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Islet function within a multilayer microcapsule and efficacy of angiogenic protein delivery in an omentum pouch graft

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Abstract. We have previously described a new multilayer alginate microcapsule system, and the goals of the present study were to assess the in vitro function of islets encapsulated in its inner layer, and the angiogenic ability of FGF-1 delivered from the external layer in an omentum pouch. Following isolation and culture, islets were encapsulated in the inner core of microspheres (500 - 600 μm in diameter) with a semi-permeable poly-L-ornithine (PLO) membrane separating two alginate layers, and both unencapsulated and encapsulated islet function was assessed by a dynamic glucose perifusion. For angiogenesis experiments, one group of microcapsules without FGF-1 (control) and another (test) containing FGF-1 with heparin encapsulated in the external layer were made. One hundred microcapsules of each group were transplanted in Lewis rats (n = 5/group) and were retrieved after 14 days for assessment of angiogenesis. Glucose perifusion of unencapsulated and encapsulated islets resulted in similar stimulation indices. The release of FGF-1 resulted in increased vascular density compared to controls. In conclusion, islets encapsulated in the core of multilayer alginate microcapsules maintain functionality and the microcapsule's external layer is effective in delivery of FGF-1 to enhance graft neovascularization in a retrievable omentum pouch.

Keywords: microcapsule; islet; angiogenesis; drug delivery; transplantation

1. Introduction

The Diabetes Control and Complications Trial (DCCT) showed that intensive insulin treatment to keep blood glucose levels in the normal range could only delay the development and progression of diabetic complications DCCT (1993). In contrast, *β*-cell replacement therapy can prevent and in some cases reverse co-morbidities associated with diabetes Kendall and Robertson (1997), Fiorina *et al.* (2008) and White *et al.* (2009), possibly because of the role played by C-peptide (Ido *et al.* 1997, Wahren and Sima 2009, Hills and Brunskill 2009). Of the two methods available for β-cell replacement in diabetic patients, pancreas transplantation is the gold standard while islet transplantation remains experimental. Still, islet transplantation engenders considerable excitement because of the ease of performing the procedure (White *et al.* 2009) and the emerging data

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showing benefits of islet transplantation to patients (Poggioli *et al.* 2006 and Tiwari *et al.* 2012). Therefore, there has been continued interest in islet transplantation (Poggioli *et al.* 2006, Tiwari *et al.* 2012, Alejandro *et al.* 1997, Shapiro *et al.* 2000, Shapiro *et al.* 2006, Harlan *et al.* 2009 and Robertson 2010) since the description of a reliable method to isolate viable human islets (Ricordi *et al.* 1988), despite the fact that the present outcome of islet transplantation has not matched expectations (Harlan *et al.* 2009 and Robertson 2010). However, like pancreas transplantation and other organ transplant procedures, islet transplantation is afflicted with two major obstacles, the severe shortage of human organs and the need to use immunosuppressive drugs to prevent transplant rejection. In particular, it has been shown that immunosuppression carries a high risk of nephrotoxicity in islet transplant recipients (Senior *et al.* 2007) even though it may allow long-term graft survival (Bellin *et al.* 2008 and Berney *et al.* 2009).

Islet immunoisolation by microencapsulation prior to transplantation was proposed as a potential solution to the severe shortage of human organs and the need for immunosuppressive drugs more than three decades ago Lim and Sun (1980). In turn, clinical application of the microencapsulation technology has been hampered by various impediments (De Vos *et al.* 2009), such as severe hypoxia caused by a lack of vascular supply and an inability to retrieve encapsulated islets transplanted in the peritoneal cavity for biopsy and subsequent evaluation (Opara *et al.* 2010). With the inherent extended hypoxia suffered by encapsulated islets transplanted in the peritoneal cavity, it is not surprising that large numbers of these islets have been routinely used in studies performed in large animals and humans (Soon-Shiong *et al.* 1992, Soon-Shiong *et al.* 1996, Sun *et al.* 1996, Dufrane *et al.* 2006, Calafiore *et al.* 2006, Elliott *et al.* 2007, Wang *et al.* 2008 and Tuch *et al.* 2009).

