

**IMPROVEMENTS TO THE BIOARTIFICIAL PANCREAS:  
CHARACTERIZING AN ENCAPSULATION MATERIAL AND  
STUDYING THE EFFECTS OF HYPOXIA ON ISLET FUNCTIONS**

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**IMPROVEMENTS TO THE BIOARTIFICIAL PANCREAS:  
CHARACTERIZING AN ENCAPSULATION MATERIAL AND  
STUDYING THE EFFECTS OF HYPOXIA ON ISLET FUNCTIONS**

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## SUMMARY

Current treatments for insulin dependent diabetes fall short of achieving the natural glyceic regulation of functional islets, resulting in the development of secondary complications. A bioartificial pancreas, comprised of insulin-secreting cells encapsulated in a hydrogel, is a promising approach to treat insulin-dependent diabetes. The cells would provide regulated insulin in response to elevated blood glucose levels, thereby providing superior glyceic regulation to exogenous insulin delivery. Two of the current challenges to the successful clinical application of a bioartificial pancreas are: 1) it does not ensure immune acceptance of xenogeneic cells and immunosuppression appears necessary and 2) the transplantation site of the bioartificial pancreas has been shown to be hypoxic compared to islet extraction area.

To address the first concern, this thesis tests the stability and insulin diffusivity to characterize an encapsulation material that is known to steric hindrance effects. This property can combat the adsorption of proteins to its surface, reducing failure of the graft by immune rejection. For the second concern, this experiment will look at total protein content, the ratio of insulin secretion to intracellular insulin content, and metabolic activity of encapsulated adult and neonatal porcine islets exposed to different oxygen levels. This will give insight to the effects of hypoxia on different functions of each islet type, and help determine which islet type is more preferred for transplantation in hypoxic regions.

Researchers emphasize on the use of an alginate material for encapsulation because this material is water soluble and does not seem to interfere with cellular

functions, but is permeable to molecules that activate immune protein production. Then, poly(l-lysine) (PLL) was used to lay on top of the alginate capsules to decrease the permeation of the unwanted molecules, however, PLL stimulates an immune response by the host cells. Several considerations were made for a material to cover the PLL layer in order to improve immune acceptance, such as another layer of alginate. Alginate-PLL-alginate (APA) beads were unsuccessful when the permeation of molecules through the outer alginate layer, made it unstable and exposed the PLL layer underneath. Now, research is concentrated on finding an encapsulation material to replace the outer layer of alginate to cover the PLL layer. Poly (ethylene glycol), or PEG, chemistry has been determined to be a strong candidate for encapsulation because it resists protein adsorption and host cell adhesion.

Using Ca-LVM as the alginate material, this study focuses on the bonding of PEG with Ca-LVM-PLL beads as a possible encapsulation material by characterizing its stability over a 35-day period and insulin diffusivity for one hour. To test the stability of the PEGylated coating, FITC tagged PEG was used to observe the density of the PEG on the Ca-LVM-PLL beads over time. The diffusivity of insulin was tested by monitoring insulin diffusion out of the capsules and into the bulk liquid. Experimental data from the insulin diffusivity test were fit to the analytical solution for Fickian diffusion to determine the effective diffusivity of insulin in each capsule type. Interestingly, the beads were found to be stable within the 35-day duration of the experiment, and the diffusivity of PEGylated beads were comparable to that of Ca-LVM and APA beads in already published data.

Encapsulated islets are transplanted into the peritoneal cavity, which has been shown to be hypoxic compared to the environment that the islets were extracted from. The second part of this thesis studied the effects of oxygen availability on the functions of islets of different ages and islets exposed to different levels of oxygen. Neonatal porcine islets were exposed to 1% and 10% at saturation (AS), and adult porcine islets were exposed to 10% AS; with a normoxic group (21% AS) for each islet group. Preliminary results showed that lower levels of oxygen has more effect on the functions of islet, but the functions of younger islets are less affected by the lower level of oxygen.

# CHAPTER 1

## INTRODUCTION

Diabetes is a metabolic disease in which a person has high blood sugar resulting from one of two ways: the pancreas not producing enough insulin to maintain normal blood sugar levels or insulin resistance. It is a major health problem that affects approximately 23.6 million people in the United States, 27% of which (about 6.4 million people) have become insulin-dependent. Without proper regulation, deviations from normoglycemia increases risk for heart disease, kidney failure, blindness, and nerve damage.<sup>1</sup>

The most common treatment for insulin dependent diabetes (IDD) patients involves self-monitoring their blood glucose level several times a day and self-injecting their bodies with insulin. This method does not continuously monitor blood glucose levels and thus, excursions from normoglycemia often occur without the patient's knowledge. Current research focuses on developing a treatment method that works within the body of IDD patients and freely "senses" the amount of glucose in the blood as well as administers an appropriate amount of insulin needed to regulate glycemic levels.<sup>2</sup>

Cell-based insulin therapy remains a promising approach to alleviate glycemic monitoring and to provide more physiologic insulin delivery for IDD patients. The development of a successful bioartificial pancreas would not only reduce the patient's regular monitoring, but also improve glycemic control, thus reducing the development of the long-term complications associated with diabetes. Researchers are currently investigating methods to produce a bioartificial pancreas which secretes insulin in

response to physiologic cues. The bioartificial pancreas is composed of encapsulated insulin-producing cells in a semi-permeable membrane. The encapsulated insulin-producing cells will respond to the blood glucose levels and secrete insulin to regulate the glycemic levels. Successful membranes will allow the delivery of insulin to IDD patients and further protecting the cells after transplantation.<sup>2</sup>

The first major concern for the development of a bioartificial pancreas is that it does not ensure immune acceptance of xenogeneic cells and immunosuppression appears necessary.<sup>2,3</sup> Suppressing the immune system increases the susceptibility to infectious disease and cancers. One way to reduce the need for immunosuppressants is refining the encapsulation material itself.<sup>4</sup> The second major concern with the bioartificial pancreas development is that the transplantation site has been shown to be hypoxic compared to islet extraction area.<sup>5</sup> The difference in oxygen availability may have effects on the function of the encapsulated islets.

