Linked Shells on Ca-Alg Capsules by Sequential Coating with Self-Cross-Linking Polyelectrolytes, Having Complementary Reactive Groups, Amino (C70) and Acetoacetate (A70)




reactive groups should not pose a toxicity concern to either cells or host. The reactive polyelectrolytes used here are based on commonly used methacrylates.[21,33,34] Their structures, along with those of sodium alginate (Na-Alg) and PLL, are shown in Scheme 1. The polycation p(MOETAC-*co*-AEMA) (C70, a 70/30 mol ratio copolymer of [2-(methacryloyloxy)ethyl]trimethylammonium chloride and 2-aminoethyl methacrylate) and polyanion p(MAANa-*co*-MOEAA) (A70, a 70/30 mol ratio copolymer of sodium methacrylate and 2-(methacryloyloxy)ethyl acetoacetate) bear amino and acetoacetate groups, respectively, that undergo a rapid covalent cross-linking reaction[35] once the polyelectrolytes are brought together by electrostatic interactions, as shown in Scheme 1. Sequential coating of Ca-Alg beads with these polyelectrolytes (Scheme 2) should lead to a permanently cross-linked yet permeable polyelectrolyte skin.

## Experimental Section

**Materials.** Na-Alg (Keltone LV, 428 kg/mol[29]) was a gift from the Nutrasweet Kelco Company (San Diego, CA). MOETAC (75 wt % solution in water), AEMA (90%, as HCl salt), methacrylic acid (MAA, 99%), MOEAA (95%), PLL (hydrobromide salt, $M_n$ = 15−30 kg/mol), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (97%), fluorescein isothiocyanate (FITC, 90%), 2-(cyclohexylamino)ethanesulfonic acid (CHES), sodium chloride, sodium citrate, calcium chloride, sodium nitrate, and trypan blue stain (0.4% in 0.85% saline) were purchased from Sigma-Aldrich (Oakville, ON) and were used as received. 2,2′-Azobis(isobutyronitrile) (AIBN) was received as a gift from Dupont (Mississauga, ON) and used as received. Ethanol, acetone, *N*,*N*-dimethylformamide (DMF), and anhydrous ethyl ether from Caledon Laboratories (Caledon, ON), sodium dihydrogen orthophosphate from BDH (Toronto, ON), ethylenediaminetetraacetic acid, disodium salt (EDTA) from Anachemia (Montreal, QC), and serum-free media (SFM) from Gibco (Mississauga, ON) were used as received. Sodium hydroxide and hydrochloric acid solutions were purchased as concentrates from Anachemia Chemical and were prepared by diluting to 1.000 or 0.100 M with deionized water.

**p(MOETAC)(C100) and (p(MOETAC-co-AEMA) [70:30; C70].** The preparation of C100 ($M_w$ 300 kg/mol) was described previously.[36] C70 was prepared in a similar fashion. MOETAC (5.59 g, 26.9 mmol), AEMA hydrochloride (2.13 g, 12.8 mmol), and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (0.209 g, 0.77 mmol, 2 mol % relative to monomer) were dissolved in 75 mL of deionized water in a 125 mL high-density polyethylene (HDPE) screw-cap bottle. The solution was bubbled with nitrogen for several minutes, and the bottle was sealed and heated at 60 °C for 24 h while being rotated (15 rpm) to provide mixing. The polymer was isolated by precipitation in acetone (1 L) and then dried to a constant weight in a vacuum oven at 50 °C. Yield: 6.35 g (83%). Alternatively, the polymer was purified by dialysis in cellulose tubing (12 kg/mol MW cutoff, Spectrum Laboratories) with deionized water maintained at pH 5 by the addition of HCl followed by freeze-drying. Yield: 6.88 g (90%). The C70 purified by dialysis was used in the permeability study and for any capsules that included cells or were implanted.

**p(MAANa) (A100) and p(MAANa-co-MOEAA) [70:30; A70].** The preparation of A70 has been described[36] and A100 was prepared in a similar manner. MAA (5.00 g; 58 mmol) and AIBN (95 mg; 0.58 mmol) were dissolved in ethanol (45 mL) in a 60 mL HDPE bottle, bubbled with nitrogen, and then heated at 60 °C for 24 h, while the bottle was rotated at 4 rpm to provide mixing. PMAA was isolated by precipitation in ether (500 mL), washed with ether, and dried to constant weight at 50 °C in a vacuum oven. Yield: 4.81 g (96%). A70 and A100 solutions were prepared by neutralizing the MAA-based polymers with a stoichiometric amount of 1 M NaOH and then diluting to the desired polymer concentration.

**FITC-Labeled Polymers, C70f and A70f.** C70 (0.108 g, 0.17 mmol AEMA) was dissolved in 10 mL of water in a 20 mL glass vial. The

pH was adjusted to 9 by adding 0.1 M NaOH. FITC (0.010 g, 0.026 mmol) dissolved in 1 mL of DMF was added to the polymer solution under stirring. The mixture was stirred for 1 h at room temperature (20 °C). The FITC-labeled copolymer C70f was dialysed against deionized water using cellulose membranes (3.5 kg/mol MW cutoff, Spectrum Laboratories). The resulting polymer solution was freeze-dried, and the polymer was dried further to constant weight in a vacuum oven at 50 °C. A70f, FITC-labeled A70, was prepared in a similar fashion by treating 0.118 g A70 (0.26 mmol MOEAA) with 0.010 g FITC (0.026 mmol) for 24 h at 20 °C. Final label contents were determined by UV/vis spectroscopy, and were 1.7 and 0.3 mol % of the total monomer units for C70f and A70f, respectively.

