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Microcapsules with Intrinsic Barium Radiopacity for Immunoprotection and X-ray/CT imaging of Pancreatic Islet Cells

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Abstract

Microencapsulation is a commonly used technique for immunoprotection of engrafted therapeutic cells. We investigated a library of capsule formulations to determine the most optimal formulation for pancreatic beta islet cell transplantation, using barium as the gelating ion and clinical-grade protamine sulfate (PS) as a new cationic capsule cross-linker. Barium-gelated alginate/PS/alginate microcapsules (APSA, diameter = 444 ± 21 μm) proved to be mechanically stronger and supported a higher cell viability as compared to conventional alginate/poly-L-lysine/alginate (APLLA) capsules. Human pancreatic islets encapsulated inside APSA capsules, gelated with 20 mM barium as optimal concentration, exhibited a sustained morphological integrity, viability, and functionality for at least 3–4 weeks *in vitro*, with secreted human C-peptide levels of 0.2–160 pg/ml/islet. Unlike APLLA capsules that are gelled with calcium, barium-APSA capsules are intrinsically radiopaque and, when engrafted into mice, could be readily imaged *in vivo* with micro-computed tomography (CT). Without the need of adding contrast agents, these capsules offer a clinically applicable alternative for simultaneous immunoprotection and real-time, non-invasive X-ray/CT monitoring of engrafted cells during and after *in vivo* administration.

Keywords

Microcapsule; Islet cell; Diabetes; CT imaging; Barium

Introduction

Microencapsulation of biological products is used for a myriad of applications, ranging from cell therapy, cell-based bioreactors, and cell biosensors, to encapsulation of DNA, protein,

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or drugs for targeted *in vivo* delivery [1–5]. In particular, engraftment of microencapsulated therapeutic cells has been explored as a means to treat pathological conditions where replacement of the diseased organ or cells is crucial, but difficult to achieve. In type I diabetes mellitus, the patient is unable to produce insulin due to the destruction of pancreatic beta islet cells by the body's own immune system. The resulting health effect is hyperglycemia that leads to cardiovascular disease, neuropathy, retinopathy, and nephropathy. Transplantation of beta islet cells has shown great promise in providing moment-to-moment insulin regulation, much like the native, healthy pancreas [6–10]. However, immune-rejection of islet grafts has been a major challenge in pancreatic beta islet cell therapy. Immunosuppressive regimes designed to overcome this issue are potentially cytotoxic to the islet grafts, and increase the patients' risk for infection and cancer. The use of immunosuppressive drugs can be circumvented by the microencapsulation strategy [4, 6, 11–13], and recently clinical trials have been initiated in type 1 diabetes patients [14].

In this approach, pancreatic beta islet cells are encapsulated inside a matrix composed of a biopolymer. These capsules are semi-permeable and allow the passage of small metabolites, such as nutrients, glucose, and insulin, while physically blocking antibodies and immune cells, and preventing opsonization of encapsulated cells. Considering the physical isolation offered by microcapsules, this approach opens new prospects for islet xenografts in addition to allogeneic transplantation. Transplantation of microencapsulated cells into a specific organ, as opposed to external biodevices, provides the physiological conditions critical to cell viability, as well as a fast response to biological signals, for example, insulin secretion in response to elevated blood glucose levels. Moreover, beta islet cells are encapsulated inside a three-dimensional supporting matrix, which closely mimics the native tissue environment.

Microcapsules with a good potential for cell therapy should be non-toxic, mechanically strong, stable for the required period of treatment, and preserve the therapeutic properties of encapsulated cells [2, 4, 15]. Spherical microcapsules are ideal due to their large surface/volume ratio, i.e., high mass transfer rate of metabolites across the capsules. In addition, non-spherical capsules were found to be more immune-reactive than spherical ones [16]. It is crucial that the surface of the microcapsules is smooth, since surface irregularities may trigger a host immune response [4]. To date, alginate has been an attractive choice for the capsule material [1, 4]. Alginate is a polymer derived from seaweed, which has been extensively used in food and pharmaceutical applications. When exposed to multivalent cations, such as calcium or barium, alginate forms a hydrogel at physiological conditions, which is favored for maintaining the viability and functionality of cells. The surface of alginate capsules is typically cross-linked with a polycationic polymer and further cross-linked with an outer layer of alginate in order to engineer the capsule pore size [17]. The outer alginate layer also traps and covers encapsulated cells, which membrane extensions may protrude out of the inner alginate spheres and can potentially activate an immune response. The electrostatic repulsion between negatively-charged immune cells and polyanionic alginate can prevent or delay the immune cells from attacking the engrafted cells [17].