The goal of this research is to develop a bioartificial pancreas that mimics the glycemic control of a non-diabetic patient and allow for the diffusion of insulin across the semi-permeable barrier to signal the body to absorb excess blood glucose. Current research is focused on using the alginate-poly(l-lysine) [AP] system as the semi-permeable barrier for encapsulating the islets. Alginate is a water soluble polymer derived from alga that has been shown to not interfere with cellular function, but is permeable to immunoglobulin G (IgG), a molecule that can bind to the encapsulated cells and activate a cascade of immune protein production to eliminate foreign bodies, including the hydrogel.<sup>6</sup> A layer of poly(l-lysine) [PLL] was added over the alginate hydrogel to resist the diffusion of IgG.<sup>7</sup> However, PLL induces an immune response from

the host resulting in an adsorption of proteins, followed by host cell adhesion, and the formation of a fibrotic capsule to its surface, leading to insufficient nutrient exchange and cell death inside the capsules.<sup>3,8,9</sup>

Researchers are forced to search for a stable alternative source for human islets due to the shortage of human donors. Although xenogeneic transplantation are immunogenic, islets derived from pigs are an attractive option because they reduce source limitations and regulate glucose levels in the same physiologic range as human islets.<sup>10</sup> Both adult porcine islets (APIs) and neonatal porcine islets (NPIs) have been shown to restore glycemic control in diabetic animal models. Although NPIs are less difficult to isolate and possess a potential for growth, they require several weeks to function post-transplantation when compared to APIs but were found to be more resistant to hypoxia.<sup>11,12</sup> Understanding the function of both types of cells under hypoxic conditions can aid in determining which type of islet is more suitable for transplantation.

Encapsulated islets are commonly transplanted in the peritoneal cavity of the abdomen. This area is wanted because it can accommodate a high volume, and the transplantation of the encapsulated cells at this site is done by a direct surgical procedure. The drawback is that this site has been shown to be hypoxic compared to the normal environment where the islets are extracted.<sup>5</sup> Furthermore, the encapsulation method prevents neovascularization of the encapsulated islets, forcing cells to depend on diffusion to be supplied with oxygen and other nutrients.<sup>13</sup>

The oxygen concentration of the transplantation sites the grafted islets or cells are distinctly below the arterial level [142 mm Hg].<sup>5,12</sup> Continuous measurements of the oxygen partial pressure (pO<sub>2</sub>) in the peritoneum of rabbits were made in *Spokane (1990)*.

They reported  $43 \pm 13$  mm Hg for the intraperitoneal  $pO_2$  of rabbits breathing normal air.<sup>14</sup> The  $pO_2$  in diffusion chambers contained 30 islets and implanted intraperitoneally in rats was  $29 \pm 12$   $\mu$ m Hg four weeks after implantation.<sup>15</sup> The low levels of oxygen in the peritoneal cavity may affect the functions of the encapsulated cells. As stated above, studying the effects of oxygen availability on encapsulated islets can enhance our understanding of the functions of islets under hypoxic conditions.

To further understand how to improve bioartificial pancreas development, this thesis focuses on two aims: 1) characterizing PEGylated beads by stability and insulin diffusivity in order to refine the encapsulation material and 2) determining the effects of oxygen availability on the function of encapsulated adult porcine and neonatal porcine islets. For the first aim, the paper hypothesizes that PEGylating, or covalently binding poly(ethylene glycol) [PEG], the AP hydrogels will help reduce the fibrotic adhesion due to the steric hindrance effects of the PEG compound. In order to prevent fibrotic overgrowth, the PEG layer must be stable. However, to achieve the main goal of the bioartificial pancreas, the PEG layer should not affect the insulin diffusivity compared with AP capsules. The second aim studied the effects of oxygen availability on encapsulated islets to enhance our understanding of the functions of islets under hypoxic conditions. This experiment will look at total protein content as a relationship to protein synthesis, the ratio of insulin secretion to intracellular insulin content, and metabolic activity of encapsulated cells exposed to different oxygen levels.

**Aim 1: Characterizing PEGylated alginate/PLL bead for PEG stability and insulin diffusivity**

Improvements to the encapsulation therapy can be done by changing the chemistry used for the beads.<sup>4</sup> It is known that PEG can reduce immune response by preventing and displacing protein and cell adsorption to its surface. A previous study determined that PEG coated alginate capsules are highly biocompatible by themselves, however, their methodology was complex and we are seeking to develop a more straightforward method, which will be widely applicable.<sup>16</sup> In this research, it is hypothesized that the PEG layer of Ca-LVM-PLL-PEG (AP-PEG) beads would be stable over time and that the PEGylation would not affect the diffusivity of small molecules within the capsules.

**Aim 2: Determining the effects of oxygen availability on the functions of encapsulated insulin-producing cells**

In this paper, encapsulated APIs and NPIs will be cultured under 21% AS (normoxia) and 10% AS (hypoxia) to determine the effects of oxygen concentration on both types of islets. Since NPIs were found to be resistant to hypoxia, another set of NPIs will be cultured under normoxia and 1% AS (acute-hypoxia) to study the effects of different oxygen concentrations on NPIs. APIs were also introduced to a re-oxygenation process by exposing them to hypoxia for 7 of days and then bringing them back to normoxic conditions for 2 hours before sample collection; this will determine the time

point that effects by hypoxia is irreversible. All groups were evaluated on days 0, 7, and 14 for insulin secretion, metabolic activity, intracellular insulin storage, and total protein content.

## CHAPTER 2

### LITERATURE REVIEW

#### **Aim 1: Characterizing PEGylated alginate/PLL bead for PEG stability and insulin diffusivity**

Previous studies have tested a range of chemical structures for the encapsulation gel, but the primary encapsulation process has been focused on alginate beads. Since these beads adsorb and are permeable to IgG, they are unsuccessful in long-term stability. Other studies have tried combining different chemical structures like alginate and PLL and alginate-PLL-alginate (APA) beads to protect the islet cells, but proved to be unsuccessful. Researchers are now exploring PEG as a molecule to cover AP beads because of its steric hindrance properties to disrupt adsorption on surfaces.

