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## Core-shell Hydrogel Microcapsules for Improved Islets Encapsulation

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Hydrogel microcapsules have been extensively investigated for encapsulation of living cells or cell aggregates for tissue engineering and regenerative medicine.<sup>1,2,3</sup> In general, capsules are designed to allow facile diffusion of oxygen and nutrients to the encapsulated cells, while releasing the therapeutic proteins secreted by the cells, and to protect the cells from attack by the immune system. These have been developed as potential therapeutics for a range of diseases including type I diabetes, cancer, and neurodegenerative disorders such as Parkinson's.<sup>4,5,6</sup> One of the most common capsule formulations is based on alginate hydrogels, which can be formed through ionic crosslinking. In a typical process, the cells are first blended with a viscous alginate solution. The cell suspension is then processed into microdroplets using different methods such as air shear, acoustic vibration or electrostatic droplet formation.<sup>7</sup> (Figure 1a) The alginate droplet is gelled upon contact with a solution of divalent ions, such as Ca<sup>2+</sup> or Ba<sup>2+</sup>. One challenge associated with alginate microcapsules for cell encapsulation, however, is the lack of control of the relative positions of the cells within the capsules. The cells can become trapped and exposed on the capsule surface, leading to inadequate immune-protection.<sup>8</sup> It has been recognized that incomplete coverage would not only cause the rejection of exposed cells but may also allow the infiltration of macrophages and fibroblasts into the capsules through the exposed areas.<sup>9</sup>

Alginate hydrogel microcapsules have been broadly investigated for their utility with pancreatic islets to treat Type I diabetes.<sup>10</sup> Numerous promising results have been reported in several animal models including rodents,<sup>11, 12, 13</sup> dogs<sup>14</sup> and nonhuman primates.<sup>15,16</sup> Clinical trials have also been performed by Soon-Shiong et al.,<sup>17</sup> Elliott et al.,<sup>18</sup> Calafiore et

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al.,<sup>19,20</sup> and Tuch et al.<sup>21</sup> In general, these clinical trials have reported insulin secretion, but without long term correction of blood sugar control, and additional challenges remain to advance these systems.<sup>22,23,24</sup> One challenge is the biocompatibility of the capsules. Upon transplantation, the foreign body responses cause fibrotic cellular overgrowth on the capsules that cut off the diffusion of oxygen and nutrients, and lead to necrosis of encapsulated islets. To this end, research groups have developed polymers to reduce the fibrotic reactions.<sup>25</sup> Another challenge is the incomplete coverage of the islets within the capsules.<sup>26, 27</sup> Islets protruding outside the capsules are more frequently observed when the islet number density in alginate solution increases or the capsule size decreases, both of which are desirable to minimize the transplantation volume.<sup>28</sup> It has been hypothesized that if even a small fragment of islet is exposed, immune effector cells may destroy the entire islet.<sup>29,30</sup> Furthermore, exposure of a small number of islets may start a cascade of events that leads to enhanced antigen-specific cellular immunity and transplant failure. A double-encapsulation process<sup>31,18</sup> has been used to improve the encapsulation and xenograft survival where very small capsules containing cells were first formed and then several small capsules were enclosed in each larger capsule. This approach required two process steps and the large size of the final capsules inevitably limited the mass transport that was essential to cell viability and functionality. Thin conformal coating of islets reduces the diffusion distance and total transplantation volume.<sup>32,33</sup> However, the process often involves multiple steps which cause damage to islets and it is not clear whether the coatings are sufficiently robust for clinical use.<sup>10,34</sup> Previous data<sup>35</sup> has suggested conformal coatings may have reduced immune-protective capacity compared with the hydrogel capsules.

We report here a new type of alginate-based hydrogel microcapsules with core-shell structures using a two-fluid co-axial electro-jetting that is compatible with the current alginate-based cell encapsulation protocols. Improved immune-protection was achieved in a single step by simply confining the cells or cell aggregates in the core region of the capsules. Both the core-shell structure and better encapsulation were confirmed by confocal microscopy. Using a type I diabetic mouse model, we also showed that the core-shell capsules encapsulating rat islets provided a significantly better treatment than the currently used, regular capsules. The composition and thickness of the core and shell can be independently designed and controlled for many types of biomedical applications.