**Characterization.** The compositions and $pK_a$ values of C70, A100, and A70 were determined by potentiometric titration. Accurately weighed samples were dissolved in about 30 mL of deionized water for C70 and PMAA or 1:1 methanol/water for p(MAA-*co*-MOEAA). A small volume (0.5−1 mL) of 0.1 M HCl was added to the solution to ensure complete protonation of the primary amine or carboxylic acid groups before titration with 0.100 M NaOH in a PC titrate automatic titrator (Man Tech Associates). The titration curves showed two end points, one for excess HCl and one for the polymer-bound groups. The $pK_a$ was taken as the pH at the midpoint (0.5 equiv NaOH) of the titration curve for the polymer-bound groups.

The degree of labeling with FITC was measured by UV−vis spectrophotometry, using 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 investigate the distribution of FITC-labeled polycation and polyanion in the microcapsules.

**MW Determination.** The MW of A100 was determined with a gel permeation chromatography system consisting of a Waters 515 HPLC pump, Waters 717 plus Autosampler, three columns (Waters Ultrahydrogel-120, -250, -500; 30 cm × 7.8 mm; 6 $\mu$m particles) and a Waters 2414 refractive index detector. The columns were maintained at 35 °C and the system was calibrated with narrow-dispersed PEG standards (Waters, Mississauga, ON). 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 1 M NaOH. The A100 solution (1%) for analysis was prepared by adding a stoichiometric amount of 1 M NaOH to the PMAA precursor followed by dilution with the mobile phase.

An Ubbelohde viscometer (viscometer constant: 0.00314 cSt/s) was used to determine the MW of C70 dissolved in 1 M NaCl at 20.0 ± 0.1 °C. Prior to the measurements, all stock solutions were filtered through a 0.45 $\mu$m membrane filter. The intrinsic viscosity $[\eta]$ was calculated by extrapolation of the Huggins plot ($\eta_{sp}/c$ vs $c$) to zero concentration. The polymer MW was calculated from the intrinsic viscosity using the relationship $[\eta] = KM^a$ with values for $K$ and $a$ found in the literature.[37]

**Spin Coating.** Planar films of Na-Alg, Ca-Alg and polyelectrolyte-coated Ca-Alg were prepared with a spin coater (Model EC101, Headway Research Inc.). A total of 1 mL of 1.5 wt % Na-Alg was placed onto a preweighed glass cover slide (2.5 cm × 2.5 cm) and spun for 30 s at 400 RPM. The resulting Na-Alg films were immediately coated with 1 mL of 1.1% calcium chloride and allowed to gel for different lengths of time before the excess solution was again removed by spinning at 400 RPM for 30 s. The resulting Ca-Alg film was washed by placing 1 mL of saline on the film for 30 s and then spinning at 400 rpm for 30 s. Subsequently, 1 mL of 1 wt % C70 was placed on the Ca-Alg film for 10 s, followed by spinning (400 RPM, 30 s) and then a saline wash, as described above. An A70 coating was added in analogous fashion using 1 wt % A70. The cover glass was weighed within 10 ± 5 s of each coating step to determine the weight of the film.

**Preparation and Coating of Ca-Alg Beads.** Ca-Alg beads were prepared and coated, as described previously.[8,12] A 1.5% aqueous solution of Na-Alg was filtered through sterile 0.45 $\mu$m Acrodisc syringe filters (Pall Corp., U.S.A.). 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 gelling bath (10× alginate volume) consisting of 1.1% calcium chloride and 0.45% NaCl causing the formation of Ca-Alg beads. These beads were washed in sequence with 4-fold volumes, relative to the concentrated bead suspension of (a) 1.1% $CaCl_2$, 0.45% NaCl for 2 min; (b) 0.55% $CaCl_2$, 0.68% NaCl for 2 min; (c) 0.28% $CaCl_2$, 0.78% NaCl for 2 min; (d) 0.1% CHES, 1.1% $CaCl_2$, 0.45% NaCl for 3 min; and (e) 0.9% NaCl for 2 min and then stored in saline.

APA capsules were prepared by exposing Ca-Alg beads (3 mL) to 0.05% PLL in saline (10 mL) for 6 min and washing them in sequence with 12 mL of wash solutions (d), (a), and (e) above. The beads were then coated with 0.03 wt % Na-Alg (10 mL) in saline for 4 min, followed by three washes with 0.9% saline (12 mL). Coating with C70 and A70 was performed in a similar fashion, except that a variety of concentrations and exposure times were explored. Unless otherwise specified, standard C70 and A70 coatings were prepared with 0.5% solutions of C70 and A70 and exposure times of 10 min.

