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Design of a bioartificial pancreas⁺

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Abstract

Introduction—In Type 1 diabetes, the β -cells that secrete insulin have been destroyed such that daily exogenous insulin administration is required for the control of blood sugar in individuals afflicted with the disease. Following the development of reliable techniques for the isolation of islets from the human pancreas, islet transplantation has emerged as a therapeutic option, albeit, for only a few selected patients largely because there are not enough islets for the millions of patients requiring the treatment, and there is also the need to use immunosuppressive drugs to prevent transplant rejection. In 1980, the concept of islet immunoisolation by microencapsulation was introduced as a technique to overcome these two major barriers to islet transplantation. Microencapsulation of islets and transplantation in the peritoneal cavity was then described as a bioartificial pancreas. However, it is difficult to retrieve encapsulated islets transplanted in the peritoneal cavity, thus making it difficult to meet all the criteria for a bioartificial pancreas. A new design of a bioartificial pancreas comprising islets co-encapsulated with angiogenic protein in perm-selective multilayer alginate-poly-L-ornithine-alginate (APA) microcapsules and transplanted in an omentum pouch is described in this paper.

Materials & Methods—The multilayer APA microcapsules are made with ultrapure alginate using poly-L-ornithine as a semi-permeable membrane separating the two alginate layers. The inner alginate layer is used to encapsulate the islets and the outer layer is used to encapsulate angiogenic protein, which would induce neovascularization around the graft within the omentum pouch.

Results—In *in vitro* studies, we found that both the wild-type and the heparin binding-growth associated molecule (HBGAM)-FGF-1 chimera can be encapsulated and released in a controlled and sustained manner from the outer alginate layer with a mean diameter in the range of 113–164 microns when 1.25% high guluronic acid alginate is used to formulate this outer layer.

Discussion—We are currently performing *in vivo* experiments to determine the ability of angiogenic proteins released from this outer layer to induce neovascularization around the grafts in the omentum pouch. We will subsequently examine the effect of co-encapsulation of islets with angiogenic protein on blood sugar control in diabetic animals. It is hoped that addition of tissue engineering to encapsulated islet transplantation will result in long-term survival of the islets and

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their ability to control blood sugar in Type 1 diabetes without the necessity to use risky immunosuppressive drugs to prevent transplant rejection.

INTRODUCTION

Diabetes mellitus represents a growing burden both on health-care expenditures and the quality of life of the afflicted individuals. Current estimates for the prevalence of diabetes indicate a global prevalence of about 285 million people ¹, about 5–6% of adults in Central Europe ², and here in the United States, available data from the Centers for Disease Control shows that about 24 million individuals are afflicted with the disease with more than 5% of them suffering from Type 1 diabetes ³. Type 1 diabetes is a significant cause of morbidity and mortality in young adults. Secondary diabetic complications include a quadrupled risk of heart attack and stroke and a significant decrease in life expectancy ^{1, 4}. The economic impact of diabetes is tremendous across the world, with a projected impact of over \$200 billion in direct annual costs in North America in 2010 ¹ and an estimated 25% of U.S Medicare annual in-patient care expenditures attributed to the treatment of diabetes and its associated complications⁵.

The current standard treatment for Type 1 diabetes is daily injections of exogenous insulin to control blood sugar. The Diabetes Control and Complications Trial (DCCT, 1993) was designed to determine whether long-term control of blood sugar using intensive insulin therapy could prevent the development of secondary complications of diabetes, and the conclusion from two cohorts of a total of 1441 patients was that intensive insulin treatment could keep blood glucose close to normal, but could only delay the onset and progression of diabetic retinopathy, nephropathy, and neuropathy, but ultimately would not prevent or reverse existing secondary complications ⁶. In addition, long-term intensive insulin therapy results in undesirable side-effects, including unwanted weight gain, ⁷ and increased episodes of hypoglycemia, which impose constant stress of frequent blood glucose monitoring ⁶.