*Sawhney et al (1993)* introduces a procedure to add a PEG-based hydrogel upon AP beads.<sup>7</sup> However, this procedure is complicated and relies on expensive equipment. PEG can decrease the immune response of fibrous overgrowth by aiding in prevention of proteins or cells attachment to the surface. In this paper, FITC tagged PEG was used to quantify the duration of constant PEG density on the AP beads. Extended research should be done to understand and explore further chemical combinations of AP with PEG.

To further look into different chemistry combinations with PEG, this research will focus on the possibility of Ca-LVM-PLL beads for covalently bonding with PEG. In order to evaluate the potential of utilizing this PEG coating, two assays will be employed: PEG layer stability and insulin diffusivity. To demonstrate that the PEG layer does not

adversely affect the favorable characteristics of the alginate microcapsules, the capsules will be tested *in vitro* by themselves. At the end of this experiment, we will conclude whether this Ca-LVM-PLL-PEG hydrogel is a worthwhile delivery material to move forward with.

**Aim 2: Determining the effects of oxygen availability on the functions of encapsulated insulin-producing cells**

Since the oxygen levels of the environment of the transplantation site is lower than that of the environment that they are extracted from, the function of the islets should be examined to observe the effects of varying oxygen levels. *Hyder (1998)* studied the effect of oxygen levels on insulin secretion of islet cells from the same species but different ages as well as islet cells of different species. The primary cell type used in this study was rat islet cells. Neonatal and adult rat islets (ARIs) were exposed to 21% O<sub>2</sub> (normoxia) and 5% O<sub>2</sub> (hypoxia) in basal and stimulated media to compare the effect of hypoxia on insulin secretion of different ages in different media environment. Results only showed that the insulin secretion of ARIs under hypoxia had a significant difference compared to ARIs in normoxic conditions for both basal and stimulated media. This suggests that islet cells of a younger age are more resistant to hypoxia.<sup>12</sup>

*Emamaullee (2006)* examined the function of NPI under oxygen-induced stress. Insulin secretion and apoptosis were evaluated after NPIs and APIs were exposed to hypoxia and hypoxia/re-oxygenation conditions. Results showed that NPIs were more successful in resisting apoptosis and maintaining insulin secretion under both oxygen-

induced stress conditions. Islet grafts of both cell types were then transplanted into chemically diabetic, immune-deficient mice. Twenty-four hours after transplantation, API grafts experienced apoptosis while NPIs showed limited apoptosis. This suggests that NPIs are more resistant to hypoxia compared to APIs.<sup>11</sup>

In order to increase our understanding of the effects of oxygen levels on porcine islets, this research will expand on the *Hyder (1998)* and *Emmanuelle (2006)* studies. This research will examine the effects of oxygen levels on insulin secretion, metabolic activity, intracellular insulin storage, and total protein content of NPIs and APIs. The functions of NPIs will be examined at three different oxygen levels (normoxia, hypoxia, acute hypoxia), meanwhile APIs will be examined at three different oxygen conditions (normoxia, hypoxia, reoxygenation). At the end of this experiment, we will receive a preliminary conclusion on the effects of oxygen concentration on API and NPI function and survival.

## CHAPTER 3

### AIM 1: CHARACTERIZING PEGYLATED ALGINATE/PLL BEAD FOR PEG STABILITY AND INSULIN DIFFUSIVITY

#### Methods

##### Stability Test

###### Purpose

To determine the effect of PEG reaction concentration on the resulting PEGylation of Ca-LVM-PLL capsules, and evaluate the stability of the PEG layer in vitro

###### Protocol

This experiment tested the bonding of FITC-PEG to the Ca-LVM beads by a concentration range. PEG capsules tested are cross-linked with PLL coated Ca-LVM alginate beads (AP-PEG beads). There were three experimental groups: 1mM AP-PEG beads, 0.5mM AP-PEG beads, and 0.05mM AP-PEG beads. The two control groups served for different reasons: the 0mM AP-PEG beads showed any background fluorescence emitted by the beads itself, and the 1mM A-PEG (PLL uncoated) beads showed us any non-specific adsorption. Under sterile environment, 100  $\mu$ L of beads were distributed into a well of a 24 non-treated well plate and the respective FITC-PEG (Nanocs) concentrations were added with the lights off, since FITC is sensitive to light. The beads were incubated with the FITC-PEG for 30 minutes on a plate shaker. The beads were then washed three times every fifteen minutes for ten washes with 6 mL of calcium chloride to get rid of any excess FITC-PEG. Before each time point, 200  $\mu$ L of the surrounding calcium chloride solution were transferred into a black 96 non-treated

well plate for reading. After the collection, the old solution was replaced with 1 mL of new calcium chloride. Fluorescent area scans and solution readings were done at day: 0, 1, 2, 3, 4, 5, 7, 8, 11, 14, 18, 21, 25, 28, 32, and 35. For the area scans, the plate reader was set to read from the bottom of the plate with excitation of 495 and emission of 525, and a gain of 125. The settings were kept the same for the solution readings except it was done from the top of the plate. The data is later evaluated for any trends. To validate the stable hypothesis, the area scans should stay constant throughout, to show that the density of PEG does not decrease and the PEG is not breaking off. A paired student's t-test was used to determine statistical significance between the first and last data points of the area scans. Furthermore, the solution readings should move towards zero to show diffusion of FITC-PEG inside the beads to their surrounding has stopped. There should also be a relationship between the concentration of FITC-PEG and the fluorescent values: the higher the concentration, the higher the fluorescence.

