The Role of Oxygen During In Vitro Culture and Immunoisolation of Islets of Langerhans

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UNIVERSITY OF MIAMI

THE ROLE OF OXYGEN DURING IN VITRO CULTURE AND IMMUNOISOLATION OF ISLETS OF LANGERHANS

By

Christopher A. Fraker

A DISSERTATION

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THE ROLE OF OXYGEN DURING IN VITRO CULTURE AND
IMMUNOISOLATION OF ISLETS OF LANGERHANS

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While clinical transplantation of islets of Langerhans for the treatment of insulin dependent Diabetes Mellitus has shown significant promise in recent years, there remains a need for procedural optimizations to improve cell viability, functionality and ultimately, graft longevity. One of the most critical factors to islet cell survival is the proper oxygenation of these highly metabolic cellular aggregates. In culture, islets experience suboptimal oxygen profiles delimited by steep gradients across culture media. When re-transplanted, they are subjected to extremes of hypoxia and anoxia, resulting in pronounced graft dysfunction and cell loss, which is further exacerbated when these cells are immunoisolated in polymer matrices. This study examined the effects of improving both in-vitro culture and immunoisolation of islet cells by optimizing oxygen mass transfer via oxygen carriers in the form of perfluorocarbons. Specifically, new systems for these applications were developed utilizing perfluoromoeities and conventional culture (polydimethylsiloxane) and immunoisolation (sodium alginate) matrices.

During in vitro culture of islet cells, the use of perfluoro-impregnated PDMS culture platforms enhanced cell recovery, viability and function over the culture period. Additionally, marginal mass transplants of the islets cultured in these novel platforms functioned better in recipients than relevant controls.
In immunoisolation, the optimization of perfluorocarbon emulsions was performed investigating the effects of combinations of surfactants and perfluorocarbons on oxygen mass transfer and cell viability. Emulsions were well characterized using particle size analysis by dynamic light scattering, perfluorocarbon inclusion by gravimetry and oxygen diffusivity measurements utilizing fluorescent optodes. A novel method was developed for the assessment of dissolved oxygen content of these emulsions. Optimal emulsions, as determined by predicted/measured oxygen transfer enhancement over relevant controls, were utilized in alginate matrices for microencapsulation of cell lines, initially, and then, islets of Langerhans. The effects of these potential improvements were assessed by in-vitro potency assays, including a novel method for assessing glucose stimulated insulin release, and in transplantation efficacy in rodent marginal mass models. While the improvements in culture were promising in cell line studies, the observed benefit did not translate in islet culture. The cause was found to be related to permeability impediments generated from the surfactant components utilized in emulsion manufacture. In addition to the development of several new methods for the characterization of oxygen containing solutions and the potency assessment of isolated islets of Langerhans, the impact of these studies is important in the field of polymer engineering. We observed that the use of Polyethylene glycol (PEG) based materials may limit transport of nutrients and oxygen critical to cells. Additionally, we developed cell culture platforms that enhance the viability, number and function of cultured islet cells, potentially impacting the clinical realm where cell preservation is critical to transplant outcome.
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CHAPTER 1: SPECIFIC AIMS

1.1 Introductory Remarks

The overall economic impact of diabetes warrants substantial scientific research into curative therapies for this disease. One fact that has come to light over the years of research into cellular transplantation approaches to curing diabetes is the exquisite sensitivity that insulin-producing cells have to local oxygen content. Typically, islets of Langerhans reside and function in environmental oxygen profiles between 0.4-40 mmHg partial pressure, where their native vasculature keeps all of the cells within the microorgan at an equivalent partial pressure. It has been demonstrated that in culture, post-isolation from the pancreas and in the transplant setting, the oxygen levels experienced by the islets can be much higher or lower than their native environment, with steep intra-islet gradients. These sub-optimal levels result in either hyperoxic or hypoxic/anoxic cell damage and death, greatly affecting viability and function. There is a clear need for the optimization of islet oxygenation both in the culture setting, where incubators are maintained at levels 4-5 times that experienced by islets in vivo, and in the post-transplant environment, where islets removed from their native vasculature are plagued by hypoxia induced cell death. It is our assertion that tailoring the oxygen environment in vitro and improving oxygen supply in vivo will enhance islet function and survival in both settings. In order to address these critical issues, the following central hypothesis will be tested and specific aims evaluated:
1.2 Central Hypothesis and Specific Aims

CENTRAL HYPOTHESIS: The use of perfluorocarbon moieties in various matrices employed for cell culture and transplantation will lead to improved viability and function in both the in-vitro and in-vivo setting due to the enhanced oxygen mass transfer afforded by the included perfluorocarbon.

Specific Aim 1:
To optimize static culture of adult endocrine pancreas (islets of Langerhans) through the use of polydimethylsiloxane/perfluorosilane oxygen permeable static culture platforms targeting the in vivo physiological niche of the cultured tissue.

Sub-aims
i) *To determine optimal parameters of culture platforms including*
   1) Determine optimal PFC for inclusion based on miscibility, volatility and diffusivity
   2) Test % v/v PFC inclusion
   3) Measure effective diffusivity of PFC/PDMS membranes

ii) *To utilize platforms in islet cell culture optimization*
   1) Measure oxygen consumption rate of islets to determine appropriate plating density
   2) Utilize finite element modeling to target physiological pO₂ in PDMS/PFC platforms and determine incubator settings.
3) Compare islet culture at physiological pO$_2$ on PDMS/PFC with standard culture conditions in standard plastic ware utilizing battery of potency assessments (GSIR, MTT, Counts, OCR, qRT-PCR)

4) Marginal mass transplantation into athymic nude mouse recipients to test differential effect on islet function of more physiological relative to standard culture.

**Rationale and hypothesis:**

Standard culture taking into account proper cell density, media depth and the oxygen consumption rate of the cultured cells still often results in hyperoxia induced free-radical damage, as the surface oxygenation is far above the physiological niche, or hypoxia/anoxia induced cell death and loss of insulin secretory function in the core regions where diffusive limitations prevail. Our working hypothesis is that *by maintaining physiological oxygen levels in the culture of adult islets of Langerhans and by delivering oxygen in a more physiological fashion, from both the apical and basal surfaces the in-vitro viability/function and ultimately, the in-vivo function of the same islets, transplanted, will be significantly enhanced*
Specific Aim 2:

To enhance conventional islet cell immunoisolation and encapsulated cell survival through the use of emulsified perfluorocarbons in polymer matrices.

Sub-aims

i) To characterize and optimize emulsions

1) Determine optimal perfluorocarbon(s) based on
   a) Effective oxygen diffusivity
   b) Volatility
2) Determine optimal surfactant(s) or combination based on
   a) Toxicity to cells
   b) Emulsion stability as gauged by particle size
3) Determine emulsion stability and optimize emulsion manufacture using
   a) Particle size analysis by DLS
   b) PFC inclusion by gravimetry
   c) Effective oxygen diffusivity through prepared emulsions

ii) To characterize/optimize PFC/Alginate gel as encapsulation material

1) Assess diffusivity of oxygen through PFC matrix relative to pure alginate
2) Assess diffusivity of glucose and proteins through PFC matrix relative to pure alginate

iii) Using alginates made from emulsions that meet specific criteria:

   assess effect of PFC matrix on cell viability/function in-vitro using

   1. Min-6 cells   2. Islets of Langerhans

1) Perform oxygen consumption measurements on cells (triplicate)
2) Use theoretical modeling to target maximal loading percentage in control configurations.

3) Mix cells with alginate (make control alginate with HBSS) and encapsulate cells at loading density based on theoretical modeling.

4) Assess daily cell growth/function/viability using a battery of metrics
   a) Glucose consumption rate
   b) Insulin production rate

5) At predetermined time points (d2, d7, d14 in Min 6 cells d2, d4, d7 in islets) perform a battery of potency assessments.
   a) MTS mitochondrial reduction (Min 6 and islets)
   b) Histological analysis and IHC (Min 6 and islets)
   c) Hypoxyprobe (islets)
   d) Glucose stimulated insulin release (islets).

**Rationale and hypothesis:**

Conventional immunoisolation techniques used with islets of Langerhans typically result in severe anoxia within the core regions of the tissue due to the increased diffusive distances and the sub-optimal oxygen transfer characteristics of the polymers utilized. Typically, mixtures of islets and low weight/volume percentages of alginate are extruded through parallel air-flow droplet generators into cationic gelating solutions, where they are formed into spherical islet-containing hydro-gels. The reduced oxygen transfer created by the semi-solid gels and the size of the capsules results in severe oxygen limitations that dictate minimal islet loading densities (volume/volume) of 1-2% within standard capsules. Loading above these densities results in pronounced loss of function.
and cell death. This presents a problem in translating micro-encapsulation into a viable clinical treatment option. The average capsule diameter is approximately 600-800 µm while the average islet cell diameter is 150 µm. A 1% loading density would translate to approximately 1-2 IEQ per capsule. Given that it takes approximately 10,000 IEQ per kg of recipient body weight to reverse diabetes in human clinical patients, approximately 350,000-700,000 capsules would need to be transplanted in an average 70 kg patient. This would result in a capsule volume of 62-125 mL necessary for the entire islet graft. Clearly, this would limit the potential sites of transplant and the number of times such a procedure could be performed without associated adverse effects. Due to the sub-optimal oxygen diffusivity in the polymer matrices currently employed in endocrine cell encapsulation and the sub-optimal oxygenation of the in vivo transplant sites, there is a substantial need for methods to improve oxygen supply and transport in immunoisolation devices, particularly in the immediate post-transplant period. Through these improvements, there is the possibility that tissue loading density could be improved to the point that a more suitable capsule volume (10-25 mL) could be utilized, thereby making the therapy more clinically relevant and allowing for the use of alternate transplant sites.

Our working hypothesis is that by improving the oxygen availability to encapsulated endocrine cells through the use of PFC impregnated polymers we can increase cell loading densities, viability and function in immuno-isolation devices.

1.3 Contents of This Dissertation

The overall objective of this dissertation is to investigate the role that proper oxygenation plays in the viability and function of islets of Langerhans, both during culture and when encapsulated in polymer matrices for immunoprotective purposes when
transplanted. A background of the field of islet isolation, transplantation and encapsulation as it related to the treatment of Type 1 Insulin Dependent Diabetes Mellitus is given in Chapter 2. Necessary for the evaluation of certain parameters of this study, namely oxygen carrying capacity of perfluorocarbon emulsions and islet potency as dictated by glucose stimulated insulin release, novel methods were developed for evaluation of both and are detailed in Chapters 3 and 4, respectively. In Chapter 5, the role of oxygen in islet culture is studied utilizing conventional culture compared to oxygen-tailored culture on perfluorocarbon/polydimethylsiloxane (PFC/PDMS) platforms.

Chapter 6 details the optimization of perfluorocarbon emulsions designed for incorporation in polymer matrices (sodium alginate) for utilization in cell transplant applications. Methods are implemented for evaluating oxygen transport (both diffusivity and solubility) as well as detailed investigation of emulsion components relating to toxicity and stability. In Chapter 7, optimized emulsions are tested in encapsulation configurations using cell lines (MIN-6, mouse insulinoma) and islets of Langerhans. The effect of the enhanced oxygen transfer is investigated and analyzed by means of potency assessments and functional assays. Histological morphological analysis is performed along with examination of tissue hypoxia.

Finally, in Chapter 8, the results are summarized and discussed in the context of application in the relevant field. Potential future research in the area is discussed with modifications that could enhance the studied strategies.
CHAPTER 2: BACKGROUND AND SIGNIFICANCE

2.1 Diabetes Mellitus and Transplantation

Diabetes Mellitus is a chronic affliction characterized by the body’s inability to maintain glucose homeostasis. This is due either to an autoimmune destruction of the beta cells in the Islets of Langerhans or to a decreased sensitivity to insulin induced by obesity or other metabolic defects. Currently, over 200 million people worldwide are afflicted with diabetes and the financial burden on the economies of developed countries is overwhelming, accounting for 9-15% of all healthcare expenses (Shapiro, Nanji et al. 2003; Nanji and Shapiro 2006). As of 2002, diabetes was classified as a condition of pandemic proportions by the CDC, as the third most common disease in the world, and the fourth leading cause of death in North America.

Of the several sub-classifications of diabetes, Type 1 autoimmune Insulin Dependent Diabetes Mellitus is generally considered the most severe and immediately debilitating form of the disease. Due to destruction of the beta cells of the pancreas by the patient’s own immune system, there is a lifelong requirement for exogenous insulin to maintain glucose homeostasis. The current standard practices in the treatment of Type 1 diabetes involve stringent regimens of intensive insulin therapy and careful monitoring of blood glycemia levels. While the past 20 years have seen substantial improvements in the treatment of the disease with the advent of insulin pumps, continuous glucose monitoring systems (CGMS) and a variety of novel insulin formulations with kinetic profiles that more closely mimic the insulin secretory kinetics of the native beta cells, there is still a great disparity between these treatments and the glycemic control provided by a properly functioning endocrine pancreas (Heinemann, Heise et al. 1993; Wiefels, Hubinger et al.
This is particularly evident in the variety of complications related to glycemic excursions that are more often the cause of mortality in diabetic patients, from a variety of angiopathies (retinopathy, nephropathy, neuropathy, coronary artery disease) to the more immediately life-threatening hypoglycemia induced insulin comas.

The current alternatives to daily insulin therapy are invasive surgical procedures. Given the relative success that intensive glycemic monitoring and multi-shot basal/bolus insulin therapy afford most patients, the associated potential complications and morbidity/mortality make these surgical procedures an option in only the most severe cases of Type 1 diabetes. In patients with end-stage renal failure brought about by secondary diabetic complications, for example, transplantation of the whole, vascularized pancreas is often coupled with a simultaneous kidney transplant to improve glycemic control in an attempt to preserve the newly transplanted kidney. Clearly, in this case, where immunosuppressive agents are already being administered to prevent rejection of the kidney, a concurrent transplant of the pancreas is justified.

Another rapidly emerging alternative is the use of endocrine cell transplantation therapies, more specifically, β-cell replacement therapy. Over the past thirty-five years, clinical islet cell transplantation has emerged as a viable therapeutic alternative to whole pancreas transplant for the treatment of the most severe cases of Type 1 Insulin
Dependent Diabetes Mellitus (Shapiro, Ryan et al. 2001; Inverardi, Kenyon et al. 2003; Shapiro, Nanji et al. 2003; Shapiro, Ricordi et al. 2003; Nanji and Shapiro 2004; Ricordi and Strom 2004; Ryan, Paty et al. 2004; Gaglia, Shapiro et al. 2005; Hatipoglu, Benedetti et al. 2005; Korsgren, Nilsson et al. 2005; Ricordi, Inverardi et al. 2005). Due to the non-invasive nature of the intra-portal infusion of isolated islet cells, where patients require only local anesthetic and the infusion is done by percutaneous catheterization of the liver, this intervention has advantages over the more dangerous solid organ transplant procedure. The less traumatic nature of this procedure also allows for application to a broader range of diabetic patients, not just those receiving simultaneous solid organ replacements. To date, however, most islet cell transplants have been limited to the most severe cases, such as those mentioned above, and to patients with hypoglycemic unawareness to such an extreme that it is debilitating to their daily existence and safety.

In its infancy, islet transplantation had limited periods of success, but overall results inferior to whole organ pancreatic transplant. This was in part due to the sub-optimal donor organ quality afforded to islet transplant centers, the cast-offs from solid organ transplant centers, but also was the direct result of diabetogenic immunosuppressive agents (glucocorticoids) utilized in the transplantation procedures. Recent advances in the techniques of islet cell processing and particularly in the development of less islet-toxic immunosuppressive regimens have led to improved clinical success in several centers around the world. As advocacy for the procedure has increased the organ quality offered to islet transplant centers has also improved. The recent long-term success rates have demonstrated the clinical feasibility of the non-invasive procedure and have brought islet transplantation from the research stage to the clinical
forefront with the formation of international islet transplant consortiums and cell-processing centers. Ultimately, a more pronounced clinical impact of curative endocrine cell transplant would require: 1.) a consistent cell source ample enough to match or surpass the growing number of diabetic patients: and 2.) a transition from the comprehensive immunosuppresion of the recipient to more experimental techniques such as immunoisolation (nano or micro scale) or the delivery of local cytoprotective/immunosuppressive agents.

2.2 Cell Preservation

Despite the impressive advances in clinical islet transplantation of the recent years, there still exist formidable obstacles to the procedure ever achieving clinical impact. A large part of this is due to a shortage of donor organs and the suboptimal culture conditions for isolated islets of Langerhans. Each year, between 6,000 and 7,000 donor pancreata become available and are offered to centers for either solid organ transplantation or islet cell processing. Of these approximately half are discarded for a variety of reasons ranging from donor serology to cold ischemia time. From the remaining 3,000 – 3,500 that are processed, approximately half result in insufficient viable tissue for successful transplantation. This translates to a 25% utilization rate of donor pancreata (Ricordi, Fraker et al. 2003). Currently, clinical islet cell transplantation requires a minimum of one and typically, two to three donor pancreata to obtain the necessary islet cell number to establish normoglycemia in diabetic recipients. This means that only 500-1000 patients, at best, undergo solid organ or islet transplantation worldwide per annum. Given that there are 30,000 new cases of type 1 diabetes diagnosed
annually in North America alone, the percentage of patients impacted by curative interventions remains less than 3%, not taking into account graft failure or rejection.

It is clear from these data that there is a large disparity between the native islet function and the suboptimal performance of a hepatic infusion of islets. Typically, the pancreas contains on the order of $1 \times 10^6$ islet cells with diabetes onset occurring when 95% of this functional mass has been destroyed. Most clinical islet patients are infused with 5-10,000 IEQ per kilogram of body weight, or approximately 10-15 times the amount of cells that should be required to reverse the hyperglycemic state. This directs to a clear need for improvement of post-isolation preservation of function and viability in isolated islets. Additionally, even if post-isolation culture interventions are successful at improving islet well being, there exists the further obstacle of maintaining this improved viability and function in the increasingly unfavorable environment of the immediate post-transplant period prior to engraftment and revascularization. **One of the most important elements critical to the maintenance of islet viability in the post-isolation period is the local oxygen concentration.**

### 2.3 The Avascular Islet: Islets and Hypoxia

Pancreatic islets are uniquely sensitive to local oxygen concentrations, both *in-vitro* and *in-vivo*. Comprising only 1-2% of the tissue volume in the whole pancreas, they utilize 10-15% of the oxygen supplied to the gland (Lifson, Kramlinger et al. 1980; Jansson 1994; Carlsson, Liss et al. 1998). Much of this can be attributed to the increased metabolic activity of islets, acting as the master regulator of glucose levels in the blood stream, thereby requiring sufficient oxygen for both continuous glucose monitoring and rapid compensatory insulin manufacture and secretion. Further, islets are not single cells,
but rather are aggregates of four distinct cell types and have on the order of 30 times the radial distance of other single cell types within the body (Cabrera, Berman et al. 2006). The *in vivo* niche of pancreatic islets is comprised of a rich capillary network, much like the glomeruli of the kidney, constantly maintaining the local pO$_2$ of the islet organoid, from surface to core, at 30-40 mmHg. When extracted from their native capillary network, the islets rely solely on diffusion for the delivery of needed oxygen. The surface to core radial distances of typically > 50 µm result in mass transfer limitations and lead to the formation of significant oxygen gradients across cultured islets. This limits the density of islet culture and results in decreased viability and function relative to islet size. This characteristic oxygen demand of pancreatic endocrine tissue, while easily handled in the native *in vivo* setting, becomes a formidable challenge in the static culture and re-implantation of islet cells isolated from the pancreas for cell therapies.

There has been extensive research into the oxygenation profiles of islets in their native environment, after isolation and additionally, transplantation (Carlsson, Palm et al. 2000; Carlsson, Palm et al. 2001; Carlsson and Mattsson 2002; Carlsson and Palm 2002). In standard culture, where the oxygen partial pressure is approximately 142 mmHg in a conventional 95% Room Air/ 5% CO$_2$ incubator, there is sufficient oxygen to maintain approximately 175-200 IEQ/cm$^2$ (uncontaminated by exocrine tissue) in a 175 cm$^2$ culture flask. At tissue loading densities above this level or in less pure preparations, as demonstrated by the theoretical modeling of Papas, Avgoustiniatos and Colton, islet equivalents begin to suffer anoxia at their core and can undergo apoptotic events leading to necrosis (Papas, Avgoustiniatos et al. 2005).
Additional studies have clearly demonstrated that hypoxia induced injury during the post-isolation culture period results in a decrease in islet secretory function and a pronounced increase in apoptotic mechanisms. A recent study by the group of Guiliani et al, demonstrated that central necrosis in cultured islets is dramatically affected by the external oxygen partial pressure and the islet size. When islets were cultured at an external pO$_2$ of 1%, equivalent to 8 mmHg, central necrosis ranged from 25% in 25-50 µm islets to 100% in islets 150 µm or larger (Giuliani, Moritz et al. 2005). A recent publication in Nature Medicine by F. Gribble showed a direct correlation between hypoxia and substantial down-regulation of glucose stimulated insulin release. Using a mouse knock-out model, she further demonstrated that glucose stimulated insulin secretion is primarily driven by pathways exquisitely sensitive to oxygen partial pressures and hypoxia (Gribble 2009).

When islets are transplanted into the liver, where the oxygen partial pressure drops to levels nearing 25-30 mmHg, the hypoxic conditions are only exacerbated, although blood flow around the islets does provide some convective alleviation of the static diffusion limitations. Typically, islets infused into the portal system lodge in capillary sinusoids of the liver and generate local emboli resulting in regions of anoxia, inflammatory response and subsequently, graft loss. This phenomenon, known as IBMIR, was described by O. Korsgren et al (Bennet, Groth et al. 2000; Johansson, Lukinius et al. 2005). It is the result of an incompatibility between recipient red blood cells and donor tissue. As the blood flow is stopped in these capillary regions, recipient platelet cells deposit on the surface of the islets and the clotting cascade continues, marking the islets for destruction. This can lead to early graft losses of up to 80-90%, making the liver a
less than ideal site for islet infusion. Korsgren and his group were able to alleviate this effect substantially by pre-treating the islets with a coating of Heparin (Cabric, Sanchez et al. 2007). A recent study by N. Sakata et al indicated that much of this IBMIR response is due to local regions of hypoxia generated in the liver tissue by the islet-induced flow blockage (Sakata, Hayes et al. 2009). This was a temporary phenomenon that peaked at 2 days post-transplantation, coinciding with a maximal non-regulated insulin release. At 28 days post-transplant, the regions of hypoxia were non-existent, indicating that the large islet loss from IBMIR opened up previously blocked blood flow. The implications of islet oxygenation are clear in that the severe diffusion gradients existing across isolated endocrine tissue can cause dramatic shifts in viability and function and, more importantly, can lead to dramatic loss in the early period post-isolation and during static culture.

2.4 Islets and Hyperoxia

There is little debate in the field of islet transplantation that culturing islets in hypoxic conditions is detrimental to their viability and metabolic function. There is, however, limited work addressing the effect of high oxygen culture on islet function and viability, and, for that matter, on the viability and function of other larger-sized cell clusters. Early work by Lacy et al. indicated that culturing islets in high oxygen concentrations resulted in improved engraftment upon transplantation, which was attributed to a loss of immunogenicity (Lacy, Finke et al. 1982). What was observed in this study was the aggregation of islets into larger tissue masses termed “mega islets”. These “mega islets” when transplanted under the kidney capsules of diabetic rats quickly restored normoglycemia and were free of rejection for longer periods than relevant islet
controls. The morphology and function of these islets in-vitro was comparable to control islets and therefore, there appeared to be no adverse effect of high oxygen levels on this tissue. Additionally, the oxygen levels used, 95%, were 5-fold that of conventional culture.

The group of Papas et al. recently furthered the assertion that high oxygen or improved oxygenation might result in better islet viability and function (Papas, Avgoustiniatos et al. 2005). In this study, conventional plastic culture flasks were replaced with flasks containing silicone membrane bottoms. Different culture densities and media volumes were utilized for comparison to conventional culture controls. It was clear from this study that improved oxygenation to islet tissue led to improved cell viability and function as assessed by cellular oxygen consumption rate and fluorescent dye exclusion (FDA/PI) staining for viability. It would seem that what may hold for single cell populations, where oxygen diffusion is not limited by cell size might not be true for larger cell clusters like islets, which depend heavily on oxygen and are oxygen diffusion limited at their core regions.

Recently, several groups have worked with post-transplant hyperbaric treatment of islet recipients with promising results. In 2003, the group of SJ Hughes et al followed intraportal infusion of 350, 500, 700 or 1000 syngeneic rat islets with environmental housing of the recipient animals at 20% (normal control), 11% (hypoxic) or 100% (hyperoxic) oxygen concentrations (Hughes, Yang et al. 2004). The results demonstrated significant improvements of marginal mass graft success in the recipients housed in hyperoxic conditions. In all groups, animals receiving 1000 IEQ grafts reverted to normoglycemia with no significant difference in reversal time. Differences in function
were first apparent in grafts of 700 IEQ, with no recipients reversing in the hypoxia group, 3 of 9 in the normoxia group and 8 of 9 in the hyperoxia group. In animals receiving 500 IEQ, no animals reverted in the normoxic group, while 5 of 8 animals reverted in the hyperoxic group. There was no reversal in any group in animals receiving 350 IEQ. Glucose tolerance studies on the recipients demonstrated that animals receiving 700 IEQ with hyperoxia treatment had equivalent tolerance to normoxic animals receiving 1000 IEQs. Strikingly, post-explant histological assessment demonstrated that hyperoxic animals receiving 700 IEQ had non-significantly different graft sizes to those receiving 1000 IEQ in the normoxic control group. This confirmed the expected early loss in the control group to compensate for the oxygen limitations.

The group of Juang JH et al also observed similar results in hyperbaric oxygen (HBO) treatments of marginal mass (150 IEQ) murine syngeneic islet transplants (Juang, Hsu et al. 2002). Controls in this study received no hyperbaric oxygen while the experimental groups were varied based on the timing of hyperbaric oxygen treatment at 2.4 atmospheres of 100% O₂. In all groups receiving hyperbaric oxygen treatment, blood glucose levels were lower and graft insulin content examined post-explant was higher. Results were comparable amongst groups with only one exception in a group receiving 6 day a week twice daily doses of HBO from day -14 to the final day of engraftment (day 28). In this group only, there was a significant difference in β-cell mass relative to all other groups. This study further demonstrated the importance of oxygen in early post-implant islet-graft survival.