Recent articles have suggested an omentum pouch as an alternative site of islet transplantation (Harlan *et al.* 2009, Robertson 2010, Berman *et al.* 2009) a site that makes the graft easily retrievable (Opara *et al.* 2010). To overcome the problems of lack of vascular supply and irretrievability of encapsulated islet grafts in the peritoneal cavity, we recently described an alginate-poly-L-ornithine-alginate (APA) multilayer microcapsule to serve the dual purpose of islet immunoisolation and angiogenic protein delivery to enhance graft neovascularization in a retrievable omentum pouch. In the new microencapsulation scheme we proposed encapsulating islets in the inner alginate layer and an angiogenic protein in the external layer of the perm-selective APA microcapsule (Opara *et al.* 2010 and Khanna *et al.* 2010a). The goals of the present study were to determine the function of islets encapsulated in the inner layer of this APA microcapsule system and to assess the efficacy of FGF-1 released from the external layer of the microcapsule in enhancing graft neovascularization in the omentum pouch.

2. Materials and methods

2.1 Materials

Low viscosity (20-200 mPa·s) ultra-pure sodium alginate with high mannuronic acid (LVM) and high guluronic acid (LVG) contents were purchased from Nova-Matrix (Sandvika, Norway). The LVM and LVG alginates were composed of molecular weights between 75-200kDa and G/M ratios of ≤1 and ≥1.5, respectively. Poly-L-ornithine (PLO) hydrochloride with molecular weight 15,000-30,000, was purchased from Sigma-Aldrich (St. Louis, Mo). Solutions for alginate microbead synthesis were made using the following chemicals: HEPES, NaCl, and $MgCl_2$ (Fisher

Scientific); $CaCl_2$ (Acros). ^{125}I-insulin was purchased from Perkin Elmer (Waltham, MA), and human fibroblast growth factor-1 (FGF-1) was purchased from Peprotech (Rocky Hill, NJ).

2.2 Animals

Male Wistar-Furth and Lewis rats were purchased from Harlan (Dublin, VA) and housed 2 rats /cage in a temperature-controlled room with a 12-hr light-dark cycle where the animals had unlimited access to food and water. Our studies were performed according to an animal protocol approved by the Wake Forest University Health Sciences Institutional Animal Care and Use Committee (IACUC).

2.3 Isolation of islets from the rat pancreas

Islets were isolated from the pancreata of male Wistar-Furth rats (300 – 400g) using the procedure of collagenase digestion of pancreatic tissue Lacy and Kostianovsky (1967) with modifications Field and Farney (1996). Following euthanasia according to the IACUC approved protocol, the common bile duct was cannulated and 10 mL of 1 mg/mL Collagenase P (Roche, Indianapolis) in HEPES-buffered Hanks balanced salt solution (HBSS) was infused to distend the pancreas prior to incubation at 37^{o}C for 23 minutes. The digestion was stopped with the addition of 20 mL ice-cold wash solution (HEPES-buffered HBSS with 10% fetal bovine serum, and then shaken for 10 seconds to dissociate the digested pancreas. The digest was filtered through a 500 μm mesh filter and then washed three times with wash solution and centrifuged at 200 *g* for 3 minutes. Islets were then purified on an Optiprep gradient, and cultured overnight at 37^{o}C, 5% CO_2 in Memphis Serum-Free Medium (MSFM).