## **Diffusivity Test**

### Purpose

Determine insulin diffusivity in PEGylated capsules compared with APA and Ca-LVM capsules

### Protocol

This experiment compared the diffusivity coefficients of linear and branched PEGylated beads to that of Ca-LVM and APA beads. The difference between the two PEG molecules is the size; the branched PEG has a higher molecular weight compared to the linear PEG.<sup>17</sup> Both PEG types were tested to ensure that the molecule does not affect insulin diffusivity, not just a specific size. 100  $\mu$ L of CA-LVM beads were distributed

into a well of a 24 non-treated well plate via strainer. The strainer was moved into another well and the solution the beads were sitting in was replaced with 400  $\mu\text{L}$  of 16.6 mg/mL bovine insulin. The plate was placed on a shaker for one hour to ensure the beads have reached equilibrium with its surrounding. The strainer was then transferred to another well plate before removing the insulin. The beads were quickly washed twice by dunking the strainer in 1 mL of unsupplemented media (8.3 g/L Dulbecco's modified Eagle's medium (DMEM D5030; Sigma) supplemented with 3.7 g/L sodium bicarbonate ( $\text{NaHCO}_3$ ; Sigma), and 0.9008 g/L of D-glucose (Sigma)) in the adjacent two wells, and the strainer will be left in the third well with 1 mL of unsupplemented media. A 10  $\mu\text{L}$  sample of the surrounding solution will be taken at minute: 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, and 60. Procedure will be replicated for the APA and PEGylated beads. After all collection, 10  $\mu\text{L}$  of each standard was placed in the next column(s) of wells. 300  $\mu\text{L}$  of room temperature Coomassie solution was added to every well. After 30 seconds on the shaker, the plate was left to settle for 10 minutes. The absorbance reading of the solutions should be done after the 10 minutes and before 30 minutes since adding Coomassie solution. The data was then plotted and the diffusivity coefficients of both beads were calculated from the following modeling equation from *Sambanis (1999)*:

$$- \frac{C_b}{C_{bo}} = \left[ \frac{1}{(1+\alpha)} \right] \left[ 1 - \sum_{n=1}^{\infty} [6\alpha(1+\alpha) \exp(-D_{\text{eff}}q_n^2 t/R^2)] / 9 + 9\alpha + q_n^2 \alpha^2 \right]$$

- Equation describes diffusion of solute from beads into the liquid bulk, initially containing no solute.
- In the equation above,  $C_b$  is the concentration of insulin at a given time in the bulk solution ( $\text{mol}/\text{m}^3$ );  $C_{bo}$  is the initial concentration of insulin within the bulk solution ( $\text{mol}/\text{m}^3$ );  $\alpha$  is the ratio of liquid volume to total bead

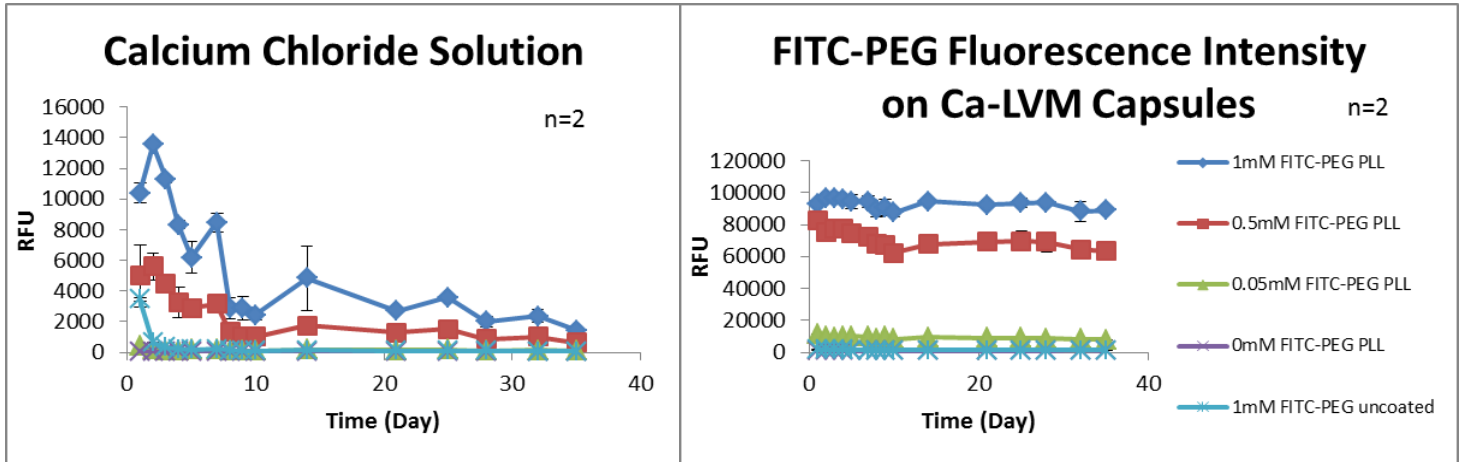
volume;  $D_{\text{eff}}$  is the effective diffusivity ( $\text{m}^2/\text{s}$ );  $R$  is the bead radius (m),  $q_n$  is the  $n$ th root of the equation<sup>18</sup>

The coefficients should be comparable between the beads to justify the hypothesis that PEG does not affect the diffusivity of AP beads.<sup>9</sup> A one way ANOVA with a p-value of 0.05 was used to determine if there were any statistically significant differences between the groups of beads.

## Results

### Stability Test

The stability of the PEG coating on AP beads were determined by analyzing the trends of the fluorescent readings of the surrounding solution and area scans of the beads. Figure A.1 shows the calcium chloride solution readings had a general decrease and bead fluorescence did not decline for both the experimental groups and control throughout the duration of the experiment. The relative fluorescence unit (RFU) of the calcium chloride solution reading for each group declined over time, but it does appear that some FITC-PEG is found in solution for several of the groups throughout the course of the experiment. Since there is huge difference in magnitude for the RFU in solution and the RFU in area scans, having a few FITC-PEG in the solution does not make the PEG layer on the beads unstable.



