Mechanically Enhanced Microcapsules for Cellular Gene Therapy

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Abstract: Microcapsules bearing a covalently cross-linked coating have been developed for cellular gene therapy as an improvement on alginate-poly(L-lysine)-alginate (APA) microcapsules that only have ionic cross-linking. In this study, two mutually reactive polyelectrolytes, a polycation (designated C70), poly([2-(methacryloyloxy)ethyl]trimethylammonium chloride-*co*-2-aminoethyl methacrylate hydrochloride) and a polyanion (designated A70), poly(sodium methacrylate-*co*-2-(methacryloyloxy)ethyl acetoacetate), were used during the microcapsule fabrication. Ca-alginate beads were sequentially laminated with C70, A70, poly(L-lysine) (PLL), and alginate. The A70 reacts with both C70 and PLL to form a ~30 μm thick covalently cross-linked interpenetrating polymer network on the surface of the capsules. Confocal images confirmed the location of the C70/A70/PLL network and the stability of the network after 4 weeks implantation in mice. The mechanical and chemical resistance of the capsules was tested with a "stress test" where microcapsules were gently shaken in 0.003% EDTA for 15 min. APA capsules disappeared during this treatment, whereas the modified capsules, even those that had been retrieved from mice after 4-weeks implantation, remained intact. Analysis of solutions passing through model flat membranes showed that the molecular weight cut-off of alginate-C70-A70-PLL-alginate is similar to that of alginate-PLL-alginate. Recombinant cells encapsulated in APA and modified capsules were able to secrete luciferase into culture media. The modified capsules were found to capture some components of regular culture media used during preparation, causing an immune reaction in implanted mice, but use of UltraCulture serum-free medium was found to prevent this immune reaction. *In vivo* biocompatibility of the new capsules was similar to the APA capsules, with no sign of clinical toxicity on complete blood counts and liver function tests. The increased stability of the covalently modified microcapsules coupled with the acceptable biocompatibility and permeability demonstrated their potential for use as immunoisolation devices in gene therapy.
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INTRODUCTION

Microencapsulated cells engineered to secrete therapeutic proteins have been effectively applied in treating mouse models of genetic disorders such as dwarfism,[1] lysosomal storage diseases,[2,3] hemophilia,[4] and cancer.[5–9] The semipermeable microcapsules allow nonautologous cells to be implanted into the host animal by protecting the cells from immune mediators yet still allowing the therapeutic protein to leave the microcapsule. The design of the microcapsules has to be optimized for permeability, stability and biocompatibility. One of the most commonly used and studied microcapsules is the alginate-poly(L-lysine)-alginate (APA) capsule. Alginate is a natural polysaccharide composed of β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues. Divalent cations, such as Ca^{2+}, can be used to cross-link G-rich regions to form a hydrogel calcium-alginate (Ca-alginate) bead. The M/G ratio, molecular weight, polydispersity index, and the ratio of homologous to heterologous chains dominate the properties of the Ca-alginate beads.[10–12] APA capsules are made by further layering Ca-alginate beads with poly(L-lysine) (PLL) and then alginate to give suitable biocompatibility and permeability for implantation.[13–15] A concern with APA microcapsules is loss of structural integrity during long-term implantation due to disruption of ionic cross-linking.[13,16] In our previous study, APA capsules that were successfully maintained in mice for over 6 months totally collapse after 14 days implantation in dogs.[13]

A number of synthetic polymers have been used to augment or replace Ca-alginate or the PLL/alginate coatings in

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attempts to improve long-term mechanical stability of the capsules. Polymers such as poly(hydroxyethyl methacrylate-*co*-methyl methacrylate), polyphosphazene, and poly(*N*-vinylpyrrolidone-*co*-sodium acrylate) have been employed during the capsule fabrication. However, there are still few formulations that greatly improve the mechanical properties while maintaining the high biocompatibility and optimal permeability characteristics of APA capsules. Our objective was to prepare a capsule with improved stability, yet similar biocompatibility and permeability to the APA capsule by forming a covalently cross-linked coating on the capsule.

In this study, two mutually reactive polyelectrolytes were used: a polycation, poly([2-(methacryloyloxy)ethyl]trimethylammonium chloride-*co*-2-aminoethyl methacrylate hydrochloride), designated C70, and a polyanion, poly(sodium methacrylate-*co*-2-(methacryloyloxy)ethyl acetoacetate), designated A70.[17] During fabrication of the modified microcapsules (designated as "4-layer" capsules), Ca-alginate beads were sequentially laminated with C70, A70, PLL, and finally alginate. Four-layer capsules were designed to improve the biocompatibility of previously described 2-layer modified capsules, avoiding the surface overgrowth seen in *in vivo* pilot studies employing Ca-alginate coated with C70 and A70 alone.[17]

The acetoacetate groups of A70 are able to react with amino groups from both C70 and PLL to form a $\sim$30 μm thick covalently cross-linked interpenetrating polymer network coating on the surface of the capsules. *In vitro* and *in vivo* tests for stability, permeability and biocompatibility were undertaken, which showed the 4-layer capsules to have permeability and biocompatibility similar to APA capsules, along with an increased stability.

MATERIALS AND METHODS

Chemicals

Sodium alginate (Keltone LV, 428 kDa) was a gift from the International Specialty Products (ISP) Co. (San Diego, CA). Poly-L-lysine (PLL, M_n = 15–30 kDa), poly(ethylene glycol) (PEG, M_v = 10, 100, 200, and 300 kDa), 2-(*N*-cyclohexylamino) ethanesulfonic acid (CHES), EDTA (ethylenediaminetetraacetic acid, disodium salt, dihydrate), sodium chloride, calcium chloride, sodium nitrate, and FITC labeled bovine serum albumin (fBSA) were purchased from Sigma Aldrich Chemical (St. Louis, MO), and were used as received. Narrow-dispersed PEG standards for GPC calibration were purchased from Waters (Mississauga, ON). Sodium dihydrogen orthophosphate was obtained from BDH, ON. DMEM (Dulbecco's Modified Eagle's Medium), Trypan blue solution, fetal bovine serum (FBS), UltraCulture serum-free media (SFM) were purchased from Gibco (Mississauga, ON), and were used as received. Sodium hydroxide and hydrochloric acid were purchased as concentrates from Anachemia Chemical (NY), and were prepared by diluting to 0.100*M* with deionized water.