**Mechanical Stability.** The mechanical stability of capsules and beads was tested by agitating the beads in the presence of hypotonic or calcium chelating solutions. The osmotic pressure test (OPT)[38] utilized six hypotonic solutions containing 0.52, 0.78, 1.19, 1.52, 3.25, and 6.50% SFM in water, with corresponding osmolarities of 1.9, 2.8, 4.2, 5.6, 11.1, and 21.3 mOsm, respectively. A total of 100 $\mu$L of capsule suspension (~200 microcapsules) and 10 mL of hypotonic solution were placed in a 15 mL polypropylene conical tube and then agitated for 3 h on an orbital mixer at 30 rpm. The capsules were stained with trypan blue and transferred to glass Petri dishes on a lightbox. The percentage of intact capsules was determined by direct visual inspection or from an image taken with a digital camera. Experiments were conducted in triplicate. In the calcium chelation test, the 100 $\mu$L capsule suspension was exposed to 10 mL of water or 0.003% EDTA solution while being agitated for 15 min before analysis, as described for the OPT.

**Permeability Studies.** Permeabilities of flat model APA and AC70A70 films were determined using a two-compartment Teflon diffusion cell where the compartments were separated by an APA film, or by a C70/A70 coated calcium alginate film, supported on a porous, polypropylene support membrane (a gift from 3 M Centre, St. Paul, MN; 4 × 4 cm, avg pore size, 5 $\mu$m). The base calcium alginate membrane was prepared by immersing the support membrane in 1.5% Na-Alg, then 1.1% $CaCl_2$ for 10 min, followed by three saline washes. The membrane was then mounted in the diffusion cell and coated on one side by exposure to (1) polycation solution (0.05% PLL or 0.5% C70) for 6 min, (2) three saline washes, (3) polyanion solution (0.03% Na-Alg or 0.5% A70) for 4 min, and (4) three saline washes. A mixture of poly(ethylene glycol) samples with viscosity-average MWs of 10, 100, 200, and 300 kg/mol (Sigma-Aldrich, 0.1 wt % each in saline) was placed into the source compartment, with an equal volume of saline loaded into the sink compartment. The assembly was maintained at room temperature (23 ± 2 °C) with stirring in both compartments for 24 h. Samples were taken from each sink side and analyzed by gel permeation chromatography (GPC), as described above, except that the mobile phase was 0.1 M $NaNO_3$. The samples from the sink compartments were concentrated by evaporation prior to analysis.

**Cell Culture.** The cell line used was a murine $C_2C_{12}$ myoblast cell line (American type Culture Collection [ATCC], Rockville, MD,

**Table 1.** Polyelectrolyte Properties

| polyelectrolyte | composition | MW (kg/mol) | $pK_a$ |
|---|---|---|---|
| Na-Alg | G/M, 40:60[a] | 428[d] | 3.20/3.38 (G/M)[g] |
| A70 | MAA/MOEAA, 70:30 (±4) [b] | 42[b] | 7.1[h] |
| A100 | | 40[e] | 7.2[h]/6.3[i] |
| PLL | | 15−30[a] | 10.5[i] |
| C70 | MOETAC/AEMA, 70:30 (±4)[c] | 167[f] | 7.3[i] |
| C100 | | 300[b] | |

[a] Given by supplier. [b] See ref 36. [c] MOETAC/AEMA determined by titration. [d] See ref 29. [e] $M_n$ obtained from gel permeation chromatography data. [f] $M_w$ obtained from viscometry data. [g] See ref 40. [h] From titration in 50% methanol. [i] From titration in water. [j] See ref 41.

U.S.A.; Catalogue 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), 100 U/mL penicillin, and 100 $\mu$g/mL streptomycin (Gibco, Grand Island, NY) at 37 °C in a water-jacket incubator (5% $CO_2$, 100% humidity).

**Encapsulation of Cells.** The cells were harvested, counted, and centrifuged at 1000 rpm for 10 min at 4 °C. The supernatant was aspirated and the cells were resuspended in ice cold normal saline. A known number of cells were centrifuged and resuspended in 50 $\mu$L of cold normal saline. Prefiltered (0.45 $\mu$m) Na-Alg solution (1.5%, 5−20 mL) was added to the cells to produce a cell loading of 2 million cells/mL of solution. The cell suspension was mixed well and then transferred to a syringe for capsule preparation as described above. Polyelectrolyte solutions used for coating were filtered through 0.45 $\mu$m syringe filters prior to use.

**Cell Viability.** The number of viable cells per capsule was determined with an Alamar Blue assay.[39] A total of 100 $\mu$L of capsules were loaded in a 24-well plate with 500 $\mu$L media. A total of 50 $\mu$L of Alamar Blue was added to each sample and the plate was incubated at 37 °C for 4 h. After incubation, 100 $\mu$L of solution 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 was determined by comparing fluorescence values with a standard curve generated from a known number of cells.

**Implantation.** Animals were treated in accordance with the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals. Freshly made capsules were kept in the supplemented DMEM described above at 37 °C for 3 days before implantation in mice, as described previously.[8] Briefly, the mice were anaesthetized with isoflurane (Anaquest, Mississauga, ON, Canada) using a small-animal anesthetic machine (Med-Vet, Toronto, ON, Canada). A total of 3 mL of capsules suspended in normal saline (4 mL total) were implanted into the abdominal cavity of mice with an 18 gauge catheter (Angiocath, Mississauga, ON). For microcapsule recovery after implantation, mice were euthanized, the peritoneal cavity was opened, and the capsules were scooped out with a spatula. The capsules were washed several times with normal saline before testing.