Figure 1b shows a schematic of the two-fluid co-axial electro-jetting for the fabrication of core-shell capsules and encapsulation of cells or cell aggregates. Related approaches have been used to encapsulate small molecule liquids into mono-disperse concentric micro/nano droplets or block copolymers into nanofibers.<sup>36,37,38</sup> Inspired by these studies, we use this two-fluid configuration to make core-shell hydrogel capsules and encapsulate living cells. Although similar core-shell hydrogel capsules have been reported previously using microfluidic flow-focusing device, the approach involved using toxic acid and mineral oil, and it is not clear if it is scalable and sufficiently robust for clinical applications.<sup>39,40</sup> In Figure 1b, the shell fluid consists of a cell-free alginate solution, while the core fluid contains the islets or other therapeutic cells. The relatively high viscosity of the two fluids and short interaction time between them prevent their intermixing. (See Experimental for details on the design of the coaxial nozzle) Under electrostatic force, microdroplets with core-shell structures are formed and converted to hydrogel capsules in the gelling bath.

First, to demonstrate the formation of core-shell hydrogel capsules, we used a fluorescently labeled alginate as the shell and a non-labeled alginate as the core. Figure 2 (a-c) shows that the thicknesses of the core and shell can be controlled by simply tuning their respective flow rates. For cell encapsulation, the size of the core can then be designed based on the desired mass of cells per capsule, while that of the shell can be adjusted according to the requirements of mechanical strength and mass transfer. The compositions of the core and

shell are also adjustable. This allows independent optimization of the material in direct contact with the encapsulating cells and the one adjacent to the host immune system when transplanted. For example, an anti-inflammatory drug (Figure 2d) or an imaging contrast reagent (e.g. iron oxide nanoparticles) can be loaded in the shell without contacting the cells in the core. It is also possible to put a different material such as Matrigel® as the core inside the alginate shell (Figure 2e). The core material, which may not be able to form mechanically robust capsules alone, can then provide a preferred local environment for the encapsulated cells.

Better cell encapsulation using core-shell capsules was demonstrated using both homogenized rat liver tissue cells (Supplemental Information, Figure S1) and rat pancreatic islets. Figure 3 shows microscopic images for both regular capsules and core-shell capsules containing islets. In both cases, a relatively low volume of alginate solution per islet (i.e. 0.27 ml solution for every 1000 islets) was used to minimize the total volume of capsules for transplantation. This is approximately a third of the volume that is typically used.<sup>27</sup> For crosslinking, a BaCl<sub>2</sub> solution<sup>41</sup> was used and the average capsule sizes were both around 500 μm. For regular capsules, the islets were randomly located within the capsules and islets protruding outside were frequently observed. (Fig 3a) In contrast, the islets in the core-shell capsules were fully encapsulated. (Fig 3b) The core-shell structure of the capsules was demonstrated by using a fluorescent alginate in the shell and a non-fluorescent alginate in the core. Figure 3 (c-e) shows fluorescent images where the islets were stained blue (i.e. nuclei staining). In some cases, the islets were close to the core-shell interface but still completely encapsulated due to the additional shell layer. It is important to note that a confocal microscopy technique was used to confirm the full encapsulation. Conventional microscopic examination may be misleading<sup>29</sup> as the relatively dense islet mass may fall at the bottom of the capsule and in line with the objective. In traditionally formed alginate capsules, the islets appeared fully encapsulated under conventional light microscope. The z-series and orthogonal views using confocal microscopy revealed however the islets were partially exposed outside the capsules. In contrast, the core-shell capsules fully enclosed the islets as viewed from all three directions. (Supplemental Information, Figure S2) Quantitatively, based on examinations of a number of islets-containing capsules using confocal microscopy, the regular capsules had about 30% with protruding islets while the core-shell capsules had none. The percentage of inadequately encapsulated islets in regular capsules was similar to that reported earlier.<sup>26</sup>

To evaluate the functionality of the coaxially encapsulated islets and compare the efficacy with regular capsules, we used streptozotocin (STZ) - induced diabetic mouse model.<sup>42</sup> Encapsulated rat islets were transplanted into the peritoneal cavity of the diabetic mice and their blood sugar was measured. Figure 4a shows the blood glucose level of the mice as a function of time post-transplantation for both core-shell capsules and regular capsules. For both types of capsules, a density of 1000 islets per 0.27 ml alginate solution was used and each mouse was transplanted with 500 islet equivalents (normalized to 150 μm size, see Experimental for details). Mice with blood glucose below 200 mg/dL are considered normoglycemic.<sup>39</sup> A couple of days after transplantation, all diabetic mice became normoglycemic, as expected. However, differences between the two types of capsules started to appear after about 2 weeks. The average blood glucose level for the mice receiving regular capsules went back above 200 mg/dL by 15 days, and that of the mice receiving core-shell capsules remained below 200 mg/dL up to 50 days. Figure 4b shows the percentage of normoglycemic mice over time. Regular capsules in all mice failed to control the diabetes by 20 days, while the core-shell ones in some of the mice did not fail until about 80 days after transplantation, indicating that the core-shell capsules improved the treatment significantly compared with the regular capsules.