Polymer Synthesis

The synthesis of A70 (42 kDa), A100 (40 kDa) and C70 (167 kDa) polymers and a fluorescein-labeled analog (C70f) is described fully in our previous publication.[17]

Encapsulation

The APA microcapsules were fabricated as previously described,[18] and the preparation of 4-layer capsules followed a similar procedure. Briefly, 1.5 (w/v) % sodium alginate in 0.9% NaCl was sterile filtered and then extruded though a 27-gauge needle with concentric airflow into a 1.1 (w/v) % $CaCl_2$ bath. For the 4-layer capsules, these beads were then coated with 0.5 (w/v) % C70 and 0.5 (w/v) % A70 for 3 min each. The Ca-alginate-C70-A70 capsules or Ca-alginate beads were then laminated with 0.05 (w/v) % PLL for 6 min, and then with 0.03 (w/v) % alginate for 4 min, to form the 4-layer capsules or APA capsules, respectively (see Figure 1). Each layering step was followed by two 0.9% NaCl washes (2 min each). For some 4-layer capsules, C70 was replaced with C70f.

For microcapsules containing cells, a C2C12 myoblast cell suspension was mixed with the sterile alginate solution to a final cell concentration of 2 millions cells/mL of alginate. Following the final wash step, the microcapsules with cells were cultured in DMEM medium (with 10% FBS and 1% penicillin/streptomycin) in a tissue culture incubator at 37°C, while empty microcapsules were left in normal saline for incubation.

Luciferase Activity Assay *In Vitro*

The expression and release of recombinant proteins from encapsulated cells was demonstrated with Luciferase vector pC3B.Luci transfected MDCK (Madin-Darby canine kidney) cells constructed in this lab (unpublished observation). The cells were encapsulated and incubated as described earlier for 24 h before samples of the media were removed. Luciferase expression by the encapsulated cells was assayed using the Luciferase activity kit (Promega, Madison, WI) using the protocol provided by the manufacturer. Approximately 100 capsules were cultured in 1 mL of media for 24 h, and 20 μL of the media was loaded into a 96-well plate. An automated injector added 50 μL of luciferase substrate mixture to each well at specified time intervals and luciferase activity in relative light units (RLUs) was measured with an automated luminometer using a total integration time of 10 s.

Pore Size Measurement

Permeability through uncoated and coated Ca-alginate membranes was measured using a two-compartment plastic permeation cell separated by an alginate-based membrane on a porous polypropylene support membrane (a gift from 3*M*, St. Paul, MN; average pore size = 5 μm). The Ca-alginate membrane was prepared by immersing the polypropyl-

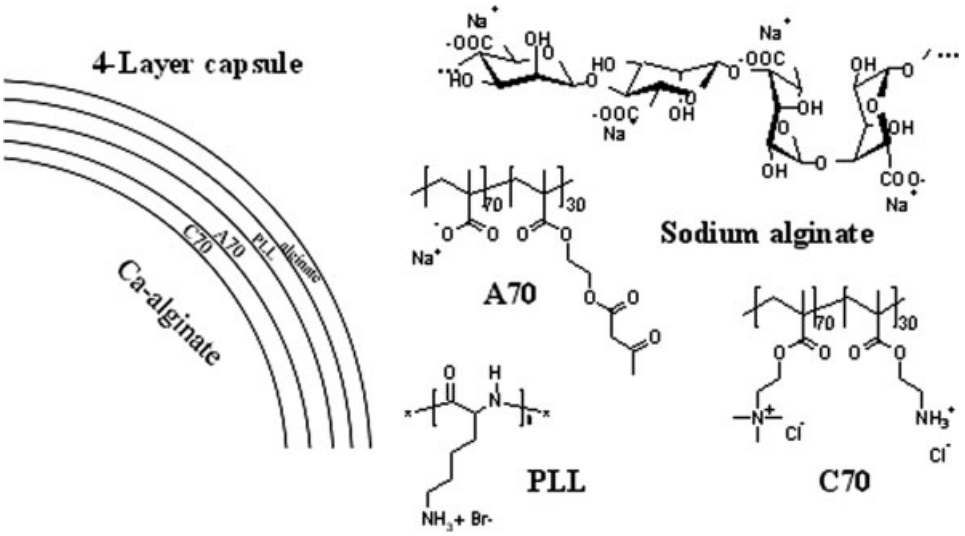

Figure 1. Construction of 4-layer-capsule.

ene support in 1.5% sodium alginate, then 1.1% $CaCl_2$ for 6 min, followed by three 0.9% NaCl washes. The supported Ca-alginate membrane was mounted in the diffusion cell and then coated on one side by exposing to polycation (6 min) or polyanion (4 min) solutions to build-up layers analogous to the microcapsule coatings. To create an APA-like membrane, the Ca-alginate was coated with 0.05% PLL and then 0.03% alginate. For the 4-layer capsule membrane, the Ca-alginate was coated with 0.5% C70, followed by 0.5% A70, 0.05% PLL and finally 0.03% alginate. After each round of coating the membrane was washed three times with 0.9% sodium chloride for 2 min. The diffusion cells containing the uncoated Ca-alginate membrane, APA membrane or 4-layer membrane, were loaded with a mixture of PEG samples [0.2 (w/v) % each of 10, 100, 200, and 300 kDa PEG in 0.9% sodium chloride] in the source compartment, and an equal amount of saline in the sink compartment. The assembly was maintained at room temperature with stirring in both compartments for 24 h.

Samples were taken from each side and analyzed by a Gel Permeation Chromatography (GPC) 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-μm particles) and a Waters 2414 refractive index detector. The columns were maintained at 35°C and the system was calibrated with molecular weight standards. Samples were eluted at a flow rate of 0.8 mL/min with a mobile phase consisting of 0.1*M* $NaNO_3$ in 0.05*M* phosphate buffer (pH 7).

Bovine Serum Albumin Uptake

FITC-labeled BSA (fBSA) was dissolved in 0.9% NaCl to form a 0.01% solution. Capsules (4-layer or APA) containing C2C12 cells were kept in an incubator at 37°C for 3 days, and then 5 mL of fBSA solution, 2 mL capsules, and 10 mL medium were added to a culture dish. After 24 h at 37°C, the capsules were then washed six times with 0.9% NaCl before examination by confocal microscopy.

Confocal Microscopy

Confocal microscopy was performed with a MRC 1024 confocal laser scanning microscope (Bio-Rad, Hemel Hempstead, United Kingdom) attached to a Microphot SA microscope (Nikon, Tokyo, Japan) equipped with a 10×, 0.3 NA (Nikon) lens. Images were analyzed with Image J1.34S (http://fbs.info.nih.gov/ij).

Stress Test

Microcapsules (100 μL) and 10 mL of 0.003% sodium-EDTA solution were loaded into a conical polypropylene tube and then agitated with an orbital mixer at 30 rpm for 15 min at room temperature. The capsules were then stained with trypan blue and transferred to glass dishes to determine the percentage of intact capsules. Each test was performed in triplicate.