The concept of microencapsulated beta islets was first introduced by Lim and Sun [18], who encapsulated pancreatic beta islets inside calcium-gelated alginate microcapsules. In their study, alginate microcapsules were cross-linked with polycationic poly-L-lysine (PLL) and an outer layer of alginate. However, these capsules were found to cause fibrosis when engrafted inside an animal, presumably due to cationic lysine branches of PLL protruding out of the outer alginate layer, which in turns, attract immune cells [19–21]. This finding, however, did not discourage the investigation of therapeutic applications of alginate microcapsules. To date, the therapeutic efficacy of alginate-encapsulated pancreatic islets to

treat diabetic animals is being investigated, but very few trials have advanced to large animal models [16, 22]. In the recent study by Tuch et al. [14], four T1DM patients were injected intra-abdominally with encapsulated islets. Islets stopped functioning between 1–4 weeks post engraftment, as concluded from undetectable c-peptide levels. However, in one multi-islet recipient, c-peptide was detected at 6 weeks after the third infusion and remained detectable for 2.5 years. Recovered capsules were found to be surrounded by fibrous tissues and contained necrotic islets. The outcome of this trial indicates the necessity of further improving encapsulated islet cell therapy.

To this end, we studied a library of capsule formulations using Ba^{2+} ions as the gelating cation and the protamine residues from clinical-grade protamine sulfate (PS) as a new alginate cross-linker. Ba^{2+} ions have a higher affinity than Ca^{2+} ions; therefore, the mixing of barium ions and alginate produces a stronger barium-alginate hydrogel [23]. The use of protamine residues as a capsule cross-linker is a clinically applicable choice, since PS is currently used as an anti-heparin agent in the clinic and as a transfection agent with a lower toxicity than that of poly-L-lysine in research applications [24, 25]. Since barium ions are more electron-dense than calcium ions, we hypothesized that barium-gelated capsules would be intrinsically radiopaque for use as an imaging agent. At present, engraftment of pancreatic cells is performed blindly with few cases using imaging guidance [26]. A robust non-invasive means to follow the delivery and tissue engraftment of transplanted cells is essential to assure the accuracy of the surgical procedures, to monitor graft post-surgery, and to study the underlying factors in graft failure [27–31]. In order to visualize alginate microcapsules, exogenous contrast agents can be co-encapsulated [32] for visualization by MR [22, 33–35], ultrasound [33–35], or X-ray/CT imaging [33–36]. Radiopaque microencapsulated cells are clinically of interest, since X-ray/CT imaging is a commonly used medical imaging modality for real-time injection of biological and cellular therapeutics. In the present study, we synthesized new microcapsule formulations based on barium-gelation and PS cross-linking, and assessed whether or not these Ba^{2+} -containing capsules can be detected by X-ray/CT imaging without the use of exogenous contrast agents.

Materials and Methods

Cell culture

Cadaveric human pancreatic islets were provided by the National Islet Cell Resource Center. The purity and viability of human islets used in the experiments were at least 90%. Human islets were encapsulated one day after extraction from the donor, and cultured using CMRL1066 medium (Mediatech, Manassas, VA), supplemented with 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA), 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO), and 1% amphotericin B (250 $\mu\text{g}/\text{ml}$, Sigma-Aldrich, St. Louis, MO). Mouse insulinoma $\beta\text{TC}-6$ cells were purchased from ATCC (Manassas, VA) and cultured in DMEM medium (ATCC), supplemented with 15% fetal bovine serum, 1% penicillin/streptomycin, and 1% amphotericin B.