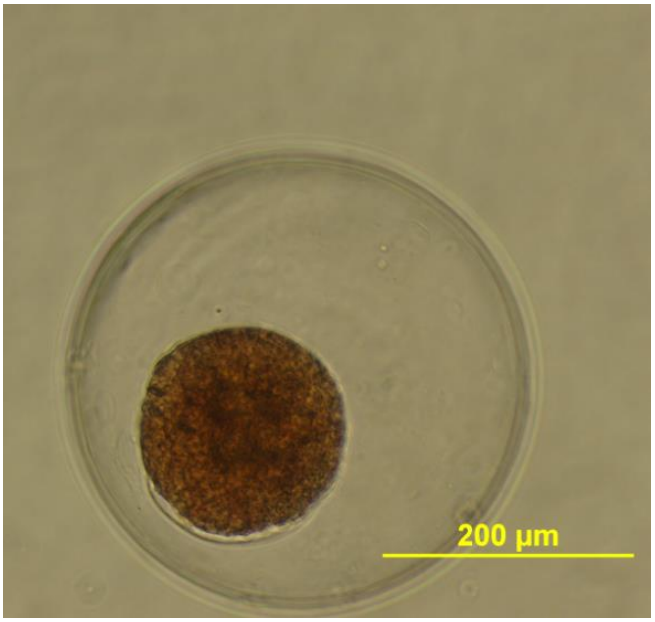


Fig. 1 Illustration of a Dithizone-stained islet encapsulated in the inner alginate core of the multi-layer microcapsule: The positive Dithizone-stain is indicative of a viable islet in the microcapsule

2.4 Microencapsulation of islets

Islets were microencapsulated by a modification (Khanna *et al.* 2010a) of the procedure that we had previously described (Darrabie *et al.* 2005) using an 8-channel microfluidic device (Tendulkar *et al. 2012*). Following purification, islets were suspended in 1.5% alginate sodium LVM alginate solution, and microspheres (500-600 μm) containing one islet/microsphere were collected in 100 mM $CaCl_2$ bath where they were gelled during 15 minutes incubation. Following two washings with normal saline, the microspheres were incubated in 0.1% (w/v) PLO for 20 minutes to provide them with perm-selectivity. The inner alginate core was then partially liquefied to enhance diffusion of oxygen and nutrients to islets by incubating the microcapsules in 55 mM sodium citrate solution for 2 minutes. After two washings in normal saline, the PLO-coated microcapsules were incubated in 1.25% LVG alginate solution for 45 minutes to synthesize the external alginate layer, as described below in section 2.6. Fig. 1 is an image of a Dithizone-stained islet encapsulated in the inner alginate core of the multilayer microcapsule.

2.5 Dynamic perifusion of islets and measurement of insulin secretion

After isolation, washing, and purification, islets were cultured overnight in Memphis Serum-Free Medium (MSFM) using standard conditions prior to encapsulation as described above. Following encapsulation and another 24 hr culture, both unencapsulated and encapsulated islets were then tested for function at 37^{o} C using the dynamic perifusion procedure with low (3.3 mM) and high (16.7 mM) glucose concentrations in a Krebs Ringer bicarbonate solution, pH 7.4, and effluent samples were collected on ice, as previously described (Garfinkel *et al.* 1998). The effluent samples collected during the perifusion tests were stored frozen at -20^{o}C until performance of the radioimmunoassay for insulin content.

2.6 Encapsulation of FGF-1 protein in the external layer of alginate microcapsules

Microcapsules were prepared aseptically in a biosafety cell culture hood using the multilayer microencapsulation procedure that we have previously described (Khanna *et al.* 2010). LVM alginate at a concentration of 1.5% was extruded through an 8-channel microfluidic device at a flow rate of 2 ml/min with an air jacket pressure of 18 psi to form microspheres, which were received into a dish containing 100 mM $CaCl_2$ solution. The microbeads were allowed to crosslink for 15 minutes before being strained through a 100 μm cell strainer, followed by rinsing with 25 ml of a mixture of 22 mM $CaCl_2$ and 0.9% NaCl. They were subsequently placed in a 0.1% PLO solution and rocked for 20 minutes. After rinsing with 25 ml 0.9% NaCl, the outer alginate layer was formed by transferring the PLO-coated microbeads into either 1.25% LVG + ultrapure water (control) or 1.25% LVG + FGF-1 (1.79 μg/100 microcapsules) and heparin (5 U/ml) for 45 minutes. The addition of 22 mM solution of $CaCl_2$ was then used to cross-link the outer layer. Two additional washes were performed with 2 mM $CaCl_2$ and 0.9% NaCl solution to remove unbound alginate. The microcapsules were finally rinsed with normal saline solution and dried prior to transplantation.