In summary, we report hydrogel microcapsules with core-shell structures and their use for improved cell encapsulation and immuno-protection. We demonstrate better islet encapsulation using the core-shell capsules at a reduced material volume per islet. Islet microencapsulation represents a promising approach to treat Type I diabetes and has been a topic of intensive research for decades.<sup>43</sup> The results from different research groups were often inconsistent. Islet quality and material properties such as biocompatibility are certainly critical factors that affect the outcome of treatment. The quality of encapsulation could also influence the results and cause inconsistency. The core-shell capsules can be made in a single step using the same encapsulation protocols that have been used to make the regular capsules and do no harm to islets. In addition to providing better immuno-protection, the opportunity to control the compositions of the shell and the core may provide additional opportunities in microencapsulation. Examples include the replacement of the alginate in the core with a different material that may enhance long-term islet survival. In addition, as the cell biology develops, insulin-producing cells derived from stem cells<sup>44</sup> or adult cells<sup>45</sup> are likely to provide a great alternative to islets. The core-shell capsules allow not only improved encapsulation but also greater freedom in the designs of the encapsulating material in the shell and that in direct contact with the cells in the core.

## Experimental

### Design of core-shell nozzle and Formation of core-shell capsules

The design of the co-axial nozzle was critical to the stable formation of uniform core-shell structures of the capsules, and complete encapsulation of islets. First, the nozzle must be concentric; any eccentricity may cause non-uniform flow of the shell fluid surrounding the core fluid, disturbing the core-shell structure. Second, the nozzle must be designed in a way that the flow of the shell fluid inside the nozzle is uniform around the core tube. Third, the inner diameter of the core tube must accommodate the sizes of islets. The co-axial nozzle has a core tube with an ID of 0.014 and an OD of 0.022 and a shell tube with an ID of 0.04 and an OD of 0.0625. The length of the shell tube that protrudes from the nozzle body is 0.5 and was tapered at the outlet tip. The core tube was placed 100 microns inward relative to the shell tube at the outlet. The flow rates of the core and shell fluids were independently adjusted by separate syringe pumps. The voltage was 6.2 kV and the distance between the nozzle tip and the surface of gelling solution was 1.8 cm. To optimize encapsulation, the solution concentration of alginate with given molecular weight must be optimized. If both the concentrations of the core and shell solutions were too small, there would be significant intermixing between the core and shell solutions leading to non-uniform core-shell structures. For the PRONOVA SLG20 alginate (Mw ~ 75-220 k, FMC Biopolymer), we found the optimal concentration ranges were from 0.8% (w/v) to 2% (w/v). Particularly for the images shown in Figure 2, all alginate solutions were at 1.4% (w/v) and the Matrigel (BD Biosciences) was used at 4 mg/ml in a 4°C cold room to avoid gelling prior to encapsulation. Besides the properties of the core and shell solutions, optimization of operating parameters was also important to obtain ideal encapsulation. Typical flow rates were 0.005 ml/min to 0.1 ml/min for the core flow and 0.1 ml/min to 0.5 ml/min for the shell flow.

### Rat Islet Isolation and Purification

Sprague-Dawley rats from Charles River Laboratories weighting approximately 300 grams were used for harvesting islets. All rats were anesthetized by a 1:20 Xylazine (10 mg/kg) to Ketamine (150 mg/kg) injection given intraperitoneally, and the total volume of each injection was 0.4ml – 0.5ml depending on the weight of rat. Isolation surgeries were performed as described by Lacy and Kostianovsky.<sup>46</sup> Briefly, the bile duct was cannulated and the pancreas was distended by an *in vivo* injection of 0.15% Liberase (Research Grade,