Despite the observed benefits of hyperoxic treatment of islets, there are observations to the contrary, demonstrating that increased oxygenation results in
increased production of toxic free radicals and consequent islet β-cell dysfunction and destruction (Modak, Parab et al. 2009). Additionally, there is a substantial body of literature that describes the negative effect of hyperoxia on tissues throughout the body, suggesting that deviations from physiological oxygen levels are detrimental to function and viability (Kazzaz, Horowitz et al. 1999; Adeghate and Parvez 2000; Fessel, Porter et al. 2002). Granted, the majority of these studies are performed on explanted cells and tissues in static culture conditions.

Islets have been shown to be greatly affected by oxygen free radicals in their local environment, with β-cell function typically adversely affected by any increased presence (Rabinovitch, Suarez-Pinzon et al. 1996). The free-radical/free-radical scavenger balance is critical to the viability and function of β-cell and, given the low intra-islet content of constitutive cellular free-radical scavengers such as catalase, even slight deviations in the local concentration of oxidative species can dramatically affect the β-cell. In hypoxic conditions, free radical concentration increases due to local intra-islet cytokine production, starting the apoptotic signaling cascade. In hyperoxic conditions, free radical populations are increased by the oxidation of lipids and other oxygen species due to the change in local oxygen partial pressure. This change overwhelms the scavenging system of the islets and can result in pronounced cell death. This is dependent, however, on the exposure time to elevated oxygen levels and the capacity of the local ROS scavenging system. Two recent studies by Matsunami et al examined the effect of HBO treatment on the mechanism of diabetes development in chemically induced diabetic rat subjects (Matsunami, Sato et al. 2008; Matsunami, Sato et al. 2010). They observed a significantly higher blood glucose level in subjects whose chemical induction was coupled with HBO
treatment. Additionally, the measured β-cell mass in HBO treated subjects was significantly reduced compared to like induced control subjects not receiving HBO. This study supports the concept that islet damage or stress is only further exacerbated by the presence of high oxygen/oxidative radical concentrations. The increased oxygen in this environment merely provides a substrate for the formation of increased amounts of damaging free radicals.

What is clear from the body of work on both hypoxia and hyperoxia and its effects on cellular function and viability, particularly related to islets of Langerhans, is that deviations in oxygen concentrations from that within the physiological niche of given cell/tissue types can be detrimental rather than beneficial to cells/tissues. In static culture, this is particularly evident, as there is no scavenging system in place to balance free-radical production, unless added as a culture supplement. There is much literature demonstrating improved islet viability and function after culture in scavenger supplemented media. In the transplant setting, where the presence of increased free-radicals due to hyper-oxygenation may be balanced by the recipient’s free-radical scavenging mechanisms, the differences are less distinguishable. It is evident that the affects, whether beneficial or detrimental, are dose dependent and rely heavily on the state of the cells prior to implantation. Most importantly, in order for cell function and graft longevity to be maximized, there is a definite need for greater understanding of the role of oxygen and for improvements in the culture and post-transplant setting to protect cells and prevent inflammatory destructive responses until they have been adequately engrafted with sufficient nutrient and oxygen delivery. Rather than simply increasing oxygen concentrations, both in vitro and in vivo, enhancing oxygen mass transfer to
better meet the demands of cultured/transplanted cells might prove a superior method for increasing long-term viability, function and engraftment.

2.5 Improving Oxygen Transfer in Static Culture: Perfluorocarbons (PFCs) and Polydimethylsiloxane (PDMS)

Conventional culture is comprised of plating cells in plastic dishes or flasks with gas impermeable bottoms and placing them in a humidified incubator with 95% room air (142 mmHg oxygen, accounting for vapor pressure differences) and 5% CO₂. The cells are sustained in culture media optimized for their growth or maintenance at a depth that will provide them sufficient nutrition for several days, while preventing hypoxia induced from large diffusion distances through the medium layer. The sole source of oxygen in conventional culture is from the top surface of the medium layer, where oxygen diffuses down reaching the tissue at a transfer rate limited by the oxygen diffusivity through the medium, the distances of diffusion, and the rate of oxygen consumption of the cells. If the cellular rate of consumption exceeds the rate of diffusion from the top surface to the cells, anoxia will develop in the cellular core regions and necrosis may result.

Equally important, if the media layer is reduced to shorten the diffusive distance, the cells may suffer from lack of nutrients or dehydration, if medium evaporation occurs. Thus, the static culture environment is a critical balance between oxygen supply and nutrient supply. Despite modifications to improve the culture conditions, there will always exist steep gradients of gas and nutrient concentration across the cell, and this is more than likely the cause of the inability to duplicate characteristic in-vivo environment, in the in-vitro culture setting.
In single cell populations, diffusion of oxygen is not a limiting factor, assuming an appropriate cell density, as diffusive distances in these cells are short. Therefore, emulating in vivo oxygen levels in static culture is not difficult, as the diffusion across an entire 10 µm cell is rapid and results in a uniform surface to core concentration. By simply lowering the oxygen content in a conventional incubator, it would be possible to mimic the native oxygen levels of these cells, with gradients from apical to basal surface, although less pronounced, still present. Culturing such cells in high oxygen levels, on the other hand, has been observed to result in increased oxidative damage and a slowing of proliferative capabilities. This seems logical given the oxygen concentrations utilized in these studies, generally 50% or greater, and the non-limited diffusion of oxygen across these cells. The resultant intra-cellular oxygen concentrations would be upward of 10-20 fold higher than the in-vivo concentrations seen by these cell types (Chase, Ocrant et al. 1979; Powers, Millman et al. 2008; Ivanovic 2009).

In the case of islets, which are much larger than single cell populations, physiological oxygen concentrations are difficult to mimic in static culture. In standard culture, the core oxygen levels are generally below or near physiological levels, while the exterior of the cultured tissue is generally substantially higher. This results in a very narrow volume of tissue where the oxygen levels are near to physiological levels. It is conceivable that this has some margin of error (± 5-10 mmHg), but even with this hypothetical region of error, the band of physiologically normoxic tissue is a small percentage of the entire tissue mass.

Unlike single cell cultures, simply lowering the external oxygen partial pressure would not result in a more physiological static culture environment. Again dependent on
culture density (tissue proximity), medium height and oxygen consumption rate, the expected outcome of lower external partial pressures would be more resultant anoxia and central necrosis and physiological oxygen levels only at the surface region of the tissue. This has been documented in several studies, as mentioned above, with dramatic necrotic cell loss due to islet culture in sub-physiological oxygen concentrations (Giuliani, Moritz et al. 2005; Emamaullee, Shapiro et al. 2006; Miao, Ostrowski et al. 2006; Lehmann, Zuellig et al. 2007). These observations in pancreatic endocrine cells contradict the observations in single cell populations, such as tumor cells of the lung or some stem cell lines, where it has been demonstrated that hypoxia, in an acute fashion, can result in enhanced survival signaling and proliferative growth (Powers, Millman et al. 2008; Zhao, Zhang et al. 2008; Ivanovic 2009). Given the sensitivity of islet cells to deviations from physionormal oxygenation, it seems that methods for culturing these cells at partial pressures of oxygen that more closely approximate their physiological niche could improve post-isolation islet well-being. However, in order for this methodology to elicit a favorable effect, deviations from physiological oxygen levels, generated by solely changing incubator pO2, should be avoided. Rather, these methods should work to improve oxygen mass transfer to the cells in their culture/transplant environment.

One means by which oxygen mass transfer could be improved in both static culture and the transplant setting is through the utilization of compounds with higher oxygen diffusive permeabilities, such as perfluorocarbons and polydimethylsiloxane. Diffusive permeability is the product of the effective diffusivity and solubility of one compound through and in another, respectively. It is the determinant of the mass transfer rate across a given diffusive distance.
Perfluorohydrocarbons, or PFCs, are chemically inert compounds constructed of long carbon chains where all hydrogens bound to the carbon sites are replaced with fluorine atoms. This unique characteristic of these compounds gives them a substantial ability to bind and transfer molecular oxygen. Functional studies of the varied pure PFCs have shown that they have oxygen solubilities approximating or surpassing hemoglobin at different oxygen saturations and about 40-50 times that of water or culture medium (at \( pO_2 \) of 760 mmHg oxygen) (Faithfull 1992). Further, they have an oxygen diffusivity that is approximately 2.5-5 fold that of water or culture medium. In clinical trials, emulsions made with perfluorocarbon moieties have been successfully utilized as parenteral blood substitutes (Clark, Wesseler et al. 1975; Bollands and Lowe 1986; Lowe 1987; Barry, Geyer et al. 1988; Johnson, Greene et al. 1988; Lowe 1990; Lowe 2000). Additionally, biphasic solutions of dense pure perfluorocarbons and conventional UW preservation solution/culture medium have been used in the field of pancreas preservation and islet cell isolation with promising results, improving islet yields per pancreas and improving viability and function during islet culture (Hiraoka, Kuroda et al. 2002; Lakey, Tsujimura et al. 2002; Matsumoto and Kuroda 2002; Matsumoto, Rigley et al. 2002; Fujino, Kakinoki et al. 2003; Tanioka, Tanaka et al. 2005; Takahashi, Tanioka et al. 2006; Goto, Tanioka et al. 2007). The mechanism by which the pure PFCs enhance oxygen transfer has been detailed extensively in the literature with varied opinions (Lowe 1987; Faithfull 1992; Shah and Mehra 1996; Riess 2005). All studies confirm the enhanced oxygen solubility in pure perfluorocarbon moieties, but there is some debate over the effect of pure PFCs on oxygen diffusion, with environmental \( pO_2 \) apparently dictating the rate of diffusion in some studies and not a factor in other studies.
Enhancement of diffusive permeability by the combined effect of increased solubility and diffusivity is the most likely cause of the observed beneficial effect of pure PFCs on oxygen delivery.

Polydimethylsiloxane (PDMS) is a polymeric organosilicon. It generally comes as two highly viscous pourable liquid parts that when mixed in specific proportions and heated, cure to form flexible solids with properties inherent of their individual chemistries. One of the intrinsic properties of all polydimethylsiloxanes is their enhanced oxygen transfer relative to other similar compounds, the result of improved oxygen solubility and diffusivity (diffusive permeability). Published values of PDMS diffusive permeability are in the range 10-200 fold higher than that of culture media or other polymers/plastics used in cell culture (Refojo 1979; Christen and Andreou 2006). Additionally, PDMS is a bioinert compound generating minimal immune response and for years has been used as a negative standard for biocompatibility tests of novel biomaterials (Keough, Mackey et al. 1985; Ciapetti, Cenni et al. 1993; Ciapetti, Granchi et al. 1995; Jewrajka, Erdodi et al. 2008; Nishikawa, Kojima et al. 2008).

The oxygen transfer benefits of PDMS were first clinically utilized in the development of improved contact lens formulations in the late 1990s. Prior to that, contact lenses had been made of oxygen impermeable variations of polymethylmethacrylate and other, more oxygen permeable hydrogels. These formulations caused many adverse effects in the eye, primarily due to cell death resultant from low gas transfer. The introduction of silicone hydrogels all but eliminated many of the problems associated with hard lenses and other hydrogel formulations, due to a pronounced increase in oxygen permeability across the lens barrier.
Recently, the oxygen permeability of PDMS has been implemented in the development of cell culture devices, microfluidic systems for well-regulated maintenance of oxygen partial pressures, and biosensors dependent on oxygen substrate levels to maintain sensor linearity, such as electrochemical glucose sensors. In 2005, the group of Papas et al implemented the use of liquid impermeable PDMS membranes in islet cell culture. They demonstrated, both theoretically and empirically, that the oxygen transfer rate across these membranes was sufficient to maximize surface area coverage (4000 IEQ/cm\(^2\)) relative to conventional culture densities (175-200 IEQ/cm\(^2\)) with improved islet function relative to comparable controls (Papas, Avgoustiniatos et al. 2005). Our group applied a similar technology to the culture of murine embryonic pancreatic rudiments at the time of maximal β-cell specification, theorizing that proper physiological oxygenation would result in improved differential expression of insulin producing cells. The results confirmed this hypothesis, with rudiments cultured on PDMS/PFC membranes having enhanced proliferation and differentiation as exhibited by fold up-regulation of endocrine genes and comparable protein expression (Fraker, Alvarez et al. 2007). Taken together, these works demonstrate the importance of proper physiological oxygenation in maintaining cells in culture. By avoiding hypoxia and hyperoxia through the use of compounds to enhance oxygen mass transfer to cultured cells, it seems reasonable that cell survival and functional maintenance will be improved.

2.6 Improving Oxygen Transfer in Islet Encapsulation: Perfluorocarbons and Alginate Microcapsules

Based on the work of TMS Chang in 1964, Sun et al in 1980 initiated the microencapsulation of islets in alginate or alginate-polycation-alginate (APA) capsules.
Since then, the field has grown into a branch of research crossing over from academia into large-scale commercial ventures (Chang 1964; Lim and Sun 1980; Lim and Sun 1981; O'Shea, Goosen et al. 1984; Campos-Lisboa, Mares-Guia et al. 2008). Over the course of greater than 25 years of experimentation in islet encapsulation techniques, researchers have strived to develop coatings that exclude both the cellular and humoral immune responses to encapsulated cells and have a nominal pore size to allow for rapid inward diffusion of metabolites, such as oxygen and glucose, and outward diffusion of insulin and waste products. Sun’s early work was the foundation for all subsequent work, with variations typically only in polymer type, concentrations, and methods of gelation/chelation. Researchers have experimented with polymers including agarose, carboxymethylcellulose, polyamides, polyacrylates, living cells, polyethylene glycol (PEG), PVA and others (Sugamori and Sefton 1989; Jain, Yang et al. 1995; Cruise, Hegre et al. 1998; Cruise, Hegre et al. 1999; Schneider, Feilen et al. 2001; Teramura, Kaneda et al. 2007; Teramura and Iwata 2009; Teramura and Iwata 2010). Despite the expansion into varied materials, alginate has remained the most utilized in microencapsulation due to demonstrated long-term graft survival in small animal models and ease of application.

Much of the early work in APA multilayer encapsulation (alginate-polycation-alginate) was hindered by a lack of biocompatible alginites. Early studies into the biocompatibility of varying alginate compositions were confounding, with some groups arguing that alginites high in mannuronic content were not biocompatible while others asserted the same for those with high guluronic content (Klock, Frank et al. 1994; De Vos, De Haan et al. 1996; Klock, Pfeffermann et al. 1997; Prokop and Wang 1997). A
clear conclusion of these studies was that capsules manufactured from alginate high in 
guluronic acid content were significantly more stable than those prepared with high 
mannuronic acid content. A seminal study by Paul DeVos et al in 1997 advanced the idea 
that biocompatibility might not be so much related to alginate composition as to the 
purity of the alginate (De Vos, De Haan et al. 1997). Contaminants in commercial 
alginites, such as endotoxin, caused pronounced inflammatory responses in 
biocompatibility studies regardless of alginate composition. DeVos and his group 
developed a method of purification including endotoxin removal and multi-step filtration 
processes. In additional studies, side by side transplants were performed into AO rat 
recipients using unpurified controls and purified alginate capsules. Explants of capsules 
were performed at up to 12 months after implantation. Typically, the unpurified alginate 
capsules, regardless of composition, exhibited a 100% overgrowth by fibrotic tissue 
capsules and cellular aggregates within one month post implantation. Conversely, of the 
capsules manufactured from purified alginates, only 10% generated any fibrotic response 
after 12 months of implantation (De Vos, De Haan et al. 1997).

Further comprehensive studies by DeVos et al investigated the differences in 
biocompatibility due to the mannuronic and guluronic composition of alginates. They 
postulated that the primary factor in this biocompatibility difference had nothing to do 
with alginate composition, but rather was the direct result of differing viscosities. Their 
assertion was that equivalent viscosities equate to equivalent capsule pore sizes, so any 
biocompatibility differences will be removed if pore-size exclusion and therefore, 
viscosity, is equivalent. The majority of the biocompatibility studies performed by this
group, however, were with capsules with outer polycationic layers (van Schilfgaarde and de Vos 1999).

Although not the first to recognize the inflammatory response to polycation layers, in 2001, the group of Weir et al demonstrated that removal of this layer could result in extended graft success (Duvivier-Kali, Omer et al. 2001). They also noted that immune response was increased to capsules with a high-G content as opposed to high-M (Orive, Tam et al. 2006). Additionally, they found that the use of BaCl$_2$ as a gelating solution provoked a reduced immune response when compared to those made with CaCl$_2$. This was later attributed to the improved capsule strength imparted by barium relative to the smaller calcium. Transplant of syngeneic and allogeneic murine islets housed in high-M alginate only capsules resulted in extended reversal of diabetes (> 350 days) in immune competent diabetic recipients (both chemically induced and autoimmune models). This clearly indicated that despite the presumed benefits of capsule stability in the APA layering, long-term structural stability and function of encapsulated islets is possible in a simple alginate capsule ionically gelated with barium chloride. This was confirmed with additional studies published by DeVos et al in 2006 and others, where their results indicated that inflammatory response was due entirely to the particular polycationic layer used to form the outer coatings of the alginate capsules and not at all due to the alginate composition utilized (King, Strand et al. 2003; De Castro, Orive et al. 2005; de Vos, Faas et al. 2006; Li, Jiang et al. 2006; Ponce, Orive et al. 2006). The recent manufacture of clinical grade ultrapure alginates and the move away from multi-layer APA capsules to alginate capsules alone has resulted in substantially improved outcomes in transplant models.
2.6.1 Encapsulation and Mass Transfer Resistances

Despite promising results in small animal and some large animal models, microencapsulation has seen limited success in the clinical realm. One reason for this is the mass transfer resistances of oxygen and other nutrients due to long, suboptimal diffusive distances within the capsules the result of the polymer matrix and the limitations of the parallel flow droplet generation. There is a necessary balance between capsule size and islet size, requiring that capsules be large enough to prevent islet protrusions out of the capsule, known as “knees and elbows”, and small enough to allow for proper metabolic supply. Based on theoretical modeling observations by several groups, this limits the loading of microcapsules to a range of 2-4\% volume to volume density. Loading densities greater than this generally result in pronounced anoxia and metabolic decline, indicated by impaired insulin secretory response to glucose (Dionne, Colton et al. 1993; Avgoustiniatos and Colton 1997; Wu, Avgoustiniatos et al. 1999; Papas, Avgoustiniatos et al. 2005). Additionally, encapsulation devices should not exceed 1 mm in thickness with spheres being the optimal geometry for suitable mass transfer given their superior surface area to volume ratio. The clinical efficacy of microcapsules is limited given these critical requirements, as the typical volume of islets needed to reverse diabetes (1-2 mL) in a human patient would require 37-100 mL of polymer volume to maintain the proper loading density. Additionally, as the typical infusion site for encapsulated islets is limited to regions such as the intraperitoneal cavity and cannot be performed safely in the vasculature of the liver due to the size of the capsules (500-1000 µm), explant recovery of capsules becomes a daunting task. There is only one claimed success in clinical islet encapsulation transplant from Soon-Shiong et al in 1994
and most trials since then, in large animals or humans, have been unable to reproduce these results (Soon-Shiong, Heintz et al. 1994; Calafiore, Basta et al. 2006). Recent results from Living Cell Technologies’ clinical trials in Sydney, Australia have claimed reversal in one clinical patient infused with encapsulated porcine islets, but this occurred only after three infusions totaling 18,000 IEQ per kilogram, nearly four-fold the dose required for unencapsulated islets. Assuming a 70 kg recipient, this would translate to a total dose of 1.25 million islets and approximately 125 mL capsule volume (Elliot 2007). This study exemplifies why many in the field fear that the inability to retrieve capsules would lead to increased sensitization to any necessary future transplants and that recipients would become a virtual waste dump of failed capsule grafts. **There is a real need, therefore, for interventions to improve mass transfer limitations in polymer matrices used for islet cell immunoprotection to reduce the transplant volume, aid in ease of retrievability and improve islet viability/function so fewer cells can be used to achieve clinically significant results.**

Perfluorocarbon emulsions, like pure perfluorocarbons, have been investigated extensively for their oxygen transfer characteristics (Navari, Rosenblum et al. 1977; Biro 1993; Shah and Mehra 1996). The methods and results of the numerous studies have differed greatly, some groups finding increased oxygen diffusivities and solubilities in perfluorocarbon emulsions and others finding outcomes in disagreement with Boyle’s law, claiming solubilities and diffusivities that vary non-linearly with environmental oxygen partial pressure. There is among this body of literature a consensus agreement that oxygen transfer is enhanced by the presence of perfluorocarbon moieties, yet all the studies pay little attention to characterization of the emulsions and to the role that the
individual components of the emulsion might have on oxygen transfer characteristics. Most groups have assessed emulsion stability and particle size by filtration through membranes of known pore diameter, conventional microscopy and earlier generation spectrophotometric techniques, perhaps underestimating their stability and micellar size. Recent advancements in the technology of dynamic light scattering (DLS) have made it increasingly robust and the method considered most accurate for assessing sub-micron particle sizes. Our recent data utilizing DLS indicates that the particle size is one critical factor in oxygen diffusivity through PFC emulsions and that oxygen transfer hinges largely on the interfacial surface area of micellar particles within the emulsion (Chapter 6). Greater particle size per unit volume reduces interfacial surface area at a rate that corresponds to the square of the particle radius. As this particle size increases, the diffusivity concurrently decreases. It is likely that some of the variations in mass transfer characteristics reported in the literature are due to a lack of accurate emulsion characterization at the time of diffusivity and solubility measurements.

Temporal emulsion stability is critical as changes in particle size result in loss of oxygen mass transfer capabilities. In parenteral emulsions used in place of whole blood for transfusion, this change in diffusive characteristics is not critical, as most are continually saturated with high oxygen pO₂ often approaching 1 atm. However, in microencapsulation for in-vivo applications where oxygen partial pressures are low (20-40 mmHg), mass transfer characteristics and particle stability become extremely important. For use in microencapsulation, a desirable emulsion should have maximal oxygen transfer characteristics and optimal stability not affecting polymer matrix strength/stability.
To date, four groups have implemented perfluorocarbon moieties within encapsulation matrices to enhance cell function and viability. Our group performed work (Inverardi 1999; Khattak, Bhatia et al. 2005; Khattak, Spatara et al. 2006; Khattak, Chin et al. 2007; Chin, Khattak et al. 2008) with encapsulated islets of Langerhans by simply sonicating pure PFCs at a given volume to volume percent into high weight percent solutions of alginate (4-8%). We found that compared to relevant controls, islets fared much better in these solutions, as assessed by static glucose stimulated insulin release, immunohistological analysis, and transplantation into immune deficient chemically induced and auto-immune diabetic murine models (Inverardi 1999). It should be noted that the alginates used in these studies were prior to the purification protocols utilized now by commercial entities to manufacture ultra-pure alginates. Additionally, the weight percent solutions utilized were necessarily higher to entrap the perfluorocarbon. No attempt was made to fully characterize these solutions, other than by conventional light microscopy. Furthermore, there was noticeable phase separation of the PFCs from the aqueous solutions, if they were not utilized within several hours.

The group of Khattak et al has published recently on the use of perfluorocarbon emulsions in alginate matrices to enhance cell viability in microencapsulated HepG2 cells (Khattak, Chin et al. 2007). Initially, this group examined various pluronic surfactants typically utilized in PFC emulsion manufacture and reported that the optimal surfactant appeared to be Pluronic F68 at concentrations less than 2% w/v, although the toxicity studies used concentrations approaching 10% w/v before toxicity was observed. Pluronic F127, another surfactant commonly utilized, was observed to be highly toxic and was excluded from further utilization (Khattak, Bhatia et al. 2005). The toxicity studies of
Khattak’s group were followed by emulsion manufacture utilizing 10% w/v (5%v/v) perfluorooctylbromide with a 1% w/v F68 surfactant concentration. These emulsions were utilized to manufacture alginate microcapsules containing HEPG2 cells and were compared in static culture at 20% and physiologically relevant 5% O₂ concentrations. Emulsion stability was examined by conventional light microscopy and histological capsule examination, only. Emulsion particle size was reported as 1-2µm in diameter. Both cellular proliferation rate by MTT and metabolic activity by glucose consumption and lactate production were examined and an enhancement of both by perfluorocarbon incorporation was reported. This was observed in both standard and physiological oxygen conditions. The effect, however, did not match theoretical predictions of diffusive permeability enhancement but did correlate with a model developed by the group.