2.7 Transplantation and retrieval of microcapsules in the omentum pouch

Under general anesthesia with isoflurane, two groups (5/group) of normal Lewis rats underwent

a mini-laparotomy and the greater omentum was mobilized and placed onto moist gauze. A 4-0 uncoated vicryl suture in a running fashion was placed along the edge of the omentum. Frequent irrigation with saline was ensured through the entire procedure to prevent the omentum from drying up. For each animal, 100 empty (control) or FGF-1-loaded (test) microcapsules were placed onto the exposed omentum, the suture was pulled up and tied, creating a pouch for the microcapsules. After hemostasis was confirmed, the surgical incision was closed using a standard surgical technique. After 2 weeks of implantation, the animals were sacrificed and immediately after the terminal procedure was performed, the animals underwent a full-laparotomy. The omentum pouch containing the microcapsules was harvested, fixed in formalin and paraffin embedded. Specimens were serially sectioned (5 μm thickness) for immunohistochemical analysis.

2.8 Immunohistochemistry and image analyses for microvessel density

Serial sections were stained for CD31, a sensitive marker of endothelial cells (ECs) (Moya *et al.* 2010a). The sections were deparaffinized and rehydrated, before being processed by steam antigen retrieval using Dako target retrieval solution (Dako, Carpinteria, CA) prior to immunohistological staining. Specimens were stained following an indirect procedure using rabbit anti-human CD31 (Santa Cruz Biotechnology, Santa Cruz, CA) and a biotinylated anti-rabbit secondary antibody using the Vectastain Elite ABC kit (Vector Labs, Burlingame, CA). Sections were digitally imaged (20x objective, 0.017 μm/pixel) using an Axiovert 200 inverted microscope. Areas stained positive for CD31 were manually tallied using Axiovision AC (Carl Zeiss, Oberkochen Germany).

2.9 Statistical analysis

Data are expressed as means ± standard error (SEM). To determine significant differences between two groups of data, Student's *t*-test was performed, and differences were considered significant for $p < 0.05$.

3. Results

3.1 Glucose stimulation of insulin secretion by islets encapsulated in the inner alginate core of the multilayer APA microcapsule

The dynamic insulin response to changes in glucose concentrations in the perifusate of un-encapsulated naked islets (control) and islets encapsulated in the inner alginate core of the multi-layer APA microcapsule system is illustrated in Fig. 2. As apparent in the figure, increasing the glucose concentration from a basal 3.3 mM to a high 16.7 mM resulted in significant stimulation of insulin secretion, which was seen to be pulsatile when effluent samples were collected at 2-minute interval during the high glucose perifusion. Insulin secretion promptly returned to basal rate in both the encapsulated and unencapsulated islet groups upon re-perifusion of islets with the low glucose solution. In the encapsulated islets the mean basal rate of insulin secretion was 107.6 ± 15 and the peak rate was 328.8 ± 54.3 pg/islet/min ($p = 0.0028$, n=6). In the naked unencapsulated islets the mean basal rate of secretion was 142.2 ± 18.8 and the peak rate was 462.9 ± 88.8 pg/islet/min ($p = 0.0055$, n =6). When the basal and stimulated insulin secretion data shown in Fig. 3 were assessed as glucose stimulation indices (SI) of insulin secretion defined