Roche) in RPMI 1640 media solution. Rats were sacrificed by cutting the descending aorta and the distended pancreatic organs were removed and held in 50ml conical tubes on ice until the completion of all surgeries. All tubes were placed in a 37°C water bath for a 30 minute digestion, which was stopped by adding 10-15 ml of cold M199 media with 10% heat-inactivated fetal bovine serum and lightly shaking. Digested pancreases were washed twice in the same aforementioned M199 media, filtered through a 450 µm sieve, and then suspended in a Histopaque 1077 (Sigma)/M199 media gradient and centrifuged at 1,700 RCF at 4°C. Depending on the thickness of the islet layer that was formed within the gradient, this step was repeated for higher purity islets. Finally, the islets were collected from the gradient and further isolated by a series of six gravity sedimentations, in which each supernatant was discarded after four minutes of settling. Purified islets were hand-counted by aliquot under a light microscope and then washed three times in sterile 1X phosphate-buffered saline. Islets were then washed once in RPMI 1640 media with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin, and cultured in this media overnight for further use.

### Liver tissue homogenization and encapsulation

Liver was obtained from sacrificed rats by dissecting the liver from the hepatic portal, system vessels and connective tissue. It was placed into a 0.9% NaCl solution and minced using surgical forceps and scissors in a petri dish. These pieces were washed with 0.9% saline twice to remove blood and then dissociated using a gentleMACS Dissociator (Miltenyi Biotec). After dissociation, the tissue sample was filtered through a 100 µm cell strainer and subsequently a 40 µm one. This filtration was repeated once more to obtain liver tissue cells for encapsulation. For single-fluid encapsulation, 0.06 ml dissociated liver tissue cells were dispersed in 4.5 ml 1.4% SLG20 alginate solution. For the two-fluid coaxial encapsulation, 0.06 ml dissociated liver cells were dispersed in 0.5 ml 1.4% SLG20 alginate solution that was used as the core fluid. A separate 4 ml 1.4% alginate solution was used as the shell.

### Islets Encapsulation and Transplantation

Immediately prior to encapsulation, the cultured islets were centrifuged at 1400 rpm for 1 minute and washed with Ca-Free Krebs-Henseleit (KH) Buffer (4.7mM KCl, 25mM HEPES, 1.2mM KH<sub>2</sub>PO<sub>4</sub>, 1.2mM MgSO<sub>4</sub>×7H<sub>2</sub>O, 135mM NaCl, pH≈7.4, osmotic pressure ≈290mOsm). After the wash, the islets were centrifuged again and all supernatant was aspirated. The islet pellet was then re-suspended in a 1.4% solution of SLG20 alginate dissolved in 0.8% NaCl solution at desired islet number density. In the case of the regular capsules, 0.27 ml alginate solution was used for every 1000 islets. For the core-shell capsules, 0.03 ml solution for every 1000 islets was used as the core fluid. Another 0.24 ml 1.4% alginate solution without islets was used as the shell fluid. The flow rate for the shell was 0.2 ml/min and that for the core was 0.025 ml/min. For the same number of islets, the total volume of the alginate solution used in both regular encapsulation and core-shell encapsulation was therefore the same. Capsules were crosslinked using a BaCl<sub>2</sub> gelling solution (20mM BaCl<sub>2</sub>, 250mM D-Mannitol, 25mM HEPES, pH ~ 7.4, osmotic pressure ~ 290 mOsm).<sup>47</sup> Immediately after crosslinking, the encapsulated islets were washed 4 times with HEPES buffer and 2 times with RPMI Medium 1640 and cultured overnight at 37°C for transplantation. As the islets had variable sizes (50 - 400 µm) and there was an inevitable loss of islets during the encapsulation process, the total number of encapsulated islets were recounted and converted into islet equivalents (IE, normalized to 150 µm size) based on a previously published method<sup>48</sup> prior to transplantation.

Immune-competent male C57BL/6 mice were utilized for transplantation. To create insulin-dependent diabetic mice, healthy C57BL/6 mice were treated with Streptozocin (STZ) by

the vendor (Jackson Laboratory, Bar Harbor, ME) prior to shipment to MIT. The blood glucose levels of all the mice were retested prior to transplantation. Only mice whose non-fasted blood glucose levels were above 300 mg/dL for two consecutive days were considered diabetic and underwent transplantation. The mice were anesthetized using 3% isoflurane in oxygen and maintained at the same rate throughout the procedure. Preoperatively, all mice received a 0.05 mg/kg dose of buprenorphine subcutaneously as a pre-surgical analgesic, along with 0.3mL of 0.9% saline subcutaneously to prevent dehydration. The abdomens of the mice were shaved and alternately scrubbed with betadine and isopropyl alcohol to create a sterile field before being transferred to the surgical field. A 0.5mm incision was made along the midline of the abdomen and the peritoneum was exposed using blunt dissection. The peritoneum was then grasped with forceps and a 0.5-1mm incision was made along the linea alba. A desired volume of capsules with predetermined number of islet equivalents were then loaded into a sterile pipette and transplanted into the peritoneal cavity through the incision. The incision was then closed using 5-0 taper tipped polydioxanone (PDS II) absorbable sutures. The skin was then closed over the incision using a wound clip and tissue glue.