## Results and Discussion

The use of the oppositely charged reactive polyelectrolytes C70 and A70 (Scheme 1) to strengthen Ca-Alg microcapsules was examined and compared with the more commonly used noncovalent coatings based on PLL/Na-Alg. The polyelectrolytes C70 and A70, and their nonreactive analogs C100 and A100, were prepared by conventional free-radical copolymerization (Table 1).

Polyelectrolyte complexes formed with the nonreactive analogs, C100 and A100, were shown earlier to dissolve when exposed to [NaCl] > ~500 mM.[36] Complexes that included C100 or A100 dissolved rapidly in 2 M NaCl. In contrast, the

**Scheme 3.** Formation of C70A70 Bilayer or Multilayer Films by Sequential Coating of Ca-Alg Hydrogel Films on Glass Slides

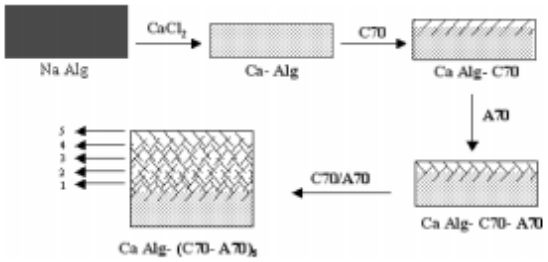



**Table 2.** Weight and Thickness of Hydrogel Films Prepared on Glass Cover Slides

| | weight of film (mg) | | thickness of wet film (μm) | |
|---|---|---|---|---|
| film | wet film | dry film | from film wt[a] | from OM[b] |
| Na-Alg | 54 ± 5 | 1.0 ± 0.1 | 86 ± 8 | 80 ± 8 |
| Ca-Alg | 19 ± 5 | 1.2 ± 0.1 | 31 ± 8 | 26 ± 3 |
| Ca-Alg-C70 | 19 ± 5 | 1.5 ± 0.1 | 30 ± 8 | 25 ± 3 |
| Ca-Alg-C70-A70 | 23 ± 5 | 1.7 ± 0.1 | 37 ± 8 | 31 ± 3 |
| Ca-Alg-(C70-A70)$_{10}$ | 54 ± 5 | 2.9 ± 0.1 | 86 ± 8 | 80 ± 8 |

[a] Assuming a film density of 1.0 g/cm.[3] [b] Optical microscopy.

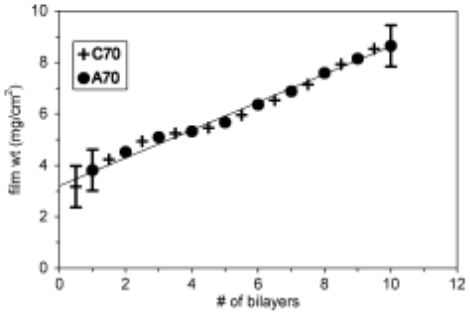



**Figure 1.** Film weights during formation of Ca-Alg-(C70-A70)$_{10}$ multilayer film on a glass cover slide.

C70/A70 gel complex formed by combining solutions of A70 and C70 in a 1:1 mol ratio swelled slightly in 2 M NaCl but did not dissolve. The survival of this complex at high ionic strength, where the electrostatic interactions are broken, is attributed to the formation of covalent cross-links. The C70/A70 complex survives with little change when challenged with 2 M NaCl within 1 min of formation, showing that the reaction between amine and acetoacetate groups in the complex is rapid at room temperature. Sequential deposition of C70 and A70 on the surface of Ca-Alg model films and microcapsules should lead to a covalently cross-linked complex that is better able to resist environmental stresses.

**Ca-Alg Model Films Coated with C70/A70.** Flat Ca-Alg model films prepared by spin coating were sequentially coated with C70/A70 (multi)layers (Scheme 3) to better understand the polyelectrolyte interactions involved. The weights and thicknesses of these films at different stages of coating are shown in Table 2.

The original Na-Alg hydrogel film had a water content of about 98%, which decreased to about 94% after gelling with calcium chloride for three minutes or longer. Except for the first C70 layer, each polyelectrolyte exposure caused gains in weight and thickness (Figure 1), a typical trend for layer-by-layer assembly.[42,43] The average incremental thickness of each

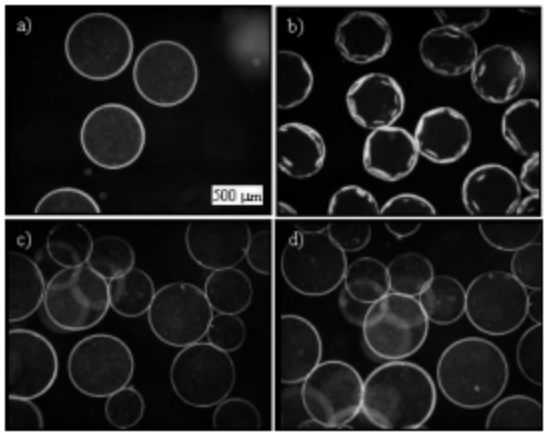



**Figure 2.** Phase contrast microscope images of Ca-Alg beads exposed to a 0.05% solution of PLL for (a) 10 min and (b) 30 min or to a 0.05% solution of C70 for (c) 10 min and (d) 30 min, followed by saline washing.

bilayer is ~5 $\mu$m, similar to results obtained by Goosen et al. for a single bilayer.[44] This large incremental layer thickness is partly due to the porous, swellable nature of the Ca-Alg gel. Polycations such as C70 may diffuse into this porous gel, complexing with alginate and displacing Ca to a certain depth. Subsequent exposure to A70 should lead to charge reversal and formation of a cross-linked layer of C70A70.