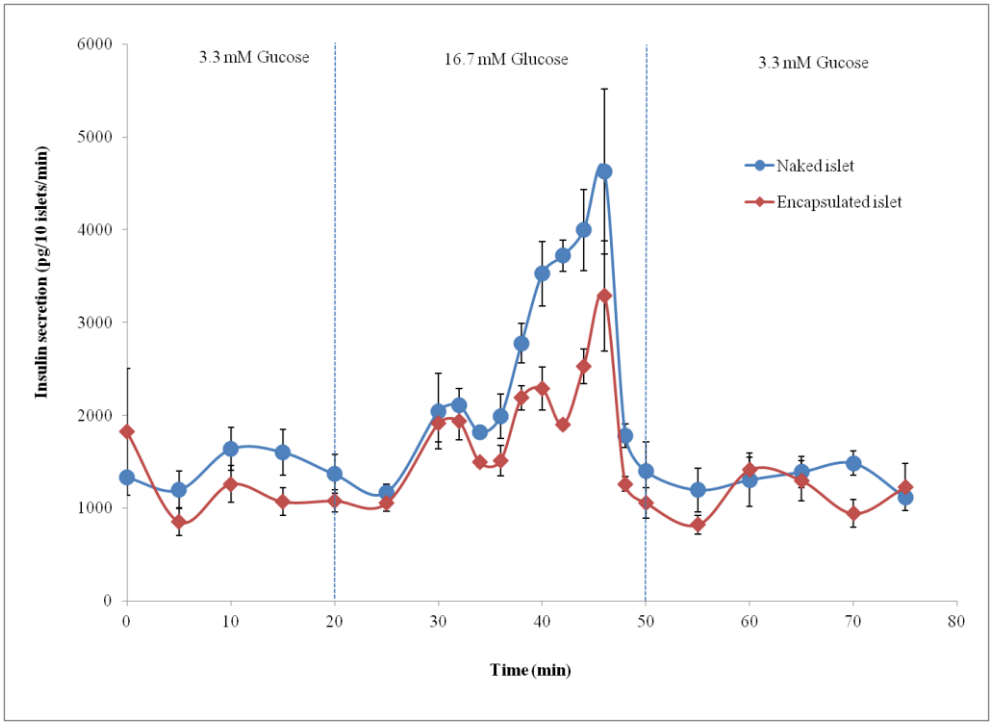


Fig. 2 Dynamic insulin secretion profile of unencapsulated islets and islets encapsulated in the inner core of the multilayer APA microcapsule: Following isolation and purification with Optiprep gradient, islets were cultured for 24 hours in a Memphis Serum-Free Medium (MSFM under standard conditions. Some islets were then encapsulated in the inner core of the APA microcapsules and cultured along with unencapsulated islets for an additional 24 hours after which 10 islets/group of either encapsulated or unencapsulated islets were loaded into a perifusion chamber and exposed to alternating low and high glucose solutions in Krebs-Ringer bicarbonate buffer maintained at pH 7.4 and gassed continuously with 95% air/5% CO_2. Effluent perifusate samples collected were stored frozen until radioimmunoassay for insulin. Data represent mean ± SEM, (n =6).

as the peak insulin secretion during the high glucose stimulation divided by the mean basal rate of insulin secretion, the calculated SI values were not statistically different between naked and encapsulated islets (SI; naked islets 3.50 ±0.35 versus encapsulated islets 2.92 ±0.48, p = 0.35).

3.2 Effect of FGF-1 encapsulated in the external layer of the APA microcapsule on vascular density in the omentum pouch

We have previously shown that the outer layer of the APA capsules can provide sustained release of FGF-1 when incorporated with heparin (Khanna *et al.* 2010a). When implanted in the omentum the effect of FGF-1 can be seen in Figs. 4(a)-(b), which shows gross images of retrieved omentum tissue. A greater number of vessels are apparent in the FGF-1 treated samples. When examining immunohistochemical stains a higher density of vessels appears in the FGF-1 treated samples relative to control omentum tissue sample (Figs. 5(a)-(b)). Capillaries containing red blood cells can be seen near the surface of the alginate microbeads. A relatively thin layer of