Clearly, there is a potential incremental benefit in oxygen mass transfer through the utilization of perfluorocarbon moieties in both static culture and encapsulation methodologies. In the use of emulsions in microencapsulation, the effect is highly dependent on emulsion stability and particle size characterization. This, in turn, is highly dependent on the perfluorocarbon utilized, as emulsion stability relies on the characteristics of emulsion “coalescence” dictated by PFC molecular weight. This work will examine all of these factors in emulsion optimization and then translate this to working in-vitro and in-vivo cell encapsulation matrices. It is expected that optimization will improve cell culture and encapsulation but that further modification will be necessary when biological applications are investigated.
CHAPTER 3- DISSOLVED OXYGEN CONTENT IN PFC EMULSIONS

3.1 Perfluorohydrocarbons as Oxygen Carriers

Perfluorohydrocarbons (PFCs) are chemically inert compounds constructed of long fluorine-saturated carbon chains with varied terminal groups. Due to this structural characteristic, PFCs have enhanced oxygen dissolving and transfer capabilities, which have been implemented in numerous applications in the clinical and research arenas. For the last half of a century, substantial research has investigated the use of pure perfluorocarbons and micellar PFC suspensions (emulsions) as oxygen carriers (Clark, Wesseler et al. 1975; Hall 1975; Rosenblum 1975; Kostrzewska 1976; Novakova 1976; Doss, Kaufman et al. 1977; Clark 1978; Riess and Le Blanc 1978; Bowman 1983; Riess 1984; Clark 1985; Gould, Sehgal et al. 1985; Waxman 1986; Biro and Blais 1987; Lowe 1987; Faithfull 1992; Spahn 1999; Lowe 2001; Riess 2006). Functional studies of various PFCs have found their oxygen solubilities to approximate or surpass hemoglobin, at 12-20 times that of water or physiological salt solutions at varied oxygen partial pressures (Navari, Rosenblum et al. 1977; Clark 1985; Biro and Blais 1987; Lowe 1987; Faithfull 1992; Biro 1993; Shah and Mehra 1996; Patel and Mehra 1998; Kaisers, Kelly et al. 2003). Additionally, it has been demonstrated that the dissolved oxygen content in some perfluorocarbons does not vary substantially with temperature (Dias 2004). For this reason, the oxygen solubility ratio of PFCs to physiological solutions at higher temperature is further increased, due to the concomitant decrease in oxygen solubility in physiological solutions. In addition to increased oxygen solubility, the effective oxygen diffusivities of PFCs are approximately 2.5-4 fold that of water or culture medium. These highly desirable properties have been shown to enhance the oxygen transfer rate (i.e.
diffusive permeability) in several clinical and bench-top studies, where they have been utilized in oxygen delivery capacities, both as parenteral blood substitutes and in organ preservation solutions. (Navari, Rosenblum et al. 1977; O'Brien, Langlais et al. 1982; Urushihara, Sumimoto et al. 1992; Holman, McGiffin et al. 1994; Johnson, Erickson et al. 1995)

The most common implementation of perfluorocarbons to enhance oxygen transfer is in the form of nano-scale emulsions/micellar suspensions. Given their hydrophobicity, PFCs are typically suspended in aqueous solutions of amphiphilic surfactants where high pressure microfluidization is used to generate stable, micro or nano scale emulsions. Given this requirement and their conventional use as intravenous oxygen carriers in medical procedures requiring transfusion, it is critical that they are manufactured in a size range that does not have the potential for causing thrombotic events in patients. Additionally, a further requirement is emulsion stability as change in particle size could adversely affect patient safety, as well as emulsion efficacy. Typical formulations of PFC emulsions are comprised of a w/v% of lipid-based (egg-yolk phospholipids, cholesterol) or PEG-based (Pluronics) surfactants, a w/v% of PFC and the remainder, a physiological salt-based aqueous phase. While substantial investigation into the individual properties of emulsion components has been conducted, there has been little published characterization of manufactured emulsions in regards to oxygen transfer capacities. Much of the literature uses theoretical estimations based on measurements in pure perfluorocarbons. It is our belief that this may be erroneous. Therefore, there is a need to fully characterize emulsion parameters including dissolved oxygen capacity, diffusivity and stability, particularly for use in clinical applications.
One potential application of perfluorocarbons, more importantly perfluorocarbon emulsions, is their use as oxygen carriers in biomaterials utilized for cellular encapsulation, particularly alginate. This application has long been explored by our group and has recently seen renewed interest by others (Fraker 2003; Khattak, Chin et al. 2007). In PFC emulsions, the benefit derived from the inclusion of oxygen carrying emulsions is the product of the increased effective oxygen diffusivity, $D_{\text{eff}}$, and inherent oxygen solubility, $S_{O_2}$, known as diffusive permeability, $P_D$. Critical to the characterization of the benefit afforded by a particular PFC emulsion is the accurate determination of both of these variables. Historically, solubility has been demonstrated in the literature to have the most substantial effect on the diffusive permeability of PFC emulsions, as the oxygen solubility of pure PFCs is an order of magnitude greater than physiological buffers (13-20 fold) (Navari, Rosenblum et al. 1977; O'Brien, Langlais et al. 1982; Clark 1985; Rosen, Sehgal et al. 1985; Biro and Blais 1987; Lowe 1987; Dias 2004). Therefore, accurate determination of the dissolved oxygen content and further the fold dissolved oxygen in PFC emulsions, which are comprised of hydrophilic and hydrophobic phases and varying PFC concentrations, is critical in determining their theoretical oxygen transfer benefit.

Several methods, both theoretical and practical, have been utilized in the measurement of dissolved oxygen in pure PFC solutions and in PFC emulsions. Most of these methods, however, require specialized laboratory equipment and cumbersome techniques that are not easily performed and are prohibitive in assessing accurate information about dissolved oxygen content in PFCs and PFC emulsions. For example, the early method of Wessler et al, where gas chromatography measurements were utilized to assess oxygen solubility in 25 pure perfluorocarbons, required numerous columns, a
molecular sieve, pressure lock injectors, sealed gassing chambers and high temperature incubation environments (70-300 °C). Herein, we present two novel and complementary methods to accurately determine dissolved oxygen content within solutions, particularly PFC emulsions, by employing a stirred oxygen micro-chamber typically utilized for oxygen consumption measurements coupled with a modification of the Trinder reaction.

The Trinder reaction is a well-characterized method originally developed for the determination of glucose concentration within plasma or other solutions (Trinder 1969). It is a two-step oxidation reduction reaction that first converts glucose and oxygen into gluconic acid and hydrogen peroxide in the presence of glucose oxidase. Subsequently, in the presence of peroxidase, a mixture of colorimetric dyes (3,5-dichloro-2-hydroxybenzoic acid (DCHBA) and 4-amino antipyrine (4AAP)) is reduced to form a visible color with a measurable optical density at 510 nm. The optical density is directly proportional to peroxide concentration and, thereby, to oxygen and glucose concentration.

In this work, we measured peroxide/dissolved oxygen concentrations in solution within a sealed, stirred oxygen micro-chamber and compared these results against specific variables obtained from oxygen consumption trace measurements. A strong correlation was observed between the fold zero order reaction rate (slope) of the oxygen consumption (PFC emulsions/control solutions) and the fold of the oxygen concentrations determined by spectrophotometric measurements of the Trinder reaction against a standard curve of known peroxide concentrations. The methods utilized in this study are technically less demanding than conventional methods for solubility measurements. Other groups have used more technically demanding permutations of this reaction for
oxygen determination in non-aqueous solutions and emulsions in the past, but measuring the consumption of glucose as opposed to oxygen (Ghosh 1970; Freire 2005)

3.2 Materials and Methods

3.2.1 Emulsion Manufacture

For the purpose of comparison with numerous literature values and its extensive characterization, Perfluorodecalin (Fluoromed LLC) was selected for testing within emulsions. Pluronic polaxamer copolymers (F-68, F-127 BASF corp) were dissolved at 2% w/v in Hank’s Balanced Salt Solution, HBSS, (Mediatech/Cellgro). A volume percent of this solution was continuously circulated through a model M 110-Y high pressure pneumatic microfluidizer processor (Microfluidics) at 5000 PSI. The entire solution was cooled through an ice-water slurry to 4-8 °C. In a step-wise fashion, a volume percent of a pure perfluorodecalin was added to obtain the desired emulsion concentration. The mixture was emulsified into a uniform micellar suspension for 8 minutes and then collected in a 250 cc conical tube. The solution was then pH adjusted to 7.35 and sterile filtered through a 0.2 µm filter.

3.2.2 Emulsion Characterization: PFC Quantification by Gravimetric Determination

Emulsion PFC content was quantified by gravimetric determination. performed by weighing 1 mL of each perfluorocarbon emulsion and comparing the observed weight to the a standard curve generated from serial 1 mL mixtures of the pure perfluorocarbon and base solution at 0, 5, 10, 20, 40 and 100% v/v.
3.2.3 Solubility Determination: Trinder Reaction in sealed oxygen micro-chamber

Stock solutions (20X) containing both 10.5 U/mL glucose oxidase (Sigma Aldrich) and 5 U/mL peroxidase (Sigma Aldrich) were prepared in dPBS w/o Ca\textsuperscript{2+}/Mg\textsuperscript{2+} (Mediatech/Cellgro). Trinder reagent dyes, 3,5-Dichloro-2-Hydroxy-Benzene sulfonic acid and 4-aminoantipyrine were solubilized in HBSS Base Solutions at concentrations of 80 mg/mL and 20 mg/mL, respectively (20X stock).

Standard curves of peroxide were prepared using serial dilutions in dPBS and a 1 mM peroxide stock. The peroxide concentration was determined using the Beer-Lambert law at 240 nm (\(\alpha = \varepsilon bc\)) and previously reported extinction coefficient of 43.6 M\textsuperscript{-1} cm\textsuperscript{-1} (Noble 1970). For each standard, 1 part of each dye was added per 18 parts peroxide standard into a 1.5 mL Eppendorf tube. The tubes were sealed and suspended in a 37 °C water bath. The reaction was allowed to continue for 10 minutes. At the end of the ten minute period, the reaction was stopped by addition of 10 \(\mu\)L of 1M NaOH per 200 \(\mu\)L of solution. The tubes were vigorously mixed on a vortex for 10 seconds and then 100 \(\mu\)L samples were read in 96 well plate at 450 nm and 620 nm. For a blank, dPBS without peroxide was utilized.

Solutions for solubility determination were prepared by adding 1 part of each dye per 18 parts of either control (HBSS + w% surfactant base solutions) or PFC emulsion. For determination of oxygen content, custom designed, stirred, thermo-regulated oxygen-sensing titanium micro-chambers (6.4 mm ID, ~200 \(\mu\)L sealed chamber liquid volume: Instech Laboratories, Plymouth Meeting, PA.) were utilized. The custom systems were designed with a ruthenium sol-gel based life-time fluorescence spot sensor (PreSens GMBH, Regensburg, Germany) housed in the chamber wall. Measurements were
performed at either 37 °C or 25 °C, controlled via a circulating water bath (Haake). For each measurement, 300 µL of solution was added to the chamber. The chamber was capped with an acrylic plug containing a narrow injection port at the top. After capping, the final internal chamber volume was approximately 200 µL. The solution was allowed to equilibrate with the internal chamber temperature and the oxygen partial pressure was monitored/recorded every two seconds to insure signal stability. At equilibration, 10 µL of the glucose oxidase/peroxidase enzyme solution was added to the chamber and the injection port immediately sealed with a custom acrylic plug. The chamber oxygen partial pressure was recorded every two seconds to monitor the reaction and for an additional ten minutes after reaching zero to insure reaction completion. The sample was then collected into a tube containing 10 µL of 1M NaOH, vigorously mixed, and centrifuged at 20,000 rpm for 30 seconds in a microcentrifuge, to phase separate the hydrophobic emulsion components from the liquid volume. Controls were also centrifuged to maintain consistent sample handling. Absorbance of the reaction solutions was measured at 450 nm and 620 nm and the transfer (abs450 - abs620) recorded. Dissolved oxygen content was determined by comparing against the standard curve. Additionally, the fold increase/decrease in dissolved oxygen relative to the base solution control was determined by taking the ratios of measured oxygen concentrations.

The oxygen traces recorded from the sensing unit were graphed and analyzed. Parameters of particular interest were: the time to reach zero oxygen (TO2), the area under the curve (AUC), and the slope of the zero order reaction rate (linear portion of the oxygen depletion) from 140 mmHg to 40 mmHg (mO2) Control solution and emulsion data were compared relative to the collected concentration data. The fold (PFC/control)
of these results was compared to the fold (PFC/control) of the measured oxygen concentration by means of the Trinder reaction.

3.2.4 Solubility Determination: Characteristic PFC curves

For measurements performed at 37 °C, perfluorodecalin emulsions of 10%, 5% and 2.5% v/v were prepared (n = 3) and the PFC concentration determined by gravimetric determination as outlined above. For measurements made at 25 °C, a single v/v % emulsion was manufactured and perfluorodecalin content determined by gravimetry. Serial dilutions of this emulsion were made at 1:2 and 1:4 with emulsion base solution. Solubility measurements were made for all emulsion solutions and the blank to generate characteristic dissolved oxygen curves at 37 °C and 25 °C. From extrapolation of the equation of these curves, the theoretical fold oxygen solubilities of the pure PFCs were determined by addition of the slope and the intercept and compared to theoretical values derived by the equation below detailed by A. L. Rosen et al (Rosen, Sehgal et al. 1985):

\[
[O_2]_{\text{emulsion}} = \{((\alpha_{\text{perc}} \cdot \%_{\text{perc}}) + (\alpha_{\text{H}_2\text{O}} \cdot (1 - \%_{\text{perc}})))) \cdot pO_2\} \quad (3-1)
\]

Here, \( \alpha \), represents the Bunsen solubility coefficient.

3.3 Results

3.3.1 Emulsion Characterization: PFC Quantification by Gravimetric Determination

Gravimetric determination of perfluorodecalin content in each emulsion was performed to insure accuracy of solubility measurements and calculations when comparing against published literature values. Additionally, these values were utilized to determine loss, if any, during the emulsification process. The emulsions utilized for
measurements at both 37 °C and 25 °C all had a measured perfluorodecalin content lower than the expected values (9.05% ± 0.035% and 6.81% ± 0.042%, respectively for 10% theoretical concentration). This is more than likely due to loss during initial priming and residual left in the system after collection. Figure 3-1, following, displays the standard curves utilized for v/v% Perfluorodecalin determination, from the measured 1mL weight of manufactured mixtures and from the theoretical values based on the published density of the pure perfluorocarbon, 1.93 (Fluoromed, L.P., product specifications http://fluoromed.com/ProductsMain.htm ), utilizing the following equation:

\[
\rho_{\text{mixture}} = \%_{\text{base}} \cdot \rho_{\text{base}} + \%_{\text{PFC}} \cdot \rho_{\text{PFC}}
\]  

(3-2)

The two lines are statistically identical (p = 0.95) with the largest deviation, 0.004 g/mL, occurring in the 20% PFC mixture. This result establishes the validity of the gravimetric method in accurately determining the emulsion PFC content.

![Figure 3-1: Gravimetric calibration curve for Perfluorodecalin. Closed diamonds represent theoretical density values based on formula; open squares represent measured values.](image)
3.3.2 Solubility Determination: Enzymatic Methods

In the presence of glucose oxidase, β-D-Glucose reacts with oxygen and water to form D-gluconic acid and hydrogen peroxide. This peroxide formation can be readily quantified in the presence of peroxidase and electron accepting colorimetric dye complexes. This reaction complex is detailed below:

\[
\text{glucose} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{glucose oxidase}} \text{D - gluconic acid} + \text{H}_2\text{O}_2
\]

(3-3)

\[
2\text{H}_2\text{O}_2 + 4\text{AAP} + 3,5\text{-DCHBA} \xrightarrow{\text{peroxidase}} \text{dye complex} + \text{H}_2\text{O}
\]

Standard curves \((n = 5)\) prepared from the serial dilutions of hydrogen peroxide were consistently linear and the inter-assay standard curves were nearly identical with less than a 4% coefficient of variation in all standard concentrations (data not shown). Standard curves were utilized to determine the concentration of peroxide, and indirectly, the concentration of oxygen in each measured solution volume.

Figure 3-2, following, shows average oxygen traces \((n = 3)\) from control solutions (HBSS base solutions), \(\frac{1}{2}\) perfluorodecalin emulsion, and full perfluorodecalin emulsion measured following the addition of glucose oxidase/peroxidase enzyme solution. Consistently, the curves were reproducible and significantly different at both 25 °C and at 37 °C. Three metrics were analyzed: \(\text{TO}_2\), time to zero oxygen; AUC, the integral of the oxygen trace (initial read to zero oxygen); and slope, \(\text{mO}_2\), of the oxygen trace from thermal equilibrium (140 mmHg) to the apparent Michaelis-Menten constant, \(K_m\), of the oxygen depleting enzyme reaction were analyzed. The fold increase of these parameters relative to control solutions was compared to the theoretical and measured fold increase in concentration, by Trinder determination.
Comparison of these variables associated with oxygen traces (TO₂, AUC and mO₂) to the measured and theoretical fold concentration indicated that the best metric of fold-oxygen solubility was mO₂. The theoretical fold concentrations were derived in the following fashion, using the values published in prior works detailed in Table 3-1. First, the solubility of the emulsion (M) is calculated based on the formula, below,

\[ SO_2^{Emulsion} = \%PFC \times SO_2^{PFC} + \%base \times SO_2^{base} \]  

(3-4)

Next, the fold of the control solubility was calculated by taking the ratio of the calculated emulsion value over the solubility value for the aqueous base solution.
Table 3-1: Reported and measured (from this study) oxygen solubilities in perfluorodecalin and perfluorodecalin emulsions.

<table>
<thead>
<tr>
<th>SOURCE</th>
<th>Solution</th>
<th>T (°C)</th>
<th>FOLD Control 100% O2</th>
<th>FOLD Control Room Air</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Saline</td>
<td>Pure PFD</td>
<td>25</td>
<td>13.24</td>
<td>13.29-16.57</td>
</tr>
<tr>
<td>Normal Saline</td>
<td>Pure PFD</td>
<td>37</td>
<td>16.29</td>
<td>16.28-20.3</td>
</tr>
<tr>
<td>Reiss</td>
<td>Pure PFD</td>
<td>25</td>
<td>16.53</td>
<td>16.53-20.6</td>
</tr>
<tr>
<td>Kaisers</td>
<td>Pure PFD</td>
<td>25</td>
<td>16.53</td>
<td>16.53-20.6</td>
</tr>
<tr>
<td>Wessler, Clark</td>
<td>Pure PFD</td>
<td>25</td>
<td>13.4</td>
<td>13.39-16.68</td>
</tr>
<tr>
<td>Measured Data (Conc.)</td>
<td>PFD Emulsions</td>
<td>37</td>
<td>17.59</td>
<td></td>
</tr>
<tr>
<td>Measured Data (Slope)</td>
<td>PFD Emulsions</td>
<td>37</td>
<td>18.35</td>
<td></td>
</tr>
<tr>
<td>Measured Data (Conc.)</td>
<td>PFD Emulsions</td>
<td>25</td>
<td>14.41</td>
<td></td>
</tr>
<tr>
<td>Measured Data (Slope)</td>
<td>PFD Emulsions</td>
<td>25</td>
<td>13.53</td>
<td></td>
</tr>
</tbody>
</table>

Clearly, from the data collected, our measured values, both slope and concentration determined by the Trinder reaction, fall within the range of published literature values. This indicates that both methods are robust for determining dissolved oxygen content in PFC emulsions and other aqueous solutions.

3.3.3 Solubility Determination: Characteristic PFC curve

The published oxygen solubility values for Perfluorodecalin range between 13-20 fold higher than water and physiological salt solutions (see table above), depending on the temperature of the measurements and the oxygen partial pressure (room air versus
pure oxygen saturation). Figures 3-3a and 3-3b, following, show the results of measured fold solubilities relative to control solutions (HBSS) for perfluorodecalin emulsions at 37 °C and 25 °C. Both the measured fold slope and the measured fold concentrations are shown. Results are expressed as mean fold control solubility ± SD.

Figure 3-3a: Plot of measured fold concentration (open diamonds) by Trinder reaction and fold measured slope (cross hairs) from oxygen traces at 37°C.
Figure 3-3b: Plot of measured fold concentration (open diamonds) by Trinder reaction and measured fold slope (cross hairs) from oxygen traces at 25°C.

Linear fits of the experimental data at 37°C were found to be $18.34x + 0.9378$ for measured slope and $16.628x + 0.9645$ for measured concentration. At 25°C, linear fits of the experimental data were found to be $12.491x + 1.042$ for measured slope and $13.369 + 1.036$ for measured concentration. $R^2$ values ranged from 0.981 for the measured slope at 25 °C to 0.997 for the measured slope at 37 °C. For Perfluorodecalin, the measured concentration from the Trinder reaction absorbance and the slope were within the literature values referenced (see Table 3-1). Using the linear regression of both the slope and measured concentration curves, the dissolved oxygen capacity of pure Perfluorodecalin was extrapolated as 17.59 for the measured concentration and 19.28 for the measured slope at 37 °C, while at 25 °C the measured fold concentration was 14.41 and the measured slope 13.53, all within the range of literature values. Figure 3-4 shows the fold slope plotted versus the fold concentration at 25 °C, demonstrating the strong
correlation between the two values. The value measured exhibited less than a 6% difference between measured fold concentration and measured fold linear slope. This indicates that the two metrics can be interchanged confidently in determining the dissolved oxygen content in perfluorodecalin emulsions.

![Graph showing measured fold slope versus measured fold concentration of perfluorodecalin emulsions relative to base solution. The difference between metrics was less than 6%, indicating reliability of both measures at predicting dissolved oxygen content in emulsions.]

3.4 Discussion

Perfluorocarbon emulsions are a promising tool for improving oxygen transfer within solutions and biomaterials; however, thorough characterization of their potential oxygen mass transfer enhancements is limited. Critical to the determination of the expected benefit of these emulsions is the measurement of the dissolved oxygen content in the emulsions and, less so, the effective oxygen diffusivity through the emulsions. The
product of these two characteristics is the diffusive permeability coefficient, which is the direct assessment of oxygen mass transfer through any system. Determination of the diffusive permeability allows for numerical optimization of emulsion preparations to maximize both characteristics of oxygen diffusive permeability and thereby, oxygen transfer.

Prior methods for the accurate determination of dissolved oxygen content within solutions often required sophisticated laboratory equipment with custom glassware and precision manometers or gas chromatographs. Additionally, many methods are quite laborious and time consuming, requiring hours to collect a single measurement (34,37). Herein, we present a novel method with complementary results that, while requiring a piece of custom equipment, is more user-friendly and efficient. Our data also illustrate that the use of recorded oxygen traces within these sealed system is as accurate as colorimetric determination via Tinder reaction, thereby allowing for further ease in measurements by oxygen traces alone. Our results demonstrate that the oxygen solubility measurements utilizing our system fall within published values collected using more intricate methodologies, thereby offering a simpler means of quantifying dissolved oxygen in any solution or micellar suspension, with some modifications. Utilization of this method permits accurate determination of oxygen content in solutions, particularly those utilized for medical applications and oxygen delivery capacities. This simple method would benefit researchers in the optimization of the manufacture of oxygen carrying solutions utilized in, for example, wound healing, blood replacement therapies and the immunoisolation of cell products for transplant therapies in the treatment of
medical conditions such as Diabetes Mellitus. We feel that this simple assay could serve a critical role in the advancement of oxygen delivery mechanisms.
CHAPTER 4- NOVEL METHOD OF ISLET POTENCY ASSESSMENT BY GLUCOSE STIMULATED INSULIN RELEASE.

4.1 Potency Testing in Isolated Islets of Langerhans

The last decade has seen substantial advances in the field of islet cell transplantation as a potential curative therapy for the treatment of Type 1 Diabetes Mellitus. One persistent obstacle to ensuring the transplant of optimal cellular products is the need for reproducible potency assessment(s) (Hanson, Steffen et al. 2008; Papas, Suszynski et al. 2009; Hanson, Park et al. 2010; Friberg, Brandhorst et al. 2011). The accepted “gold standard” for assessing islet function, concomitant renal sub capsular transplant in immunodeficient mice, is not ideal, since the results are only available post-transplantation. In addition, the data is often difficult to extract regarding islet potency, given the variations in IEQ number transplanted, islet purity, the number of donors used for transplantation, and clinical recipient. However, correlations have been demonstrated when single donors are utilized (Caiazzo, Gmyr et al. 2008; Papas, Suszynski et al. 2009). Despite these limitations, it is accepted by most in the field agree that reversal of hyperglycemia is a strong predictor of function in patients barring immunological complications or other issues. Clinicians, however, are still seeking a quick and simple in vitro assay that is predictive of transplant success and can be performed before and analyzed prior to the transplant procedure.

Historically, viability staining, metabolic activity, and insulin release and have been employed as benchmarks of islet function and well-being, although recent evidence suggests viability staining is a poor predictor of clinical outcome (Barnett, McGhee-Wilson et al. 2004; Papas, Suszynski et al. 2009; Yamamoto, Horiguchi et al. 2009). Novel fluorescent/luminescent probes and flow cytometry have also been used to
determine islet viability through membrane integrity/apoptosis staining, ATP quantification, cell subset analysis and gene upregulation of secretory products. However, despite promising results, no single measurement has emerged as a totally reliable indicator of islet transplant outcome (Ichii, Inverardi et al. 2005; Goto, Holgersson et al. 2006; Armann, Hanson et al. 2007; Adewola, Lee et al. 2010; Hanson, Park et al. 2010; Omori, Mitsuhashi et al. 2010).

A more recently proposed in-vitro assay for the assessment of islets is metabolic assessment via the measurement of oxygen consumption rate (OCR) (Fraker, Timmins et al. 2006; Papas, Colton et al. 2007; Papas, Pisania et al. 2007; Sweet, Gilbert et al. 2008). While OCR has been shown to be highly predictive in the hands of several groups, it is still limited due to the complexity of the measurement systems and ease of use for the unskilled operator.

The static incubation, or glucose stimulated insulin release (GSIR) assay, has been utilized since the inception of islet research to observe the insulin output from a representative aliquot of islets in both basal and elevated glucose concentrations. Methods have varied substantially over the years, with discrete variations in the medium utilized, basal and elevated glucose concentrations, and the overall assay methods. Traditionally, results have been informative concerning islet function but not predictive with a high degree of sensitivity and specificity (Bretzel, Alejandro et al. 1994; Vandewalle, Douillard et al. 1999; White, James et al. 2001; Linetsky and Ricordi 2008). Reasons for the lack of predictive value have been proposed, such as the need to use a complete medium, the need for IEQ normalization, washout of residual insulin during challenges, and the sensitivity of islets to insulin dumping resulting from mechanical
stimulation by handling and washing. In this work, we introduce a novel and highly predictive method for the assessment of glucose stimulated insulin release that is performed in a Sepharose slurry to minimize mechanical stimulation of the islets, prevent aspiration of islet particles and precisely control washes and collection times and volumes. We explore the ability of this simple method and combinations of this method with another potency metric, fractional β-cell viability, to serve as an accurate predictor of islet function in the standard mouse model of potency. Implications of the use of this method for predicting islet quality are discussed.

4.2 MATERIALS AND METHODS

4.2.1 Islet Isolation and Culture

Islets were isolated using a modified version of the Ricordi automated method (Ricordi, Lacy et al. 1988) and were allowed to recover for 24 hours in a 37 °C incubator in conventional islet medium (MM1, Cell-Gro) before transplantation and GSIR/Fractional Beta Cell viability assessment. On the day of potency assessments and transplantation, islet equivalents (IEQ) were counted based on conventional dithizone staining using an inverted stereo-microscope with a graded reticule.

4.2.2 Glucose Stimulated Insulin Release

Static glucose stimulated insulin release (GSIR, Static Incubation) was performed on 36 consecutive islet preparations using a GSIR column method as described, herein. A modified Kreb’s buffer with 26 mM sodium bicarbonate, 25 mM HEPES and 0.2% w/v bovine serum albumin and either 2.2 mM (low glucose) or 16.7 mM (high glucose) was prepared and warmed to 37 °C in a standard 95% RA/5% CO₂. Approximately 5 g of
Sepharose G-10 (molecular weight cutoff < 700D, GE Healthcare) was added to a 50 mL beaker containing 20 mL of dPBS w/o Ca\(^{2+}\),Mg\(^{2+}\) (Cellgro). and boiled for 30 minutes to swell the beads. Triplicate 10 mL Poly-Prep columns (Biorad). Each column was individually labeled at the 400 µL and 1 mL increment to allow for ease in filling. 1 mL of modified Kreb’s low glucose buffer was added to each column. After the beads were cooled to room temperature, the slurry was slowly added to the level of 400 µL in the column. At this point, based on the IEQ counts, 100 IEQ aliquots free in 1 mL of modified Kreb’s low glucose buffer were added to each column. An additional 600 µL of bead slurry was then added to each column to bring the final slurry volume to 1 mL. During this addition, the islets were gently mixed within the slurry to distribute them throughout the beads and prevent islet aggregation. Well packed, the void space of the bead slurry contains approximately 350 µL of liquid.