Cell Viability

The viable cell number per capsule was determined using an Alamar Blue Assay (Trek Diagnostic Systems, Cleveland, OH).[19] Briefly, a known number of capsules (in 100 μL suspension) were loaded in a 24-well plate containing 500 μL medium and 50 μL Alamar Blue. The plate was incubated at 37°C for 4 h. After the incubation, 100 μL of the supernatant was transferred to a 96-well plate and checked for fluorescence ($\lambda_{ex}/\lambda_{em}$ = 535/590 nm) with a TECAN GENios plate reader (Australia). The number of viable cells was determined by comparing fluorescence values with a standard curve generated from a known number of un-encapsulated cells.

In Vivo Assessment of Capsules

The animals were treated in accordance with Canadian Institutional Animal Care guidelines. C57BL/6 mice

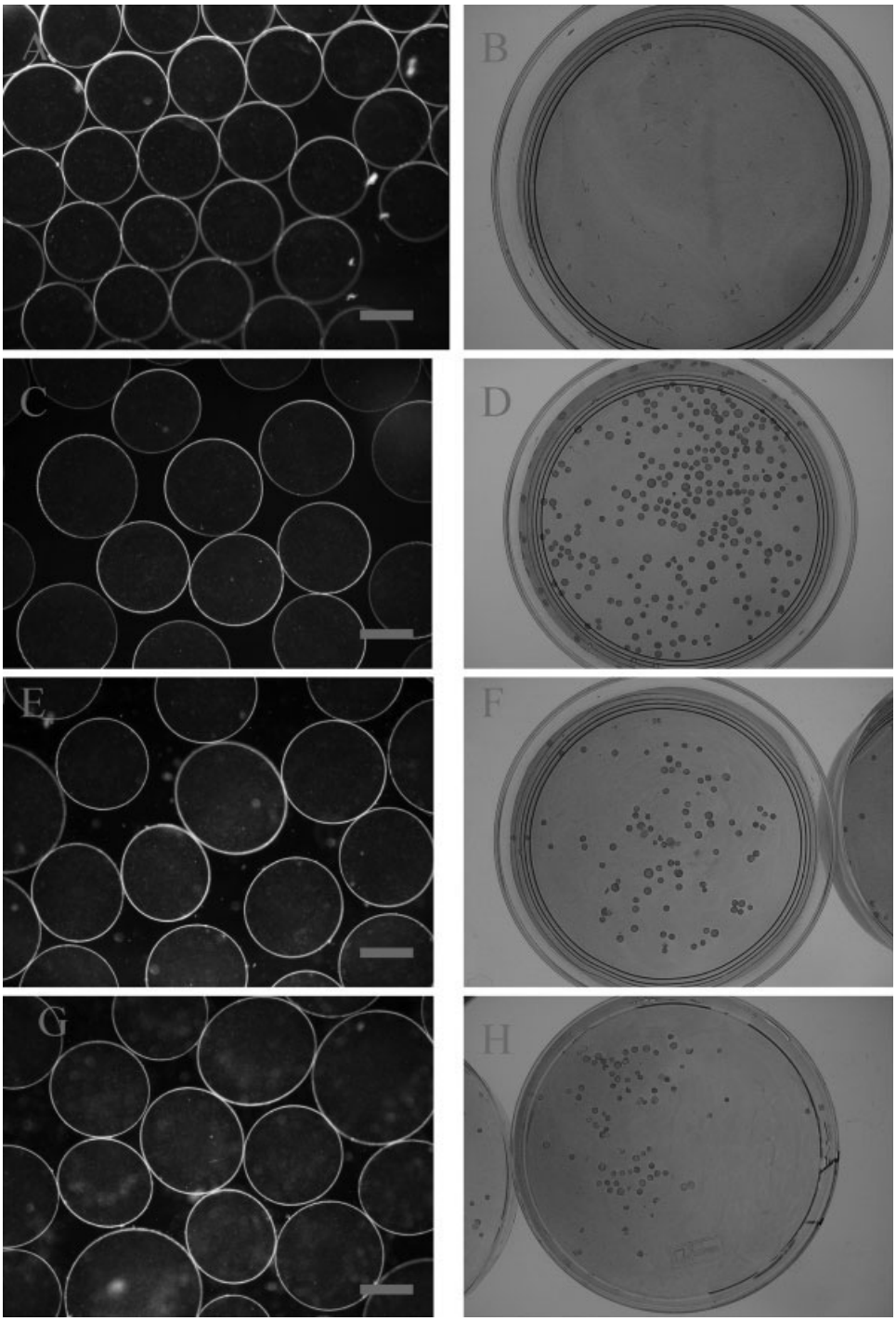

Figure 2. Light microscopy phase contrast images (A, C, E, G) of capsules before stress test, and optical pictures (B, D, F, H) of Petri dishes containing the same capsules shaken in 0.003% EDTA solution for 15 min and then stained with trypan blue. (A,B) Empty APA capsules, stored in saline for 3 days. (C,D) Empty 4-layer capsules, stored in saline for 3 days. (E,F) empty 4-layer capsules, retrieved from mice after 1-week implantation. (G,H) empty 4-layer capsules, retrieved from mice after 4-weeks implantation.

(Charles River, Montreal QC) were anaesthetized with isofluorane (Anaquest, Mississauga, ON) before a suspension of 3 mL microcapsules in normal saline (total volume 5 mL) was implanted into the intraperitoneal cavity of mice under sterile conditions using a 20-gauge catheter (BD, Oakville, ON). Blood samples from mice implanted with capsules for 1 and 4 weeks were collected by orbital bleeding. Hematological and biochemical liver function tests

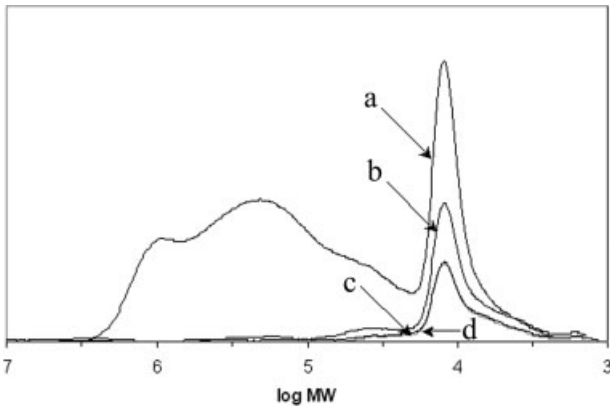

Figure 3. GPC traces of: (a) the original PEG solution; and the solutions in the receptor compartments at $t = 24$ h for (b) Ca-alginate membrane; (c) Ca-alginate-PLL-alginate membrane; and (d) Ca-algiante-C70-A70-PLL-alginate membrane.

were performed by VITA-TECH Canada (Mississauga, ON). At the end of the experiment mice were sacrificed and the capsules retrieved as previously described.[20] Three mice were tested for each kind of capsules at each time point.