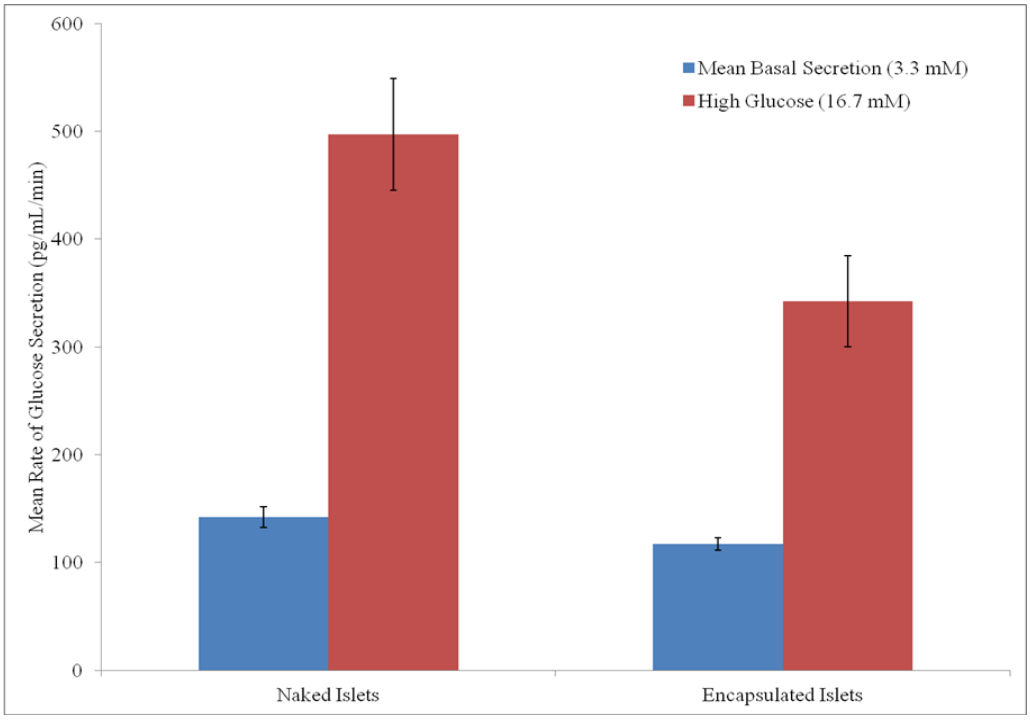
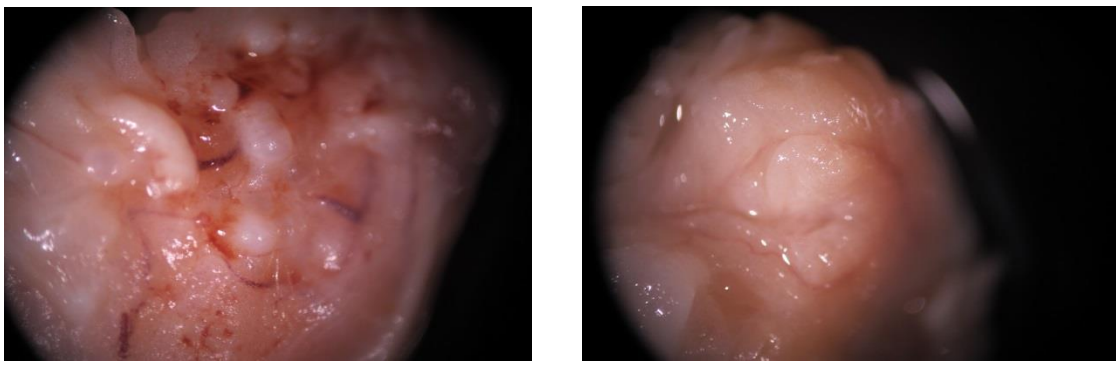


Fig. 3 Assessment of glucose stimulation index of insulin secretion: The mean basal rate of insulin secretion in low glucose was assessed along with the peak insulin response to high glucose stimulation by islets during exposure to changing levels of glucose in the perifusate secretion/minute in order to calculate the glucose stimulation index in each group of islets. Data represent mean ± SEM (n=6).



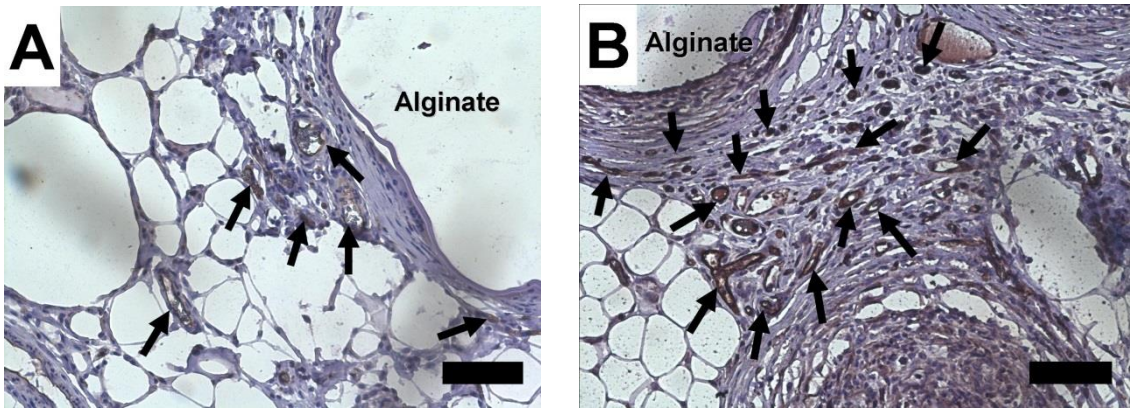
(a) (b)