Exposure of the Ca-AlgC70A70 film to sodium citrate (170 mM) to extract the calcium led to isolation of a thin cross-linked C70A70 film. This film was stable in NaCl (2 M), confirming its cross-linked nature. In the following section, this approach is used to coat Ca-Alg beads.

**Polyelectrolyte Coating of Ca-Alg Microcapsules.** The nature of the coating formed by interaction of polycations with Ca-Alg beads depends on factors such as polycation concentration, MW, and exposure time.[4,44–46] The work by Bysell and Malmsten[41] on the interaction of PLL with poly(acrylic acid) microgels provides a good model for the present systems: at pH 7, they found low MW PLL (1−10 kg/mol) throughout the particle, together with significant but uniform shrinkage of the microgel. Medium MW PLL (28−84 kg/mol) gave less shrinkage, while high MW PLL (84−170 kg/mol) gave the least shrinkage, but a wrinkled and dense outer layer. It was believed that the intermediate MW PLL could redistribute itself at the hydrogel surface during deswelling, while the high MW PLL formed a strong surface polyelectrolyte complex that could not redistribute itself, hence, leading to a wrinkled surface layer.

In our case, Ca-Alg beads exposed to PLL or C70 appeared unchanged when examined by optical microscopy (OM) but trypan blue staining, as well as phase contrast OM (Figure 2a,c), indicated that the polycations were confined to the surface region. This is consistent with other studies that showed that polycations with MW greater than about 15 kg/mol are restricted to the surface region of Ca-Alg beads.[44,46,47] Longer exposure times caused only slight increases in shell thickness as seen in phase contrast microscopy (Figure 2d vs c), however, in a number of cases, the beads became wrinkled (Figure 2b).

Ca-Alg beads were coated with C70 concentrations ranging from 0.001 to 1%. Capsules coated at the lowest concentrations had incomplete or very thin shells and fared poorly in subsequent stability tests. Intermediate concentrations (0.01−0.1%) often led to capsule aggregation during coating. When coating was done at higher concentrations (0.5−1%), no aggregation occurred although wrinkling of the capsules was more likely as the concentration and, presumably, the osmotic pressure increased. Most subsequent experiments were performed using 0.5% C70 for coating because this avoided capsule aggregation and wrinkling. The C70 concentration is higher than the standard 0.05% PLL concentration but it does not produce shells that are significantly thicker (vide infra) because penetration of the high MW C70 is limited. In the future, the coating process can likely be optimized (i.e., by addition of $CaCl_2$ or a surface stabilizer) to allow coating at lower C70 concentrations.

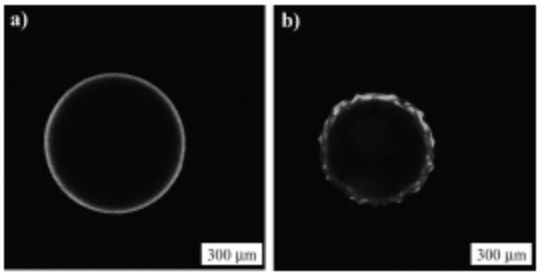



**Figure 3.** Confocal laser scanning microscopy image showing an equatorial section of representative Ca-Alg capsules exposed to a 0.5% solution of C70f for (a) 10 min and (b) 120 min.

The C70 solutions used during coating acquired free calcium ions, revealed by the precipitation as $CaCO_3$ following treatment with NaOH and $Na_2CO_3$. In addition to binding with free charges on alginate, C70 displaces some $Ca^{2+}$ from the hydrogel. The displacement of calcium by the polycation has been mapped in detail using scanning transmission X-ray microscopy and will be reported separately.

Penetration depth of the polycation was further studied using confocal laser scanning microscopy (CLSM)[41,48–50] and the fluorescein-labeled polycation C70f. Ca-Alg beads exposed to a 0.5% C70f solution showed a shell increasing from a few microns in thickness after 30 s exposure (image not shown) to approximately 25 ± 2 $\mu$m after a 10 min exposure (Figure 3a). Continued exposure to 120 min leads to a wrinkled surface but no significant further increase in skin thickness or penetration (Figure 3b).

The coating process was further studied using A70f, a fluorescein-labeled version of A70. Capsules exposed to C70f and then A70f, each for 30 s, showed a fluorescent shell approximately 7 ± 1 $\mu$m thick (Figure 4a) that grew to approximately 26 ± 2 $\mu$m thick when each exposure was increased to 10 min (Figure 4b). The thickness of the fluorescent layer was similar to that formed by C70f alone (Figure 3a) or to that formed when only one of the two polyelectrolytes was fluorescently labeled (Figures 4c,d), indicating that the A70f is approximately coincident with the C70f (Figure 4e).