RESULTS

Capsule Structure and Strength Testing

Empty capsules (without cells) were kept in saline in an incubator at 37°C for 3 days and then implanted into mice. Optical images of the capsules before implantation and those retrieved after implantation for 1 or 4 weeks are shown in Figure 2(C,E, and G). The capsules were subjected to the stress test. After shaking in EDTA, all of the APA capsules were dissolved or broken [Figure 2(B)]. However, most (>95%) of the 4-layer capsules were intact [Figure 2(D)]. Four-layer capsules retrieved from mice after 1 week [Figure 2(F)] and 4 weeks [Figure 2(H)] showed similar results where the majority of the capsules remained intact after the EDTA test. Previous studies[21,22] have shown that the strength of APA capsules decreases after mouse implantation.

The molecular weight cut-off of Ca-alginate, APA, and 4-layer membranes was tested by PEG diffusion across model flat membranes. Free diffusion through the membrane would result in source and receptor compartments with half the concentration of the original PEG mixture. The GPC spectrum in Figure 3 shows that higher MW PEG is excluded from the receptor compartments, while the 10 kDa PEG (log MW = 4) shows up in all receptor compartments. The transfer of intermediate MW PEG was partially retarded by all membranes, with an apparent pore-size order of Ca-alginate > APA ≈ 4-layer. The apparent MW cut-off for the APA and 4-layer membranes is ~70 kDa.

From the aforementioned data it appears that the pores for the APA and 4-layer membranes are close in size. A similar result was observed when the diffusion of fBSA into microcapsules was examined (Figure 4). Confocal images for 4-layer capsules or APA capsules incubated with fBSA (MW 67 kDa) showed that both kinds of capsules allowed fBSA through the surface membrane to interact with the encapsulated C2C12 cells. After 24-h incubation there are less fluorescent cells in the core of the 4-layer capsules, suggesting the rate of diffusion into the 4-layer capsules is less than that of the APA capsules.

The *in vivo* stability of the 4-layer capsule was assessed by fluorescently labeling the capsules with C70f. After incubation for 3 days, the labeled 4-layer capsules were implanted into six mice. Capsules were retrieved from three mice 1 week after implantation and from the remaining three mice after 4 weeks and were examined with confocal

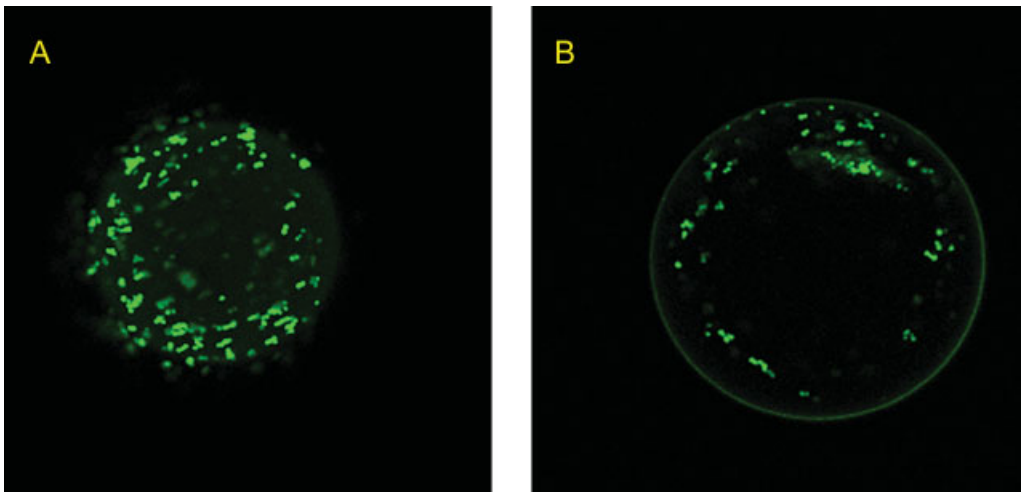

Figure 4. Confocal images of APA (A) and 4-layer (B) capsules containing C2C12 cells that were incubated with fluorescently labeled BSA at 37°C for 24 h. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

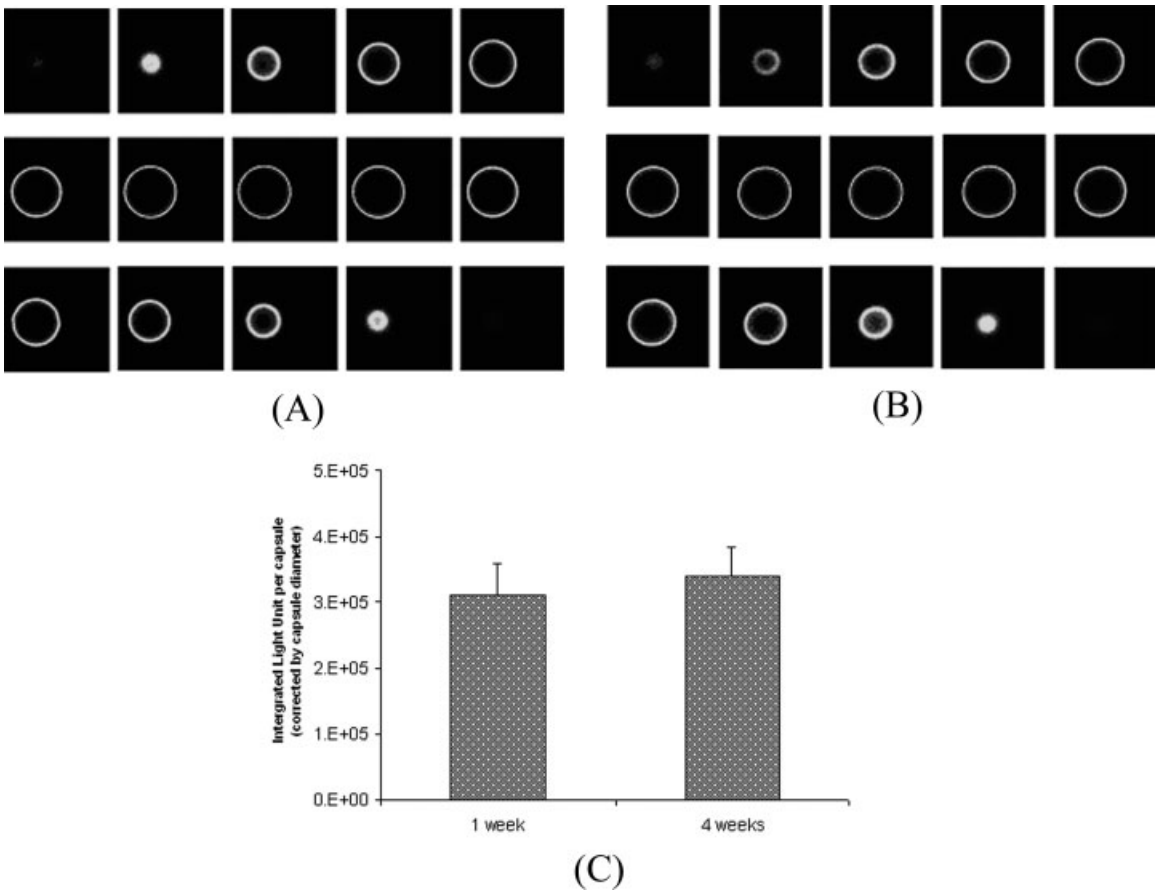

Figure 5. A series of optical slices taken with a confocal microscope from the top to the bottom of a 4-layer-capsule labeled with C70 before (A) and after (B) 4-weeks implantation in mice. The fluorescence intensity (corrected for capsule diameter) of capsules retrieved from mice after implantation for one and 4 weeks (C).