Microcapsule synthesis

Ultrapurified alginates were obtained from Nova Matrix (Sandvika, Norway). LVG alginate is a low-viscosity alginate rich in guluronate blocks, while LVM alginate is abundant in mannuronate blocks. Human pancreatic islets (16,000–17,000 islets per ml of alginate to obtain approximately 1–2 islets per capsule) or mouse insulinoma $\beta\text{TC}-6$ cells (15 million cells per ml of alginate) were suspended in 2% w/v Pronova UP LVG [32]. Alginate microcapsules were formed using an electrostatic droplet generator at a flow rate of 0.1 ml/min, and were immediately gelated in a divalent cationic bath. The capsules were washed twice with 0.9% w/v NaCl + 10 mM HEPES, and subsequently cross-linked with 0.05% w/v

or 0.1% w/v polycationic polymer for <5 min or 10–15 min. After saline washing, the outer surface of the capsules was cross-linked with 0.15% w/v Pronova UP alginate (LVM or LVG) for 5 min, followed by a final saline washing. Solutions were prepared in 0.9% w/v NaCl + 10 mM HEPES. PS was purchased from NovaPlus (APP Pharmaceuticals, Schaumburg, IL) and poly-L-lysine hydrobromide from Sigma-Aldrich (P-7890, Mw= 15–30 kDa, St. Louis, MO). Nine different formulations of capsules were synthesized by varying the divalent cation type, concentration of gelating cation, gelation time, type of cross-linker (polycation), concentration of polycation, and type of alginate outer layer (see Table 1).

The concentration of non-gelled, free Ba^{2+} ions in the BaCl_2 gelling bath after capsule synthesis was measured using inductively coupled plasma mass spectrometry (ICP-MS, Microbac Laboratories Inc., Baltimore, MD). The concentration of Ba^{2+} ions bound to the alginate LVG inner capsule was calculated from ICP-MS data.

In vitro assessment of capsule mechanical strength: Osmotic pressure test

One hundred capsules were washed with picopure water and incubated in 5 ml of picopure water at 37°C for 1 hour. Capsules were then washed with 0.9% saline/10 mM HEPES and stained with 0.5% trypan blue for five minutes to facilitate visualization of capsule fracture. After subsequent saline washing, fractured or swollen capsules, as well as intact capsules, were counted and visualized using an inverted light microscope (Olympus IX71, Center Valley, PA).

In vitro assessment of capsule mechanical strength: Bead agitation test

Microcapsules were suspended in 1.0 ml of 0.9% saline/10 mM HEPES containing 0.05 g of inert glass beads (diameter 0.425–0.6 mm, Sigma-Aldrich, St. Louis, MO). The suspension was centrifuged for 3 hours at 13,200 rpm at room temperature using an Eppendorf Minispin microcentrifuge (Brinkmann, VWR LabShop, Batavia, IL). Ruptured, fractured, and intact capsules were counted as described above.

In vitro assessment of capsule mechanical strength: Time stability test

Capsules were incubated in 0.9% saline/10 mM HEPES at 37°C with and without a physiological concentration of Ca^{2+} (1.3 mM of Ca^{2+} , i.e., similar to blood plasma). The capsule dimension and morphology were examined immediately after capsule synthesis (Day 0), and at 3, 7, and 30 days after synthesis. The data are presented as the ratio of the final diameter to the initial diameter (Day 3/Day 0, Day 7/Day 0, and Day 30/Day 0).

In vitro assessment of cell viability

Mouse insulinoma cells encapsulated in alginate microcapsules were stained with 0.156 mM Fluorescein Diacetate (FDA) and 1.47 μM propidium iodide (PI) for three min and subsequently imaged using a LSM 5 Zeiss confocal microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY). Images of 100 optical slices per capsule were obtained. The intensities of stained live (FDA-stained) and dead (PI-stained) cells in each slice image were measured by a manually-calibrated, pixel-counting python computer program and converted into the percentage of live cells inside the capsules [34].

In vitro assessment of cell functionality

Three hundred capsules containing human islets were cultured in a six-well plate. Equal numbers of unencapsulated (naked) islets were also cultured for comparison. The culture medium was changed and collected every 3 days. Aliquots of the culture medium were stored at –80°C. The functionality of encapsulated and naked human islets was determined

by measuring the human C-peptide secretion level of encapsulated and naked islets using a C-peptide EIA ELISA kit (Alpco Diagnostics, Salem, NH). The data are presented as the average $\pm$ SD of three samples.