Following bead loading, the bottom seals were removed from each column and an additional 4 mL of low glucose buffer solution was added to each column to pack the beads and assure that flow was unimpeded through each column. Additional bead slurry was added, if necessary, to reach 1 mL packed bead volume. Flow in the columns ceased when the liquid level reached the surface of the beads, thereby keeping the fluid volume in each column constant and the slurry/islet mixture fully hydrated.

Islet/slurry filled columns were then incubated in a standard 95% RA/5% CO\(_2\) 37 °C incubator (for 1 hr for pre-incubation, followed by a 4 mL wash with low glucose KREB’s to wash out insulin secreted during the assay set-up and manipulation). Subsequently, the first step of the glucose challenge (Low1) was initiated. After 1 hour, low glucose KREBs was exchanged with high glucose (16.7 mM) KREBs to begin step
two of the challenge (High1). After this hour, high glucose was exchanged with low glucose KREB to begin step three of the challenge (Low2). During each exchange, 1 mL of the respective KREBs solution was added and the 1mL eluate was collected in tubes and stored at –80 °C for later analysis. Insulin was quantified using the Mercodia Human Insulin ELISA (Mercodia, Upsalla, Sweden).

4.2.3 IEQ Enumeration

After the incubation periods were completed, the slurry and islets were washed from the columns into an 8.5 cm petri dish by forceful air ejection via a syringe luer-locked to the bottom of the column. 10 mL of PBS was added to each dish (n = 3) to dilute dithazone and slurry. By gentle swirling, islets were centered in the dish and excess slurry was aspirated. Dithizone was added in a drop wise fashion onto the centered islets. The stained islets were counted and quantified in IEQ number under a graded reticule. This method was performed in 16 of the 36 preparations. Results were compared to earlier preparations (n = 20) where standard aliquoting was utilized for islet enumeration. This was done in order to ascertain whether or not normalization is a critical variable in the GSIR when IEQ aliquoting is done by a single test performer.

4.2.4 Beta cell Fractional Viability

β-cell viability was assessed as previously described (Ichii, Inverardi et al. 2005). Briefly, islet aliquots were gently dissociated using a non-enzymatic buffer. Single cell suspensions were incubated with 1 µM Newport Green (NG) and stained with 7-aminoactinomycin D (7AAD, Invitrogen, Carlsbad, CA) for the identification and quantification of β-cells and live/dead cells, respectively. Islet β-cell viability was
characterized as NG+/7AAD− staining. As a marker of mitochondrial membrane potential and a further determination of viability, 100 ng/mL of tetramethylrhodamine ethyl ester (TMRE, Molecular Probes, Eugene, OR) was added. Flow cytometric analysis was performed on all samples and viable β-cells were expressed as TMRE+ cells on gated NG+ 7AAD− cells. This method allows for the detection of early apoptosis and is predictive of islet function in vivo.

4.2.5 In vivo assessment of islet function

Animal protocols were approved and monitored by the University of Miami IACUC (Ichii, Pileggi et al. 2005). As described Male athymic nu/nu (nude) mice were purchased from Harlan Laboratories (Indianapolis, IN) and housed in virus- and antigen-free rooms in micro-isolated cages at the Division of Veterinary Resources of the University of Miami. Animals were rendered diabetic via a single intravenous administration of 200 mg/kg of Streptozotocin (Sigma-Aldrich, St. Louis, MO). Non-fasting blood glucose was assessed by glucometer (Elite, Bayer; Tarrytown, NY or OneTouchUltra2, LifeScan, Milpitas, CA) and mice with sustained hyperglycemia (> 300 mg/dl) were designated for islet transplant. Standard sized grafts (2,000 IEQ per recipient) were transplanted under the kidney capsule (1-3 per preparation. For impure islet preparations, the cells were transplanted across both sides of the kidney to avoid oxygen limitations caused by contaminating tissue mass. After transplantation, non-fasting blood glucose values were assessed daily for the first week and then 3 times a week, thereafter, up to 100 days. Reversal of diabetes was defined as stable, non-fasting blood glucose < 200 mg/dl. A “good” islet preparation was defined as one where reversal of hyperglycemia occurred rapidly (<= 5 days) and a “poor” preparation, one where
reversal occurred more slowly (> 5 days). For the purpose of analysis, non-reversal was assigned an arbitrary time point of 100 days. Nephrectomy of the graft-bearing kidney was performed to confirm return to hyperglycemia and exclude residual function of the native pancreas in animals achieving and maintaining normoglycemia after transplantation. Animals with residual function after nephrectomy were excluded from the analysis as technical failures.

4.2.6 Data Analysis

The stimulation index (ratio of insulin produced in high glucose to insulin produced in low glucose #1), the total insulin production (low glucose #1 insulin + high glucose insulin + low glucose #2 insulin) and the “delta” insulin (high glucose insulin – low glucose #1 insulin) were all examined for the predictive ability in determining graft outcome. Data was expressed as insulin output per 100 IEQ in both the cases where IEQ were and were not counted. In a subpopulation of pancreata (n = 24), the above metrics were coupled with the fractional β-cell viability to evaluate whether or not a combination of tests would serve as a better indicator of islet potency relative to each test individually. In these cases, the data was expressed in the following fashion:

\[
\frac{\Delta \text{Insulin}}{\% \text{Viable } \beta - \text{cells}}
\]

(4-1)
as insulin per viable beta cell. All data was expressed as mean values ± SD of the mean. Additionally, in another subpopulation (n = 16), islet enumeration was compared with assumed aliquot values of 100 IEQ. Again, in both, data was expressed as insulin per 100 IEQ.
4.2.7 Statistical Analysis

Receiver operating characteristic curve (ROC) analysis was performed on all of the individual metrics and on the coupled metrics, as well. ROC analysis is used to determine if a diagnostic test has the ability to predict an outcome of interest and was used to evaluate the ability of the above metrics to predict graft success/failure (Sweet, Gilbert et al. 2008). ROCs were generated by plotting the sensitivity [true positives/ (true positives + false negatives)] vs. 1 – specificity [true negatives / (true negatives + false positives)]. The area under the curve (AUC) value was used as the indicator of metric predictability with an AUC of 1 if the correct classification was 100%, and 0.5 (line of unity) if the test had no predictive ability. Paired ROC curves (enumeration vs. no enumeration, multi-metric potency versus single metric potency, index versus delta and viable beta cell index versus delta) were also compared and examined for statistical significance using the method described by Hanley et al. (1983 Radiology 148: 839-843).

4.2.8 Survival Analysis

From the ROC analyses, a potency test “cutoff” value was obtained (maximum sensitivity and specificity). The potency metrics utilized were the delta for all preparations and a comparison of the delta versus the delta/VBI for the respective subpopulation study. The calculated cutoff was implemented in Kaplan-Meier graft survival analysis. Curves of reversal percentage were plotted against time in days for potency values above and below the determined cut-off value to evaluate its effectiveness at classifying “good” and “poor” islet preparations. Curves were analyzed for significant difference utilizing the Log-rank (Mantel-Cox) test and the Gehan-Breslow-Wilcoxon test in the GraphPad Prism software package. As an indicator of improved predictive
power in the subpopulation study, the hazard ratio, which predicts the ratio of the slopes of the two survival curves above and below the cutoff (rate of diabetes reversal), was utilized. A greater hazard ratio was indicative of improved predictive power.

4.3 RESULTS

4.3.1 Glucose Stimulated Insulin Release

Thirty-six human islet preparations were assessed in this study. In the first 20 preparations, an IEQ of 100 was assumed, based on standard aliquoting following counting. For the latter 16 preparation, enumeration was performed via an additional IEQ count after the GSIR assay (see Methods) Table 4-1, following, details the mean values and ranges for all the data analyzed including IEQ counts, raw insulin values and potency metrics. As expected, the raw values and metric values had large variability, inherent with islet preparations.

<table>
<thead>
<tr>
<th>IEQs</th>
<th>LOW 1 (uU/mL)</th>
<th>HIGH (uU/mL)</th>
<th>LOW 2 (uU/mL)</th>
<th>INDEX</th>
<th>TOTAL INSULIN (uU/mL)</th>
<th>DELTA (uU/mL)</th>
<th>VBI</th>
<th>Delta/VBI</th>
<th>Reversal Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIN</td>
<td>84.44</td>
<td>14.12</td>
<td>55.67</td>
<td>25.51</td>
<td>1.08</td>
<td>111.56</td>
<td>5.67</td>
<td>0.06</td>
<td>148.92</td>
</tr>
<tr>
<td>MAX</td>
<td>129.00</td>
<td>730.83</td>
<td>4700.54</td>
<td>1636.48</td>
<td>20.83</td>
<td>7067.85</td>
<td>3969.70</td>
<td>0.62</td>
<td>34519.17</td>
</tr>
<tr>
<td>MEAN</td>
<td>105.95</td>
<td>173.60</td>
<td>737.03</td>
<td>262.01</td>
<td>5.29</td>
<td>1158.08</td>
<td>563.43</td>
<td>0.24</td>
<td>3798.46</td>
</tr>
<tr>
<td>SD</td>
<td>13.32</td>
<td>156.24</td>
<td>855.98</td>
<td>304.91</td>
<td>4.30</td>
<td>1266.75</td>
<td>741.29</td>
<td>0.14</td>
<td>7593.21</td>
</tr>
<tr>
<td>CV</td>
<td>12.6%</td>
<td>90.6%</td>
<td>116.1%</td>
<td>116.4%</td>
<td>81.2%</td>
<td>109.4%</td>
<td>131.6%</td>
<td>59.8%</td>
<td>199.9%</td>
</tr>
</tbody>
</table>

Table 4-1: Range and mean values of IEQ counts, incubation period insulin outputs and investigated potency metrics.

Figure 4-1, following, displays the GSIR results from a representative “good” and “poor” islet preparation.
The mean stimulation indices for the preparations, bad and good, were 5.20 ± 0.76 and 8.82 ± 1.20, respectively. The delta (total insulin produced in high glucose – total insulin produced in low 1) for both preparations was 58.74 ± 6.86 µU/mL and 2,255.74 ± 488.72 µU/mL respectively. The graft reversal times were 8,11 and no reversal, out of three transplants from the bad preparation and six out of six animals on day 1 for the good preparation. All values were highly significantly different amongst the two groups (p << 0.01). This figure also demonstrates the wide range of values associated with islet preparation variability.

4.3.2 IEQ Enumeration

The post-assay dithazone staining method was sufficient for obtaining column IEQ counts after glucose stimulation. The mean column IEQ count for the 16 islet
preparations where enumeration was performed was 105.6 ± 13.3 IEQ. The maximum mean IEQ number was 129 and the minimum, 84.4. The coefficient of variation between all counts was 12.4% demonstrating that if counts are performed by a single person, the variation between counts is low enough as to not significantly impact the potency results. This 12.4% variation, when applied to measure potency values, had no effect on the predictive capacity of any of the metrics.

4.3.3 Beta cell Fractional Viability

Twenty-four consecutive random islet preparations were analyzed for beta cell fractional viability. The potency metric analyzed was the viable beta cell index (VBI), previously validated by our group (Ichii, Inverardi et al. 2005). The mean VBI for the analyzed preparations was 0.24 ± 0.14. The maximum measured VBI was 0.62 and the minimum, 0.06.

4.3.4 Data Analysis/Statistical Analysis

ROC analysis is conventionally utilized to determine the predictive ability of a diagnostic test of a disease state, positive or negative, or, in this case, human islet graft success or failure after transplantation in diabetic nude mice. The ROC curve is generated by plotting the test’s sensitivity, the ability to predict “true” positive disease state against 1-specificity, the specificity representing the test’s ability to predict “true” negative disease state. In this case, for the purpose of proper graphing, graft failure (reversal time > 5 days) was designated as the “true” positive state (x-axis) and graft success (reversal time <= 5 days) was designated as the “true” negative (y-axis). The area under the generated curve (AUC) is used as a measure of the test’s predictive ability, with 1 being
complete correct classification, meaning the test predicts every test case correctly, and 0.5 being no predictive ability, also called the line of unity. The line of unity means that the test has equal likelihood of measuring positive or negative for each test subject. Generally, a test with an area under the curve of 0.70 - 0.79 is considered a “fair” predictor, between 0.8 and 0.89 a “good” predictor and > 0.9 and “excellent” predictive test. Of critical importance to ensuring the accuracy of our analysis, we sought to include all grafts, including those that failed to achieve normoglycemia during the course of the implant. Therefore, all animals that remained hyperglycemic were assigned a reversal time of 100 days (the total time course for the mouse implant studies). In this manner, failed grafts were included in the analysis. From the ROC analysis, AUC values were acquired to assess predictive power of the various metrics examined, additionally, the potency value at the point of maximal sensitivity and specificity was utilized for further survival analysis for the most promising potency metric, the delta insulin.

Figure 4-2, following, shows the ROC curves for the delta insulin, stimulation index and the total insulin output for all 36 islet preparations. For 24 of the 36 preparations, VBI was also performed
Figure 4-2: ROC curves for the Delta Insulin (closed circles, solid line), Stimulation Index (open triangles, dotted line) and Total Insulin Output (cross hairs, dashed line) for all 36 pancreata.

Figure 4-3a-c, the three graphs following, shows the ROC curves for the VBI, the delta and the delta/VBI, for the 24 preparations termed “VBI Subpopulation”.
Figure 4-3a-c: ROC curves for the VBI, delta and delta/VBI of the 24 human islet preparations where both potency tests were performed.

Figure 4-4, the two graphs following, shows the ROC curves for the delta of the 16 islet preparations where post-assay islet enumeration by dithazone staining was performed, termed “Enumeration Subpopulation”. The ROC curves shown are for the delta values per 100 IEQ using either assumed aliquot values of 100 IEQ or the actual mean IEQ counts.
Figure 4-4: ROC curves of Delta Insulin comparing assumed aliquot numbers (top) to mean IEQ counts (bottom) in a subpopulation of 16 islet preparations.

Table 4-2, following, displays the AUC values, the maximum sensitivity and specificity values and the cutoff points used for survival analysis for all pancreata and the two investigated subpopulations (VBI and Enumeration).
Table 4-2: AUC, maximum sensitivity/specificity and cut-off values determined from maximum sensitivity and specificity for all potency metrics in all pancreata and the two described subpopulations.

<table>
<thead>
<tr>
<th>Metric</th>
<th>MAX Sensitivity</th>
<th>MAX Specificity</th>
<th>Cut-off Value</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ALL HP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stimulation Index</td>
<td>80%</td>
<td>75%</td>
<td>4.92</td>
<td>0.7781</td>
</tr>
<tr>
<td>Total Insulin</td>
<td>92%</td>
<td>84.38%</td>
<td>995.2</td>
<td>0.9019</td>
</tr>
<tr>
<td>Delta Insulin</td>
<td>81.25%</td>
<td>88%</td>
<td>425</td>
<td>0.9319</td>
</tr>
<tr>
<td><strong>VBI</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Index Subpopulation</td>
<td>76.47%</td>
<td>78.95%</td>
<td>4.92</td>
<td>0.7523</td>
</tr>
<tr>
<td>Delta Subpopulation</td>
<td>88.24%</td>
<td>94.74%</td>
<td>274.3</td>
<td>0.9381</td>
</tr>
<tr>
<td>Viable Beta Cell Index</td>
<td>88.24%</td>
<td>73.68%</td>
<td>0.1475</td>
<td>0.7755</td>
</tr>
<tr>
<td>Delta/VBI</td>
<td>94.12%</td>
<td>84.21%</td>
<td>1803</td>
<td>0.9505</td>
</tr>
<tr>
<td><strong>Enumeration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Delta Enumeration</td>
<td>80%</td>
<td>83.33%</td>
<td>241.6</td>
<td>0.8</td>
</tr>
<tr>
<td>Delta Aliquot</td>
<td>80%</td>
<td>83.33%</td>
<td>274.3</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Based on the data analysis, the delta insulin value of the glucose stimulated insulin release appears the most predictive. The delta had an AUC value that was higher than both stimulation index and total insulin content (0.9319 vs. 0.7781 and 0.9019, respectively). Relative to the index and the total insulin output, the delta ROC curve was significantly different (p < 0.01). Additionally, in the subpopulation study where two potency metrics were utilized, VBI and delta insulin, the AUC value of the delta insulin was higher than the VBI and the index in predicting rapid reversal (<= 5 days) for full mass transplants within this subpopulation (0.9381 vs 0.7523 and 0.7755, respectively). Relative to the index and the VBI in this subpopulation, the delta ROC curve for the same subpopulation was significantly different only compared to the index (p < 0.01 and p = 0.078, respectively.) Interestingly, when the delta value was normalized against the fractional beta cell viability in the following fashion:
the predictive power of the combined potency metric was superior to each, individually (0.9505 vs. 0.9381 and 0.7755, respectively). The ROC curve of this combined metric was significantly different relative to the delta but not to the VBI ($p << 0.01$ and $p = 0.053$, respectively).

In the subpopulation study investigating aliquot IEQ number relative to enumerated IEQ number in 16 preparations, the ROC analysis showed that there was no difference affecting predictive power of the delta potency metric (and therefore, total insulin or index) due to IEQ enumeration. In fact, the AUC for both ROC curves was identical, 0.80, and the test for statistical significance between the two curves indicated that they were statistically indistinguishable ($p = 0.15$, NS).

4.3.5 Survival Analysis

Survival analysis was performed utilizing the optimal cutoff values predicted by ROC analysis. The survival analysis was an additional method used to indicate the predictive ability of the developed assay and the corresponding metrics. Figure 4-5, the three graphs following, displays the survival curves for the delta of all islet preparations ($n = 36$), the delta for the multi-test subpopulation ($n = 24$) and the delta/VBI for the same subpopulation, respectively.
Figure 4-5: Survival curves for the delta analysis for all islet preparations (n = 36, cutoff > 425 µU/mL), delta analysis for the multi-test subpopulation (n = 24, cutoff > 274.3 µU/mL), and the delta/VBI (n = 24, cutoff > 1803 µU mL⁻¹ viable beta cell⁻¹).
The cutoff points utilized were 425 µU/mL for the first, 274.3 µU/mL for the second and 1803 µU mL\(^{-1}\) viable beta cell\(^{-1}\), for the final, combined metric analysis (VBI Subpopulation). In all three cases, the cutoff values generated by ROC analysis were strong indicators of graft success or failure, with highly significant differences between values above and below the cutoff point (p << 0.01 in all three cases). In the subpopulation study comparing the delta alone to the delta/VBI, the delta/VBI proved a better metric of survival analysis based on an increased hazard ratio. The hazard ratio of the delta/VBI was 8.42 compared to a hazard ratio of 7.265 for the delta alone. This increased hazard ratio indicates a more rapid rate of separation between the reversal rates above and below the calculated cut off points and verifies the increased sensitivity of the test as a predictor of graft outcome.

4.4 DISCUSSION

While promising advances have been made in the last decade of islet/cellular transplantation therapies for T1DM, there is still the lack of a product release criterium of measurable potency necessary for FDA approval as a clinical treatment (Bretzel, Alejandro et al. 1994; Fiorina, Shapiro et al. 2008; Linetsky and Ricordi 2008; Yamamoto, Horiguchi et al. 2009; Friberg, Brandhorst et al. 2011). While many promising methods have been proposed for quantifying islet cell potency, including OCR, ATP production, β-cell specific viability with measure of mitochondrial membrane potential and the “gold standard” concomitant full mass sub-renal capsular transplant in immunodeficient mice, none has emerged as a rapid, ubiquitously easy to perform assay that could be implemented in any clinical cell transplant laboratory (Ichii, Inverardi et al. 2005; Fraker, Timmins et al. 2006; Goto, Holgersson et al. 2006; Armann, Hanson et al. 2011).
2007; Hanson, Steffen et al. 2008; Hanson, Park et al. 2010). Additionally, with the exception of β-cell specific viability, the majority of the tests look at all the islet cell subsets, perhaps under or over estimating the viability of the preparation and its capacity to reverse hyperglycemia. To that end, a logical measure of specific β-cell function is the glucose stimulated insulin release. Physiological insulin release in this historical assay has been linked to proper cell signaling between the subset populations of the islets and also closely related to the metabolic machinery of the β-cells. In this way, this test provides information about the overall health of all cells within the islet, but, most importantly, the insulin producing β-cells and their secretory capacity. The use of dynamic perifusion systems in which islets are exposed to changes in glucose concentrations and samples are collected sequentially at tight time intervals (every minute) has confirmed the value of GSIR in determining islet potency, but the test is demanding as it requires dedicated apparatuses (Sweet, Cook et al. 2002; Cabrera, Berman et al. 2006; Cabrera, Jacques-Silva et al. 2008) and the costs are higher due to the multiple samples to be assessed for insulin release. More simple methods of ‘static’ GSIR in which islets are incubated in differing glucose concentrations for extended periods of time to measure the cumulative concentration of insulin are more practical and less expensive. Prior methods of the GSIR have involved extensive mechanical manipulation and complex DNA extractions to normalize results. Additionally, recently utilized multi-center methods have implemented only two incubation periods performed in parallel rather than sequential incubations of the same aliquots in order to accelerate the collection of results. This assay did not demonstrate reliability as an indicator of

By utilizing a stabilizing agent, sepharose beads, to minimize mechanical perturbations during glucose incubations and a third incubation period to demonstrate return to basal insulin levels, we obtained results that correlated significantly in ROC curve and survival analysis with rapid diabetes reversal in immunodeficient mouse full mass transplants. Additionally, as the data in this study was collected by one test performer, we discovered that in the hands of a single operator where counting methods and errors are identical in every islet preparation, it is not necessary to normalize data against IEQ number or DNA content. Given the difficulty in accurately extracting genomic DNA from lyophilized samples, a part of the multi-center protocol previously utilized, this is an added benefit of our method.

Most importantly, we found that a combination of potency tests proved even more predictive than a single potency metric, as has been demonstrated by other centers (Hanson, Park et al. 2010). When using fractional β-cell viability, we found that insulin values divided by the viable β-cell content improved the ability of both tests to predict graft outcome. This was particularly apparent in the case of a few preparations where viable β-cell content was relatively low (<10%), but the insulin output for these cells surprisingly high (δ > 1000 µU/mL). The combination of the two metrics indicated potency is not necessarily related to cell content, but more to the insulin output of said cells. In this case, the β-cell fractional viability would have predicted graft failure while the delta insulin success, but with the reversal time ranging from 1 - 5 days. The
combination of the two tests resulted correlated with a reversal rate of 1 day for all the animals transplanted.

Previous studies have shown that combination of multiple parameters of human islet assessment may increase the ability to predict islet potency after transplantation in diabetic immunodeficient mice (Hanson, Park et al. 2010). However, unlike our approach, the methods utilized in previous studies did not discriminate the contribution of beta cells and that of other cells in the human islet preparation. We postulate that the best metrics for islet cell potency relate to insulin production thereby evaluating the relevant $\beta$-cell population. Ultimately, the ability of islets to produce insulin is what dictates graft success or failure, as glucose metabolism is directly dependent on the quantity of insulin present to aid in glucose uptake by cells. This is, of course, a simplification of the process that occurs in fully immune competent patients. This is why a measure of rapid reversal is perhaps better than simply distinguishing between reversal/no reversal. In the clinical setting, where immunosuppressive agents and cytotoxic immune response further lessen the likelihood of graft success, a reversal time in nude mice of > 50 days relative to < 5 days would seem to be clinically irrelevant.

The method presented herein provides a rapid assay for the determination of human islet potency after the establishment of a correlation to the in vivo bioassay based on immunodeficient mouse full mass transplant (< 6 hours). In addition, the coupling of this method with other potency assessments, in this case $\beta$-cell fractional viability, increases the discriminatory power of both assessments and gives an improved potency sensitivity and specificity. In the future, work will be undertaken to develop a multi-
pronged assessment method utilizing currently applied or novel methods, to develop a strong potency algorithm for predicting islet function in the clinical setting.
CHAPTER 5: IMPROVING ISLET VIABILITY AND FUNCTION THROUGH THE USE OF GAS PERMEABLE CULTURE PLATFORMS

5.1 The Role of Oxygen in the Culture of Islets

Conventional culture is comprised of plating cells in plastic dishes or flasks with gas impermeable bottoms and placing them in a humidified incubator with 95% Room air (142 mmHg oxygen, accounting for vapor pressure differences) and 5% CO2. The cells are sustained in culture media optimized for their growth and maintained at a depth that provides them sufficient nutrition for several days, while preventing hypoxia induced from large diffusion distances through the medium layer. The sole source of oxygen in conventional culture is from the top surface of the medium layer, where oxygen diffuses down reaching the tissue at a transfer rate limited by the oxygen diffusivity through the medium, the distances of diffusion, and the rate of oxygen consumption of the cells. If the cellular rate of consumption exceeds the rate of diffusion from the top surface to the cells, anoxia will develop in the cellular core regions and necrosis may result (Sher 1990; Hyder, Laue et al. 1998).

Equally important, if the media layer is reduced to shorten the diffusive distance, the cells may suffer from lack of nutrients or dehydration, if medium evaporation occurs. Thus, the static culture environment is a critical balance between oxygen supply and nutrient supply. Despite modifications to improve the culture conditions, there will always exist gradients of gas and nutrient concentration across the cell, depending on cell size. This undesirable environment is more than likely the cause of the inability to duplicate characteristic in vivo environments in the in-vitro culture setting.
In single cell populations, diffusion of oxygen is not a limiting factor, assuming an appropriate cell density, as diffusive distances in these cells are short. Therefore, emulating in-vivo oxygen levels in static culture is not difficult, as the diffusion across an entire 10 µm cell is rapid and results in a uniform surface to core concentration. By simply lowering the oxygen content in a conventional incubator, it is possible to mimic the native oxygen levels of these cells, with gradients from apical to basal surface, although less pronounced than standard culture oxygen tensions. Culturing such cells in high oxygen levels, on the other hand, has been observed to result in increased oxidative damage and a slowing of proliferative capabilities. This seems logical, given the oxygen concentrations utilized in these studies, generally 50% or greater, and the non-limited diffusion of oxygen across these cells. In high oxygen culture conditions the resultant intra-cellular oxygen concentrations can be upward of 10-20 fold higher than the in-vivo concentrations seen by these cell types.