An alternative treatment modality for Type 1 diabetes is the replacement of the missing β -cells through transplantation of whole pancreas, which in contrast to insulin administration is capable of achieving normoglycemia along with the prevention and even reversal of certain secondary diabetic complications, such as nephropathy and atherosclerosis ⁸. The advantageous effects of β -cell replacement therapy on diabetic complications compared to insulin treatment may be attributed to the role played by the byproduct of pro-insulin cleavage, named C-peptide, during insulin processing in the β -cell ^{9–12}, albeit, the benefits of cell replacement therapy may be masked by collateral risks associated with the use of immunosuppressive drugs to prevent transplant rejection in transplant recipients ⁸. Unfortunately, whole pancreas transplantation is a complex surgical procedure that is fraught with significant morbidities and technical issues including the drainage of exocrine secretions from the transplanted pancreas ⁸. For many years, a preferred β -cell replacement option has been islet transplantation ^{13–16}. This approach was energized by the first report of a method for isolating islets from the rat pancreas ¹⁷, which was followed by other reports showing the successful isolation of islets from the human pancreas using different modifications of the original pancreatic tissue digestion with collagenase ^{18–20} and new improvements in the collagenase enzyme activity characterization ²¹. However, successful islet transplantation in diabetic patients remained elusive ²² until the introduction of the glucocorticoid-free immunosuppressive regimen by the Edmonton group about a decade ago ²³. Still, the need to use long-term immunosuppressive drugs and the severe shortage of human pancreas remain major barriers to clinical islet transplantation ^{13–16, 23–27}. According to the UNOS figures (www.unos.org) in the ten years preceding 2000, transplant registrants quadrupled to nearly 80,000 patients, whilst the number of donors remained stable at less than 20,000 per annum. The search for alternative sources of islets for transplantation has continued, and there are currently attempts to bioengineer stem cells to pseudoislets and neoislets both *in vitro* and *in*

vivo. If any of these efforts to bioengineer insulin-producing cells becomes successful, another major hurdle presented by the need for immunosuppression to prevent transplant rejection would still need to be overcome.

One of the alternative approaches to overcome the two major barriers to islet transplantation indicated above is the technique of islet immunoisolation by microencapsulation prior to transplantation^{14, 28–30}. The principle behind this technique is illustrated in Figure 1, and Figure 2 shows rat islets encapsulated in alginate microbeads. Since the introduction of this technique in 1980²⁸, there have been several reports of successful pre-clinical studies with microencapsulated allo- and xenoislets in non-immunosuppressed large animals^{31–35}. Several pilot clinical trials have also been performed with variable success^{36–38}, and a large clinical trial is currently being performed by Living Cell Technologies, Ltd., in Russia and New Zealand³⁹.

Routinely, these microencapsulated islet transplantations have been performed in the peritoneal cavity, where the grafts have little or no chance to vascularize. It is very well known that revascularization of islet grafts is critical for their long-term survival and function⁴⁰, which may explain why microencapsulated islet transplants may fail in the absence of fibrotic overgrowths around the graft in the peritoneal cavity⁴¹. Also, a continual problem with intraperitoneal transplantation of microencapsulated islets is the inability to retrieve them after extended periods of transplantation, which represents an inadequacy of the grafts to fulfill the criteria for a bioartificial pancreas. To qualify as a bioartificial pancreas, the transplantation should be performed with little or no immunosuppression; should be performed with technical ease; the graft should be capable of long-term function, and should be retrievable for biopsy, if and when desired. We have designed a new model of microencapsulated islet transplantation to meet these criteria for a bioartificial pancreas, which should be distinguished from various models of mechanical insulin delivery devices referred to as the artificial pancreas⁴².