**Microcapsule Stability.** The physicochemical stability of the Ca-Alg microcapsules was probed in several ways. As described earlier, a measure of the resistance to chemical degradation can be obtained by challenging the capsules with sodium citrate and high ionic strengths, which can lead to dissolution of the capsule core, and of the surface coating. Uncoated Ca-Alg beads are stiff gels at physiological salt concentrations. Upon treatment with 170 mM sodium citrate, the beads rapidly dissolved as the $Ca^{2+}$ was lost. When APA, AC70A70 or AC70A100 capsules (Figure 5, left column) were treated with citrate to liquefy the cores, the capsules swelled but the polyelectrolyte shells survived (Figure 5, center column). The shells of APA

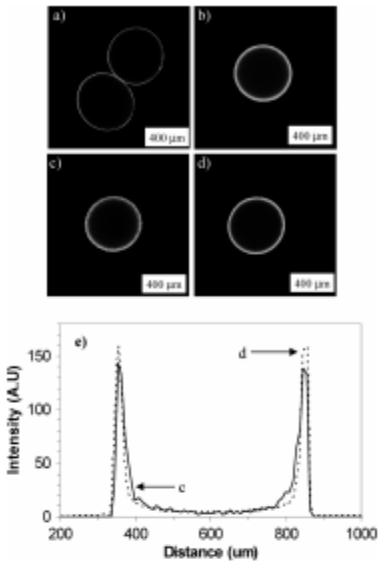



**Figure 4.** CLSM optical sections in the equatorial region of Ca-Alg beads exposed to 0.5% solution of polycation and polyanion for (a) C70f and A70f; 30 s each, (b) C70f and A70f; 10 min each, (c) C70f and A70; 10 min each (d) C70 and A70f; 10 min each, (e) intensity line profile of microcapsules c and d showing the distribution of C70f and A70f.

and AC70A100, which are held together by ionic interactions alone, disappeared when further exposed to 2 M NaCl (Figure 5, right column, top/bottom), while the covalently cross-linked shell present in the AC70A70 capsules resisted this challenge unless punctured (Figure 5, right column, middle).

Mechanical stability was examined by exposing the capsules to osmotic pressure in a test developed by Chang and co-workers[38] or to the gradual loss of $Ca^{2+}$ using an EDTA solution. Microcapsules were tumbled in hypotonic or EDTA solutions and the fraction of intact capsules was determined. APA and AC70A70 capsules that were empty (containing no cells) were subjected to the tests and the results are shown in Table 3. All of the capsules survive tumbling in SFM but the standard APA capsules are largely broken in the hypotonic or EDTA solutions after tumbling. APA capsules prepared with a 10× higher PLL concentration (0.5%) proved to be stable in distilled water (0% SFM) but 40% were broken in the EDTA solution. This higher PLL concentration would likely create a thicker Alg/PLL polyelectrolyte complex shell since at least a fraction of the 15−30 kg/mol PLL will be able to penetrate into the Ca-Alg gel. In contrast, capsules made with 0.5% C100 showed similar stability to the standard APA capsules. C100, which has a considerably higher MW than PLL, will be restricted to the surface of the Ca-Alg gel and the shell that is formed is no stronger than that formed by 0.05% PLL. Similarly, C70 is restricted to the surface due to its high molecular weight, and the greater stability in the hypotonic and EDTA solutions compared to APA capsules is attributed to the formation of covalently cross-linked shells around the AC70A70 capsules.

The OPT was used to examine the stability of capsules containing cells. APA (0.05/0.03) and AC70A70 (0.5/0.5) capsules containing myoblast $C_2C_{12}$ cells were prepared and then incubated for times ranging from 4 to 72 h before the OPT (Figure 6). Both types of capsules showed reduced stability in comparison to the analogous empty capsules (Table 3). This effect of encapsulated cells on mechanical stability has been observed previously and was attributed to the interruption of the Ca-Alg gel by the cells.[38] The fraction of intact APA capsules decreased on moving from 6.5 to 0.52% SFM, with no effect observed for varying the duration of storage before the OPT. The AC70A70 capsules again performed better than the APA control capsules (Figure 6). In addition, the fraction of intact AC70A70 capsules increased with increasing storage time before the OPT, suggesting that the cross-linking reaction continues for several days after coating. While the reaction between amine and acetoacetate groups is rapid in freely diffusing systems, it will slow as the movement of the polyelectrolytes becomes restricted. The change in the extent of cross-linking is probably not substantial after the first few hours because the polyelectrolytes become locked in place by electrostatic and covalent interactions. However, it appears that the additional curing time can lead to noticeable changes in the capsule strength. This extended curing reaction was not considered in our initial tests of capsule stability, which were typically conducted within 4−24 h of capsule preparation. The AC70A70 capsules might have shown even greater strength in these tests, had they been allowed to cure for a longer period of time.