X-Ray/micro-CT imaging

One hundred phantom microcapsules in 0.9% saline were imaged using an XSPECT Gamma Medica imager (Gamma Medica-Ideas Inc., Northridge, CA), with 75.16 kV and 240.3 μ A tube settings, a 64 mm detector setting, and a number of projections=512. CT images were processed using Image J software. Using a syringe with a 18G needle, 3000–4000 empty capsules in 1.0 ml 0.9% saline were engrafted subcutaneously into the abdomen of male C57Bl/6 mice, aged 8–10 weeks (n=3). Mice were anesthetized with 1–2% isoflurane anesthesia, and scanned with micro-CT one day post-transplantation, using the same settings as described above for the phantom imaging. Animal experiments were approved by our Institutional Animal Care and Use Committee.

Statistical analysis

Statistical differences were calculated by a two-tailed, unpaired, unequal variance student's t-test at a significance level of $P < 0.05$.

Results

Microcapsule formulations

We hypothesized that the mechanical strength of alginate microcapsules may be improved by: 1) varying the concentration of Ba^{2+} in the gelation bath; and 2) strengthening the outer layer of the capsules by varying the concentration of the cationic alginate cross-linker when using LVG alginate as the final capsule layer. A library of 9 different capsule formulations was synthesized (Type I–IX, Table 1). We expected that stronger alginate bonds would result in the formation of more robust capsules, however, such a dense alginate matrix may decrease the passage of metabolites and nutrients, and eventually starve encapsulated cells. For Type I–IV capsules, the concentration of Ba^{2+} used to gel the inner alginate matrix of the capsules was therefore increased accordingly in order to increase the gelation degree of the alginate hydrogel. In order to further enhance the degree of alginate gelation, Type V capsules were gelled in 20 mM Ba^{2+} for 10–15 min, i.e., two to three times longer than the typical gelation time (< 5 min). Type VI capsules were crosslinked with twice the amount of PS. By increasing the concentration of PS, we expected to obtain capsules with a stronger outer layer. However, the capsule pore size might shrink and potentially obstruct unhindered passage of small molecules across a semi-permeable membrane. Type VII capsules are formulations where the surface of the capsules was crosslinked with LVG alginate rather than LVM alginate. LVG alginate is rich in guluronate monomers, while LVM alginate is abundant in mannuronate monomers. Multivalent cations can only link regions of subsequent guluronate blocks with another homopolymeric guluronate region; therefore, we hypothesized that, by using LVG alginate as the outer layer, we could thicken the outer shell and strengthen the capsules. To fully compare and understand the effect of gelation by using Ba^{2+} instead Ca^{2+} , we included Type VIII capsules as a control, having the same make up as conventional APLLA capsules except that PS was used as capsule cross-linker. Finally, Type IX capsules were prepared in which conventional APLLA was gelated with Ca^{2+} .

Appearance of microcapsules

Each capsule formulation was carefully examined using a light microscope immediately post-synthesis. Representative images of each capsule formulation are shown in Figure 1. Capsules I, II, III, V, VIII, and IX (Figs. 1a–f) were spherical with a smooth surface which is favored, given that a non-spherical capsule shape or irregular surface can activate the host

immune response [4, 16]. Although Type IV capsules were spherical in shape, at this high Ba^{2+} concentration salt crystals were deposited on the capsule surface (Fig. 1g), which might compromise the biocompatibility of the capsules. We speculate that these crystals are Ba_2SO_4 , representing the precipitates of Ba^{2+} and sulfate residues from PS. The crystals could not be removed by repeated washes or prolonged incubation in saline or culture medium, or incubation in 6 mM Na_2SO_4 solution in order to dissolve the crystals. To a lesser extent, the crystals were also found on the surface of Type VI capsules (Fig. 1h). We observed that Type VI capsules were darker in appearance, in concordance with the higher PS concentration that was used to cross-link the capsule surface, forming a thicker outer layer. However, the individual Type VI capsules were found to vary in their degree of darkness, presumably due to an uneven cross-linking of protamine residues. In addition, Type VI capsules were less dispersed than other capsule formulations with capsules adhering to each other, which is undesirable as free *in vivo* oxygen diffusion at the transplantation site may become compromised. For Type VII capsules, we observed a thick alginate layer surrounding the surface (Figs. 1i–j). The thick alginate layer was unstable and became dislodged a few days post-synthesis. This can be explained by the use of guluronate-rich alginate (LVG), which has more binding sites as compared to mannuronate-rich alginate (LVM). LVG may form polyelectrolyte complexes with polycations, such as protamine residues from PS, which are partially bound to the inner alginate capsules.