MATERIALS AND METHODS

Chemicals

Low viscosity (20–200 mPa·s) ultra-pure sodium alginate with high mannuronic acid (LVM) and high guluronic acid (LVG) content were purchased from Nova-Matrix (Oslo, Norway). LVM and LVG alginates were reported by the manufacturer to have molecular weights 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). Fluorescein conjugated albumin from bovine (FITC-BSA), was obtained from Invitrogen (Eugene, OR). FITC goat anti-mouse IgG (FITC-IgG) was purchased from AbD Serotec (Martinsried, Germany). Solutions for alginate microbead synthesis were made using the following chemicals: HEPES, NaCl, and $MgCl_2$ (Fisher Scientific); $CaCl_2$ (Acros).

Multilayer microbead synthesis

The procedure used for generating multilayer microbeads is a modification of our routine method of islet microencapsulation⁴³. This modified procedure, which has been recently described⁴⁴, involves first synthesizing PLO-coated alginate microbeads followed by the generation of an outer alginate layer. The inner alginate core was synthesized using the original conditions previously shown to support functional islet encapsulation⁴³. Droplets of 1.5% (w/v) LVM alginate were formed with a two-channel air jacket microencapsulator (at air jacket pressure of 15 psi and alginate jacket pressure of 12.5 psi) using a 25-gauge needle. The droplets were expelled into a 100mM $CaCl_2$ crosslinking solution and allowed to incubate for fifteen minutes. After several washings of the microbeads with calcium-supplemented saline solution, and the PLO-coating⁴³, the external alginate layer was synthesized⁴⁴. The concentration of

alginate used to make the outer layer was varied between 1% and 1.5% for both LVG and LVM alginate. In some cases 5 Units/ml heparin was incorporated into the outer layer by directly adding it to the outer alginate solution prior to addition of the microbeads. Immediately after synthesis, the microbeads were imaged using an Axiovert 200 inverted microscope (Carl Zeiss MicroImaging, Inc.) with a 10x objective (1.08 $\mu\text{m}/\text{pixel}$), and the size of the outer alginate layer quantified with AxioVision AC Rel. 4.6.

Stability of the outer alginate layer

The microbeads were incubated in saline solution containing a physiological concentration of calcium (2mM) and placed in an incubator maintained at 37°C to monitor the stability of the outer layer. The microbeads were imaged daily and the thickness of the outer layer was quantified as described in the previous section. The surrounding solution was replenished with fresh solution after imaging.

Radiolabeling of FGF-1

FGF-1 was purchased from Peptotech (Rockyhill, NJ) and radiolabeled with ^{125}I isotope using Iodo-beads (Pierce Chemical Co.). The Iodo-beads were washed with reaction buffer, placed in a buffer solution containing ^{125}I (1mCi, Perkin Elmer), and allowed to incubate for five minutes. A solution of 50 $\mu\text{g}/\text{ml}$ FGF-1 was added to the vial and allowed to react for fifteen minutes, with intermittent mixing. The Iodo-beads were removed from the vial, and the solution was diluted to a final volume of 3 mL before being dialyzed against de-ionized water for 48 hours to remove any unincorporated ^{125}I .

Protein encapsulation and release

Radiolabeled FGF-1 was added to the outer layer alginate solution prior to addition of the PLO-coated alginate microbeads. Encapsulation efficiency was determined by quantifying the fraction of FGF-1 encapsulated in the microbeads measured immediately after constructing the outer layer, relative to the total amount of FGF-1 added to the alginate solution used to create the outer layer. After formation of the outer layer, the microbeads were transferred into 2mM CaCl_2 solution and incubated at 37°C. The entire incubation medium was removed at pre-determined time points and replaced with fresh solution. Initially, samples were taken every twenty minutes, and then hourly, and daily, as the rate of protein release decreased. Protein content was determined using a gamma-counter (Perkin-Elmer Packard Cobra II Auto Gamma). The percent FGF-1 release was calculated as the sum of FGF-1 released at a particular time point relative to the total amount encapsulated.