**Permeability of AC70A70 Capsules.** Capsules used for immuno-isolation of nonautologous cells must have well-defined wall permeabilities, capable of preventing in-diffusion of immune modulators, but permitting any therapeutic protein to escape the capsule. This usually requires MW cut-offs for diffusion through the capsule shell of about 150 kg/mol.[8] The size exclusion properties of the covalently cross-linked membranes were tested by allowing a poly(ethylene glycol) mixture to diffuse through flat APA or AC70A70 model membranes made by coating one side of a Ca-Alg gel held in a porous, polypropylene support.[29] Figure 7 shows the GPC traces for the original PEG mixture and for concentrated samples from sink compartments for both membranes after 24 h of equilibration. It clearly shows that in both cases the high MW fraction of the PEG remains excluded from the sink, while the low MW fraction (<∼20 kg/mol) can diffuse through the membrane. Above ∼20 kg/mol, the PEG permeates the membrane more slowly, and above ∼170 kg/mol (vertical line in Figure 7), little or no PEG has passed through the membrane. These results indicate that the cross-linked AC70A70 capsule walls have MW cut-offs similar to those of APA control capsules and are, hence, suitable for encapsulation of therapeutic nonautologous cells. Both sink solutions were concentrated by evaporation, and the higher intensity of curve b may be due to differing degrees of evaporation rather than greater diffusion.

**Cell Viability in Microcapsules with Cross-Linked Shells.** The viability of myoblast $C_2C_{12}$ cells in APA and AC70A70 capsules was examined. Cell-containing AC70A70 capsules incubated for 1 week are shown in Figure 8 and were similar in appearance to APA capsules. The number of living cells per capsule was monitored versus time of in vitro incubation (Figure 9). APA capsules contained about 90 living cells each after incubation for 1 day, rising to 240 cells/capsule after 7 days. Capsules coated with C70 and A70 contained significantly fewer living cells/capsule after incubation for 1 day. As the incubation continued, the number of cells/capsule rose in each case, but the rise was most rapid for the capsules exposed to the lower C70/A70 concentrations. This trend continued when still lower C70/A70 concentrations of 0.1% were used, though the data are not shown in Figure 9 because some capsule aggregation occurred making the capsules tested less representative of the

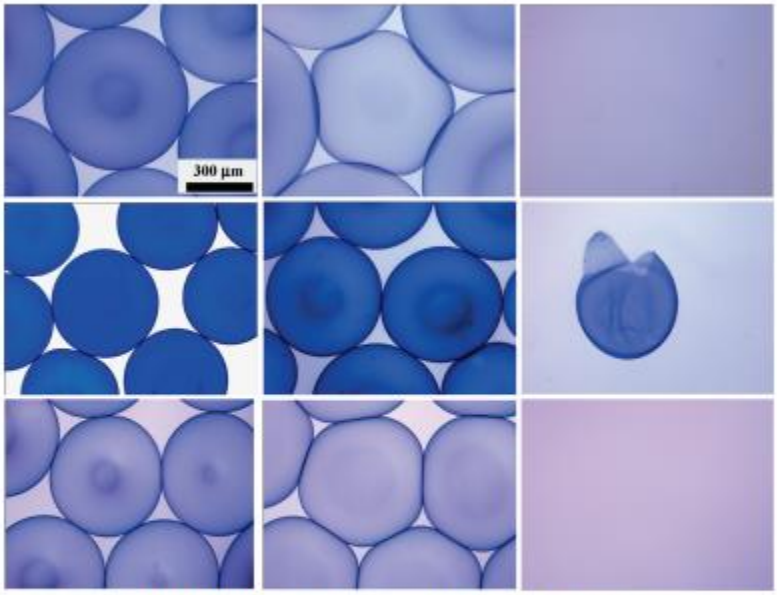



**Figure 5.** Optical microscope image of APA (0.05/0.03) (top row), AC70A70 (0.5/0.5) (middle row), and AC70A100 (0.5/0.5) (bottom row) microcapsules in saline (left column), after exposure to 170 mM sodium citrate (center column), and then 2 M NaCl (right column). The concentrations of polyelectrolytes (wt %) used to coat the capsules are shown in brackets. Capsules were stained with trypan blue to facilitate observation.

**Table 3.** Performance of Empty APA and AC70A70 Microcapsules in the OPT and Calcium Chelation Test

| | percent intact capsules[b] | | | |
|---|---|---|---|---|
| microcapsules[a] | 100% SFM | 0.52% SFM | water | 0.003% EDTA |
| APA (0.05/0.03) | 99 ± 1 | 15 ± 10 | 0 | 0 |
| APA (0.5/0.03) | | | 100 | 60 |
| AC100A (0.5/0.03) | 99 ± 1 | 15 ± 10 | | |
| AC70A70 (0.5/0.5) | 100 | 100 | 100 | 100 |

[a] Concentrations in brackets refer to polyelectrolyte concentrations (wt %) used for coating. [b] Capsules were tumbled for 3 h in SFM solutions or 15 min in water or EDTA solution.