Fig. 4 Effect of FGF-1 released from the external alginate layer on new blood vessel formation: Following encapsulation of recombinant human FGF-1 in the external layer of some multi-layer APA microcapsules, the FGF-1 containing-microcapsules and control empty microcapsules with no protein were separately transplanted in omentum pouches created in two groups of normal Lewis rats. The omentum pouches were retrieved after 14 days for microscopic assessment. Representative sample of retrieved omentum containing microbeads with FGF-1 is shown in Fig. 4(a), and Fig. 4(b) represents a sample obtained from control empty microcapsule implants. More vessels are visible in the FGF-1 treated samples

Table 1 Quantitative analysis of vessel density

Control	FGF-1
128.9 ± 10.9 vessels/mm^2	198.8 ± 59.2 vessels/mm^2

Data are significantly different from one another ($p < 0.05$). Data have been presented previously (McQuilling et al. 2011).



(a) (b)

Fig. 5 Effect of FGF-1 released from the external alginate layer on vascular density assessed by CD-31 staining: Following retrieval of the omentum pouches after 14 days, samples were stained for CD-31 as a measure of new blood vessel formation. A representative sample of CD-31 stain obtained from control microcapsule implants is shown in Fig. 5(a), and Fig. (b) represents a sample obtained from FGF-1 loaded microcapsule implants. Arrows indicate some locations of CD31 positive vessels.

fibrous encapsulation can be seen at the surface of the microbeads separating the vascularized tissue from the bead. As reported previously in an extended abstract (McQuilling *et al.* 2011) the density of vasculature is increased in response to the microcapsules containing FGF-1 in the outer layer relative to control empty microcapsules without FGF-1 (see Table 1).

4. Conclusions

We have shown in this study that the multi-layer APA microcapsule can be successfully used for the dual purpose of immunoisolating functional islets and release of an angiogenic protein for enhanced graft neovascularization. Specifically, we have shown that islets encapsulated in this inner layer of the multi-layer alginate microcapsule retain their function as assessed by their response to changes in glucose concentrations *in vitro*. Our data from the dynamic perifusions of islets encapsulated in the inner alginate core of the multilayer APA indicating a comparable level of glucose stimulation of insulin secretion to that observed with un-encapsulated naked islets suggest that this encapsulation system does not adversely affect islet function. In particular, it is of interest to see that the pulsatility with a periodicity of approximately 5 minutes in insulin release, which we have previously described in perifused naked islets, Opara and Atwater (1988), is preserved following the encapsulation of islets in the new APA microcapsule system. The significance of this observation is that previous studies had shown that oscillatory insulin delivery is more efficient for blood glucose disposal (Tolic *et al.* 2000).

As previously reported (McQuilling *et al.* 2011), we have also shown that the encapsulation of FGF-1 in the outer layer of the multi-layer APA microcapsule can be released to stimulate an increase in vascular density around a graft at a retrievable site such as an omentum pouch. This provides evidence that the external layer of the APA microcapsule system can be effectively used as a drug delivery device for the purpose of enhancing neovascularization of a graft. Previous studies had shown that FGF-1 can be released from ordinary alginate microbeads with no perm-selective coating to induce angiogenesis (Moya *et al.* 2010a, Moya *et al.* 2009, Wee and Gombotz 1998, Gu *et al.* 2004 and Moya *et al.* 2010b). The uncoated alginate microbead used in those previous studies represents the inner alginate layer of the multi-layer APA microcapsules where we have encapsulated islets for immunoisolation in the present study.