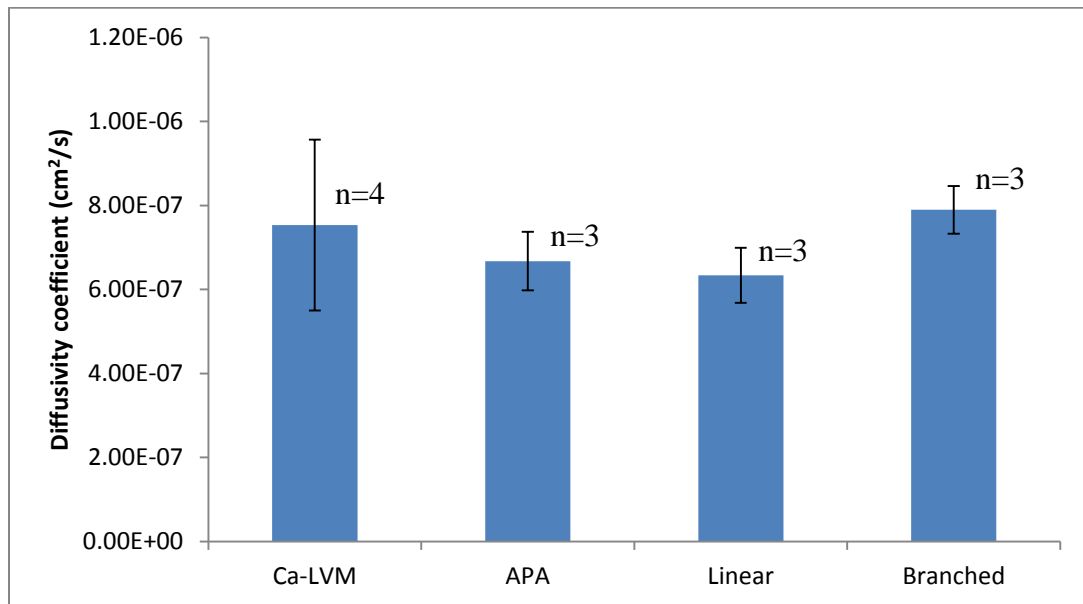
**Figure A.1: Fluorescent solution readings and area scans of all groups at predetermined time points.** The experiment tested three different concentrations groups against two controls. Average fluorescence of the area scans was recorded in order to quantify the amount of PEG density on the surface of the Ca-LVM beads. Solution readings were recorded to observe the amount of FITC-PEG diffusing from inside the beads into the solution surrounding the beads. The fluorescence of both solution readings and area scans are used to determine the stability of the beads by tracking them over time. Errors bars show standard deviation between n=2 groups.

The FITC-PEG fluorescence readings show a relationship between the concentration of FITC-PEG and fluorescence; the higher the concentration, the higher the fluorescence. As the reaction concentration of FITC-PEG increases, so does the RFU of the beads, indicating that a greater density of PEG was grafted onto the surface. Shown as a negligible RFU in the 1mM A-PEG, the data suggest that PEG bonds with the PLL layer. As seen in Figure A.1, the fluorescent readings for all groups stayed consistent throughout the 35-day duration, meaning that the PEG layer was bonded to the AP beads for the entire experiment. Since the AP beads did not lose anymore PEG for 35 days and the PEG layer stay bonded to the AP beads for that time span, the PEG layer can be concluded as stable for the duration of the experiment. To statistically determine

significant results, a student's t-test was used for the first and last data point for each group. A p-value of 0.2208 was obtained, showing no statistical significant difference between the RFU at the beginning and end of the experiment.

### Insulin diffusivity

The diffusivity of the Ca-LVM, APA, linear PEG and branched PEG beads were determined by comparing the calculated and measured diffusivity coefficients of the beads. Figure A.2 shows the diffusivity coefficients of all four bead groups. The diffusivity coefficients were calculated for each trial individually using the equation provided by *Sambanis (1999)* and then the average of each group is graphed in figure A.2. The results show that diffusivity coefficients are similar for all four hydrogel materials. Using a one-way ANOVA, a p-value of 0.786322 was calculated, confirming that there are no statistically significant differences between the four groups.



**Figure A.2: Diffusivity coefficients of different hydrogels.** Different hydrogels were placed in a solution with bovine insulin and until an equilibrium was reached. After

transfer of the beads into solution containing no insulin, samples of the solution were collected at various time points and the concentration of insulin was analyzed. Using the equation in *Sambanis (1999)*, diffusivity coefficients for each group was calculated. The average diffusivity coefficients are shown above. \*Bars indicate standard deviation between n groups.

## Discussion

Based on Figure A.1, the solution readings display a general decrease in RFU within all groups. The decreasing trend shows that the amount of FITC-PEG inside of the bead is lessening. The average bead fluorescence displays a correlation between concentrations of FITC-PEG to RFU expression. The 0mM AP-PEG beads were used as a control to detect any background fluorescence, while the 1mM A-PEG beads were set to observe non-specific adsorption. Since both controls have statistically similar RFUs and therefore we can conclude that minimal non-specific adsorption occurred. With the straight line trends in average bead fluorescence and decreasing solution readings, PEGylated AP beads are determined to be stable within the 35-day time frame of the experiment.

The data in Figure A.2 shows that both linear and branched PEGylated beads have similar diffusivity coefficients as compared to Ca-LVM and APA beads. A one way ANOVA was used to determine if there were any statistically significant differences between the diffusivity coefficients of all groups of beads. After calculations, a p-value of 0.786322 obtained, showing no statistically significant differences between the diffusivity coefficients. This confirms that the insulin diffusivities of linear and branched PEG are comparable to Ca-LVM and APA diffusivities in already published findings, and PEG did not hinder the insulin diffusivities of these beads.<sup>19</sup>

Similar to *Sawhney et al (1993)*, this experiment has successfully PEGylated AP-beads. The difference between the two experiments derives from the method used to PEGylate beads, this project simply crosslink PEG to the surface of AP beads, while *Sawhney et al (1993)* utilizes photopolymerization, a difficult method requiring expensive equipment. The PEG layer is found to be stable over a 35-day period and generally does not affect the insulin diffusivity of the AP beads. In order to introduce PEGylated beads as an encapsulation material, they must be tested to see if they elicit an immune response that may lead to islet cell death. *Sawhney et al (1993)* found that PEGylated beads are less likely to elicit an immune response than AP beads. Since insulin diffusivity was not evaluated in previous studies, these results are important as a deciding factor for PEGylated beads as an encapsulation material. Furthermore, the stability of these beads will determine the duration that PEGylated beads can function as an insulin therapy, if adapted.