The average diameter of the capsules immediately post-synthesis is given in Table 1. For hepatic transplantation of capsules via intraportal vein injection, a capsule diameter around 500 μm is ideal. When the Ba^{2+} concentration in the gelating solution was increased (from 10–100 mM for Type I–IV capsules, respectively), the capsule generally became smaller due to an increasing degree of alginate being gelled by Ba^{2+} . During the synthesis process, we observed that the diameter of capsules would gradually change. The capsules underwent several washes with physiological saline, during which time water molecules enter or exit the capsule as a result from osmotic pressure differences, making the capsules swell or shrink. For a given Ba^{2+} concentration, a longer gelation time (Type V capsule) or higher PS concentration (Type VI capsule) resulted in larger capsules (compared to Type II). When Ca^{2+} -gelled capsules are compared to Ba^{2+} -gelled capsules, we observed a much larger diameter for Type VIII and IX capsules (compared to Type IV). As the interaction between Ba^{2+} and alginate molecules is stronger, these capsule types are more resistant to water osmosis.

In vitro mechanical strength and stability

We assessed the mechanical strength of the various capsule types by performing three sets of *in vitro* experiments: 1) an osmotic pressure test to simulate the worst case scenario by exposing capsules to pure water; 2) a bead agitation test to simulate the shear force of flowing blood; and 3) a time stability test to study the possible degradation of capsules over time. Capsules subjected to the glass bead treatment were visibly collapsed or dented (Fig. 2a,b), while those subjected to osmotic pressure gradients were swollen or fractured (Fig. 2c,d). Capsules that were not affected in any way were counted as intact capsules. The results of the glass bead and osmotic pressure tests are summarized in Fig. 2e. Type I, II, and V capsules were found to best withstand these tests of mechanical strength. Unexpectedly, Type III capsules were less stable compared to capsules gelled with lower Ba^{2+} concentrations. It is possible that the higher barium concentration may have created an ionic repulsion between the molecules, decreasing the degree of gelation, and weakening the capsules. We did not include the Type IV formulation due to its heavy presence of crystals on the surface. All Type VI capsules were collapsed or dented after glass bead treatment, while nearly all Type VIII and IX capsules were adversely affected after exposure to osmotic pressure gradients.

To assess the stability of capsules over time, capsules were incubated at 37°C in regular saline (Fig. 2f) or saline containing a physiological concentration of 1.3 mM Ca^{2+} (Fig. 2g), which mimics the physiological constitution of blood plasma. Capsule morphology and diameter were monitored over a period of 1 month. None of the capsule formulations showed morphological degradation over the one month-period; however, their diameters changed over time. The change in diameter occurred within 1 week of incubation and then the capsules stabilized as a result of equalization of osmotic pressure inside and outside the capsules. The Type I, II and V capsules were the most promising formulations in terms of stability over time, while the Type VI and VIII capsules appeared least promising.

In vitro cell viability

Mouse insulinoma βTC6 cells were encapsulated inside Type I, II, and III capsule formulations, with Types VIII and IX as control, and their cell viability was determined (Fig. 3). A shorter gelation time is preferable to preserve the viability of fragile pancreatic islets during the capsule synthesis process. We selected Type I and II capsules for the *in vitro* cell viability test since prolonging the gelation time (Type V) did not appear to improve the capsule mechanical strength. Type III capsules were included to study the effect of higher Ba^{2+} concentration on encapsulated cell viability. Type VIII capsules were used to measure the effect of Ba^{2+} vs. Ca^{2+} on cell viability. We compared the viabilities of cells encapsulated in our new capsule formulations with that of cells encapsulated in conventional APLLA capsules (Type IX).