The influence of heparin on the rate of release of FGF-1 was also investigated. FGF-1 with 5 U/mL of heparin was incorporated into the outer alginate layer, and release was quantified in the same manner as described above.

Permeability

The permeability of the microbeads was examined by incubation of the beads in 2mM CaCl_2 solutions containing either FITC-IgG (Stokes radius (r_s) ~ 5.5 nm) or FITC-BSA ($r_s \sim 3.6$ nm) at a concentration of 2.5 $\mu\text{g}/\text{mL}$. Confocal images of the beads were taken at time zero, 5 hours, and 3 days. All images were obtained using a confocal laser-scanning microscope with a low pass filter of 505 nm and an excitation at 488 nm.

Protein activity

Fifty microbeads with an outer layer formed using 1.25% LVG were synthesized under two conditions: one in which 15 $\mu\text{g}/\text{mL}$ of FGF-1 alone was incorporated into the outer alginate solution prior to addition to the microbeads, and the other in which 15 $\mu\text{g}/\text{mL}$ of FGF-1 and 5

U/mL heparin were incorporated into the outer layer. The beads were placed in 1mL of Hank's Buffered Saline Solution (HBSS) containing 5 U/mL Heparin. Samples were taken at 12 hours, and the biological activity of released FGF-1 determined using an enzyme immunoassay for quantification of proliferating human umbilical vein endothelial cells (HUVEC). For this assay, HUVECs were seeded in a 96-well plate and grown in endothelial basal media containing 2% fetal bovine serum (FBS), bovine brain extract, gentamicin, hydrocortisone, and EGF, for three days. Cells were then cultured in 0.5% serum in basal media with gentamicin for 24 hours, prior to exposure to release samples for another 24 hours. Bromodeoxyuridine (BrdU), a synthetic nucleoside incorporated into newly synthesized DNA strands, was added to the wells following the 24-hour exposure to release samples. Proliferation was determined 24 hours after addition of BrdU by measuring BrdU incorporation using a BrdU cell proliferation assay kit purchased from Calbiochem.

Omentum Pouch Model of Microbead Implantation

We have previously described the omentum pouch procedure for transplantation of alginate microbeads ⁴⁵⁻⁴⁶. Briefly, Lewis rats were anesthetized using isoflurane and beads were implanted in an omental pouch via laparotomy. The alginate beads were placed on the surface of the surgically exposed omentum. The omentum pouch was created by a purse-string suturing along the edges of the omentum and then tying the sutures. The entire intact omentum was removed from each rat between 7 days and 6 weeks post implantation, fixed in formalin and paraffin embedded. Specimens were serially sectioned (5 µm thickness) for immunohistochemical analysis.

Immunohistochemistry and Image Analyses

Serial sections were stained for CD31, a sensitive marker of ECs, and smooth muscle alpha actin (SMA), a mural cell marker ⁴⁷. Deparaffinized and rehydrated sections underwent 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) or rabbit anti-alpha smooth muscle actin (Abcam) 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 selected using Axiovision AC (Carl Zeiss, Germany). The percent vessel area was calculated using the following formula:

$$\% \text{ vessel area} = \frac{\text{total CD31 stained area}}{\text{total tissue area}} \times 100\%$$

Four images (328 µm × 485 µm) per section were quantified for both CD31 and SMA analysis. Blood vessels per tissue view area were manually counted for CD31 as well as SMA stained sections. SMA coverage was calculated using the following formula.

$$\text{SMA Coverage} = \frac{\text{number of SMA stained vessels}}{\text{total tissue area}}$$

Viability staining

Following islet microencapsulation, rat islets were fluorescently labeled with carboxyfluorescein diacetate (CFDA) for 15 minutes followed by propidium iodide (PI) for

two minutes to demonstrate live and necrotic cells respectively, prior to fixation with 4% paraformaldehyde.