## **CHAPTER 4**

### **AIM 2: DETERMINING THE EFFECTS OF OXYGEN AVAILABILITY ON THE FUNCTIONS OF ENCAPSULATED INSULIN-PRODUCING CELLS**

#### **Methods**

To study the effects of oxygen availability on different cell types, NPIs and APIs were encapsulated separately in Ba-LVM, alginate that is solubilized easier than Ca-LVM, capsules and exposed to a hypoxia level of 10% AS. Each cell type had a normoxic (21% AS) group to represent as the control at each time-point. On the other hand, to study the effects of different levels of oxygen availability, another group of NPIs were exposed to 1% AS and had a normoxic control group for each time-point. APIs were also subjected to a reoxygenation process, placing them in hypoxic conditions for 7 days then normoxic conditions for 2 hours before collecting samples. This was done to study the response of API functions after a traumatic exposure. All groups (hypoxic and normoxic) associated with the 10% AS were evaluated for insulin secretion, metabolic activity, intracellular insulin storage, and total protein content on days 0, 7, and 14. The normoxic and hypoxic groups related to the 1% AS were evaluated for the aforementioned parameters on days 0, 7, and 15.

During the study, samples were collected at the time-points and placed in -80°C until assays and micro-plate readings were performed. Hypoxic groups also received media and HBSS that was placed in respective oxygen levels at least 24h prior to use to

allow solutions to acclimate to the hypoxic levels. An alamarBlue protocol and fluorescence readings were used to determine the metabolic activity of the encapsulated cells. On the days of evaluation, beads were given fresh media and alamarBlue reagent was added to the wells at a 1:9 dilution of reagent to media for a 2h incubation period. Samples for the metabolic activity test were collected after the incubation period in respective oxygen levels then the beads were washed with HBSS. After receiving new media, a t=0 sample was collected for the insulin secretion test. After a two hour incubation in respective oxygen levels, a t=2 sample was collected. The concentration of secreted insulin was determined using a porcine insulin ELISA kit and was calculated by subtracting the initial quantity of insulin from the final quantity of insulin.

When all samples for insulin secretion have been collected, beads were solubilized and cell pellets were collected through centrifugation. Cells were then lysed with mammalian cell lysis buffer to perform assays for total protein content and intracellular insulin storage. Total protein content samples were prepared using a Micro BCA protein assay kit and absorbance readings were taken to determine the quantity of protein inside the cells. The porcine insulin ELISA kit above was also used to quantify the amount of insulin in the intracellular storage through fluorescence readings.

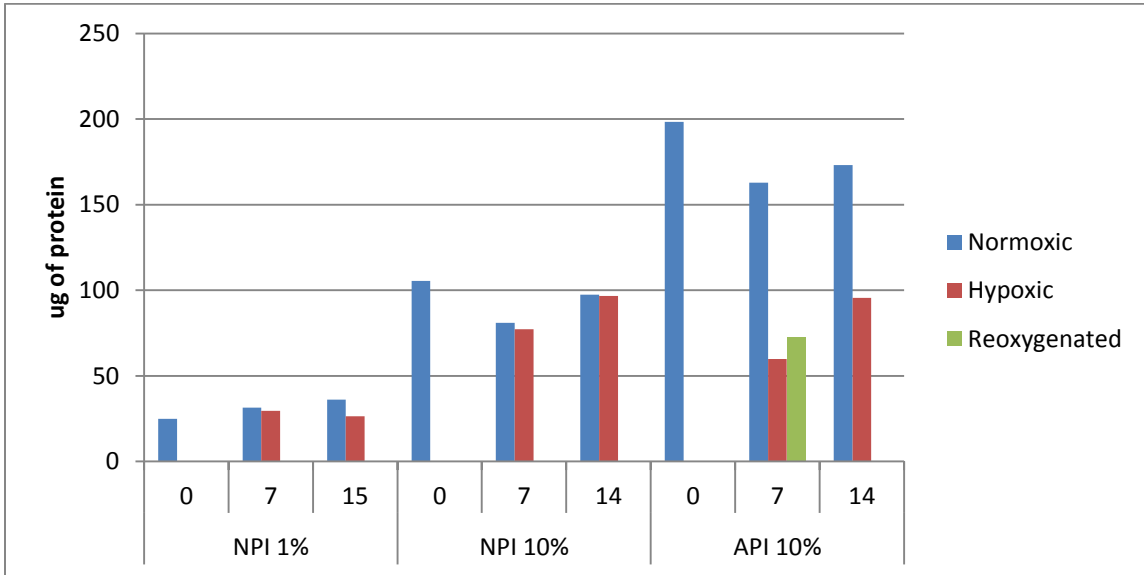
General trends of each function were observed to assess the effects of oxygen on islet functions. For easier understanding, insulin secretion and intracellular insulin storage will be represented as the ratio of insulin secretion to intracellular insulin content. This means that the data will denote percent of insulin secreted compared to the amount of insulin stored inside the beads. Since this is the first time this experiment has been done, these results should be viewed as preliminary and no definite conclusions can be made.

## Results

Neonatal porcine islets were exposed to 1% and 10% AS oxygen levels, while adult porcine islets were exposed to 10% AS oxygen. Parameters of total protein content, secretion percentage, and metabolic activity of each hypoxic islet group and its normoxic counterpart were measured. Figure B.1 shows the data that was collected from the total protein assays with results graphed as quantity of protein. The secretion to storage ratio percentage of each group was calculated by:  $\frac{\text{insulin secretion}}{\text{intracellular insulin storage}} \times 100$  and is shown in Figure B.2. The metabolic activity of the islets were also tracked and graphed in Figure B.3. The general trends of each function parameters will be compared between groups, this result analysis will not investigate the trends within an individual group. To determine the effect of hypoxia on the functions of different cell types, the parameters of the NPIs and APIs exposed to 10% AS should be compared. Similarly, to determine the effect of different levels of hypoxia on the functions of the same cell type, the parameters of both the NPIs exposed to 1% and 10% AS should be compared.