microscopy. Figures 5(A,B) show typical confocal images of labeled 4-layer capsules before and 4 weeks after implantation (1 week data, not shown, was similar). The integrated fluorescence intensity per capsule, which reflects the density of C70f on the capsule, remained stable after 1 or 4 weeks' implantation in mice [Figure 5(C)].

Biocompatibility Tests

The use of C70 and A70 and the presence of the cross-linked shell formed by C70/A70/PLL might impact the viability of encapsulated cells. Figure 6 shows the cell number per capsule in either 4-layer capsules or in APA capsules after 1, 7, or 14 days of *in vitro* incubation at 37°C. On the day after the capsule fabrication, the average cell number in 4-layer capsules was about 85% of that in APA capsules indicating some disadvantage in survival during the 4-layer capsule fabrication. The cell density in the 4-layer capsules increased at the same rate as in the APA capsules after 1 or 2 weeks *in vitro* incubation, which confirms the suitability of the internal environment of the modified capsules for cell growth.

In contrast to the empty 4-layer capsules [Figure 2(E–H)], the *in vivo* performance of these capsules with cells was sub-optimal. One week after implantation, the modified capsules were opaque in appearance [Figure 7(A)] likely indicating an inflammatory or immune reaction. It was surmised that this reaction was related to the A70 or C70, since in previous work with APA capsules containing the

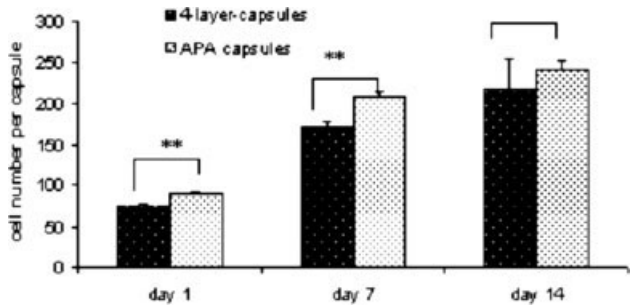

Figure 6. Effect of the C70 and A70 coating on survival of encapsulated cells. C2C12 myoblasts were encapsulated in 4-layer-capsules or APA capsules. The viable cell number per capsule was assayed after incubation for up to 14 days. (**$p < 0.01$; no statistical difference between the two kinds of capsules at day 14).