Capsule formulations that exhibited >80% viability are the Types II, III, and VIII. Since Ba^{2+} ions may be potentially toxic to cells, the low viability of ~20% for the formulation gelled with the lowest concentration of Ba^{2+} (Type I) was unexpected. Overall, our new barium APSA formulations (Type II and III) resulted in a statistically significant higher viability compared to conventional APLLA capsules ($P < 0.05$, Fig. 3). Previous studies have indicated that the exposed cationic lysine branches of PLL may attract immune cells, resulting in fibrosis [19, 20]. It is possible that these cationic lysine branches also adversely affected the viability of encapsulated cells.

In vitro functionality

In order to study the functionality of our new barium APSA formulation, human pancreatic islets were encapsulated in Type II microcapsules, which appeared to be the most promising formulation, based on our mechanical strength and viability tests. Representative images of a human pancreatic islet encapsulated inside a Type II capsule are shown in Figs. 4a,b. Capsules were spherical with a smooth surface and a diameter of $444.18 \pm 20.52 \mu\text{m}$, fully enclosing the islets for complete immunoprotection. Each capsule can encapsulate 1–2 human islets, which have a mean diameter of $150 \mu\text{m}$. Encapsulated human islets retained their islet structure for at least 30 days in culture (Figs. 4a,b). In contrast, unencapsulated islets broke into single cells after being in several days in culture (Fig. 4c,d). Encapsulated human islets secreted C-peptide for at least 24 days *in vitro*, which was several days longer than that observed for unencapsulated islets (Fig. 4e). We measured <1 nM of barium that was actually bound to the LVG alginate. This concentration is much lower than the published concentration of free Ba^{2+} (5–10 mM) [37], which may potentially be toxic to cells by disrupting potassium channel membrane activity.

Micro-CT imaging

Barium ions are much more electron-dense than calcium ions, and we found that barium-gelled capsules are radiopaque enough to be visualized by CT imaging. An example is shown in Fig. 5a,b for Type II capsules. This radiopacity lasted for at least 15 months *in vitro*, indicating that barium ions do not dissociate from alginate for a long period of time. In

contrast, calcium-gelled capsules (Type VIII and IX) were not visible on micro-CT (Fig. 5c,d). When Type II capsules were transplanted s.c into the mouse abdomen, they could be easily detected (Fig. 5e,g).

Discussion

We employed an established capsule preparation method using an electrostatic bead generator [2] to produce different formulations of spherical alginate capsules, all have an approximate diameter of 500 μm . Most capsules were found to have a smooth surface, which is essential for their biocompatibility. In order to effectively protect engrafted cells against the immune system *in vivo* for the required period of treatment, microcapsules must possess sufficient mechanical strength to withstand the shear force of blood circulation and *in vivo* osmotic gradients. The best transplantation site for encapsulated cells is considered to be intraportal injection into the liver due to its high-perfusion rate, assuring high oxygenation and supply of nutrients. In this scenario, encapsulated cells are constantly exposed to blood flow. When the capsule integrity is compromised, encapsulated cells are exposed to the immune system, which eventually will lead to rejection and failure of cell therapy.

For successful encapsulated cell therapy, it is also important for capsules to be free of erosion. This can be caused, for example, by dissociation of barium ions or protamine residues from the alginate as a result from prolonged exposure to osmotic pressure gradients. In addition, alginate-bound Ba^{2+} or Ca^{2+} ions may also exchange with Ca^{2+} ions that are naturally abundant in blood plasma and can weaken the capsules. We therefore performed a series of *in vitro* assays in order to measure the mechanical strength of capsules to withstand *in vivo* physical forces, as well as the stability of capsules over time. Barium-gelled capsules (Type I and II formulations) were found to be superior compared to calcium-gelled (Type VIII and IX) capsules. We postulate that this arises from the stronger alginate binding affinity of Ba^{2+} compared to Ca^{2+} . Calcium-gelled capsules were particularly susceptible to osmotic pressure changes, with the Type VIII capsules being more fragile than the Type IX capsules. Continuous swelling of capsules may cause them to burst, while shrinking of capsules may lead to exposure of encapsulated cells. We found that the protamine cross-linker is best combined with barium ions. The viability study showed that the Type II, III, and VIII formulations exhibited the highest cellular viability (>80%), while the conventional Type IX capsules had a cell viability of ~60%. Since Type II capsules are also superior in mechanical strength compared to Type III and Type VIII, we chose to perform further experiments with this type of capsules.