In the case of islets of Langerhans, physiological oxygen concentrations are difficult to mimic in static culture, primarily due to the large cell cluster size (50 – 350 µm). In standard culture, the core oxygen levels are generally below or near physiological levels, while the exterior of the cultured tissue is generally substantially higher. This results in a very narrow volume of tissue where the oxygen levels are near to physiological levels. It is conceivable that this has some margin of error (± 5-10 mmHg), but even with this, the band of physiologically normoxic tissue is a small percentage of the entire tissue mass. In seminal literature regarding the effect of local oxygen partial pressure on islet viability and function, both hyperoxic and hypoxic environments have
been demonstrated to be substantially detrimental to islet viability and function (Chase, Ocrant et al. 1979; Tiedge, Lortz et al. 1997; Pileggi, Molano et al. 2001; Ribeiro, Klein et al. 2003).

Unlike single cell cultures, simply lowering the external oxygen partial pressure in cell aggregate/islet culture would not result in a more physiological static culture environment. Again dependent on culture density (tissue proximity), medium height and oxygen consumption rate, the expected outcome of lower external partial pressures would be anoxia and central necrosis in the majority of the tissue with physiological oxygen levels only at the surface region of the cell clusters. This has been documented in several studies, as mentioned above, with dramatic necrotic cell loss due to islet culture in sub-physiological oxygen concentrations (Giuliani, Moritz et al. 2005; Emamaullee, Shapiro et al. 2006; Miao, Ostrowski et al. 2006; Lehmann, Zuellig et al. 2007; Ko, Ryu et al. 2008). These observations in pancreatic endocrine cells contradict the observations in single cell populations, such as tumor cells of the lung or some stem cell lines, where it has been demonstrated that hypoxia, in an acute fashion, can result in enhanced survival signaling and proliferative growth (Powers, Millman et al. 2008; Zhao, Zhang et al. 2008; Ivanovic 2009). Given the sensitivity of islet cells to deviations from physio-normal oxygenation, it seems that methods for culturing these cells at partial pressures of oxygen that more closely approximate their physiological niche could improve post-isolation islet well-being. However, in order for this methodology to elicit a favorable effect, deviations from physiological oxygen levels, generated by solely changing incubator pO$_2$, should be avoided. Rather, these methods should work to improve oxygen mass transfer to the cells in their culture/transplant environment.
One means by which oxygen mass transfer could be improved in both static culture and the transplant setting is through the utilization of compounds with higher oxygen diffusive permeabilities, such as perfluorocarbons and polydimethylsiloxane. Diffusive permeability is the product of the effective diffusivity through and solubility, dissolved content in, of one compound in another. It is the determinant of the mass transfer rate across a given diffusive distance.

Perfluorohydrocarbons, or PFCs, are chemically inert compounds constructed of long carbon chains where all of the hydrogens bound to the carbon sites are replaced with fluorine atoms. This unique characteristic of these compounds gives them a substantial ability to bind and transfer molecular oxygen. Functional studies of the varied pure PFCs have shown that they have oxygen solubilities approximating or surpassing hemoglobin at different oxygen saturations and about 40-50 times that of water or culture medium (at \(pO_2\) of 760 mmHg oxygen) (Faithfull 1992; Faithfull 1992; Faithfull 1992). Further, they have an oxygen diffusivity that is approximately 2.5-5 fold that of water or culture medium. In clinical trials, emulsions made with perfluorocarbon moieties have been successfully utilized as parenteral blood substitutes. Additionally, biphasic solutions of dense pure perfluorocarbons and conventional UW preservation solution/culture medium have been used in the field of pancreas preservation and islet cell isolation with promising results, improving islet yields per pancreas and improving viability and function during islet culture (Sutherland, Farrar et al. 1984; Fujino, Kuroda et al. 1991; Kuroda, Fujino et al. 1991; Kuroda, Morita et al. 1993; Matsumoto and Kuroda 2002; Matsumoto, Rigley et al. 2002; Tsujimura, Kuroda et al. 2002). The mechanism by which the pure PFCs enhance oxygen transfer has been detailed extensively in the literature.
with varied opinions (Navari, Rosenblum et al. 1977; Riess 1984; Biro and Blais 1987; Riess 1991; Shah and Mehra 1996; Patel and Mehra 1998; Riess 2005). All studies confirm the enhanced oxygen solubility in pure perfluorocarbon moieties, but there is some debate over the effect of pure PFCs on oxygen diffusion, with environmental $pO_2$ apparently dictating the rate of diffusion in some studies and not a factor in other studies. Enhancement of diffusive permeability by the combined effect of increased solubility and diffusivity is the most likely cause of the observed beneficial effect of pure PFCs on oxygen delivery.

Polydimethylsiloxane (PDMS) is a polymeric organosilicon. It generally comes as two highly viscous pourable liquid parts that when mixed in specific proportions and heated, cure to form flexible solids with properties inherent of their individual chemistries. One of the intrinsic properties of all polydimethylsiloxanes is their enhanced oxygen transfer relative to other similar compounds, the result of improved oxygen solubility and diffusivity (diffusive permeability). Published values of PDMS diffusive permeability are in the range 10-200 fold higher than that of culture media or other polymers/plastics used in cell culture (Refojo 1979; Christen and Andreou 2006; Nishikawa, Kojima et al. 2008). Additionally, PDMS is a bio-inert compound generating minimal immune response and for years has been used as a negative standard for biocompatibility tests of novel biomaterials (Keough, Mackey et al. 1985; Ciapetti, Cenni et al. 1993; Ciapetti, Granchi et al. 1995; Jewrajka, Erdodi et al. 2008).

The oxygen transfer benefits of PDMS were first clinically utilized in the development of improved contact lens formulations in the late 1990s. Prior to that, contact lenses had been made of oxygen impermeable variations of
polymethylmethacrylate and other, more oxygen permeable hydrogels. These formulations caused many adverse effects in the eye, primarily due to cell death resultant from low gas transfer. The introduction of silicone hydrogels all but eliminated many of the problems associated with hard lenses and other hydrogel formulations, due to a pronounced increase in oxygen permeability across the lens barrier.

Recently, the oxygen permeability of PDMS has been implemented in the development of cell culture devices, microfluidic systems for well-regulated maintenance of oxygen partial pressures (Fraker, Alvarez et al. 2007). In 2005, the group of Papas et al implemented the use of liquid impermeable PDMS membranes in islet cell culture. They demonstrated, both theoretically and empirically, that the oxygen transfer rate across these membranes was sufficient to maximize surface area coverage (4000 IEQ/cm²) relative to conventional culture densities (175-200 IEQ/cm²) with improved islet function relative to comparable controls (Papas, Avgoustiniatos et al. 2005). Our group applied a similar technology to the culture of murine embryonic pancreatic rudiments at the time of maximal β-cell specification, theorizing that proper physiological oxygenation would result in improved differential expression of insulin producing cells. The results confirmed this hypothesis with rudiments cultured on PDMS/PFC membranes having enhanced proliferation and differentiation as exhibited by fold up-regulation of endocrine genes and comparable protein expression (Papas, Avgoustiniatos et al. 2005). Taken together, these works demonstrate the importance of proper physiological oxygenation in maintaining cells in culture. By avoiding hypoxia and hyperoxia through the use of compounds to enhance oxygen mass transfer to cultured cells, it seems reasonable that cell survival and functional maintenance will be improved.
In this study, we examined the use of novel PFC/PDMS culture platforms in the culture of human islets of Langerhans compared to standard culture methodologies. The PFC/PDMS platforms allowed us to tailor environmental oxygen levels to better match the physiological niche of isolated islets and we theorized that doing so would improve islet viability and function. We investigated the effect of physiological oxygenation by comparing overnight loss, oxygen consumption rate, glucose stimulated insulin release, quantitative real time RT-PCR of inflammatory and apoptosis markers and in vivo efficacy via marginal mass (1,000 IEQ) sub-renal capsular islet transplants in immunodeficient mice. Our hypothesis was that culture at more physiological oxygen $pO_2$ (~90 mmHg compared to 142 mmHg) would result in improved islet viability and function as measured by our potency tests.

5.2 Materials and Methods

5.2.1 35 mm PFC/Si dish manufacture

We set out to build a mass-producible, disposable 35 mm diameter prototype for the experiments described herein. The inner and outer rings are held together by an interference fit, and the latter has three legs that elevate the membrane 1 mm off the surface to allow circulation of air from underneath. Membranes were extruded by Specialty Silicone Fabricators (Paso Robles, CA) according to our specifications. Using their proprietary knife coating micro-film technology, a two-part polydimethylsiloxane (PDMS), RTV615 Part A (938.7 g), RTV615 Part B (312.9 g) (GE Silicones) and Perfluoroctyltriethoxysilane (Alfa Aesar)(408.9 g) were mixed to obtain a 0.012” (305 µm +/- 12 µm ) x 13” (33 cm) x 600” (1,524 cm) roll, from which discs could subsequently be punched. Rapid prototyping was done with the Objet Eden 250 3D
printing system. Prototypes were manufactured with VeroWhite resin to check fit and function. Advanced models were made by Computer Numerical Control (CNC)-assisted precision machining. Injection molding was done with Noryl HNA033, an unfilled, modified polyphenylene ether resin designed to withstand several autoclave cycles and gamma irradiation sterilization. The injection molding of the inner and outer rings of the 35 mm prototype was done at ProtoMold (Maple Plain, MN). The assembly process was done at Biorep Technologies (Miami, FL). For quality control, the assembled dish was placed on a tension meter jig and tension read with a Newman SR-Meter1. Tension had to be within the 16-40 N/cm range. The final product is shown in Figure 5-2.

5.2.2 Diffusivity studies

Oxygen spot sensors (PreSens GmBH, Regensberg Germany) were affixed with epoxy to the center of the bottom surface of wells in a standard 24 well culture plate (VWR Inc, Marietta, GA). Pieces of pre-fabricated PDMS and PFC/PDMS membranes (305 μm +/- 12 μm) were fit into the wells atop the sensors, using quick-drying PDMS along the outer edges to insure an air-tight seal. As a control, three sensor wells were filled with 603 μL (3mm height) Hanks Balanced Salt Solution (HBSS, Invitrogen, CellGro). After equilibration at 38mmHg O₂ (5%), the entire sensor rigging was transferred to the upper stage of an incubator set to standard 95% Room Air/ 5% CO₂ culture conditions. Taking into account vapor pressure differences, this translates to a pO₂ of 142 mmHg. Care was taken not to agitate the well-plate to avoid convective disturbances. The time of exposure to the temperature difference between the incubator and the room (approximately 12 °C) was < 5 sec, to minimize thermal effects. Oxygen
partial pressure was measured in the system until the signal reached an equilibrium point at the new pO\textsubscript{2} setting of 142 mmHg. Data points were recorded every 2 sec for the duration of the experiment.

Diffusion was modeled as that through a single surface of a slab. Modeling assumed that (a) diffusion was 1D through the height of the measured compound, as the sides were treated as impermeable to O\textsubscript{2}; (b) effects due to diffusion through plastic and the edge seal were minimal and (c) temperature shifts from moving the apparatus were negligible.

Membrane thicknesses utilized for the models were the mean, minimum (mean - SD) and the maximum (mean + SD) estimated membrane thicknesses. From this, the mean, maximal and minimal effective diffusivities were tabulated and fold control effective diffusivity determined. Transient solutions of concentrations were generated by iterative numeration. The diffusion coefficient was determined using least squares curve fitting of measured oxygen concentration to the theoretical model:

\[
C = C_s + \frac{4}{\pi} (C_o - C_s) \sum_{n=0}^{\infty} \frac{(-1)^n}{2n + 1} \cos \left[ \frac{(2n + 1)\pi z}{2a} \right] \exp \left[ \frac{-D(2n + 1)^2 \pi^2 t}{4a^2} \right]
\]  

(5-1)

Where, \( C \) is the concentration at time, \( t \); \( C_s \) is the max pO\textsubscript{2} measured; \( C_o \) is the starting pO\textsubscript{2} measured; \( z \) is the height of the sensor above the dish bottom; \( a \) is the solution height; \( D \) is the calculated diffusivity; and \( n \) is the number of iterations used in the series (\( n = 20 \)). The boundary conditions utilized were the \( C_s = 142 \text{mmHg} \), which was assumed to remain constant at the air-solution interface, and zero flux at the plate bottom surface. Saline solutions were used as controls to test the accuracy of the system.
5.2.3 Islet Size Distributions

Prior to experimentation with islets, retrospective analysis of islet size distributions from 184 human isolations was performed. The purpose of this analysis was to determine the size range responsible for the majority of tissue volume from typical isolations in order to target oxygen profiles close to physiological within the majority of the cultured tissue. Total volume was determined as described below. In counting, islet sizes are typically broken down into ranges of 50 micron increments: 50-100, 101-150, 151-200, 201-250, 251-300, 301-350, 351-400 and 401-450. The average radial value from each size range was utilized to calculate volumes for each size range as follows:

\[ V_p = \left( \frac{M_v}{2} \right)^3 \times \pi \times \frac{4}{3} \]  

(5-2)

From this, the total volume for each count was calculated as the sum of the number from each range, \( \# \), multiplied by the volume from each range, as follows:

\[ V_T = \sum_{50-100}^{401-450} \# \times V_p \]  

(5-3)

The contribution of each size range to the total count volume was then calculated as the quotient of the size range volume over the total count volume.

5.2.4 Finite Element Modeling of Islet Oxygen Gradients

Islets from 13 individual preparations were utilized after overnight culture in conventional settings. The oxygen consumption rate of the islets was assessed using triplicate aliquots of 500 IEQ in a stirred chamber oxygen measurement device (Instech Labs, Plymouth Harbor, PA). Two dimensional diffusion/reaction theoretical modeling
the governing species conservation diffusion/reaction equation.

\[
\frac{dc}{dt} - D\nabla^2 c = R 
\]  

(5-4)

where \(c\) represents concentration, \(D\) is the diffusion coefficient and, \(\nabla^2 c\), the Laplacian operator (the second derivative of concentration with respect to one spatial direction, since each geometry is symmetric about a plane, line or point) and \(R\), the reaction rate or oxygen consumption rate of the tissue. \(R\), the rate of oxygen consumption per unit volume of tissue, is expressed by a simplified representation of the dependence of oxygen consumption on the concentration of oxygen. This is described by Michaelis-Menten kinetics:

\[
R = R_{max} \times \frac{c}{c + K_m}, \text{ when } c \geq K_m , \text{ otherwise if } c \leq K_{critical} \quad R = 0 
\]  

(5-5)

where \(R_{max}\) is the maximal consumption rate per unit volume of tissue determined by our experimental OCR measurements. Figure 5-1, following displays a sample oxygen trace from a baseline OCR measurement on human islets:
Figure 5-1: Representative oxygen trace of islet cells measured in a stirred oxygen consumption microchamber.

\( K_m \) is the Michaelis-Menten constant: the oxygen concentration where the consumption rate is \( \frac{1}{2} \) of \( R_{\text{max}} \). The \( K_m \) utilized, 0.44 mmHg, was derived from the value for mitochondrial oxidative phosphorylation corrected for the solubility dependence on temperature. This value has been utilized in prior diffusion reaction modeling of endocrine tissue (Papas, Avgoustiniatos et al. 2005; Avgoustiniatos, Hering et al. 2008). This value was also applied in the determination of anoxia. Tissues with oxygen levels below the \( K_m \) value were considered to be approaching anoxic conditions. A tissue \( \text{pO}_2 \) of <0.1 mmHg was designated as \( K_c \) and anoxic, i.e. not contributing to the cellular oxygen consumption rate (OCR = 0).

The unique solution was determined through the application of two boundary conditions which were dependent on the particular culture system schematic setup. For the control system, the boundary conditions utilized were:
\[ c = c_0 \]  

(5-6)

for the top culture medium surface and insulation/symmetry along the plastic sides and bottom of the culture device to represent the no flux boundary condition

\[- D \nabla c = 0.\]  

(5-7)

The modeling was carried out, to the time of system equilibrium, depending on the height of culture medium applied. The time to solution in the time dependent solver was estimated by the ratio of the path length of diffusion squared (cm\(^2\)) to the diffusivity of oxygen through the culture medium (cm\(^2\)/s),

\[ \frac{L^2}{D}. \]  

(5-8)

To account for material flux differences, dictated by the partition coefficient, the diffusivity was expressed as the product of the effective oxygen diffusivity (literature values) and the oxygen solubility of each material. The solubility factor dictates the partition coefficient, which is typically expressed as the ratio of one material solubility to another. Modeling was performed in terms of oxygen partial pressure in mmHg rather than concentration values, expressed in mol/m\(^3\). This method has been validated in prior modeling performed by Avgoustiniatos et al in similar culture systems (Avgoustiniatos, Hering et al. 2008). Table 5-1, following outlines the coefficients utilized in the modeling:

<table>
<thead>
<tr>
<th>Coefficient</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCR</td>
<td>4E-02 mol/m(^3) s</td>
</tr>
<tr>
<td>( K_m )</td>
<td>0.44 mmHg</td>
</tr>
<tr>
<td>( K_C )</td>
<td>0.1 mmHg</td>
</tr>
<tr>
<td>( P_{\text{tissue}} )</td>
<td>1.3E-12 mol/m\cdot mmHg\cdot s</td>
</tr>
<tr>
<td>( P_{\text{media}} )</td>
<td>3.9E-12 mol/m\cdot mmHg\cdot s</td>
</tr>
<tr>
<td>( P_{\text{PFC/PDMS}} )</td>
<td>1.64E-10 mol/m\cdot mmHg\cdot s</td>
</tr>
</tbody>
</table>

Table 5-1: Coefficients utilized for finite element modeling (FEM) of islet culture systems
The PFC/PDMS systems were modeled identically to the control systems with the only difference being a concentration boundary condition on the bottom surface, representing the oxygen permeability of the PFC/PDMS. This model displayed the advantage of the proposed experimental system providing oxygen from both the apical and basal surfaces, with the greatest oxygen supply coming from below the cells. Theoretical modeling was performed to determine incubator oxygen settings for the low oxygen groups, targeting physiological core levels in the maximal tissue volume in the PFC/PDMS group with a constrained culture density not exceeding 200 IEQ/cm$^2$, the standard.

5.2.5 Pre and Post Culture IEQ Enumeration and Loss Determination

Prior to culture experiments, IEQ number was assessed by dithizone staining and counting with an inverted stereo microscope using a graded reticule. Aliquots of equal IEQ number per group were utilized for culture. At the end of the overnight culture period utilizing experimental conditions, all IEQ from each group were collected and enumerated in the same fashion, individually. IEQ loss was expressed as a percentage of the initial IEQ number. These values were averaged amongst all preps and expressed as mean % IEQ loss ± SEM. Further, individual IEQ counts were expressed as fold control. These values were averaged amongst all preps and expressed as mean fold control ± SD. There was a difference in $n$ for each group, as culture in physiological oxygen on conventional plastic and on PFC-PDMS in standard oxygen was eliminated to maximize islets.
5.2.6 Pre and Post Culture Oxygen Consumption Rate

Oxygen consumption rate measurements were performed using the BD Oxygen Biosensor system, as previously described (Fraker, Timmins et al. 2006). Briefly, triplicate aliquots of 500 IEQ (200 µL) were plated in wells of low and high glucose (2.2 and 22 mM) modified Kreb’s buffer containing 25 mM Hepes buffer and 0.2% BSA. Kinetic fluorescence measurements were made every 5 minutes for 3 hours and recorded values were corrected against “dry” (air) and “wet” (buffer containing). Additionally, 100 mM sodium sulfite solutions were plated as a maximum consumption positive control. After the kinetic read, IEQ aliquots were collected and solubilized in AT-extraction solution for DNA analysis using the picogreen dSDNA detection kit. OCR in low and high glucose solution was determined by the initial slope of the fluorescence change, as previously described, and the index of high slope relative to low slope was used an islet potency metric, shown to significantly correlate with subrenal capsular transplant outcome in diabetic athymic nude mice in prior work. The indices from individual preps were expressed as fold control index for all groups. These values were averaged amongst all preps and expressed as mean fold control ± SEM.

5.2.7 Pre and Post Culture Glucose Stimulated Insulin Release (GSIR)

As a measure of islet secretory function, triplicate aliquots of 100 IEQ were suspended in a Sephadex G10 bead slurry and added to 10 mL polyprep microchromatography columns (Biorad). Additional beads were added to a volume of 1 mL and the islet/bead slurry was packed by addition of a modified Kreb’s low glucose buffer (2.2 mM) containing 25 mM Hepes, 26mM Sodium Bicarbonate and 0.2% BSA. Columns were placed in a standard 37 °C 95% RA/ 5% CO₂ incubator for a one hour pre-incubation period to allow the cells to recover from loading/manipulation and
adjust to culture settings. After the first hour, an additional 4 mL of Kreb’s buffer was added to elute the pre-incubation volume and begin the glucose challenge. For the next three hours, sequential incubations and collections in low, high and low glucose were performed. Samples were immediately frozen at -80 °C for later insulin analysis using the Mercodia Insulin Elisa (Mercodia, Inc). Other work has demonstrated that the delta insulin (insulin produced in high glucose – insulin produced in low glucose) per 100 IEQ has a high sensitivity and specificity in predicting transplant outcome in sub-renal capsular transplants in diabetic athymic nude mice (see Chapter 4). Based on the pre-culture glucose stimulated insulin results coupled with the pre-culture oxygen consumption assay, islets were classified as sub-optimal/marginal or good quality. This classification allowed us to better interpret transplant outcome in athymic nude mice. The individual delta values from each islet preparation were expressed as fold control delta. These were averaged amongst all islet preps and expressed as fold control ± SEM.

5.2.8 Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction

Real-Time quantitative reverse transcription polymerase chain reaction was performed on three preparations, where comparison was done between optimized groups (control versus PFC-PDMS physiological pO₂). Total RNA was purified using Qiagen kits (QIAshredder, RNeasy, and DNase-free; Qiagen, Hilden, Germany). The First-Stand system (Roche Diagnostics, Basel, Switzerland) was used to generate cDNA (random oligomers). Relative gene expression was quantified using TaqMan Low Density Array (TLDA) Cards designed for the measure of inflammation and apoptosis markers in a 7500 Fast Real Time polymerase chain reaction (PCR) cycler (Applied Biosystems, Foster City, CA). The ΔCt method for relative quantification was deemed optimal for this application after discussion with Applied Biosystems researchers. All assays were designed to span exon-exon junctions, thus eliminating the
possibility of genomic DNA contamination. Quantitative reverse transcription (qRT)-PCR results are the average of several independent experiments. In addition, each marker utilized was measured in triplicate. Gene expression was normalized against 18S and beta actin ribosomal RNA. Both endogenous controls have been validated in our system and proven extremely stable and more accurate over varying oxygen concentrations than other potential housekeeping gene standards.

5.2.9 Marginal Mass Sub-Renal Capsular Islet Transplants In Athymic Nude Mice

After initial in-vitro studies, two groups were used for further in-vivo investigation: 1) control culture in conventional plastic ware and 95%RA/5% CO₂; and 2) PFC/PDMS culture in an optimal pO₂ as determined by oxygen consumption measurements and finite element modeling. Animal protocols were approved and monitored by the University of Miami IACUC. As previously described, athymic nu/nu (nude) mice were purchased from Harlan Laboratories (Indianapolis, IN) and housed in virus- and antigen-free rooms in micro-isolated cages at the Division of Veterinary Resources of the University of Miami (Ichii, Pileggi et al. 2005). Animals were rendered diabetic via a single intravenous administration of 200mg/kg of Streptozotocin (Sigma-Aldrich, St. Louis, MO). Non-fasting blood glucose was assessed by glucometer (Elite, Bayer; Tarrytown, NY or OneTouchUltra2, LifeScan, Milpitas, CA) and mice with sustained hyperglycemia (> 300 mg/dl) were designated for islet transplant. Marginal mass grafts (~1,000 IEQ per recipient) were transplanted under the kidney capsule (3 per group). For impure islet preparations (<85% observed purity), the cells were transplanted across both sides of the kidney to avoid oxygen limitations caused by contaminating tissue mass. Marginal mass islet transplantation leads to the reversal of hyperglycemia, but with measurable delay from the time of implantation. An
effective experimental treatment should lead to a reduction in the time to reversal. This model allowed us to assess the effects of pre-transplantation culture (24 h) of human islet preparations using PFC/Si devices.

After transplantation, non-fasting blood glucose values were assessed daily for the first 2 weeks and then 3 times a week thereafter. Reversal of diabetes was defined as stable non-fasting blood glucose < 200 mg/dl for 5 consecutive days. For the purpose of analysis, non-reversal was assigned a time point of 100 days, the typical length of post-transplant follow-up. Nephrectomy of the graft-bearing kidney was performed to confirm return to hyperglycemia and exclude residual function of the native pancreas in animals achieving and maintaining normoglycemia after transplantation. Animals with residual function after nephrectomy were excluded from the analysis as technical failures. Analysis examined differences in time to reversal.

5.2.10 Statistical Analysis

For in vitro islet culture analysis, all data was expressed as the mean fold control value ± SEM. The Kruskal-Wallis non-parametric one-way analysis of variance was performed for comparison amongst all groups, assuming that the data was not normally distributed. The Dunns post hoc test was used to determine statistically significant differences between the groups. A p value < 0.05 was considered significant. Values of p < 0.01 were considered highly significant. Kaplan-Meier survival analysis with log-rank test for significance was performed on transplant data.
5.3 Results

5.3.1 35mm PFC/Si dish manufacture

Figure 5-2(a), following, displays the general premise of our device design, highlighting the delivery of oxygen at both the apical and basal surface. The computer aided design (CAD) drawings in Figure 5-2(b) illustrate the design prototype of the mass-produced 35 mm culture surfaces used in the islet experiments, highlighting the two-piece snap assembly that draws the bottom membrane to a maximum tautness. Figure 5-2(c), shows the print-mold rapid prototypes of the support platform for the PFC-PDMS membranes.

Figure 5-2: (a) Schematic representation of PFC-PDMS culture platform compared to conventional culture. (b) CAD drawings of 35mm PFC-PDMS design. (c) Printed rapid prototype of PFC-PDMS frame.
5.3.2 Diffusivity studies

Theoretical calculations and functional studies have shown that oxygen solubility in our dishes to be about 13-20 fold that of conventional dishes and culture media (data not shown). Furthermore, the O₂ diffusivity through the bottom membrane was theoretically calculated as several orders of magnitude greater than through conventional plastics utilized for standard culture platforms. The measured fold mean diffusivity of PFC-PDMS films relative to PDMS alone was 1.53 ± 0.31. The minimal fold difference between the two groups (MIN PFC-PDMS/MAX PDMS) was 1.31 ± 0.27 and the maximal fold difference (MAX PFC-PDMS/MIN PDMS) was 1.78 ± 0.37. The maximal and mean fold differences were statistically significant by student’s t-test (p = 0.014 and p = 0.033, respectively), but the minimal fold difference was not (p = 0.105). This measured increased effective diffusivity, coupled with a theoretical increased solubility of approximately 1.2 fold, afford the PFC-PDMS platforms a 1.6-2 fold increase in diffusive permeability. While not a dramatic difference when thin membranes are utilized, the PFC-PDMS membranes demonstrated both by computerized mathematical modeling (COMSOL), as well as direct in vitro measurements (Fraker, Alvarez et al. 2007; Fraker 2008) maximize the volume of tissue exposed to physiological pO₂ concentrations.