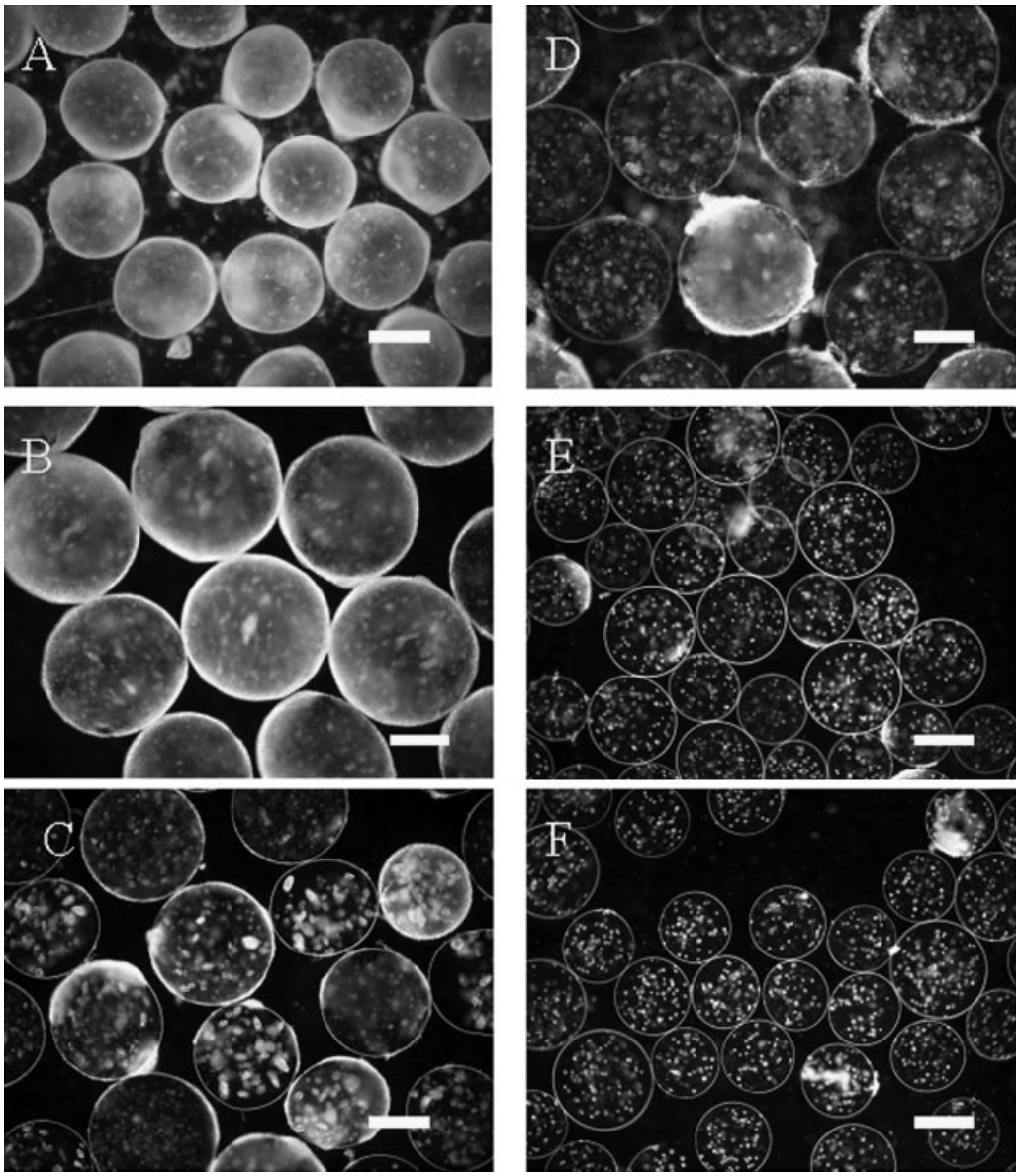

Figure 7. Phase contrast images of capsules retrieved from mice after one-week implantation. (A) 4-layer capsules (Ca-alginate beads sequentially coated with 0.5% C70, 0.5% A70, 0.05% PLL, 0.03% alginate). (B) "0.50% C70" capsules (Ca-alginate beads sequentially coated with 0.50% C70, 0.03% alginate). (C) "0.10% C70" capsules (Ca-alginate beads sequentially coated with 0.10% C70, 0.03% alginate). (D) "0.50% A70" capsules (Ca-alginate beads sequentially coated with 0.05% PLL, 0.50% A70, 0.05% PLL, 0.03% alginate). (E) "0.10% A70" capsules (Ca-alginate beads sequentially coated with 0.05% PLL, 0.10% A70, 0.05% PLL, 0.03% alginate). (F) "0.05% A70" capsules (Ca-alginate beads sequentially coated with 0.05% PLL, 0.05% A70, 0.05% PLL, 0.03% alginate). Data for "0.05% C70" capsules (Ca-alginate beads sequentially coated with 0.05% C70, 0.03% alginate) are absent because C70 concentrations lower than 0.1% cause the beads to clump together. Bar = 200 μm.

same concentration of C2C12 cells showed excellent biocompatibility in mice in *in vivo* tests.[20]

The effect of A70 and C70 and their respective concentrations was examined by fabrication of microcapsules in which only A70 or C70 was present. The images of these capsules retrieved from mice after 1-week implantation are shown in Figure 7(B–F). From these images, it is clear that 0.5% C70 or A70 alone are sufficient to initiate a host reaction that leads to the coating on the capsule surface. It appears that presence of C70 is more important than A70 for initiating the host response. A semiquantitative scoring was devised for the retrieved capsules: 200 capsules from each experiment were evaluated under microscopy, and the approximate percentage of the capsule surface covered by

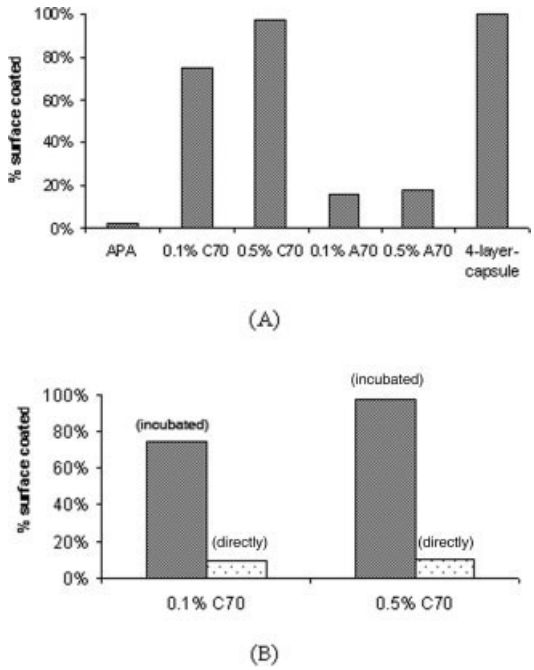

Figure 8. Summary of the surface analysis of capsules retrieved from mice that were implanted after 3-days in culture media (A) or implanted directly after manufacture (B, light bars). Capsules (~200) were randomly selected and the percentage of visible surface area coated by opaque materials was visually determined by light microscopy and the mean values are shown. 4-layer-capsules: Ca-alginate beads sequentially coated with 0.5% C70, 0.5% A70, 0.05% PLL, 0.03% alginate. "0.50% C70" capsules: Ca-alginate beads sequentially coated with 0.50% C70, 0.03% alginate. "0.10% C70" capsules: Ca-alginate beads sequentially coated with 0.10% C70, 0.03% alginate. "0.50% A70" capsules: Ca-alginate beads sequentially coated with 0.05% PLL, 0.50% A70, 0.05% PLL, 0.03% alginate. "0.10% A70" capsules: Ca-alginate beads sequentially coated with 0.05% PLL, 0.10% A70, 0.05% PLL, 0.03% alginate. "0.05% A70" capsules: Ca-alginate beads sequentially coated with 0.05% PLL, 0.05% A70, 0.05% PLL, 0.03% alginate.

growth was recorded. This data, summarized in Figure 8(A), agrees with the initial impression from the images shown in Figure 7.

A critical difference between the empty capsules and those containing cells was the culture conditions. Capsules with cells were kept in culture medium containing 10% FBS in an incubator for 3 days while empty capsules were incubated in normal saline for the same time period. Capsules implanted directly after manufacture [Figure 8(B)] showed less overgrowth. To examine the possibility that the C70 or A70 on the capsule surface absorbed proteins from the culture medium or from cell debris from the encapsulation procedure, empty capsules were incubated with fBSA immediately after fabrication. The 4-layer capsules have a fluorescent layer of fBSA on the surface, whose thickness depends on the amount of C70 and A70 used [Figure 9(A–B)]. This layer was absent in APA capsules [Figure 9(C)].

The dependence of fBSA binding on C70 and/or A70 was probed by preparing 4-layer capsules in which C70 was replaced by PLL and/or A70 was replaced by A100 [poly(methacrylic acid, sodium salt)], an analog of A70 that lacks acetoacetate groups. The capsules were exposed to 0.05% fBSA for 24 h at 20°C, washed five times with saline and then examined by confocal microscopy. The APA capsule [Figure 10(A)] shows a weak and uniform distribution of fBSA throughout. A similar intensity and distribution is seen for a 4-layer capsule when the C70/A70 layers were replaced by PLL/A100 [Figure 10(B)]. When A70 is present, some trapping of fBSA at the surface is observed [Figure 10(C)]. A capsule containing C70 but lacking A70 showed very strong fBSA binding [Figure 10(D)], similar to that exhibited by the 4-layer capsule [Figure 10(E)].

To further confirm the role of culture medium, serum-free medium (SFM) was used to replace the regular DMEM medium (containing FBS) for culture of the 4-layer capsules after fabrication. Figure 11 shows the resulting C2C12-containing 4-layer capsules retrieved from mice after one-week implantation. Exclusion of FBS from the process eliminates the reaction of the host against the 4-layer capsules, with the capsule surface remaining optically transparent. Further, the average cell number per capsule increased from 120 immediately after fabrication to 850 after 1-week *in vivo*.

Protein Expression and Release From Encapsulated Cells

pC3B.Luci transfected MDCK cells were encapsulated in APA and 4-layer capsules. Encapsulated cells were then cultured and the growth medium was collected for a luciferase assay 24 h after capsule fabrication. There was secretion and release of luciferase from pC3B.Luci transfected cells in both kind of capsules, but significantly less luciferase was released by the 4-layer capsules (27 ± 5 RLU/thousand cells for APA capsules; 13.4 ± 0.2 for 4-layer capsules, $p < 0.01$). As a control, untransfected MDCK cells were encapsulated and tested in the same way, and no luciferase activity was detected.