Human pancreatic islets were encapsulated inside Type II capsules and tested for functional C-peptide secretion over a period of one month. Capsules were spherical with a smooth surface and a diameter of $444.18 \pm 20.52 \mu\text{m}$, which is ideal for hepatic transplantation of capsules via injection through the intraportal vein. For engraftment sites other than the liver, however, smaller capsules may be desirable, since a smaller diameter offers less restrictive diffusion of small metabolites compared to larger capsules. For Type II capsules, that were synthesized in the presence of 20 mM barium in the gelation bath, we found that less than 1 nM of barium ions were actually bound to the alginate, which is much lower than the cytotoxic level of free barium ions (5–10 mM) [37]. Moreover, while barium is toxic in its free ionic form, they are bound here to the alginate polymer. Indeed, we were unable to observe any toxicity for barium-APSA encapsulated islets. They maintained their structural integrity for at least 30 days *in vitro*, with the three-dimensional supporting matrix provided by the alginate polymer acting like an extracellular matrix that is present in native pancreatic tissues. In contrast, unencapsulated, naked islets broke down into single cells within a few days of culture. Encapsulated islets secreted C-peptide levels of 0.2–160 pg/ml/islet over a

24-day period. Except for days three and six, these levels were not statistically different than those of unencapsulated islets. Thus, the semi-permeable alginate membrane in Type II capsules allows a sufficient passage of nutrients, glucose, and insulin, and does not have detrimental effects on the viability and functionality of encapsulated human islets.

Unlike calcium-gelated capsules, our most important finding in this study was that barium-gelated capsules have radiopacity which is a unique, intrinsic property that may be exploited for clinical applications. Of note is that this radiopacity persisted for at least 15 months *in vitro*. This property of barium-APSA capsules can significantly reduce, and perhaps eliminate, the use of typical X-ray/CT contrast agents (i.e., gold nanoparticles, barium sulfate, and iodine) in image-guided applications. When these conventional contrast agents are co-encapsulated [32], there is a potential risk of *in vivo* release of agents in the case of capsule rupture, and some of these CT contrast agents can be quite toxic in the liver and elsewhere [38, 39].

At present, the fate of islets engrafted in diabetic patients can only be assessed through indirect means of functional activity, i.e. measuring circulating insulin and C-peptide, and/or biopsies and subsequent histopathology. These methods are often inaccurate and patients are being subjected to invasive, risky, and sometimes repetitive biopsy procedures. Our barium-APSA capsule formulations may offer a non-invasive and reliable new way for monitoring islet transplantation in a clinical scenario. Since X-ray/CT is the most commonly used imaging method in image-guided interventional surgeries, encapsulated cells may be imaged in real-time to guide the transplantation process and to ensure that the cells are accurately being delivered to the targeted site.

Conclusion

Barium-APSA microcapsules, optimally gelated at 20 mM of barium, were mechanically stronger and supported higher cell viability than conventional calcium-gelated APLLA capsules. Human islets demonstrated functional c-peptide secretion levels over a sustained period. Due to its intrinsic radiopacity, barium-APSA capsules have excellent properties for immunoprotection and non-invasive visualization of cells using X-ray/CT imaging without the use of contrast agents.

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Figure 1.

Light microscopic images of capsule formulations (see Table 1 for Roman Numeral notation) immediately post-synthesis. Note that all capsule types are spherical, with Type I, II, III, V, VIII, and IX capsule formulations having smooth surfaces. (**g,h**) Crystals were formed on the surface of Type IV (**g**) and VI (**h**) capsules. (**h**) Lighter (*) and darker (**) capsules were observed in the Type VI capsule population, presumably due to an uneven coating of protamine. (**i**) Type VII capsules were coated with thick shells of LVG gel. (**j**) After 2 days post-synthesis, the shell started to shed. Black bars=200 μ m.