All animal protocols were approved by the MIT Committee on Animal Care and all surgical procedures and post-operative care were supervised by MIT Division of Comparative Medicine veterinary staff.

### Confocal imaging of encapsulated islets

Pancreatic islets were stained with Hoechst dye (2 $\mu$ g/ml) and encapsulated in fluorescent, regular capsules or core-shell capsules with a fluorescently labeled shell. The capsules were placed on chambered glass coverslips (LabTek) and allowed to settle. The encapsulated islets were then imaged under a 10X objective using a Laser Scanning Confocal Microscope 710 (LSM710). Multiple confocal slices were imaged from bottom to top of sample to construct a Z-stack. An orthogonal image and a 3D rendering were performed using LSM browser software to visualize the islet-containing capsules.

### Blood glucose monitoring

Blood glucose levels were monitored three times a week following the transplant surgery. A small drop of blood was collected from the tail vein using a lancet and tested using a commercial glucometer (Clarity One, Clarity Diagnostic Test Group, Boca Raton, FL). Mice with unfasted blood glucose levels below 200 mg/dL were considered normoglycemic. Monitoring continued until all mice in the experimental group had returned to a hyperglycemic state at which point they were euthanized.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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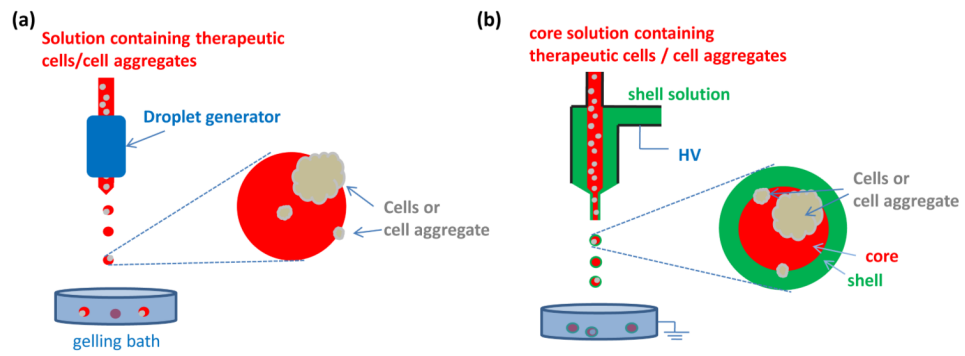
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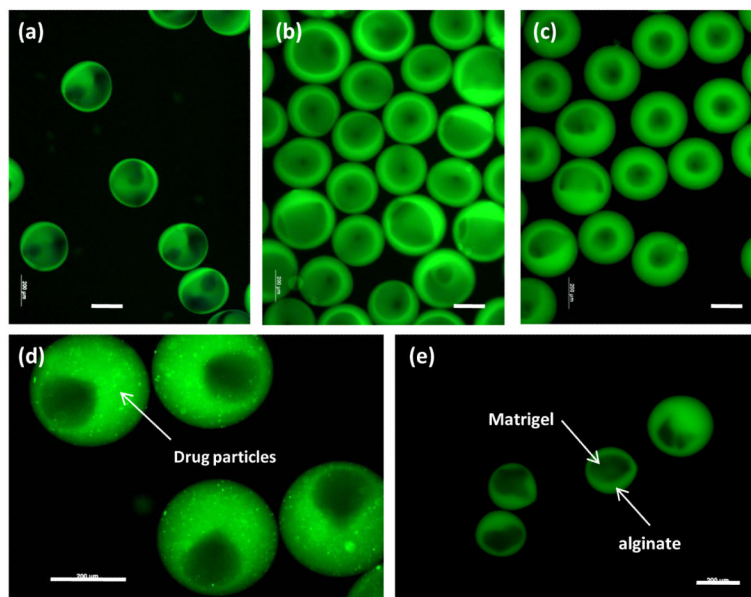
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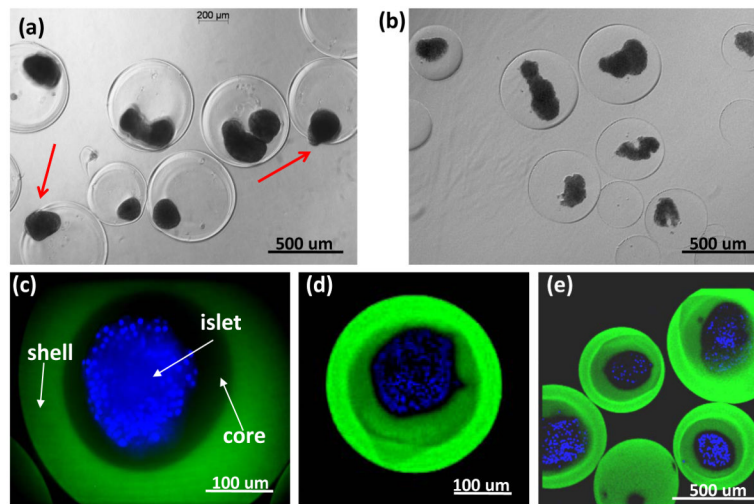