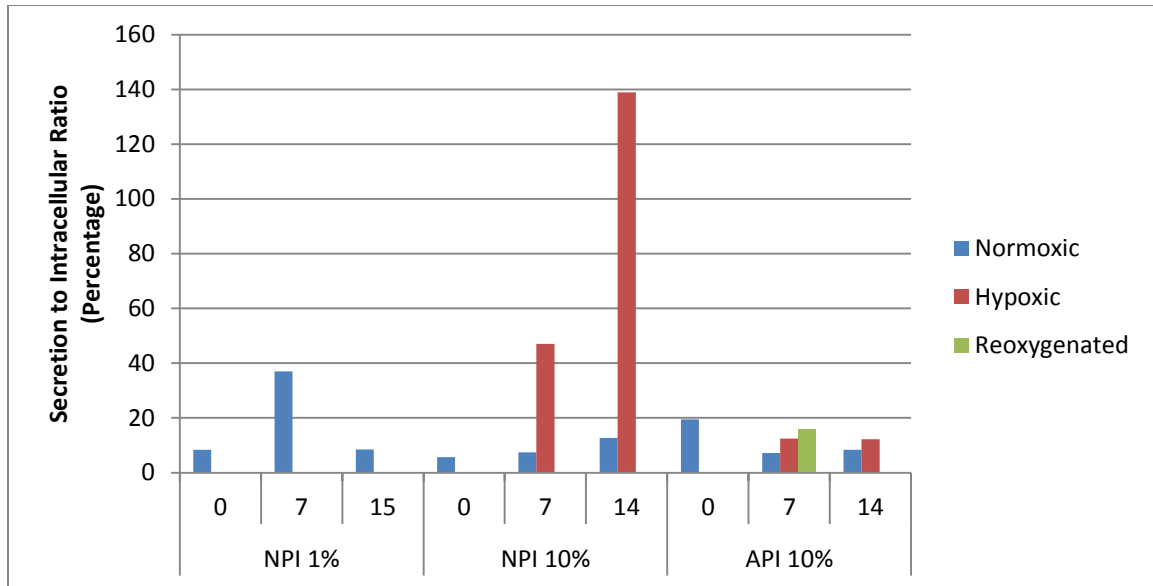
Since the two are from the same group, and their normoxic groups were exposed to the same condition, the data suggests that the number of cells encapsulated in the NPIs at 1% AS may have been lower than that of the 10% or there possibly might be another factor that cannot be determined by this data alone. APIs, on the other hand, shows a 200% increase of total protein content compared to NPIs exposed to the same condition. Since the hypoxic groups of the NPIs show similar results as their normoxic counterparts, total protein content of NPIs seem to not be affected by the different level of oxygen. However, the total protein content for hypoxic and reoxygenated groups of the APIs is

reduced, and therefore affected by the oxygen level. Furthermore, these two groups of the APIs are similar, meaning that total protein content of APIs is comparable for different oxygen-induced stress conditions.



**Figure B.1: Effect of hypoxia on total protein content of encapsulated porcine islets.** Encapsulated NPIs and APIs were exposed to different levels of hypoxia. Total protein content samples were collected at three time points and analyzed using a Micro BCA total protein assay kit.

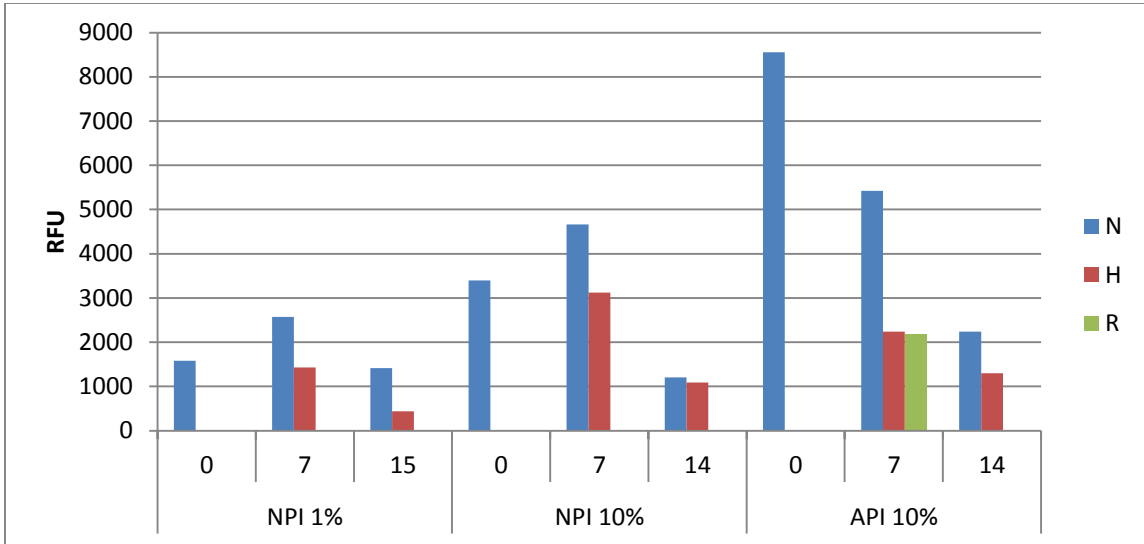
As seen in Figure B.2, the normoxic groups of both cell types secrete around the same percentage of insulin. For the data is missing for the NPIs exposed to 1% AS, possibly resulting from error in cell pellet collection. The NPIs exposed to 10% AS shows an increase in insulin secretion percentage to 140% . This suggests that the islets secreted all of its intracellular insulin content and more. Since this data was collected for the first time, this data point shows a source of possible error. However, APIs exposed to 10% AS also showed an increase in insulin secretion ratio. Based on the data presented, it is unclear whether this increase was due to an increase of insulin secretion or a decrease on intracellular insulin content.



**Figure B.2: Effect of hypoxia on the ratio of insulin secretion to intracellular insulin content.** Encapsulated NPIs and APIs were exposed to different levels of hypoxia. Ratio was determined by dividing insulin secretion by intracellular insulin storage, value was multiplied by 100 to convert into percentage.