Figure 2.

Representative light microscopic images of a collapsed Type IX phantom microcapsule (a) or a dented Type I capsule (b) after glass bead treatment, and a swollen Type IX capsule (c) or a fractured Type II capsule (d) after an osmotic gradient test. Capsules were stained with trypan blue to facilitate visualization in (c) and (d). Arrows indicate dent (b), swelling (c) or fracture (d). (e) Percentage of intact microcapsules after glass bead treatment (black bars) and osmotic pressure test (white bars). (f,g) Ratio of transient microcapsule diameter at different time points post-synthesis relative to the initial capsule diameter in 0.9% saline immediately post-synthesis. Shown are data for capsules suspended in 0.9% saline (f), and in 0.9% saline containing a physiological concentration of 1.3 mM Ca²⁺ (g).

Figure 3.

(a) Percentage of viable mouse insulinoma β TC6 cells encapsulated in different capsule formulations. A representative example is shown for live (FDA, green) and dead (PI, red) cell staining for cells encapsulated in Type II (b) and conventional Type IX (c) microcapsules. Scale bar=200 μ m. Asterisks indicate where the viability of cells encapsulated in new capsule formulations was statistically different from Type IX capsule ($P<0.05$).

Figure 4.

Light microscopic images of a human pancreatic islet encapsulated in a Type II microcapsule after 1 day (a) and 14 days (b) in culture. Images of unencapsulated (naked) islets are shown on day 1 (c) and 14 (d) for comparison. Scale bars=200 μm in (a-c) and 100 μm in (d). (e) *In vitro* human C-peptide secretion levels of unencapsulated islets (white bars) and islets encapsulated in Type II capsules (black bars). Asterisks indicate the days when the C-peptide levels of encapsulated islets were statistically different from unencapsulated islets ($P < 0.05$).

Figure 5. Micro-CT images of 100 Type II phantom microcapsules in saline one day (**a**) and 15 months (**b**) post-synthesis. Type II capsules were intrinsically radiopaque, while Type VIII (**c**) and IX (**d**) capsules (100 capsules per tube) could not be visualized due to the lack of Ba²⁺. Axial (**e**), sagittal (**f**) and 3-dimensional, volume-rendered (**g**) micro-CT images of Type II capsules transplanted s.c. in mouse abdomen. Yellow arrows=bladder, white arrows=capsules.

Table 1

Library of capsule formulations. Different capsules were synthesized by varying the divalent cation type (Ba^{2+} or Ca^{2+}), concentration of gelating cation, gelation time, type of crosslinker (polycation, PS=protamine sulfate, PLL = poly-L-lysine), concentration of polycation, and type of alginate outer layer (LVM or LVG). For Type VII capsules, both the inner (I) and outer (O) diameter are given.

Capsule type	Gelating cation	[Cation] (mM)	Gelation time (min)	Polycation	[Polycation] (%)	Outer layer	Diameter (μm)
I	Ba^{2+}	10	<5	PS	0.05	0.15% LVM	517 $\pm$ 9
II	Ba^{2+}	20	<5	PS	0.05	0.15% LVM	441 $\pm$ 5
III	Ba^{2+}	50	<5	PS	0.05	0.15% LVM	497 $\pm$ 19
IV	Ba^{2+}	100	<5	PS	0.05	0.15% LVM	421 $\pm$ 17
V	Ba^{2+}	20	10 –15	PS	0.05	0.15% LVM	498 $\pm$ 8
VI	Ba^{2+}	20	<5	PS	0.10	0.15% LVM	471 $\pm$ 11
VII	Ba^{2+}	20	<5	PS	0.05	0.15% LVG	492 $\pm$ 13 (I) 690 $\pm$ 52 (O)
VIII	Ca^{2+}	100	<5	PS	0.05	0.15% LVM	613 $\pm$ 16
IX	Ca^{2+}	100	<5	PLL	0.05	0.15% LVM	616 $\pm$ 38