5.3.3 Islet Size Distributions

Figure 5-3, following, shows the histogram of the islet volume distribution of the 184 consecutive islet isolations used for analysis. The data is expressed as mean volume percent for each size range ± SD.
The majority of the tissue volume (77% pre-ficoll and 80% post purification in layer 1) fell in the range of 100-300 µm, with the largest percentage falling between 150-200 µm. This volume distribution was utilized for finite element modeling to maintain the largest tissue percentage (IEQs between 100-300 µm) at or near physiological pO\textsubscript{2} while minimizing anoxia and hyperoxia (>0.1 mmHg and <100 mmHg pO\textsubscript{2}).

5.3.4 Finite Element Modeling

Figure 5-4, following, shows the results of theoretical modeling for all four culture groups (conventional culture on plastic ware, conventional culture on PFC/PDMS, physiological pO\textsubscript{2} on plastic ware and physiological pO\textsubscript{2} on PFC/PDMS).
The letters A-E represent the increasing IEQ size ranging in 50 µm increments from 100 µm to 300 µm.

Figure 5-4: FEM modeling results of IEQ of all size ranges (100-300µm) in every culture setting (Control in conventional 95% Room Air/5% CO₂, PFC/PDMS in conventional 95% Room Air/5% CO₂, Control Low O₂, PFC/PDMS Low O₂), where A-E represent the increasing IEQ size ranging in 50µm increments from 100µm to 300µm.

Theoretical models of islets cultured in the PFC/PDMS indicated that optimal physiological tissue oxygenation occurred in an environmental pO₂ ranging from 65-95 mmHg, depending on the islet oxygen consumption rate (ranging from 8.2-12.5% in this study). The model results were examined based on three parameters: percentage of anoxic tissue volume; percentage of hyperoxic tissue volume; and percentage of tissue in
designated physiological range. Only the ~80% of IEQs in the size range from 100-300 µm were measured using this algorithm. Table 5-2, following details the outcome of modeling, when the theoretically determined optimal external pO₂ of 95 mmHg was utilized.

<table>
<thead>
<tr>
<th>Group</th>
<th>% anoxic tissue (pO₂ &lt; 0.1mmHg)</th>
<th>% hyperoxic tissue (pO₂ &gt; 100mmHg)</th>
<th>% physiological tissue (5mmHg&lt;pO₂&lt;100mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12.6%</td>
<td>0%</td>
<td>52.0%</td>
</tr>
<tr>
<td>(pO₂ = 142mmHg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>22.4%</td>
<td>0%</td>
<td>41.8%</td>
</tr>
<tr>
<td>(pO₂ = 95mmHg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PFC</td>
<td>0%</td>
<td>42%</td>
<td>37.4%</td>
</tr>
<tr>
<td>(pO₂ = 142mmHg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PFC</td>
<td>1.2%</td>
<td>0%</td>
<td>74.5%</td>
</tr>
<tr>
<td>(pO₂ = 95mmHg)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5-2: *Estimations of anoxic, hyperoxic and physiologic tissue percentages in all experimental culture conditions. Results based on FEM modeling calculations.*

In all three criteria, the theoretical models favored islets cultured at reduced pO₂ (65-95 mmHg) on PFC/PDMS relative to all other groups. In the PFC/PDMS group at 95 mmHg, anoxic tissue was almost eliminated, the largest percentage of tissue was within the physiological range, and no hyperoxic tissue was predicted in this optimized culture setting. Conversely, all other groups either had a higher percentage of anoxic tissue (control plastic ware, and standard and reduced pO₂ culture conditions) and a
reduced physiological tissue region, or contained a large hyperoxic portion of tissue (PFC/PDMS standard culture conditions).

5.3.5 Pre and Post Culture IEQ Enumeration and Loss Determination

Figure 5-5, following, shows the results of IEQ enumeration following overnight culture. The results are expressed as mean fold control cultured at standard pO$_2$ ± SEM.

![Fold Control Overnight Loss](image)

* $p < 0.05$ vs control

Figure 5-5: IEQ enumeration following overnight culture. Expressed as mean fold control cultured at standard 95% RA/5% CO$_2$. The red line represents mean control values (always expressed as 1) and the red asterix signifies mean fold values significantly different from the control ($p < 0.05$).

The results show a significant reduction ($p = 0.011$) in overnight loss when islets are cultured in physiological oxygen levels on our PFC-PDMS platforms when compared to standard culture conditions. The mean loss in this group was 40% of the loss
experienced in culture in conventional plastic ware in conventional incubator conditions. Islets cultured in conventional plastic ware at physiological pO$_2$ exhibited and expected increased loss (132% of control) which is in line with reduced oxygen and more severe diffusion gradients. The islets cultured in PFC-PDMS platforms at standard pO$_2$ also had reduced islet loss relative to the control condition (92%), although not significant.

5.3.6 Pre and Post Culture Oxygen Consumption Rate

Figure 5-6, following, shows the fold standard culture condition oxygen consumption indices for all culture groups. Values are expressed as mean fold ± SEM.

![Fold Control OCR Index](image_url)

* * p < 0.05 vs control

Figure 5-6: Mean fold oxygen consumption indices relative to control culture conditions on plastic ware in 95%RA/5% CO$_2$ incubator. The red line represents mean control values (always expressed as 1) and the red asterix signifies mean fold values significantly different from the control (p < 0.05).
The islets cultured in physiological oxygen levels on the PFC-PDMS culture platforms were significantly superior to all other culture groups (p << 0.01). Those cultured in physiological oxygen levels on standard plastic culture ware were significantly lower than the relevant control (p << 0.01). Islets cultured at standard culture oxygen levels on PFC-PDMS platforms also had better oxygen consumption indices, but the comparison with conventional plastic ware was insignificant (p = 0.38).

5.3.7 Pre and Post Culture Glucose Stimulated Insulin Release (GSIR)

Figure 5-7, following, displays the fold standard culture GSIR delta values for all culture groups. Values are expressed as mean fold control GSIR delta ± SEM.

![Figure 5-7: Fold standard pO\textsubscript{2} control GSIR Delta for all culture groups. The red line represents mean control values (always expressed as 1) and the red asterix signifies mean fold values significantly different from the control (p < 0.05).](image)

As with the overnight loss IEQ enumeration and the oxygen consumption index, the islets cultured in physiological pO\textsubscript{2} had significantly improved insulin secretion in
response to glucose challenge compared to the conventional culture condition, as measured by the delta metric (p << 0.01).

5.3.8 Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction

The TLDA cards utilized were designed to quantitatively measure the expression of a panel of 94 genes involved in apoptosis and, in one preparation, inflammation. In one preparation, HP1915, apoptosis was significantly down regulated in islets cultured on PFC-PDMS platforms at physiological pO\textsubscript{2}. Figure 5-8, following details the fold expression (control relative to PFC) of 12 genes particularly integral in the apoptosis cascade including BCL2, BCL2 like protein 11, several proteins of the caspase cascade, (Caspases 1, 2, 4, 6, 7, 9 and 10), DEDD 2 (DNA-binding death effector domain-containing protein 2, targets CASP 8 and CASP 10 to the nucleus), FAS ligand and PMAIP 1 (Phorbol-12-myristate-13-acetate-induced protein 1, promotes activation of caspases and apoptosis).
In the two other preparations analyzed, HP1927 and HP1970, apoptosis was comparable in the two groups analyzed (control conventional culture and PFC/PDMS physiological pO\textsubscript{2}). In one islet preparation, HP1927, several of the genes analyzed had lower level of expression in the control group relative to the PFC-PDMS group. In the other preparation, HP1970, all apoptotic genes were down-regulated, but not nearly as significantly as exhibited in HP1915. Figure 5-9, following, details the results of apoptosis analysis of both of these preparations.
Figure 5-9: Fold expression of apoptosis markers in islets culture on conventional plastic-ware relative to those cultured on PFC-PDMS (HP1927 solid bars, HP1970 gradient bars).

In HP1970, to address this observed reduction in apoptosis down-regulation, baseline expression levels prior to the varied cultures were performed. We observed higher absolute levels of expression in HP1970 vs. HP1915, even in comparison of baseline values of HP1970 with post-culture values of HP1915, suggesting that, for the most part, these islets were of lower quality than those of HP1915. Additionally, absolute expression levels of HP1927 were higher than those of HP1915 after overnight culture, indicating probable irreversible apoptosis.

In addition to genes associated with apoptosis, genes related to inflammation were also investigated for HP1970. Interestingly, nearly all genes associated with inflammation were up-regulated in the control group relative to islets cultured in PFC-PDMS and ranged from 1.57-55.44 fold. Additionally, inflammatory gene expression for
all markers was higher in the baseline samples than in the PFC-PDMS group, perhaps indicating that this preparation was suboptimal and irreversibly damaged even before our experimental culture intervention.

5.3.9 Marginal Mass Sub-Renal Capsular Islet Transplants In Athymic Nude Mice

Based on our observations during in vitro studies, we examined the results of animal studies using retrospective potency analysis as a means of segregating the transplanted preparations into “good” and “poor” performance classification. Our basis for classification was either or both of the following potency assessments: oxygen consumption rate index, where we previously demonstrated that a cutoff value of 1.27 ((Fraker, Timmins et al. 2006) delineates rapid versus slow reversal in the nude mouse bioassay; glucose stimulated insulin release delta value, where we showed that a cutoff value of 425 µU/mL also acts as a predictive marker nude mouse bioassay transplant outcome (see Chapter 4).

Five preparations were transplanted: HP1912, HP1915, HP1927, HP1970, and HP1977. Of these five preparations, based on our potency criteria, three were retrospectively classified as “poor” preparations and two, as “good” preparations. Table 5-3, following, details the results of the potency tests performed. In two preparations, HP1927 and HP1970, only one potency assessment, GSIR, was utilized as there was a technical malfunction in the performance of the OCR assay of both preparations.
Based on the potency results, survival analysis was performed on the “good” and “poor” preparation sub-classifications, separately. Figures 5-10a and 5-10b, following detail the results of survival analysis performed on the “good” and “poor” preparations, respectively.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>GSIR Delta (μU/mL)</th>
<th>Cutoff Value (μU/mL)</th>
<th>OCR Index</th>
<th>Cutoff Value</th>
<th>Good/Poor</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP1912</td>
<td>572 ± 130</td>
<td>425</td>
<td>1.95 ± 0.49</td>
<td>1.27</td>
<td>Good</td>
</tr>
<tr>
<td>HP1915</td>
<td>337 ± 58</td>
<td>425</td>
<td>1.19 ± 0.07</td>
<td>1.27</td>
<td>Poor</td>
</tr>
<tr>
<td>HP1927</td>
<td>283 ± 17</td>
<td>425</td>
<td>N/A</td>
<td>1.27</td>
<td>Poor</td>
</tr>
<tr>
<td>HP1970</td>
<td>148 ± 128</td>
<td>425</td>
<td>N/A</td>
<td>1.27</td>
<td>Poor</td>
</tr>
<tr>
<td>HP1977</td>
<td>2184 ± 601</td>
<td>425</td>
<td>2.01 ± 0.26</td>
<td>1.27</td>
<td>Good</td>
</tr>
</tbody>
</table>

Table 5-3: Potency assessment values for preparations utilized for nude mouse bioassay

Figures 5-10a: Survival analysis of marginal mass transplants (1,000 IEQ) from “good” preparations. The control islet grafts are represented by the dashed lines and those cultured at physiological pO₂ on PFC/PDMS culture platforms by the solid lines.
The survival analysis correlated with our *in vitro* studies. There was no significant difference in reversal time in the “poor” preparation group (p = 0.66), as in all previous studies of potency assessment and gene expression analysis. This supports our hypothesis that our platform is unable to “rescue” islets that are suboptimal at the start of intervention. There was no significant difference in the survival curves in the “good” preparation group (p = 0.34), perhaps due to the small n observed (n = 6). There was a trend toward improved survival and perhaps with a greater number of experiments, this will be demonstrated. It should be noted that an animal in each group was excluded from analysis, one due to non-return to hyperglycemia after nephrectomy, the other because of potential failure due to infection.
5.4 Discussion

Taken together, our data indicate that culture at physiological pO$_2$ on gas permeable platforms capable of enhancing oxygen mass transfer is superior when compared to conventional culture in standard incubator conditions (95%RA/5% CO$_2$). There is a clear improvement of islet function and viability both in vitro and in vivo. There were several take away messages from this battery of work, as well.

First, critical in assessing the value of utilizing a preparation for transplantation, multiple potency assessments should be performed, as no one test is a clear indicator of graft success. We had results in vitro that were contrary to expectations (lower IEQ count in HP1977 PFC/PDMS) with outstanding in vivo results. Conversely, we had exceptional in vitro results (HP1912) where there were inexplicable graft failures (potential infection in one PFC/PDMS, return to hyperglycemia in another after 22 days normoglycemic). This collected data indicates the necessity of several reliable tests run concurrently in determining islet function prior to culture.

Second, it is clear that proper determination of pO$_2$ level prior to culture on PFC-PDMS (based on baseline OCR) is critical to the experimental outcome. In one of the four transplant preparations (HP1970), pre-plating OCR was not performed and the incubator was set to 8%, which is the lower end of our culture range and is designed for an islet preparation with a reduced oxygen consumption rate ($< 4E-02$ mol/m$^3$ s$^{-1}$). It is likely that this lower pO$_2$ was insufficient to maintain physiological pO$_2$ in the PFC-PDMS group, resulting in impaired function and viability as measured by our potency metrics and bioassay transplant outcome.
Finally, and perhaps, most importantly, it was clear from our studies that while PFC-PDMS culture is beneficial to “good” islets with potency indicators (GSIR, OCR) associated with rapid reversal in full mass transplants and gene profiles associated with low levels of apoptosis and inflammation, the same culture has no or minimal beneficial effect on marginal or suboptimal preparations, often resulting in decreased potency and increased/comparable up-regulation of apoptosis and inflammation gene expression compared to islets in standard culture conditions.

In our future work, we hope to continue investigating the correlation between the culture of “good” preparations and determine if islet number can be reduced in grafts, given the improved function and gene profile associated with these islets when cultured on our novel platforms. It is clear that rapid assessment of potency and a careful tailoring and/or study of oxygen pO$_2$ levels could further enhance our outcomes. Ultimately, there is a clear need for improving islet culture to optimize post-culture clinical outcome. Future studies will seek to further optimize our device to aid in this endeavor.
6.1 Perfluorocarbon Emulsions as Oxygen Carriers

Critical to sustaining cell viability and function during culture and, further, in transplantation applications, is the maintenance of adequate oxygen supply, as mentioned in Chapter 5. Typical cell culture in plastic dishes is suboptimal requiring supra-physiological environmental pO$_2$ to prevent the formation of oxygen gradients and deprivation across cultured cells. This lack of adequate oxygen transfer in culture medium, with oxygen solubility and diffusivity coefficients nearly identical water, results in limitations in plating density, medium depth, and cell dimensions to prevent tissue anoxia. Particularly, in cell aggregates with dimensions an order of magnitude greater than single cell populations (150-300 µm compared to 5-10 µm), such as islets of Langerhans, oxygen mass transfer limitations often dictate survival in culture and graft success or failure. Further, in tissue engineering applications, where cells/organoids are often encased in protective materials for immunoprotective purposes, oxygen mass transfer limitations are severely exacerbated. There is a substantial need for methods, therefore, that enhance oxygen mass transfer in all areas of cell harvest, culture and transplant, ideally, maintaining adequate oxygenation of cellular products and doing so in a physiological fashion more closely approximating the in vivo niche.

One potential material for improving oxygen mass transfer in all the aforementioned applications, is though the use of perfluorocarbons. Perfluorocarbons, or PFCs, are chemically inert compounds constructed of long carbon chains with terminal
fluorine atoms. This unique characteristic gives them a substantial ability to bind and transfer molecular oxygen. Functional studies of the varied pure PFCs have shown that they have oxygen solubilities approximating or surpassing hemoglobin at different oxygen saturations and about 20 times that of water or culture medium (at pO$_2$ of 760 mmHg oxygen) (Lowe 1987; King, Mulligan et al. 1990; Faithfull 1992). Further, they have an oxygen diffusivity that is approximately 2.5-5 fold that of water or culture medium. These mechanisms, by which the pure PFCs enhance oxygen transfer, has been described extensively in the literature as an improved oxygen diffusive permeability, P$_D$, the product of oxygen solubility and effective oxygen diffusivity, D$_{eff}$. (Papas, Hering et al. 2005; Avgoustiniatos, Hering et al. 2006).

The oxygen dissolving and shuttling capacity of PFCs creates an oxygen sink, when in pure form, that provides a substantial reservoir of oxygen for biological applications. For example, biphasic solutions of dense pure perfluorocarbons and conventional UW preservation solution/culture medium have been used in the field of pancreas preservation and islet cell isolation with promising results, improving islet yields per pancreas and improving viability and function during islet culture (Matsumoto and Kuroda 2002; Matsumoto, Rigley et al. 2002; Ricordi, Fraker et al. 2003).

Due to their hydrophobicity, immiscibility, and density, the most common form for PFCs in biomedical applications is as nano-scale emulsions/micellar suspensions. PFCs are typically suspended in aqueous solutions of amphiphilic/nonionic surfactants, where high pressure microfluidization or sonication are used to generate stable, micro or nano scale emulsions. Surfactant selection is based on a property known as the hydrophilic/lipophilic balance or HLB, an inherent characteristic of the different regions
of the molecule. A simple formula developed by WC Griffin in 1954 calculates the HLB number as follows:

\[
HLB = 20 \times \frac{M_h}{M}
\]

where, \(M_h\), is the molecular mass of the hydrophilic portion of the molecule and, \(M\), is the molecular mass of the entire particle. Using this formula, surfactants are categorized as in Table 6-1, following:

<table>
<thead>
<tr>
<th>HLB #</th>
<th>Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-3</td>
<td>Anti-Foaming Agent</td>
</tr>
<tr>
<td>4-6</td>
<td>Water in Oil (W/O) Emulsifier</td>
</tr>
<tr>
<td>7-9</td>
<td>Wetting Agent</td>
</tr>
<tr>
<td>8-18</td>
<td>Oil in Water (O/W) Emulsifier</td>
</tr>
<tr>
<td>13-15</td>
<td>Detergent</td>
</tr>
<tr>
<td>10-18</td>
<td>Solubilizer or Hydrotrope</td>
</tr>
</tbody>
</table>

Table 6-1: *HLB number classifications of surfactant and their common description*

In selecting perfluorocarbons, the body of work has demonstrated that volatility, oxygen solubility and diffusivity, and ease of emulsion formulation dictate the commonly utilized PFCs.

As most PFC emulsions are classified as oil in water emulsions (hydrophobic core), typical formulations are manufactured by the microfluidization of high HLB# surfactants such as PEG-based copolymers and phospholipids/cholesterols combined with a given weight or volume percentage of PFC and an aqueous physiological base solution. The most commonly utilized PFCs, due to the above criteria, are Perfluoroctyl bromide (PFOB), Perfluorodecalin (PFD) and Perfluorotributyl/ethylamine(s) (FC-43, for example) (Riess 1984; Biro and Blais 1987; Lowe 1987; Faithfull 1992; Biro 1993; Lowe
2000; Riess 2005). Studies into the characteristics of these emulsions have demonstrated that they maintain the oxygen dissolving and transfer characteristics of their pure counterparts, dependent on the concentration of PFC utilized and the environmental pO$_2$ (Biro and Blais 1987; Faithfull 1992; Biro 1993; Lowe 2000).

Originally, these emulsions were designed to serve as intravenous artificial blood for transfusion, a desirable alternative given the recent risks associated with whole blood transfusions. In numerous clinical trials, these emulsions have demonstrated equivalent and even superior results in traumatic injury studies with few reported adverse side effects (Riess 1984; Biro and Blais 1987; Lowe 1987; Faithfull 1992; Biro 1993; Patel and Mehra 1998). What has been reported in these studies is the requirement for emulsion stability and sub-micron particle size to prevent thrombotic injury to transfusion recipients.

As mentioned before, PFC emulsions have also been investigated extensively for their oxygen transfer characteristics, but typically only in the context of transfusion applications where elevated pO$_2$ (nearing 760 mmHg) is maintained to maximize transfusion benefit in critically injured patients (Junker, Hatton et al. 1990; Junker, Wang et al. 1990; Ju, Lee et al. 1991; Shah and Mehra 1996; Patel and Mehra 1998; Patel and Mehra 1998; Perevedentseva, Zaritskiy et al. 1998). The methods and results of the numerous studies have differed greatly, some groups finding increased oxygen diffusivities and solubilities in perfluorocarbon emulsions and others finding outcomes in disagreement with Boyle’s law, claiming solubilities and diffusivities that vary non-linearly with environmental oxygen partial pressure. There is, among this body of literature, a consensus agreement that oxygen transfer is enhanced by the presence of
perfluorocarbon moieties, yet one omission from many of the studies is full characterization of the emulsions and the role that individual components of the emulsion might have on oxygen transfer characteristics. Most groups have assessed emulsion stability and particle size by filtration through membranes of known pore diameter, conventional microscopy and earlier generation spectrophotometric techniques, perhaps underestimating their stability and micellar size (Millard 1994; Lowe 2000; Riess 2005; Khattak, Spatara et al. 2006; Khattak, Chin et al. 2007). Additionally, most characterization of diffusivity and solubility in the literature are based on theoretical calculations using data acquired from pure perfluorocarbons. There has been little evidence of accurate characterization of PFC content in manufactured emulsions in the literature, with most groups assuming little loss during processing, despite the well-documented volatility of PFCs (Freire, Dias et al. 2005; Khattak, Chin et al. 2007; Chin, Khattak et al. 2008; Johnson, O'Sullivan et al. 2011). It is likely, therefore, that some of the variations in mass transfer characteristics reported in the literature are due to a lack of accurate emulsion characterization. To date, there is no detailed study of the effect of emulsion stability and particle size on the temporal oxygen mass transfer characteristics of PFC emulsions.

One area where PFC emulsions could have a potential impact is the field of cell and tissue engineering. Unlike immiscible pure PFCs, emulsions are easily mixed and implemented in aqueous culture and transplant settings. Particularly in the transplant setting, where \( \text{pO}_2 \) is far below the environment of the standard culture incubator and where cells suffer hypoxic injury, PFC emulsions could ameliorate this initial stress and increase cell viability/function in the immediate post-transplant period. Studies utilizing
PFC emulsions have demonstrated this effect with improved cell growth rates and viability/function in bacterial bioreactors and engineered cardiac tissue constructs (Junker, Hatton et al. 1990; Junker, Wang et al. 1990; Radisic, Deen et al. 2005; Iyer, Radisic et al. 2007).

Another application in tissue engineering where PFC emulsions could have great benefit is the field of cellular encapsulation. Oxygen transfer limitations in this area of research are only exacerbated by the impeded diffusion through the hydrogel polymer constructs typically utilized (polyethyleneglycol, alginate, agarose). Additionally, the typically macro-scale devices implemented result in suboptimal cell loading densities and pronounced anoxia/necrosis in larger 3D aggregates, both in vitro and in vivo. This limits the clinical scale up and applicability of many promising therapies that have worked in smaller animal and pre-clinical models. Clearly, therefore, there is a need for the development and optimization of techniques and technologies for enhancing oxygen mass transfer to isolated cells and tissues.

In this study, we sought to carefully examine the critical factors that affect PFC emulsion stability, including PFC type, surfactant combinations, temperature, emulsification pressure and emulsification time. In addition, we fully evaluated the capacity of these PFC emulsions to enhance oxygen mobility, specifically through the characterization of oxygen diffusive permeability, $P_D$.

Based on prior literature, the perfluorocarbons selected for this study were Perfluorooctylbromide (PFOB), Perfluorodecalin (PFD) and Perfluorotributylamine (FC-43). All have been utilized in a variety of clinical capacities and some in FDA approved applications (Riess 1984; Faithfull 1992; Lowe 2000; Matsumoto and Kuroda 2002;
Matsumoto, Rigley et al. 2002; Riess 2005). Additionally, all PFCs are well characterized in regards to critical properties such as volatility, density, dissolved oxygen content and oxygen diffusivity. As initial surfactant choices, two PEG co-polymer based Pluronics were utilized, Pluronic F-127 and Pluronic F-68. These two compounds have been studied extensively in “oil in water” and PFC emulsion manufacture and are known for their non-ionic, amphiphilic properties and high HLB# making them ideal for the manufacture of stable emulsions. While other PEG co-polymers surfactants exist, we chose these two for several reasons. One is due to their higher molecular weights (~12,000 and 8,000, respectively). We sought to minimize impairment of diffusion observed in PEG co-polymers due to network aggregation, a characteristic desired in their use in controlled release devices. Additionally, while we attempted other surfactants of biological origin, such as phospholipids and oils, they were excluded as we found them to be unstable and to impair diffusion, in addition to the risk of their component immunogenicity.

We feel that the accurate characterization of PFC emulsions will allow for the fabrication of reproducible, stable emulsions suitable for tissue and other biomedical engineering applications.

6.2 Materials and Methods

6.2.1 PFC and Surfactant Materials:

The following PFCs were tested: Perfluorodecalin (PFD, Fluoromed, Round Rock, Tx.), Perfluorotributylamine (FC-43, 3M Corp., Minneapolis, MN.) and Perfluorooctylbromide (PFOB, Fluoromed, Round Rock, Tx and Sigma Aldrich Co., St. Louis, MO). The following surfactants were utilized: Pluronic F-68 and Pluronic F-127,
Additionally, one lipid based solution, Intralipid (Baxter Corp., Deerfield, IL) was utilized for diffusion comparisons. As a base solution (water phase) for all emulsions, Hank’s Balanced Salt Solution w/o Ca\(^{2+}\), Mg\(^{2+}\) was utilized, using D-Mannitol to correct for osmotic balance in all solutions (~320 mOsm).