Statistical Analysis

Data are expressed as means $\pm$ standard error. To determine significant differences between two groups of data, student's *t* test was performed. For evaluation of data requiring multiple comparisons, significant differences were determined by performing an analysis of variance (ANOVA) with a Student-Newman-Keuls post-test. In all cases, differences were considered significant for $p < 0.05$.

RESULTS

Following successful formulation of the multilayer APA microcapsules, as illustrated in Figure 3, we performed *in vitro* studies to examine the structural characteristics of the external alginate layer and to determine the kinetics of release of both wild-type FGF-1 and HBGM-FGF-1 chimera from that layer. We found the external layer to be stable with a mean thickness between 113–164 μm , increasing with alginate concentration and G-content. The outer layer was able to encapsulate and release FGF-1 for up to thirty days, with 1.25% of high G alginate displaying the most sustained release. The released FGF-1 retained its biologic activity in the presence of heparin, and the addition of the outer layer did not alter the perm-selectivity of the PLO membrane. Current *in vivo* studies are focused on examining the angiogenic effects of the released growth factor as well as the combined effect of encapsulating islets in the inner layer and the angiogenic protein in the external layer on blood glucose control in diabetic animals. We had previously shown that angiogenic protein can also be encapsulated and released from alginate microbeads, representing the inner alginate core, to induce angiogenesis in the omentum pouch ^{45,46,48}.

DISCUSSION

Multiple issues are involved in the development of a bioartificial pancreas using the approach of islet microencapsulation. These include the need for a retrievable graft with sustained function, scale up devices for microencapsulation, an unlimited islet donor source, as well as efficient islet isolation, purification, and storage techniques. In our studies over the last decade, we have been examining different strategies to address these issues. In this paper, we describe a new design of the encapsulated islet transplantation that hopefully will meet all the criteria for a bioartificial pancreas. As shown in Figure 4, the omentum pouch containing the encapsulated islets and protein is easily retrievable, thus lending the graft to easy biopsy and *in vitro* functional, histochemical, and histopathological assessments. We have found that these alginate microbeads can be used to control the local delivery of growth factors, which can induce new blood vessel formation around the islet grafts in the omentum pouch ^{45,46,48}, and would thus enhance graft viability through improved supply of oxygen and nutrients.

Microencapsulation of islets prior to transplantation is designed to obviate the need for immunosuppression of transplant recipients while expanding the donor source of islets so as to make this treatment modality widely available to diabetic patients. However, clinical application of this technology has been impaired by a number of factors, prominent among which is the death of large proportions of encapsulated islet grafts owing to severe hypoxia, resulting in the need for large quantities of islets to achieve normoglycemia in experimental diabetic animals. Although islets constitute approximately 1% of the pancreas, they receive about 6–10% of the blood flow to this gland ^{49,50}, indicating a disproportionate level of perfusion in which islets receive and consume large amounts of oxygen. The usual high oxygen requirement of islets is interrupted during the process of islet isolation and purification when used for transplantation, and studies have shown that hypoxia has significant deleterious effects

on the survival and function of islets^{51–53}. In the immediate post-transplant period, isolated islets are forced to depend upon diffusion of oxygen and nutrients through peripheral perfusion from the surrounding tissue within the site of transplantation⁵³, until the islet transplants are revascularized by angiogenesis, a process that requires 7–10 days⁵⁴. However, when isolated islets are microencapsulated and transplanted in the unmodified peritoneal cavity, no revascularization takes place, thus subjecting the islet grafts to extended periods of hypoxia and eventual death. Currently, clinical islet transplantation is performed through intraportal vein injection in the liver, where the islets are able to revascularize after several days, following an initial loss of nearly 80% of their vascular density within 24 hours of transplantation⁵⁵. What is desperately needed for long-term islet graft survival and function is an optimal procedure that provides a continuum of oxygen supply from the time of islet isolation through the immediate post-transplant period prior to the islets being integrated into a functional blood supply that will take over the supply of nutrients and oxygen to the graft. This need is currently being addressed by our group using a combination of nanotechnology and tissue engineering.