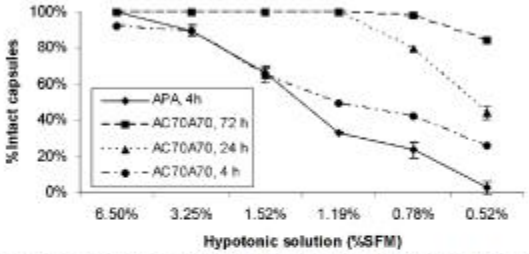



**Figure 6.** Percentage of intact cell-containing APA (0.05/0.03) and AC70A70 (0.5/0.5) capsules after osmotic pressure test. Values in brackets denote polyelectrolyte concentrations (wt %) used during coating. AC70A70 capsules were stored in saline for 4, 24, or 72 h between coating and the OPT.

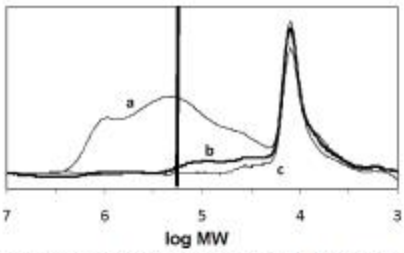



**Figure 7.** GPC traces of (a) the original PEG mixture and the solution in the sink compartment of the stirred diffusion cells separated by (b) AC70A70 (0.5/0.5), and (c) APA (0.05/0.03) membranes after 24 h at room temperature (23 ± 2 °C). The sink samples were concentrated prior to analysis. The vertical line at about 170 kg/mol indicates the approximate MW cutoff.

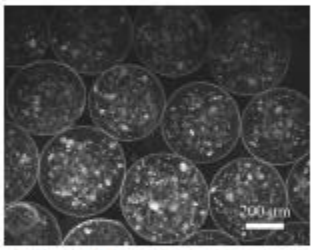



**Figure 8.** Phase contrast microscope image of $C_2C_{12}$ cells encapsulated in AC70A70 after in vitro incubation for 1 week. Polyelectrolyte coatings prepared with 0.5% solutions and 10 min exposure times.

whole sample. The reduced viability and proliferation, scaling with polyelectrolyte concentration, could be due to suboptimal ionic strength or osmotic pressure during coating. While low MW impurities were removed from C70 by dialysis, the C70 likely contains shorter chains able to diffuse into the capsule interior where they may prove to be cytotoxic. Alternately, the formation of a coating that inhibits the in-diffusion of nutrients to the cells could be responsible. Although coating with C70/A70 has an initially negative impact on cell viability, the cells that survive the coating process remain viable and do begin to proliferate. The coating process with C70/A70 remains to be optimized further, and it should be possible to find conditions that are less harmful to the cells while still forming a robust coating and avoiding capsule aggregation.

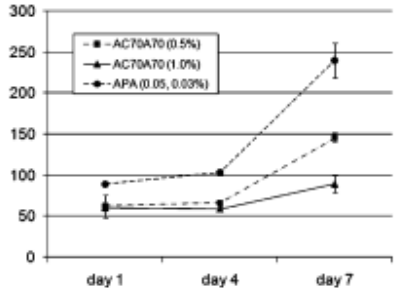



**Figure 9.** In vitro cell viability [live cells/capsule] for $C_2C_{12}$ cells encapsulated in APA and AC70A70 microcapsules. Values in parentheses refer to C70/A70 or PLL/Na-Alg concentrations used to coat the capsules.

Initial results with cell-containing AC70A70 (0.5/0.5) capsules indicated significant fibroid overcoating on many capsules after implantation in mice for 1 to 2 weeks. This overcoating indicates an immune response of the host and would limit viability of the encapsulated cells. The immune response appears to be related to the coating because cell-containing APA capsules prepared from the same Na-Alg do not show this immune response. Further experiments are currently underway to study the causes of this response and to improve the in vivo performance of the new capsules.[51]

## Conclusion

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-labeled analogs of these polyelectrolytes indicates that they are concentrated near the surface of the Ca-Alg bead. Fluorescent layers of similar thickness are observed irrespective of which polyelectrolyte is labeled, indicating the presence of a homogeneous mixture of the two polyelectrolytes near the surface of the alginate bead. The thickness of this cross-linked shell increases moderately from about 7−25 $\mu$m upon increasing the exposure time, but appears to be ultimately limited by diffusion. The cross-linked nature of this outer shell was demonstrated by its resistance to citrate and 2 M sodium chloride. These capsules with cross-linked shells have greater resistance to osmotic pressure changes, and to mechanical stress tests, compared to APA control capsules. The cross-linked polyelectrolyte coating was found to have a MW cutoff of about 170 kg/mol, similar to that of an APA membrane and a value suitable for cell encapsulation. $C_2C_{12}$ cells were viable within Ca-Alg capsules coated with the new polyelectrolytes. The number of live cells within the capsules was somewhat lower than in APA capsules, but the number of live cells remained stable or increased with extended incubation. Preliminary implantation studies in mice revealed a fibroid overgrowth on many capsules. This immune response is related to the presence of the new polyelectrolytes and current studies are focused on reducing or eliminating the response.

In summary, these results describe a promising new approach to cell encapsulation. We believe that covalent reinforcement through use of self-cross-linking polyelectrolytes provides a fundamental advantage in the long-term strengthening of Ca-Alg beads, combining the inherently low toxicity of polymer-bound reactive groups with the ability to modify coating thickness, and possibly permeability, through changes in the polyelectrolytes or the coating process. Further studies of the in vivo use of these and related capsules are being conducted and will be reported shortly.

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