**6.2.2 Cell Culture for Optimization Studies:**

Mouse insulinoma (MIN-6) cells between p30-40 were utilized for all experiments. Typically, >80% confluent flasks yielded 2.5-3.0E+07 cells per flask. At the onset of each cell culture experiment, freshly trypsinized (Trypsin-EDTA 1X, 0.05% Trypsin, 0.53mM EDTA, Cell Gro, Manassas, VA) cells were counted via trypan blue exclusion and aliquoted at cell densities required for each experiment. For each experiment, aliquots were distributed in 6 well plates to perform an MTT cell viability assessment and generate a standard curve of cell # vs. optical density for use in later culture assessment. Briefly, serial aliquots of 1E+06, 5E+05, 2.5E+05, 1.25E+05, 6.25E+04 were plated in individual wells in a 12-well plate in 0.9 mL of complete DMEM high glucose (Cell Gro, Manassas, VA). To each well, 100 µL of 10X MTT (Sigma-Aldrich, St. Louis, MO) dye stock (5 mg/mL) was added to each well, giving a final concentration of 0.5 mg/mL. The cells were incubated in the solution for 1 hour in a 37 °C 95% RA/5% CO\(_2\) standard incubator. At the end of the incubation period, the contents of each well was collected in a 15 mL conical tube and centrifuged for 3 minutes at 1000 rpm to pellet the cells. The supernatant was aspirated and a 1 mL volume of DMSO was added to each tube to solubilize the converted MTT dye from the cells. Duplicate 200 µL aliquots from each tube and from blank DMSO were pipetted into a 96-well plate and a spectrophotometric reading performed at 570 nm-620 nm, to correct for
background debris. An additional correction was performed by subtracting the OD measured in the blank DMSO. The transfer, 570 nm-620 nm-DMSO blank, OD was plotted versus cell number to generate a standard curve and equation for cell quantification. Dilutions were performed prior to reading, when necessary, and accounted for in the quantification. Individual standard curves were used in their respective cell toxicity experiments.

6.2.3 Perfluorocarbon Optimization: Toxicity Culture

Mouse insulinoma (MIN-6) cells were plated into 1 cm Millcell™ (Millipore, Inc.) inserts in 160 µL of DMEM high glucose (450 mg/dL) media or, as an additional control, HBSS, supplemented with 10% FBS, 1% L-glutamine and 1% Penicillin/Streptomycin, at a density of 160,000 cells per well and a media height of 2 mm within the insert. For toxicity assessment, 300 µL of pure PFC (PFD, FC-43, or PFOB), was added to the exterior of each insert giving a liquid height of 1.5 mm. DMEM (300 µL) added to the exterior of the insert was used as the control condition. Media was changed every day for the 7 day experiment duration.

6.2.4 Surfactant Optimization: Toxicity Culture

Mouse insulinoma (MIN-6) cells were plated 1 cm Millcell™ (Millipore, Inc.) inserts in 160 µL of either DMEM high glucose (450 mg/dL) media, Hank’s balanced salt solution (HBSS) or Hank’s supplemented with 2% w/v Pluronic surfactant solutions (2% F-68, 2% F-127, 1% F-68 + 1% F-127), all supplemented with 10% FBS, 1% L-glutamine and 1% antibiotic/antimycotic. Cells were cultured at a density of 160,000 cells per well and a media height of 2 mm within the insert. Exterior to the insert, 300 µL of complete DMEM was added. This experimental setup was utilized with the idea that
exterior medium would be similar to the initial in-vitro or in-vivo environment encountered by microcapsules manufactured from the respective surfactant/control solutions. All solutions were changed daily and the cells were cultured for 14 days.

6.2.5 Component Optimization: MTT Assay of Cell Viability

MIN-6 cell cultures were carried on for 7 days for PFC tests and 14 days for surfactant testing, with cell viability assessment via MTT metabolic assay (Sigma-Aldrich) performed at specific time points. On the day of MTT assessment, in the case of pure PFC cultures where MTT dye is insoluble in PFCs, 16 µL of MTT dye was added to each well containing perfluorocarbon (144 µL media content) and 46 µL to each media control (416 µL media), to maintain constant 0.5 mg/mL dye concentration. In the case of surfactant solution experiments, 46 µL of MTT dye was added to each well, controls and experimental. Inserts were incubated for 1 h in a 37 °C 95% RA/5% CO₂ standard humidified incubator. At the end of the incubation period, the media/dye was removed from the inserts by gently wicking the solution from the insert bottom using a Kimwipe. The inserts were transferred to individual wells of a 12 well plate and 1mL of DMSO was added to each well to solubilize the converted MTT. Aliquots were performed as above in a 96-well plate and the corrected optical densities measured. Measured, corrected OD was compared to the previously generated standard curve to obtain cell number per well. These values were expressed as mean percentage ± SD of the initial cell number.

6.2.6 Emulsion Fabrication Optimization:

Chilled mixtures of physiological base solution, surfactant, and PFC were passaged through a model 110Y microfluidizer (Microfluidics). For optimization studies, the variables examined in emulsion manufacture were emulsification time (ranging from
2 - 10 minutes) and emulsification pressure (ranging from 3000 - 15,000 PSI). Following
emulsification, particle sizes of the resulting emulsions were assessed via dynamic light
scattering (see method below). Following these studies, all subsequent experiments used
an emulsification time of 8 min and an emulsification pressure of 5,000 PSI. The majority
of the emulsions manufactured utilized the optimal Pluronic-based surfactant
combination indicated by the toxicity results, equal parts of F-68 and F-127. Studies were
also performed using similar w/v % concentrations of phospholipid-based surfactants
using previously documented methods (Johnson, O'Sullivan et al. 2011).

6.2.7 Gravimetric Determination of PFC Content

Emulsion PFC content was quantified by gravimetric determination performed by
weighing 1mL of each perfluorocarbon emulsion and comparing the observed weight to a
standard curve generated from serial 1 mL mixtures of the pure perfluorocarbon and base
solution at 0, 5, 10, 20, 40 and 100% v/v. The solutions were manipulated using a
precision 1 mL Hamilton syringe to insure accuracy of fluid volume. The measured
densities of the serial dilutions were compared to theoretical values based on the known
densities of the pure perfluorocarbons determined by the following equation:

$$\rho_{mixture} = V\%_{PFC} \times \rho_{PFC} + V\%_{BASE} \times \rho_{BASE}$$ (6-2)

6.2.8 DLS Particle Size Analysis: Initial and Long-Term Measurements

For particle size analysis, 5µL samples from each emulsion manufactured were
diluted 200X in comparable base solution and analyzed by dynamic light scattering
(Wyatt Dyna-Pro Titan) examining size distribution and polydispersity. Following
emulsion optimization studies, long term DLS particle size analysis was performed on
emulsions made from 2% w/v surfactant and ~10% v/v PFC concentrations. The Pluronic surfactants, F-127 and F-68, were studied individually and together, at 1% w/v each for a total 2% w/v concentration. For those emulsions whose initial particle size (Day 0) fell below 0.22 µm, mean particle diameter and polydispersity were recorded over a period of 14 days and greater. Emulsion stability was examined at relevant temperatures of 37 and 25 °C.

6.2.9 DLS Particle Size Analysis: Mathematical Modeling of Emulsion Stability

Mathematical modeling of size increase (if any) was performed using two previously described models of PFC emulsion coarsening (Freire, Dias et al. 2005). Additionally, we utilized a one phase association exponential fit that better fit the experimental data. In prior studies, it was suggested that PFC interfacial tension, diffusivity and solubility in the liquid phase, along with PFC molecular weight are the critical factors dictating the rate of particle size increase in PFC emulsions (Freire, Dias et al. 2005). The two models proposed are based on (1) the Van den Tempel Theory

$$\bar{\alpha}_t^3 = \bar{\alpha}_0^3 \exp(kt)$$

where $\alpha_0$ is the initial average particle radius, $\bar{\alpha}$ is the average particle radius at time $t$, and $K$ is the coalescence constant, and (2) the Lifshitz- Slyozov-Wagner (LSW) theory.

$$\frac{d}{dt} (\bar{\alpha})^3 = \frac{8CD\gamma V_m^2}{9RT}$$

Here, C and D represent the solubility and the diffusion coefficient of the dispersed phase in the continuous medium; $V_m$ is the molar volume of the dispersed substance (PFC); $\gamma$ (gamma), the interfacial tension between the aqueous and PFC phases; $R$ is the gas constant; and $T$ is the absolute temperature. According this model, an increase in the
particle’s volume is directly proportional to the solubility, the diffusion coefficient and the interfacial tension of the dispersed phase (PFC) in the continuous phase (base solution). Emulsions can, therefore, be theoretically be stabilized by decreasing one or all of these factors. Table 6-2, following, details the coefficients utilized for the LSW model at 25 °C:

<table>
<thead>
<tr>
<th></th>
<th>PFD</th>
<th>FC-43</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (mol/L)</td>
<td>9.90E-09</td>
<td>1.98E-09</td>
</tr>
<tr>
<td>D (m²/s)</td>
<td>5.61E-10</td>
<td>4.42E-10</td>
</tr>
<tr>
<td>gamma (N/m)</td>
<td>0.055</td>
<td>0.038756477</td>
</tr>
<tr>
<td>Molar</td>
<td>0.406779661</td>
<td>0.283121489</td>
</tr>
</tbody>
</table>

Table 6-2: **Coefficients at 25 °C used for Lifshitz-Slylov-Wagner modeling of particle coalescence.**

Estimate values for diffusivity were calculated using the Wilke-Chang equation and values for solubility and interfacial tension for FC-43 were estimated from published values for perfluorodecalin and the published technical values (solubility 25ppm vs 5ppm and surface tension 19.3 dynes/cm vs 13.6 dynes/cm for PFD relative to FC-43) (Kabalnov 1990). Molar volume was calculated as:

\[
\left( \frac{mL_{PFC} \times \rho_{PFC}}{MW_{PFC}} \right) \times \left( \frac{1000mL}{V_{emulsion}} \right)
\]  

Our proposed model uses a one-phase association exponential curve model where the rate of particle size change, \(K\), (initially rapid followed by slower rate until plateau), is driven by the above factors. The equation following details the model:

\[
Y = Y_0 + (Y_{max} - Y_0) \times (1 - \exp(-Kt))
\]
In this case, $Y$ is the cube of the particle radius at timepoint, $t$, $Y_0$, the cube of the particle radius at time zero, $Y_{\text{max}}$, the largest cube of the particle radius (the plateau), and $K$, the coalescence constant, determined through the curve fitting. Modeling was performed using GraphPad Prism (version 5.0a for Windows GraphPad Software, San Diego California USA, www.graphpad.com).

6.2.10 Oxygen Diffusivity: Pure PFCs and Emulsions

Calibrated 5mm diameter oxygen spot sensors (PreSens GmBH, Regensberg Germany) were used for real-time oxygen measurements in solutions (2mm liquid height) within a 12 well plate in a custom chamber (Biospherix) stored within a standard 37°C humidified incubator. Oxygen was purged from the system by injecting pure nitrogen. One sensor was designated to monitor chamber environment and the others were utilized for oxygen measurement in the individual solutions. In every experiment, several wells were utilized for base solution (HBSS + surfactant at 2% w/v concentration) or physiological salt solution controls to ensure sensor accuracy. At equilibrium, sensor logging was initiated and the chamber door was opened to allow influx of air containing oxygen. When the chamber sensor reached room air concentration (158.8 mmHg), the door was closed. Oxygen partial pressure was measured (2 sec/data point) until equilibration with room air. Liquid volumes were recorded to document evaporative loss, if any. Diffusion was modeled as that through a single surface of a slab. Transient solutions of concentrations were generated by iterative numeration. The diffusion coefficient was determined using least squares curve fitting of measured oxygen concentration to the theoretical model (Crank 1956):
\[ C = C_s + \frac{4}{\pi} (C_0 - C_s) \sum_{n=0}^{\infty} \frac{(-1)^n}{2n + 1} \cos \left( \frac{(2n + 1)\pi t}{2a} \right) \exp \left[ -\frac{D(2n + 1)^2 \pi^2 t}{4a^2} \right] \]  

(6-7)

Here, \( C \) is the concentration at time, \( t \), \( C_s \) the max \( \text{pO}_2 \) measured, \( C_0 \) the starting \( \text{pO}_2 \). The boundary conditions utilized were the \( C_s \) value, assumed to remain constant at the upper membrane surface, and the assumption of zero flux at the plate bottom surface. The same methods were utilized to assess diffusion through emulsions post-manufacture and to assess dynamically the effective diffusivity with changing emulsion particle size.

Emulsion base solutions and HBSS were used as controls to test the accuracy of the system based on published literature values. Corrections for solution height differences due to varying solution surface tensions were performed using a spectrophotometric algorithm software equating solution well height to known path-length cuvette measurements through the same solution detailed by the equation below:

\[ \frac{\text{Height}_{\text{well}}}{\text{Length}_{\text{cuvette}}} = \frac{OD_{1000\text{well}} - OD_{900\text{well}}}{OD_{1000\text{cuvette}} - OD_{900\text{cuvette}}} \]  

(6-8)

As the typical cuvette path length is 1 cm, this simplifies to:

\[ \text{Height}_{\text{well}} = \frac{OD_{1000\text{well}} - OD_{900\text{well}}}{OD_{1000\text{cuvette}} - OD_{900\text{cuvette}}} \]  

(6-9)

For pure PFCs, a fluorescent label was utilized for the same measurements and fluorescence output at wavelengths \( x \) and \( y \) replaced absorbance at 1000 nm and 900 nm. For emulsions, these measurements could not be performed due the opacity of the solutions. Therefore, it was assumed that the emulsions had the height of the relevant surfactant containing base solutions.
Additionally, temporal diffusion studies were performed to assess the effect of changing particle size in prepared emulsions (if any) on the effective diffusivity. First, PFC emulsion particle size was determined using DLS. This, in conjunction with the gravimetrically determined PFC content was used to determine the number of particles in 1mL of emulsion solution utilizing the relationship below:

$$\text{Particle#} = \frac{\% \text{PFC} \times 1 \text{mL}}{P_v(\text{mL})} \quad (6-10)$$

where $P_v$ is the calculated spherical volume of a single emulsion particle and is based on the DLS data.

Next, the total particle number and the single particle surface area were utilized to determine the total PFC diffusive surface area in 1mL of emulsion solution in the following fashion:

$$\text{SA}_{\text{total}} = (\text{Particle #}) \times \text{SA}_{\text{Particle}} \quad (6-11)$$

Measured diffusivity was compared to this calculated PFC diffusive interfacial surface area. Additionally, diffusivity per unit surface area (units of $\text{s}^{-1}$) was examined as a characteristic of the two remaining perfluorocarbons (PFD and FC-43) studied. Diffusivity was also examined in emulsions manufactured in previous publications where lipid-based emulsification agents were utilized (Johnson, O'Sullivan et al. 2011).

6.2.11 Stirred Oxygen Microchamber Determination of Dissolved Oxygen Content:

We have recently developed a method for the determination of dissolved oxygen content in solutions based on a modification of the Trinder Reaction in a sealed, stirred oxygen sensing microchamber as outlined in Chapter 3. Our data demonstrates that there is a strong correlation between measured oxygen concentration within the solution
measured and the zero-order enzymatic reaction rate in the chamber oxygen depletion. Both metrics are utilized to calculate fold oxygen solubility in PFC emulsions relative to the relevant control base solutions. For select manufactured emulsions, particularly those utilized in diffusivity studies, fold dissolved oxygen content was assessed.

6.2.12 Modeling of Mass Transfer Benefit From PFC Inclusion

Two dimensional finite element modeling was performed using Comsol v3.5a. A thin (500 µm) emulsion representative of our optimal configuration was modeled to assess the maximal mass transfer rate of oxygen through the emulsion compared to the control solution (physiological salt solution). The modeling parameters utilized are listed in Table 6-3, following:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial pO₂ (mmHg)</td>
<td>0</td>
</tr>
<tr>
<td>Boundary Condition 1</td>
<td>142 mmHg</td>
</tr>
<tr>
<td>Boundary Condition 2</td>
<td>Symmetry (0 Flux)</td>
</tr>
<tr>
<td>S&lt;sub&gt;control&lt;/sub&gt;</td>
<td>1.32E-03 mol/m&lt;sup&gt;3&lt;/sup&gt; mmHg</td>
</tr>
<tr>
<td>S&lt;sub&gt;PFC&lt;/sub&gt;</td>
<td>3.64E-03 mol/m&lt;sup&gt;3&lt;/sup&gt; mmHg</td>
</tr>
<tr>
<td>D&lt;sub&gt;control&lt;/sub&gt;</td>
<td>2.88E-09 m&lt;sup&gt;2&lt;/sup&gt;/s</td>
</tr>
<tr>
<td>D&lt;sub&gt;eff&lt;/sub&gt;</td>
<td>D&lt;sub&gt;l&lt;/sub&gt; = 1.42E-09x – 1.70E-05 m&lt;sup&gt;2&lt;/sup&gt;/s</td>
</tr>
<tr>
<td>X</td>
<td>calculated total particle surface area in 1mL of emulsion</td>
</tr>
<tr>
<td>Particle Growth Equation</td>
<td>Y = Y₀ + (Y&lt;sub&gt;max&lt;/sub&gt; - Y&lt;sub&gt;min&lt;/sub&gt;)*(1-e&lt;sup&gt;-kt&lt;/sup&gt;)</td>
</tr>
</tbody>
</table>

Table 6-3: Coefficients and conditions used in FEM modeling of emulsion oxygen mass transfer properties
Here, the starting oxygen concentration within the emulsion was designated as 0 mmHg. The boundary conditions were 142 mmHg concentration above the emulsion and no flux/symmetry on the bottom and side surfaces, representing a thin layer of emulsion in a plastic culture dish. The assumptions associated with this design are that there is negligible diffusion through the materials of the dish and that the emulsion is uniformly distributed throughout the aqueous volume. $S_{control}$ and $S_{PFC}$ are the oxygen solubility coefficients of the control solutions and the PFC emulsions, respectively. $D_{control}$ is the effective diffusivity through the control solutions from our measurements, 2.88E-09 m$^2$/s. $D_{eff}$ is the effective diffusivity coefficient as calculated using the linear fit of our measured emulsion diffusivity data.

Variation in mass transfer rate observed in the perfluorodecalin emulsions resultant from corresponding variations in particle size was also modeled. For these models, $D_{eff}$ decreased as particle size increased and this calculated value was used to model the effect of decreasing diffusive permeability, $D_p$, on mass transfer rate over time, in days.

6.2.13 Statistical Analysis

All data was either expressed as the mean ± SD or as the mean fold control value ± SD. The standard deviation of ratios was calculated as detailed in chapter 5 section 5.2.10. Additionally, standard or ratio t-tests were performed to assess significant differences based on the methods in the same aforementioned section. F-tests were performed on all group comparisons to determine if the sample variance was equal or unequal. A non-significant $p$ value in the F-test is indicative of unequal variance. This $p$ value was utilized to determine which t-test to implement, equal variance, unequal variance or paired, if the n in the compared groups was the same. Additionally, all t-tests
performed were 2 tailed. A $p$ value $\leq 0.05$ was considered significant. Those less than 0.01 were considered highly significant.

6.3 Results

6.3.1 Perfluorocarbon Optimization: Toxicity Culture

Figure 6-1, following, shows the results of the toxicity studies performed utilizing pure perfluorocarbons under the cell-culture inserts containing MIN-6 cells ($n=3$). Values are expressed as mean cell # $\pm$ SD.

![Figure 6-1: PFC toxicity studies utilizing plated MIN-6 cells. Initial loading density 160,000 cells/well, 7 days of culture with medium changed daily. The groups are, from left to right: DMEM (control), HBSS (control), PFOB from Fluoromed, PFOB from Sigma-Aldrich, PFD, and FC-43.](image)

Amongst the various groups, there was a notable, but expected, decrease in cell number comparing complete medium (DME) to supplemented HBSS. This is more than likely due to the lack of essential amino acids and other growth factors in the HBSS
solution. Amongst the perfluorocarbons, there was non-significant difference between FC-43 and PFD relative to DMEM, although there was a trend toward improved cell number at later time points. The significant finding was a pronounced MIN-6 toxicity observed in PFOB. This was independent of the brand utilized, as both Fluoromed and Sigma-Aldrich brands exhibited comparable substantial loss in viability as early day 2 post-plating (~75-80% loss). Due to this observed toxicity, PFOB was excluded from further use.

6.3.2 Surfactant Optimization: Toxicity Culture

Figure 6-2, following, summarizes the results (n=3) of the surfactant toxicity MIN-6 culture studies. Results are expressed as mean cell number ± SD at each time point.

![Figure 6-2: Surfactant toxicity studies utilizing plated MIN-6 cells. Initial loading density 160,000 cells/well, 14 days of culture with medium changed daily. The groups are, from left to right, DMEM (control), HBSS (control), 2% F-68 surfactant, 2% F-127 surfactant, and 1% F-68/1% F-127 surfactant.](image-url)
While the differences were not significant between the surfactant combinations and the control at most time points, there was a trend demonstrating that the surfactant combination 1% F-68/1% F-127 had an observed lower toxicity and consistently had measured cell viability comparable or better than the control.

6.3.3 Emulsion Manufacture:

Results from examination of emulsification time and pressure are listed in Table 6-4, following.

<table>
<thead>
<tr>
<th>Time</th>
<th>Size (nm)</th>
<th>SD</th>
<th>Pressure (PSI)</th>
<th>Size</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>238.3</td>
<td>11.92</td>
<td>3000</td>
<td>259.76</td>
<td>12.57</td>
</tr>
<tr>
<td>4</td>
<td>223.32</td>
<td>4.49</td>
<td>5000</td>
<td>206.66</td>
<td>11.78</td>
</tr>
<tr>
<td>6</td>
<td>219.64</td>
<td>7.61</td>
<td>10000</td>
<td>220.18</td>
<td>9.99</td>
</tr>
<tr>
<td>8</td>
<td>212.4</td>
<td>11.02</td>
<td>15000</td>
<td>217.38</td>
<td>5.13</td>
</tr>
<tr>
<td>10</td>
<td>221.38</td>
<td>8.24</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 6-4: Effect of emulsification time and pressure on emulsion characteristics. Values are particle size in nanometers ± SD.

The settings that produced the optimal emulsion particle sizes, regardless of perfluorocarbon type or v/v% concentration were 8 - 10 minutes of continuous emulsification at 5000 PSI. These settings were utilized for investigation of the effects of perfluorocarbon type and concentration and surfactant type and concentration on emulsion characteristics. Figure 6-3a and 6-3b, following, show the effects of surfactant and perfluorocarbon concentration on emulsion particle size.
Figure 6-3a: Effect of surfactant concentration on initial emulsion particle size. Solid bars represent equal parts F-68 and F-127 with PFD. Empty bars represent equal parts F-68 and F-127 with FC-43.

Figure 6-3b: Effect of volume % perfluorocarbon on initial emulsion particle size. All emulsions were made with 1% F-68/ 1% F-127 as the surfactant. Solid bars represent PFD. Empty bars represent FC-43.
Surfactant concentrations from 0.25% w/v – 4% w/v total surfactant concentration and from 5% v/v - 40% v/v total PFC content were examined. Surfactant concentrations below 2% w/v resulted in emulsions that were difficult to filter or could not be filtered. Concentrations higher than 2% w/v were eliminated due to toxicity associated with increased wt % of each individual component, as observed in our assessments. Therefore, a surfactant concentration of 2% w/v was considered the most optimal for cell-based applications.

PFC concentrations higher than 10% v/v resulted in emulsions with sub-optimal particle size, as well. Therefore, 10% v/v PFC content was utilized for further emulsion study and characterization.

6.3.4 Gravimetric Determination of PFC Content

Figures 6-4a and 6-4b, following, show the gravimetric standard curves of the PFC content and the comparison to theoretical values.
Figure 6-4a: Gravimetric standard curve for PFD compared to expected values (Theoretical). Open squares are the measured values from PFC mixtures. Closed diamonds are the theoretical values based on density.

Figure 6-4b: Gravimetric standard curve for FC-43 compared to expected values (Theoretical). Open squares are the measured values from PFC mixtures. Closed diamonds are the theoretical values based on density.
There was no significant difference (p = 0.94) between the measured and theoretical values, as indicated by the visible overlap of the two plots. The largest deviation, 0.004 g/mL, occurred in the 20% PFC mixture. This demonstrated the validity of the method in determining PFC content in solutions.

6.3.5 DLS Particle Size Analysis: Initial and Long-Term Measurements

All emulsions made with all Pluronic surfactant types at 2% w/v surfactant and ~10% v/v PFC concentration had initial particle sizes below the filtration cut-off of 0.22 µm (220 nm). Those made with egg-yolk phospholipids did not (> 450 nm). The initial polydispersity of manufactured emulsions was typically under 10-15%, indicating monodisperse or nearly monodisperse preparations.

Long-term analysis of particle size indicated that the critical factor in emulsion stability was the perfluorocarbon utilized. All emulsions made from perfluorodecalin increased in average particle diameter by 186.7 ± 22.5 nm at 25 °C and by 265.3 ± 14.3 nm at 37°C over the 14 day period. All emulsions made with perfluorotributylamine (FC-43) exhibited no significant changes in particle size (<8 nm), irrespective of surfactant utilized or incubation temperatures tested (25 °C and 37 °C). The initial sizes of all emulsions ranged from ~187-202 nm with emulsions made with 1% F-68/1% F-127 and 10% FC-43 having the smallest initial particle size. Figures 6-5a and 6-5b show the 14 day particle size measurements at 25 °C and 37 °C in emulsions (n=3) made with 10% PFD and 10% FC-43, respectively.
Figure 6-5a: 14 day particle size analysis of emulsions made with 10% PFD. Open diamonds represent emulsions stored at 25 °C and closed squares those stored at 37 °C.

Figure 6-5b: 14 day particle size analysis of emulsions made with 10% FC-43. Open diamonds represent emulsions stored at 25 °C and closed squares those stored at 37 °C.
At all time points, the emulsions made with FC-43 were significantly smaller than those made with PFD (p < 0.05), by students paired t-test. The observation that FC-43 made the most stable emulsions was in agreement with previously published studies that indicated PFC molecular weight and therefore, diffusivity, solubility and interfacial tension, as the critical factors dictating emulsion stability (Freire, Dias et al. 2005).

6.3.6 DLS Particle Size Analysis: Mathematical Modeling of Emulsion Stability

Figures 6-6a and 6-6b, following, show representative modelings of the particle size analysis for emulsions made from 10% PFD and 10% FC-43, respectively, using 1% F-68 and 1% F-127 and stored at 25°C.