DISCUSSION

Cellular microencapsulation based on Ca-alginate beads typically requires several coating layers to confer the desired characteristics for *in vivo* implantation. These characteristics pertain to three areas: stability, permeability, and biocompatibility. Most applications require the highest stability possible without compromising the other two areas. In Ca-alginate beads, guluronate residues can be bound with divalent cations (i.e., Ca^{2+}, Ba^{2+}) and form chain-chain associations[23] allowing formation of the hydrogel core. However, leaching of calcium from the capsules can cause capsule failure. *In vivo*, this may happen by equilibration of calcium in the capsule with the large body pool of calcium that is bound by proteins (i.e., albumin) and

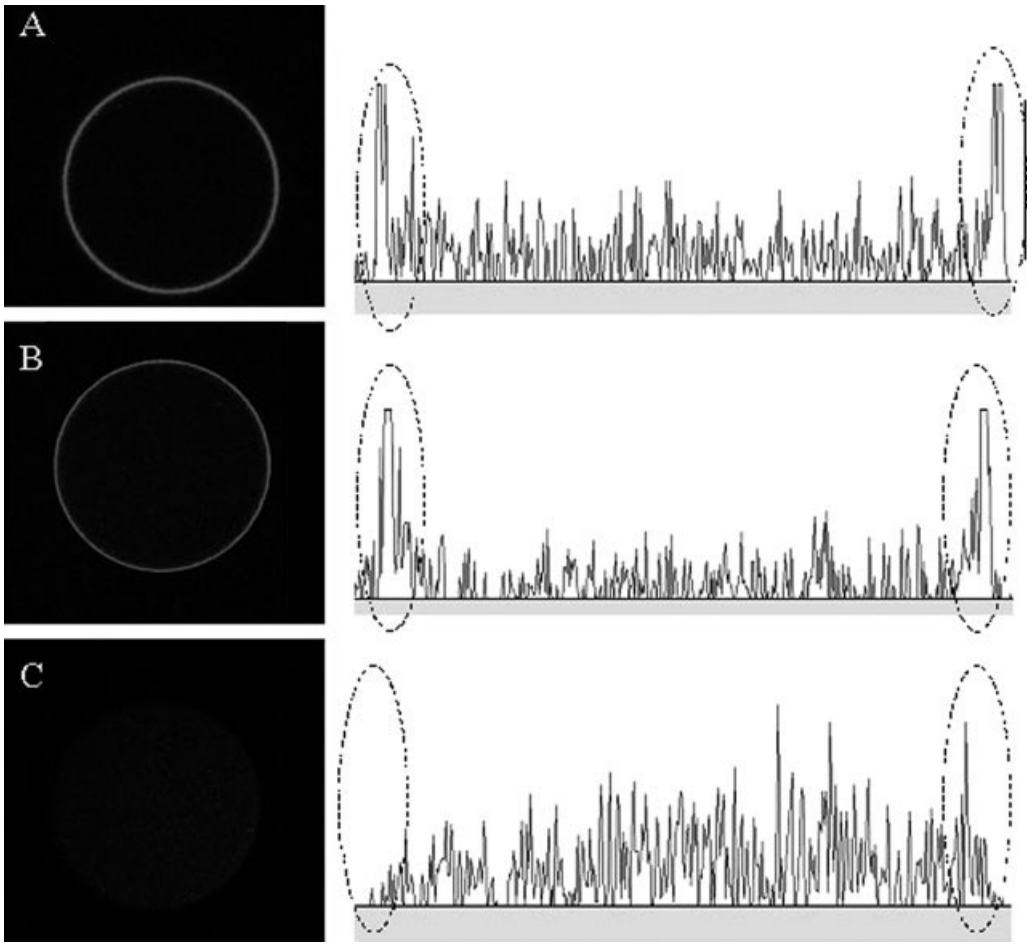

Figure 9. Confocal image through middle of capsules incubated in 0.05% FITC labeled BSA solution at 37°C for 24 h. Line profiles are shown to the right for each kind of capsule. (A) 4-layer-capsules (Ca-alginate bead sequentially coated with 0.50% C70, 0.50% A70, 0.05% PLL, 0.03% alginate). (B) Reduced C70/A70 4-layer-capsules (Ca-alginate bead sequentially coated with 0.10% C70, 0.10% A70, 0.05% PLL, 0.03% alginate). (C) APA capsules.

tightly regulated by hormone systems. Covalently-linked coatings may be able to resist dissolution and survive the loss of the underlying Ca-alginate gel leading to improved capsule stability. However, it is important to test the effects of capsule modifications on permeability, and biocompatibility in addition to stability.

Stability

In the 4-layer capsules, a reaction between C70 and A70 builds up a covalently linked network on the surface of Ca-alginate. The beads are then coated with PLL to further strengthen the capsule and to control the permeability, and finally with alginate to "hide" the other polymers and maximize biocompatibility (Figure 1). An *in vitro* assay, which we have called the stress test, mimics the *in vivo* effect of calcium leaching from the capsules at the same time as exposing the capsules to osmotic and mechanical stress. Figure 2 shows that the 4-layer capsules can survive the stress test, whereas APA capsules are totally destroyed. This is true of microcapsules maintained *in vitro* or *in vivo* in mice for up to 4 weeks before the stress test. Additionally, studies with fluorescently labeled C70f (Figure 5) showed that the fluorescence intensity of the capsule did not diminish after 4 weeks' implantation in mice, indicating the stability of the C70 component of the 4-layer capsules.

Permeability

There is a wide range reported for the permeability of alginate-based capsules.[20,24] One advantage of the typical APA capsule is that the PLL layer can be manipulated to provide a desired permeability. This allows the permeability to be selected to block immune mediators while still allowing the therapeutic secretion of recombinant proteins. The permeability of the chemically modified capsules was probed by PEG diffusion, fluorescently-labeled protein (fBSA) uptake and luciferase secretion from encapsulated cells. PEG diffusion analysis showed that the pore size of the 4-layer membrane was shown to be much smaller than Ca-alginate but similar to that of the alginate-PLL-alginate (Figure 3). This method is able to test the permeability to a large MW