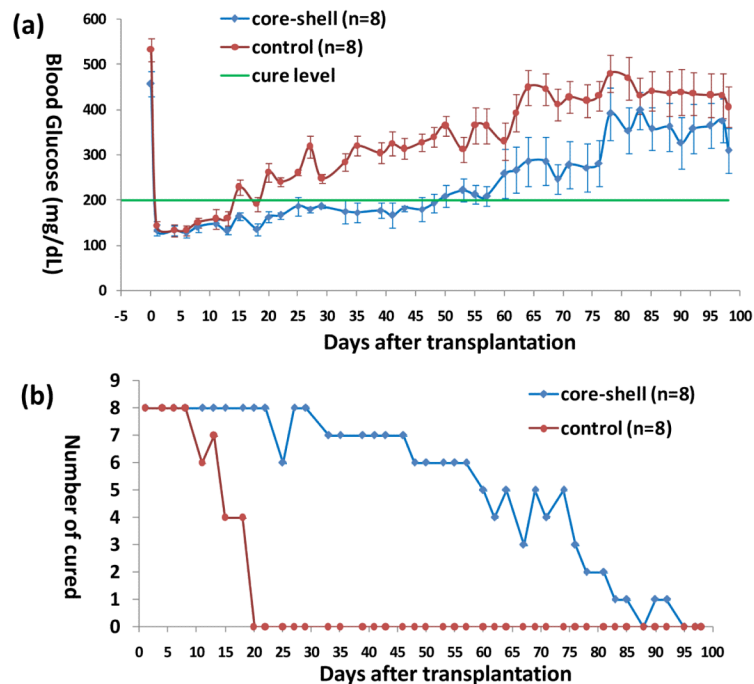
**Figure 1.** Schematic depictions of (a) a conventional cell encapsulation approach and (b) the two-fluid co-axial electro-jetting for core-shell capsules and cell encapsulation.



**Figure 2.** Hydrogel microcapsules with core-shell structures. The shell is an alginate partially modified with FITC, while the core is unlabeled alginate or Matrigel. The thicknesses of the shell and the core are controlled by tuning their respective flow rates: (a) 0.1 ml/min and 0.1 ml/min; (b) 0.15 ml/min and 0.05 ml/min; (c) 0.2 ml/min and 0.02 ml/min. The compositions of the core and shell can also be independently controlled. (d) The shell alginate contains particles of an anti-inflammatory drug, curcumin. Note that the drug particles are self-fluorescent. (e) The shell is fluorescent alginate and the core is non-fluorescent Matrigel. The scale bars in all images are 200 microns.



**Figure 3.** Comparison of islets encapsulated in (a) regular capsules and (b-e) core-shell capsules. (c-e) 3D reconstructed confocal fluorescent images of islets encapsulated in core-shell capsules. The islets were stained blue, while the shell was labeled green.



**Figure 4.** Comparison of the control, regular capsules and core-shell capsules in treating Type I diabetes. (a) Blood glucose data of STZ-induced diabetic mice after the transplantation of 500 encapsulated rat islet equivalents. The error bars represent standard errors. (b) The number of normoglycemic mice as a function of time. 8 replicates were used for both types of capsules.