Shown below in Figure B.3 is the metabolic activity of the encapsulated cells exposed to different levels of oxygen over time. NPIs exposed to 1% AS and its normoxic group had a metabolic activity of around 50% of the NPIs exposed to 10% AS and its normoxic group. Similarly to the total protein content data, this suggests that the number of cells encapsulated in the NPIs at 1% AS may have been lower than that of the 10% or there possibly might be another factor that cannot be determined by this data alone. When comparing the metabolic activity of same islets under different levels of oxygen, NPIs exposed to hypoxia shows a general 50% decrease compared to their normoxic counterparts, therefore metabolic activity of NPIs seem be affected by the different level of oxygen. Under normoxia, APIs have a metabolic activity that is generally twice that of the normoxic group of NPIs exposed to the same hypoxic conditions, suggesting there were more cells in the API capsules. The metabolic activities

for hypoxic and reoxygenated groups of the APIs are reduced by half of its normoxic counterpart, and are affected by the oxygen level. Furthermore, these two groups of the APIs are similar, meaning that metabolic activity of APIs is comparable for different oxygen-induced stress conditions.



**Figure B.3: Effects of hypoxia on metabolic activity.** Encapsulated NPIs and APIs were exposed to different levels of hypoxia. Metabolic activity was determined by fluorescent readings with alamarBlue reagent.

### Discussion

Since this experiment has only been done once, these results should be viewed as preliminary and may be used to inform the design of future studies. To see statistically significant results from this experiment, it must be repeated a minimum of 2 additional times. Furthermore, this data is incomplete, as seen in the intracellular insulin storage results for NPIs exposed to 1% AS. The hypoxic data is at zero for all time-points, suggesting that the cell pellet may have not been collected properly.

Based on the parameters of total protein content, secretion percentage, and metabolic activity, our preliminary data suggests several things about the functions of islets exposed to different levels of oxygen. The first is that only the total protein content of APIs is affected by different levels of oxygen, possibly indicating that the oxygen has an effect on protein synthesis for APIs. The second suggestion is that islets of both ages may secrete more insulin compared to its intracellular insulin content when under an oxygen-induced stress condition (hypoxia or reoxygenation). This can be that these cells are not producing enough insulin to restore its intracellular content, or the cells are no longer responding properly to physiological cues. The reader must keep in mind that this suggestion was made without data for NPIs exposed to acute hypoxia. Third, the metabolic activity of all three experimental groups show around a 50% decrease, statistical analysis may be required to show which group's metabolic activity was more affected by the oxygen levels. Fourth, our data suggests that functions of APIs respond similarly for different oxygen-induced stress conditions.

There are currently little published findings on the effects of hypoxia on neonatal porcine islets. However, this study is similar to that of *Hyder (1998)*, in that it compares the effects of hypoxia on islet cells of the same species at different ages but this study goes further in trying to determine the effects of different levels of hypoxia on islets. Although, the other paper tested the effects of hypoxia on functions of rat islet, both papers suggest that functions of neonatal islets are less affected by low levels of oxygen. This conclusion is reaffirmed by *Emamaullee (2006)* demonstrating that NPIs have a natural resistance to hypoxic environments. Overall, this confirms that NPIs may be the

preferred cell type for the encapsulation method, due to higher stability of functions under hypoxic conditions.

Even though the functions NPIs are concluded to be less affected by hypoxia, NPIs will eventually mature and develop in APIs, it is possible that they may become less resistant to hypoxia. This means that NPIs will only be resistant to hypoxia before they reach maturity. As mentioned before, NPIs also require several weeks to function post-transplantation when compare to APIs, cutting down the time span that hypoxia-resistant NPIs are used effectively.

## **CHAPTER 5**

### **CONCLUSION**

As research expands to provide a superior treatment for insulin-dependent diabetes, cell-based insulin therapies continue to be the most widely sought after insulin delivery method. To further improve this method, new encapsulation chemistry should be tested for its stability and insulin diffusivity. The results in this experiment show that PEG is stable for 35-days and does not affect the insulin diffusivity of AP beads. These results also showed that there were negligible fluorescent readings for PEG over the 35-day time period when the beads were not coated with PLL, suggesting that PEG covalently bonds to the PLL layer of AP beads.

Further research on the second aim will improve our understanding of the effects of low levels of oxygen on the encapsulated insulin-producing cells. These preliminary results showed that lower levels of oxygen had a greater affect on islet functions of total protein content, insulin secretion, intracellular insulin storage, and metabolic activity. They also suggest that NPI functions are more resistant to hypoxia than APIs, similar to published conclusions. Using this information, researchers can develop strategies to help improve the function and survival of transplanted islets.

## **CHAPTER 6**

### **FUTURE WORK**

#### **Aim 1: Characterizing PEGylated beads by stability and insulin diffusivity in order to refine the encapsulation therapy**

To further characterize the PEGylated AP capsules, additional experiments need to be performed including assessment of the permeability of the PEG to specific molecules like IgG, assessment of the cytotoxicity of the PEGylation procedure, and in vivo evaluation of the capsule. Permeability defines the types of molecules that can be exchanged in and out of the beads, which can be targeted for wanted molecules or help prevent unwanted ones. To make sure the encapsulation material does not kill the cells inside, the cytotoxicity assay determines if the material will affect the viability of encapsulated cells. To quantify the amount of cells adsorption, histology can identify the fibrotic density on the surface of the beads.

In the permeability assay similar to *Kulseng (1997)*, AP-PEG capsules should be exposed to IgG to conclude whether the PEG layer allows for IgG permeation and if PEG affects the permeability of the AP beads. Histology of the adsorption on the surface of AP-PEG beads can be done by exposing the beads to cells and then staining to quantify the amount of cells attracted to the surface of the beads. The last assay will use the metabolic activity of the islets to determine survival rate of encapsulated cells in the beads, subsequently determining cytotoxicity of the PEGylation process.

**Aim 2: Determining the effects of oxygen availability on the functions of encapsulated insulin-producing cells**

A major improvement to this experiment is during the cell pellet collection process to ensure that all data points are present. As discussed in Chapter 4, if statistically significant results are desired, this experiment should be repeated a minimum of 2 additional times. However, the goal of obtaining preliminary results for this experiment has been achieved, and the experiment is completed, therefore future work will not be planned. Continuing this work will better the understanding of the effects of oxygen levels on islet functions. Since the peritoneal cavity is an appealing site for transplantation, furthering this research will give insight on how to improve the encapsulation therapy to accommodate for the hypoxic transplantation environment.

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