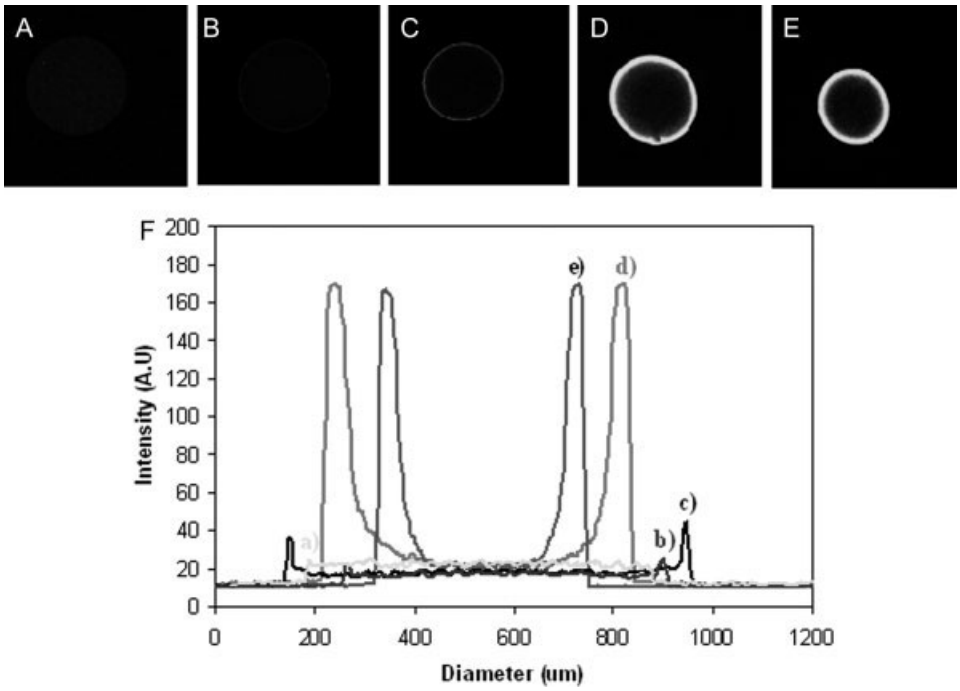

Figure 10. Top—Confocal images through the middle of (A) APA, (B) APA100PA, (C) APA70PA, (D) AC70A100PA, and (E) 4-layer (AC70A70PA) microcapsules exposed to 0.05 w/v % BSA-FITC at 20°C for 24 h. Bottom (F) Line profiles of microcapsules shown at top.

range, but is not able to detect small differences between capsule permeability. Protein/enzyme uptake and secretion, however, allow a more detailed examination of the permeability to a mono-dispersed MW molecule. Using these methods, the 4-layer capsule appears to have slightly smaller pore size than the APA capsule, as indicated by reduced fluorescence of encapsulated cells during fBSA uptake (Figure 4) and reduced amount of luciferase detected in media from encapsulated recombinant cells. Both of these latter tests examine permeability characteristics for proteins with MW near 65 kDa, and the luciferase experiment, in particular, provides proof of principle for encapsulated cellular therapy applications. Varying the molecular weight, concentration, and exposure time of PLL and/or the C70/A70 would allow further modifications of the pore size in the modified capsule to "fine-tune" the capsule for a particular application.

Biocompatibility

There are two considerations for microencapsulated cells that we include under "biocompatibility": first is the survival of the encapsulated cells within the microcapsule, and second is the reaction of the host to the microcapsules/microencapsulated cells after implantation.

The first is relatively easy to quantify by measuring cell survival after the encapsulation process and after a period of time *in vitro* or *in vivo*. Survival during the encapsulation process can be affected by many factors, such as exposure to reactive/harmful chemicals and length of time spent in sub-optimal tissue culture conditions (i.e., sub-optimal nutrient, gas and/or temperature levels). Our results show that the 4-layer capsules have a slightly negative effect on the cells, but that this seems to be mostly related to the encapsulation process itself. Survival and expansion of cells after the initial encapsulation is robust (Figures 6 and 11).

It is more complex to measure the host reaction to the microcapsules and the encapsulated cells. Liver function and CBC tests (data not shown) suggest that capsule im-

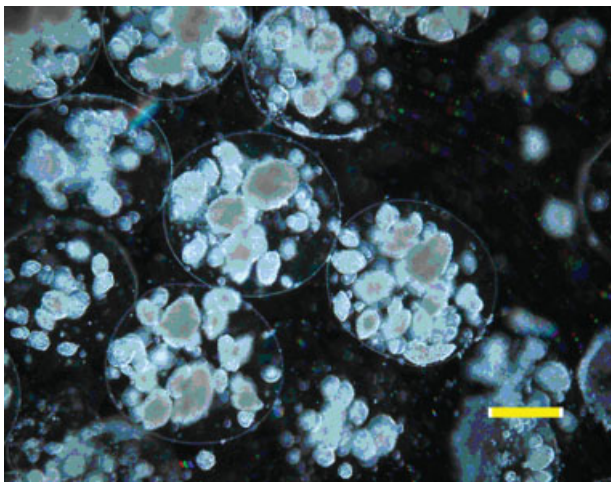

Figure 11. Four-layer capsules (cultured in serum-free medium for 72 h in advance of implantation) were retrieved after 1 week of mouse implantation. The average cell number per capsule was 850. Bar = 200μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

plantation, regardless of capsule type or whether the capsules contain cells or not, did not produce systemic toxicity to the mice. Another gross estimate of biocompatibility is to examine the microcapsules after implantation. Clearly the 4-layer capsules had greatly decreased biocompatibility when compared with the APA capsules, as evidenced by what appears to be a fibrotic coating on the surface of the retrieved microcapsules containing cells. Yet, 4-layer capsules without cells and APA microcapsules with or without cells did not show the same reaction.

These findings made us suspect the A70 and/or C70 were binding to cell debris from cells that died during encapsulation or to components of the culture media. Culturing the microcapsules before implantation is intended to allow the encapsulated cells to recover from the fabrication process and to reduce the load of necrotic and marginally surviving cells implanted. However, if cell debris or foreign proteins are trapped on the microcapsule surface, they would have an immune-stimulating effect upon implantation. By varying the nature or the concentration of the polymers used to make the coating (Figures 7, 8, and 10), it was determined that the C70 was the major contributor to binding of media proteins to the microcapsule surface, whereas A70 had less effect. The binding of media proteins was lessened by reducing the concentration of C70 or A70, but was eliminated by using serum-free media to culture the microcapsules post-fabrication. This suggests that the media components had the largest effect on inducing a fibrotic reaction. We postulate that a covalent linkage between the acetoacetate groups of A70 and amine groups on media proteins or a polyelectrolyte complex between C70 and negatively charges proteins (e.g., albumin) was formed, fixing the proteins on the capsule surface (supported by the fBSA results, Figures 9 and 10). Fortunately, the relatively simple measure of replacing the regular medium with serum-free medium for incubation after fabrication eliminated the host reaction as shown in Figure 11.

In summary, we have constructed an alginate-based microcapsule with a covalently cross-linked shell that shows improved stability on chemical testing. The 4-layer capsule maintained good biocompatibility characteristics and had permeability similar to APA capsules.

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