The original design of the microencapsulated islet construct involved immunoisolating islets in the alginate core matrix followed by generation of a perm-selective coating of the microbead using an amino acid biopolymer. A final coating of the biocompatible alginate is used to mask the positive charges on the amino acid polymer so as to prevent electrostatic interactions with cells and proteins that may lead to fibrosis of the microcapsule implant in the body. This alginate coat on the external surface of the microcapsule is usually not cross-linked and therefore prone to degradation with consequent exposure of those positive charges on the amino acid polymer and concomitant implant fibrosis and islet graft failure. We believe that the stability of the external coat of the APA microcapsule is enhanced by the redesigned procedure of cross-linking an external alginate layer of the APA microcapsule to obtain a layer with a thickness ~100 μm in diameter as we have previously reported (Opara *et al.* 2010 and Khanna *et al.* 2010a) thus decreasing the potential for developing fibrosis around the implant and enhancing graft longevity. We observed minimal firbosis with only a thin layer of fibrous encapsulation (< 50 μm) around the microbeads after implantation in the omentum. This layer is similar to what is observed with pure alginate (Moya *et al.* 2010a).

We have previously shown that the permselective APA microcapsule has a permeability appropriate for excluding immune cells and antibodies that would otherwise destroy islet grafts in the absence of immunosuppressive drugs (Darrabie *et al.* 2005) and that the addition of the external alginate layer for protein encapsulation does not alter the permeability of the APA microcapsule (Khana *et al.* 2010a). We believe that this multi-layer APA microcapsule system could also be used for compartmentalized encapsulation of multiple angiogenic proteins in order to achieve dual growth factor delivery, which has previously been shown to induce rapid formation and maturity of vascular network (Richardson *et al.* 2001). The external layer presents an initial burst release followed by a sustained release of low concentrations of protein (Khana *et al.* 2010a, and Khana *et al.* 2010b), suitable for temporal dose and spatial distribution of growth factor, which has been shown to be effective for angiogenesis Silva and Mooney (2010). To use the multilayer APA in the dual growth factor delivery scheme, a growth factor such as FGF-1 or VEGF can be encapsulated in the external layer for rapid release to initiate the angiogenic process, and another growth factor such as PDGF can be encapsulated in the inner layer for sustained release and maintenance of angiogenesis and maturity of the new blood vessels (Richardson *et al.* 2001). Furthermore, it has recently been shown that co-encapsulation of islets with bioengineered IGF-II-producing cells promotes islet cell survival (Jourdan *et al.* 2011). Therefore, our multi-layer microcapsule system may also be used to encapsulate the IGF-II protein in the external layer while the islet is immunoisolated in the inner core for improved islet function after transplantation.

In summary, we have shown that the APA multi-layer microcapsule system that we recently described (Opara *et al.* 2010 and Khann *et al.* 2010a) can be successfully used for the dual purpose

of islet cell immunoisolation and as a drug delivery device for angiogenic protein in order to enhance neovascularization of the graft at a retrievable transplant site such as the omentum pouch. It is noteworthy that the retrievability of the graft would easily lend it to a variety of post-transplant analyses. The ability to immunoisolate functional islets in a drug delivery device for angiogenic protein to enhance neovascularization of graft at a retrievable site represents a promising strategy to prolong the survival and efficacy of encapsulated islet grafts. Previous studies have shown that hypoxia has significant deleterious effects on the survival and function of islets (Dionne *et al.* 1993, Davalli *et al.* 1996 and Mendoza *et al.* 2005). It has also been recently shown that the function of encapsulated islets can be significantly improved by enhanced oxygen supply (Ludwig *et al.* 2012). Therefore, based on its potential for promoting the supply of oxygen and nutrients to increase islet cell survival, we believe that the enhanced neovascularization of the graft in the omentum pouch will result in the use of decreased numbers of encapsulated islets to achieve normoglycemia compared to the large numbers used in previous studies with encapsulated islets transplanted in the peritoneal cavity of diabetic large animals and humans (Soon-Shiong *et al*. 1992, Soon-Shiong *et al.* 1996, Sun *et al.* 1996, Dufrane *et al.* 2006, Calafiore *et al.* 2006, Elliott *et al.* 2007, Wang *et al.* 2008, and Tuch *et al.* 2009). Further studies are now required to determine the effect of the co-encapsulation of islets and angiogenic protein in the multilayer APA microcapsule on graft function after transplantation in the omentum pouch and other sites.

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