The pig pancreas has long been proposed and examined as an alternative source of islets for transplantation^{14,42}, albeit, initial concerns were raised about the possible transmission of the porcine endogenous retrovirus (PERV), which now appear to pose little or no risk to humans receiving pig islet transplantation^{56–58}. There had also been initial reports of difficulties associated with isolating pig islets⁵⁹, but our group⁶⁰ and many others have since described reliable techniques for the isolation of pig islets, thus making this species a viable donor source. We have also previously described an effective procedure for long-term storage of islets by cryopreservation⁶¹. Other potential sources of unlimited supply of insulin producing cells for transplantation include the differentiation of stem cells into cells with β -cell-like characteristics, and genetic engineering of adult cells for secretion of recombinant insulin⁴².

Another area of urgent need required in the development of a bioartificial pancreas is the availability of scale-up devices for microencapsulation. The two most widely used devices for microencapsulation are the air-syringe pump droplet generator⁶² and the electrostatic bead generator⁶³. Each of these devices is fitted with a single needle through which droplets of cells suspended in alginate solution are produced and cross-linked into spherical microbeads. A major drawback in the design of these instruments is that they are incapable of producing sufficient numbers of microcapsules in a short-time period to permit mass production of encapsulated and viable cells for transplantation in large animals and humans⁶⁴. A prolonged process of encapsulation of cells adversely affects the viability of the cells. A multi-needle approach to producing more than one encapsulated cell at a time as a scale up of the process has also been described with four needles⁶³. While this scale up is a step forward in accelerating the production of encapsulated cells, production rates at several orders of magnitude higher are required to meaningfully produce sufficient quantities of encapsulated and viable cells to serve millions of patients requiring cell transplantation. For instance, for transplantation in human subjects, it has been estimated that for the 1 million islets needed for transplantation in a diabetic human subject, about 100 hours would be required to complete the encapsulation of this number of islets, assuming one islet/microcapsule. In practice, it has actually been estimated that the duration of the process would be closer to 200 hours because of the additional steps involved in the encapsulation procedure, following the generation of the initial cell-containing alginate microspheres⁶⁴, as outlined earlier in this paper. This situation raises an urgent need for a radically different approach to rapidly producing viable encapsulated cells in sufficient quantities for routine application in human cell therapy. We have designed and are currently testing the efficiency of new prototypes of scale-up devices for cell encapsulation.

In conclusion, β -cell replacement therapy through islet transplantation remains a very promising treatment option for Type 1 diabetes, albeit, it is still work in progress. We believe that a combination of nanotechnology, tissue engineering, use of more user-friendly transplant

sites and improved surgical techniques would result in the creation of a truly effective bioartificial pancreas that can be routinely used in diabetic patients.

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Figure 1. Illustration of the principle of immunoisolation by cell encapsulation

Following encapsulation of islets in alginate microbeads, the beads are coated with perme-selective poly-L-ornithine (PLO) membrane that is permeable to small molecules such as nutrients, oxygen, and water, but impermeable to large molecules such as immune cells and antibodies. The PLO membrane is then covered by a thin coat of alginate to prevent electrostatic interactions between the positively charged PLO and the negatively charged proteins and cells *in vivo*.

Figure 2. Rat islets encapsulated in alginate microbeads in phase contrast with corresponding images for viability stains

Live cells are shown in green and dead cells are shown in red in the viability staining for carboxyfluorescein diacetate (CFDA) and propidium iodide (PI) respectively.

Figure 3. Illustration of the multilayer alginate-poly-L-ornithine-alginate microcapsules
The external alginate layer is used to encapsulate angiogenic protein, and islets are encapsulated in the inner alginate core.

Figure 4. Picture of the new model of a bioartificial pancreas

The picture shows microcapsules (40X) under the microscope 1 week after transplantation in the omentum pouch.