Figures 6-6a: Representative modelings of emulsion coalescence at 25°C in emulsions made from PFD with 1% F-68 and 1% F-127. Closed diamonds represent measured values, cross hairs represent the one-phase association model, closed squares the Van den Tempel model and closed triangles the LSW model
Figures 6-6b: Representative modelings of emulsion coalescence at 25°C in emulsions made from FC-43 with 1% F-68 and 1% F-127. Closed diamonds represent measured values, cross hairs represent the one-phase association model, closed squares the Van den Tempel model and closed triangles the LSW model.

The rationale behind using the one-phase association model was as follows: in the initial emulsion, the concentration of PFC micelles, the diffusion of these micelles and the chances for micelle collision or “binding” is high. As coalescence starts to occur, the concentration drops and, additionally, the diffusivity of micelles slows and the chances of interactions lessen. This follows the binding kinetics of one phase association between a ligand and its receptor, or a substrate and an enzyme. During each time interval a certain fraction of the unoccupied receptors become occupied. As time advances, fewer receptors are unoccupied so fewer ligands are able to bind and the curve plateaus.

Based on the sum of squares differences, the one-phase association model was superior to the other two methods for estimating particle size change over time. In models of the perfluorodecalin emulsions, the K values varied with temperature, as previously
reported. In the Van den Tempel model, a purely exponential fit, the K values increased with temperature increase at a rate that was nearly proportional to the ratio of the temperatures (1.37 fold vs. 1.48). Conversely, the K values decreased in the One-Phase Association model, as expected, at a rate, again, nearly proportional to the ratio of the temperatures (1.25 versus 1.48). In the FC-43 emulsions, the K variation had no relation to temperature, as there was little discernible particle change and the emulsions stored at 37°C trended toward decreased particle size over time. Therefore, there was an observed increase in the K value for the One Phase Association model.

Of particular interest was the comparison between One Phase Association and Van den Tempel K values of the PFD emulsions relative to the FC-43 emulsions at 25°C. In the Van den Tempel model, the ratio of the K values (0.1419 versus 0.008, ratio = 17.74) was directly proportional to the product of the ratio of all five coefficients in the LSW model (molar concentration, molecular weight, diffusivity, solubility and interfacial tension), which was calculated as 18.4. In the One Phase Association model, the ratio of the K values was directly proportional to the ratio of the products of the of the molar concentration, the diffusivity and the interfacial tension (2.30 vs 2.59, respectively).

This emulsion stability study indicated that the most critical factors in generating stable emulsions were the PFC concentration, the molecular weight of the PFC, the diffusivity of the PFC in the aqueous phase, and the interfacial tension between the two phases. In the case of FC-43, which has lower effective diffusivity, a lower interfacial tension, and a higher molecular weight, the emulsion stability was superior to those manufactured using perfluorodecalin. In designing emulsions, users should carefully investigate all of these parameters before embarking on manufacture.
6.3.7 Oxygen Diffusivity: Pure PFCs and Emulsions

The measured oxygen diffusivities through the pure perfluorocarbons were 1.88E-04 cm²/s ± 1.84E-05 cm²/s for pure perfluorodecalin and 1.55E-04 cm²/s ± 2.41E-05 cm²/s for perfluorotributylamine. The measured values were not significantly different. The mean measured oxygen diffusivities through physiological salt solutions and the emulsion base solution (2% w/v surfactant dissolved in physiological salt solution) over all the experiments were 2.94E-05 cm²/s ± 3.94E-06 cm²/s and 2.98E-05 cm²/s ± 2.95E-06 cm²/s. This is in close agreement with the expected diffusivities through aqueous solutions at this temperature (2.68 – 3.02E-05 cm²/s) (Grote 1967; Zycinski and Luczak 1975; Navari, Rosenblum et al. 1977; Zycinski and Luczak 1982; Subczynski and Hyde 1984; Biro and Blais 1987; Lowe 1987; Shah and Mehra 1996; Avgoustiniatos and Colton 1997; Helmer, Han et al. 1998; Patel and Mehra 1998; Patel and Mehra 1998; Riess 2005; Johnson, O'Sullivan et al. 2011).

Given that emulsions made from FC-43 did not change in particle size over time and, accordingly, the measured oxygen diffusivities through single emulsions varied insignificantly over the same time frame. In order to show variation with particle size, these emulsions had to be manufactured with variations in emulsification time to make larger initial particles, which was done by performing emulsification without cooling.

Figures 6-7a and 6-7b, following, show the results of effective oxygen diffusivity measured through emulsions manufactured using equal parts F-68 and F-127 (2% total surfactant) and 10% v/v of either PFC and the dependence of this measured diffusivity on emulsion interfacial surface area.
Figure 6-7a: Effective oxygen diffusivity versus interfacial surface area in 10% v/v PFD emulsions. Best-fit equation $y = 1.42E^{-09}x - 1.70E^{-05}; R^2 = 0.87$

Figure 6-7b: Effective oxygen diffusivity versus interfacial surface area in 10% v/v FC-43 emulsions. Best-fit equation $y = 1.74E^{-09}x - 2.92E^{-05}; R^2 = 0.95$
The effective diffusivity through emulsions manufactured from both perfluorocarbons varied linearly with interfacial surface area of the emulsion particles. (Note that FC-43 emulsions were intentionally made to have different initial particle size, given that minimal coalescence occurs in these emulsions). This demonstrates the importance of emulsion stability and small particle diameter (size) in maximizing oxygen transfer benefits. At smaller interfacial surface areas, which are the result of large emulsion particle diameter (size), the measured effective diffusivities were lower than that measured through physiological buffer solutions, thereby cancelling out the mass transfer enhancement afforded by the increased diffusive permeability (product of effective diffusivity and solubility). Additionally, as there was no observed impediment to oxygen diffusion in the emulsion base solutions relative to physiological salt solutions, it is likely that the amphiphillic phase of the surfactant/PFC interfacial surface is responsible for this observed effect on the effective oxygen diffusivity and larger particles with some possible agglomeration of the PEG-based particles may form a diffusion impairing network. The measured effective diffusivities per unit surface area were 8.32E-10 s$^{-1}$ ± 1.65E-10 s$^{-1}$ and 8.03E-10 s$^{-1}$ ± 1.10E-10 s$^{-1}$ for PFD and FC-43, respectively. These two measured values were not statistically significantly different, but follow our measured values through the pure perfluorocarbons where effective diffusivity through PFD was faster than that through FC-43.

In emulsions manufactured utilizing lipid-based emulsifiers, the measured effective oxygen diffusivity was greatly reduced compared to control physiological buffers and therefore, to optimal PEG based emulsions (1.69E-05 cm$^2$/s ±1.06E-06 cm$^2$/s versus 2.94E-05 cm$^2$/s ± 3.94E-06 cm$^2$/s) (Johnson, O'Sullivan et al. 2011). Typically, the
average particle size in these emulsions was significantly larger than those manufactured from our PEG based surfactants (particle radius = 581.5nm ± 36.7nm) and the PFC content v/v% was substantially higher (~36.5% v/v) potentially the reason for reduced oxygen effective diffusivity.

During the course of the experiments, no evaporative losses were observed and most measurements were completed within several hours. In accounting for solution height differences due to surface tension variations of the solution, the greatest height variations from the expected height values were observed in basal salt solution controls (0.69 mm ± 0.11 mm). Surfactant base solutions and emulsion containing PFC had little height change. Pure perfluorocarbons, known to have minimal surface tension and classified as ideal wetting solutions, also exhibited no measureable variation from the expected height. The use of the corrective absorbance measurements allowed us to accurately account for these height differences.

6.3.8 Stirred Oxygen Microchamber Determination of Dissolved Oxygen Content:

Figures 6-8a and 6-8b, following, show the measured fold oxygen solubilities in PFC emulsions manufactured with PFD and FC-43, respectively, at 37°C. The plots detail the measured fold solubilities determined from the oxygen concentrations by Trinder reaction and the slope of oxygen consumption in the closed microchamber system for serial dilutions of emulsions made from differing initial PFC content. The solubilities in both pure PFCs were extrapolated from the slopes of both measured parameters (concentration and slope of oxygen consumption). In both cases, the extrapolated values were in close agreement with the published literature values. Measurements using the
Trinder reaction are not feasible in pure perfluorocarbons, as the dyes utilized are only soluble in aqueous solutions of a hydrophilic nature.

Figure 6-8A: Theoretical and measured fold control oxygen solubilities (concentration by Trinder reaction and oxygen consumption slope) in emulsions made from Perfluorodecalin. Cross hairs represent the measured fold slope of the oxygen depletion rate. Open diamonds represent the fold measured concentration by Trinder reaction.
The measured fold solubilities of the pure PFCs were approximately 17-18.5 fold for the perfluorodecalin and 15-16 fold for the FC-43, for both measured fold slope (m) and fold concentration (c). This data clearly demonstrates the utility of this method in assessing the dissolved oxygen content in any low v/v% PFC emulsion. This is useful coupled with the measured oxygen effective diffusivity in determining the overall enhancement to diffusive permeability ($D_{\text{eff}} \times \text{Solubility}$) and therefore, oxygen mass transfer.
6.3.9 Modeling of Mass Transfer Benefit From PFC Inclusion

Figure 6-9, following, displays the results of finite element modeling of oxygen mass transfer rates in PFC emulsions made from perfluorodecalin and 1% F68/1% F127 (optimal and temporal analysis) compared to a control physiological solution.

Figure 6-9: Finite element modeling of oxygen mass transfer rates through PFC emulsions, optimal and over time, compared to a control physiological solution. Thin dashed line represents unchanging mass transfer rate through FC-43 emulsions. Thick dashed line represents mass transfer rate through control solutions. All other lines represent changing mass transfer rate through emulsions made of PFD caused by particle coalescence and change in particle interfacial surface area.

The results demonstrate that while there is an initial fold benefit in oxygen mass transfer rate that is identical to the fold diffusive permeability, $P_D$, as expected. As particle size increases over time with emulsion coalescence, however, the observed benefit decreases. By day 4 in this model, all benefit of the perfluoro-emulsion in oxygen mass transfer is gone. As time progresses, the perfluoro-emulsion has considerably impaired oxygen mass transfer relative to the control physiological solution. This data
demonstrates the critical importance of emulsion stability in maintaining efficacy of the enhanced oxygen mass transfer. Selection of proper emulsion components, particularly the perfluorocarbon, is, therefore, of the utmost significance. The modeled values for emulsions made from FC-43 and the same surfactant, based on our temporal particle size measurements, do not change over time maintaining increased mass transfer rate relative to control physiological solutions.

6.4 Discussion

Perfluoro-emulsions have the potential to enhance numerous biomedical applications, due to their inherent ability to enhance oxygen mass transfer. Potential implementations of this technology include culture systems, perfusion technologies and cell/tissue engineering devices, where the low oxygen tensions of the body often limit the efficacy of implanted cell/tissue based devices. Despite the potential impact of these oxygen carrying moieties, little work has been done to thoroughly characterize the variables dictating their oxygen transfer capacities and their long-term stability and function.

In this work, we have established methods for the optimization of the manufacture and characterization of perfluorocarbon emulsions, thereby maximizing their oxygen mass transfer potential. We have detailed the parameters important in the production of stable, monodisperse emulsions, including volume % perfluorocarbon, weight % surfactant, perfluorocarbon type, surfactant type, emulsification time and emulsification pressure. Of these parameters, we found that the fluorocarbon selected is critical to the long-term stability of manufactured emulsions, determined by the molecular weight, interfacial tension and diffusivity of the perfluorocarbon. Additionally, we found that
oxygen mass transfer, the product of the effective oxygen diffusivity and oxygen solubility, is highly dependent on the stability of the emulsion, as variations in particle size dramatically affect the oxygen diffusivity. Changes in the effective oxygen diffusivity from particle size increase rapidly eliminate any observed benefit in oxygen mass transfer (4 days or less in 10% v/v perfluorodecalin emulsions).

Our optimal configurations were emulsions made from perfluorotributylamine (FC-43) at 10% v/v and a total 2% w/v surfactant concentration of equal parts Pluronic F-68 and Pluronic F-127. Toxicity studies indicated that for each of these surfactants concentrations of greater than 2% w/v had some observed toxicity; however, at lower concentrations, they were not sufficient to make emulsions of desired particle size (<220nm). These emulsions typically had effective diffusivities slightly higher or equivalent to the measured diffusivity in control physiological salt solutions (>2.88E-05 cm²/s) and a solubility of approximately 2.75 fold that of the control. The stability of these emulsions ensured that observed mass transfer benefit (P_D) was constant throughout at least the 14 days of our studies.

The implications of these findings are many: first and most importantly, emulsions made with either lipid or pluronic based surfactants with particle sizes >0.5µm are, by our measurements, of no benefit in oxygen mass transfer, as their suboptimal particle sizes result in effective oxygen diffusivities that cancel out the benefit of the enhanced oxygen solubility inherent to the perfluorocarbon component illustrated by our initial diffusivity measurements in lipid-based emulsions and our temporal measurements in pluronic emulsions. Second, careful examination of all emulsion components is critical to the development of manufacturing protocols that result in stable and optimally sized
perfluoro-micelles. Finally, thorough examination of the mass transfer properties of each manufactured emulsion is of the utmost importance, as assuming that mass transfer properties follow theoretical models, irrespective of particle size and emulsion properties, is incorrect and can lead to misleading results in application of the emulsions (Khattak, Chin et al. 2007; Johnson, O'Sullivan et al. 2011).

Ultimately, the benefit afforded by perfluorocarbon emulsions is incremental dependent on the volume of perfluorocarbon utilized in the emulsion. Our studies were limited to 10% v/v emulsions, as greater amounts of PFC resulted in emulsions with suboptimal particle sizes (>220 nm). In future work, optimization of surfactants to contain greater volumes of PFC, maintain optimal particle size and minimize inhibition of diffusivity could result in superior oxygen carrying solutions with broader applicability to biomedical cell and tissue engineering.
CHAPTER 7: THE INCLUSION OF PERFLUOROCARBON EMULSIONS IN CELL ENCAPSULATION MATRICES TO ENHANCE OXYGEN MASS TRANSFER.

7.1 Islet Microencapsulation

The past forty years have seen substantial advances in the field of cell encapsulation (immunoisolation) for the treatment of chronic, debilitating afflictions. There have been increasingly promising results in many research areas, such as the treatment of Parkinson’s disease with encapsulated dopamine secreting cells, the treatment of chronic pain with encapsulated adrenal chromaffin cells and, most prominently, the treatment of Type 1 Diabetes Mellitus with encapsulated islets of Langerhans (Klomp, Ronel et al. 1979; Tresco, Winn et al. 1992; Lindner, Francis et al. 2000; Lindner, Francis et al. 2000; Shingo, Date et al. 2002; Calafiore, Basta et al. 2006; Moustafa, Girod et al. 2006; Beck, Angus et al. 2007; Teramura, Kaneda et al. 2007; Campos-Lisboa, Mares-Guia et al. 2008; Liu, Nothias et al. 2010; Veriter, Mergen et al. 2010). Despite encouraging ongoing results, there remain substantial obstacles to significant implementation of encapsulation as a therapy in the clinical realm (Cotton 1996; Morris 1996; Uludag, De Vos et al. 2000; Visted, Bjerkvig et al. 2001; Visted and Lund-Johansen 2003; Thanos and Emerich 2008). First and foremost of these challenges is the mass transfer limitations, particularly for oxygen, imposed by the characteristically large dimensions of polymer microcapsules (Avgoustiniatos and Colton 1997). Typical microcapsule sizes, on the order of 600µm to 1mm in diameter, dictate low v/v % cell loading densities (on the order of 1-2%) to maintain adequate cell viability and function both in vitro and in vivo.
In the case of islets of Langerhans, whose oxygen/metabolic demands exceed other somatic cell types by often several orders of magnitude, cell loading is critically important. Typically, islets are constructed of aggregates of 5 cell types: α, β, γ, δ and pancreatic polypeptide cells with a cell cluster diameter in the range of 50 µm to 450 µm. In their native environment, pancreatic islets have a rich, vascular network that provides them adequate oxygenation and nutrient supply. In fact, despite only comprising 1-2% of the total tissue of the pancreas, islets receive nearly 25% of the pancreatic blood flow and consume nearly 10-15% of the oxygen supplied (Lifson, Kramlinger et al. 1980; Jansson 1994). Islet encapsulation, therefore, is particularly limited by oxygen supply and loading density (Cotton 1996; Avgoustiniatos and Colton 1997).

Currently, the clinical requirements for successful islet transplantation are 10,000 IEQ per kilogram of recipient body weight. In a typical 70 kg patient, that would translate to 700,000 IEQ. Conservatively, this tissue occupies a volume of approximately 1.23 mL, fully packed. If this number of cells was encapsulated in optimal polymer spheroids of 600 µm at the required loading density of 1-2% v/v, only 0.7-1.2 IEQs could be loaded per capsule. This means that no less than 580,000 capsules would be required to hold the entire islet mass. Further, this would translate to a polymer volume of approximately 66 mL. Given the volume required, few sites would be available for implantation and, retrieval of such a large volume would be nearly impossible if complications arose or graft failure occurred. There is a substantial need, therefore, for methods that reduce or eliminate this mass transfer limitation.

As mentioned earlier (Chapters 2, 3, 5 and 6), one potential means of addressing this problem is through the use of perfluorocarbons (PFCs). PFCs are well-known for
their enhanced oxygen transfer capabilities, the product of increased oxygen solubility and effective diffusivity relative to typical aqueous solutions (Navari, Rosenblum et al. 1977; Lowe 1987; King, Mulligan et al. 1990; Faithfull 1992; Riess 2005). As PFCs are highly immiscible and hydrophobic, a necessary emulsification in surfactant-based salt solutions is required to suspend the PFCs in stable, micellar, nano-scale droplets. Perfluoroemulsions, as they are called, have been extensively investigated as oxygen carrying parenteral blood substitutes, culture solutions for perfusion systems, organ preservation solutions and even in microencapsulation (Riess and Le Blanc 1978; Riess 1984; Biro and Blais 1987; Riess 1991; Riess 1991; Faithfull 1992; Riess, Dalfors et al. 1992; Biro 1993; Shah and Mehra 1996; Patel and Mehra 1998; Patel and Mehra 1998; Matsumoto and Kuroda 2002; Matsumoto, Rigley et al. 2002; Ricordi, Fraker et al. 2003; Khattak, Chin et al. 2007; Chin, Khattak et al. 2008).

Our group was the first to investigate the use of perfluorocarbons in cell encapsulation matrices, using pure PFCs in concentrated solutions of sodium alginate (4% w/v) to enhance oxygen delivery to encapsulated islets of Langerhans (Inverardi 1999). Since then, the introduction of highly purified alginates has greatly improved the outcome of transplanted encapsulated cells, and has reinvigorated investigation into immunoprotection (De Vos, De Haan et al. 1996; De Vos, De Haan et al. 1997; De Vos, De Haan et al. 1997; Duvivier-Kali, Omer et al. 2001; de Vos, Hamel et al. 2002; de Vos and Marchetti 2002; de Vos, Faas et al. 2006; de Vos, Spasojevic et al. 2010). Other groups have investigated the use of PFC emulsions implemented in polymer matrices to enhance cell viability with some success, but there has been a glaring lack of thorough characterization of these emulsions and their mass transfer capabilities (Khattak, Chin et
al. 2007; Johnson, O'Sullivan et al. 2011). Oxygen transfer benefit in these systems has
been based on theoretical values of the pure compounds utilized in the emulsion
manufacture, which can lead to gross overestimation/underestimation of oxygen transfer
benefit. We have demonstrated that the emulsification process often alters these
properties in an undesirable fashion and that adequate characterization of mass transfer
variables is critical in determining the benefit afforded by a manufactured emulsion
(Chapter 6).

In this work, we present methods for the inclusion of PFC emulsions
(manufacture detailed in Chapter 6) in sodium alginate polymer matrices, optimizing
methods for the assessment of mass transfer benefit and the viability and function of
encapsulated cells. Our data demonstrates that choice of surfactant and PFC is critical to
the manufacture of proper emulsions and further, that the emulsion characteristics
directly affect the benefit, if any, to the encapsulated cells.

7.2 Materials and Methods

7.2.1 Emulsion Manufacture

Chilled mixtures of physiological base solution (HBSS, Invitrogen), PEG-based
Pluronic surfactants (Pluronic F-68, Pluronic F-127, and a novel PEG-PPS surfactant
generously donated by the laboratory of Jeffrey Hubbell) and PFC (Pefluorodecalin,
PFD, and Perfluorotributylamine, FC-43) were passaged through a model 110Y
microfluidizer (Microfluidics). Based on our prior work examining particle size and
stability (Chapter 6), optimal settings for emulsification were 8 minutes at 5000 PSI and
continuous cycling using 10% v/v PFC and 2% w/v total surfactant. The desired endpoint
for emulsification was a particle size of < 0.22 µm and a polydispersity less than 20%.
Emulsions meeting the end point criteria were sterile filtered after manufacture through a 0.22 µm filter for use in encapsulation matrices.

7.2.2 Gravimetric Determination of PFC Content

Emulsion PFC content was quantified by gravimetric determination performed by weighing 1 mL of each perfluorocarbon emulsion and comparing the observed weight to a standard curve generated from serial 1 mL mixtures of the pure perfluorocarbon and base solution at 0, 5, 10, 20, 40 and 100% v/v. The solutions were manipulated using a precision 1mL Hamilton syringe to insure accuracy of fluid volume. The measured densities of the serial dilutions were compared to theoretical values based on the known densities of the pure perfluorocarbons determined by the following equation:

\[
\rho_{\text{mixture}} = V\%_{\text{PFC}} \times \rho_{\text{PFC}} + V\%_{\text{BASE}} \times \rho_{\text{BASE}}
\]

Only emulsions where the measured PFC content was greater than 8.5% v/v and less than 12% v/v were utilized for further study.

7.2.3 Alginate Preparation

Ultra pure medium viscosity guluronic (UP-MVG) and ultra pure low viscosity mannuronic (UP-LVM) alginates were obtained from FMC Biopolymer. Portions were weighed out in sterile 50 mL conicals and then, dissolved overnight in sterile, double distilled water at a concentration of 10 mg/mL. This solution was sterile filtered through a 0.22 µm filter and aliquoted into 15 mL conical tubes to obtain a total of 48mg/ tube, in the case of UP-MVG and 99mg/tube, in the case of UP-LVM. All tubes were frozen at -80 °C and then, lyophilized in a freeze drier until completely dehydrated (typically 48-72
hours). These prepared portions were utilized for the preparation of 3 mL aliquots of alginate.

In the case of UP-MVG, 3mL of sterile HBSS w/o Ca\textsuperscript{2+},Mg\textsuperscript{2+} or 3.3 mL of sterile PFC emulsion prepared in the same HBSS base solution, was added to 48 mg of lyophilized alginate. 3.3 mL was utilized in the case of the emulsions to account for the non-aqueous portion taken up by the PFC/surfactant. It was our observation that when 3 mL was utilized, the solution was far more viscous and concentrated than those made with 3.3 mL. Viscosity measurements confirmed this, demonstrating that 3.3 mL of emulsion gave an equivalent solution and indicating that the PFC volume should be disregarded in alginate w/v\% calculations. The final w/v\% alginate concentration of all solutions was 1.6%.

For UP-LVM alginate, again 3 mL of sterile HBSS w/o Ca\textsuperscript{2+},Mg\textsuperscript{2+} or 3.3 mL of sterile PFC emulsion prepared in the same HBSS base solution was utilized, but to dissolve 99 mg of alginate, giving a final w/v\% concentration of 3.3%. Both w/v\% concentrations were selected based on average published literature values (Duvivier-Kali, Omer et al. 2001; Khattak, Chin et al. 2007; Johnson, O'Sullivan et al. 2011). Alginates were utilized within 24-48 hours of preparation.

7.2.4 Initial Particle Size Determination in PFC Alginates

From each PFC alginate manufactured, 5 µL samples were diluted 200X in comparable base solution and analyzed by dynamic light scattering (Wyatt Dyna-Pro Titan). Only alginates whose initial particle size fell below 0.22 µm and whose polydispersity was less than 20% were utilized for encapsulation study.
7.2.5 Perfluorocarbon Labeling

For the purpose of studying perfluorocarbon leakage from formed alginate microcapsules, perfluorocarbons were labeled with a fluorescent dye used for laser media, pyrromethene, (Exciton) which allowed for quantification of PFC externalization, if any, during long term culture. Pyrromethene (10mg) was dissolved in 1 mL of hexane and then was quickly added to 10 mL of perfluorocarbon, at a final dye concentration of ~1 mg/mL. In perfluorodecalin, the resulting solution was bright orange and free of insoluble particles. In perfluorotributylamine, the solubilization of the dye was less complete, resulting in some particulate matter. As a result, the FC-43 solution was less bright in visible color. All PFC-dye solutions were filtered through a 0.22 µm filter prior to use to remove all insoluble, particulate matter. Determined by spectral sweeps using a monochromater (Molecular Devices Spectramax), the optimal excitation and emission wavelengths for dye quantification were 510 and 560 nm, respectively.

7.2.6 Alginate Microcapsule Formation

Under sterile conditions in a laminar flow hood, parallel flow air droplet generation was utilized in the manufacture of microcapsules with a target diameter of 1 mm. Sterile room air mixture (95% RA/ 5% CO₂) gas was supplied by an exterior tank passaged through a 0.22 µm filter into a sterile tubing set connected laterally to the polymer fluid path (A), distributing vertically in a chamber external to the polymer fluid path upon entering the encapsulation chamber (Biorep Technologies) and parallel to the polymer flow. The airflow was further distributed into four equivalent channels designed to focus the polymer stream at the tip of a needle (C), generating oscillations that result in stream break-up and microcapsule formation. The polymer was introduced from the top
of the device through a luer-locked fitting designed to prevent introduction of contaminants and polymer leakage (B). Figure 7-1, following, details the encapsulator design:

![Figure 7-1: Schematic of parallel air flow encapsulator. Air flow is introduced through, A, generating parallel air currents to the polymer flow, B. The air is focused in multiple chambers to the point of the needle, C, thereby generating oscillations of the needle tip and causing jet breakup forming droplets.](image)

The generated polymer droplets were gelated in an osmotically balanced solution of barium chloride (50 mM) for ten minutes, washed twice in HBSS w/ Ca$^{2+}$, Mg$^{2+}$ to maintain capsule integrity and remove excess barium and then utilized in experimentation. Throughout the course of this work, microcapsules were made containing PFC emulsions, labeled with fluorescent dye for leakage studies, glucose and 10K fluorescein isothiocyanate (FITC) for diffusivity studies, with and without PFC emulsions, and finally, with both MIN-6 cells and islets of Langerhans for in vitro cell culture studies. Optimal encapsulation settings differed in the PFC group relative control alginates. Due to the increased density and the faster settling rate of the PFC containing
polymers, the height of the needle above the gelation solution had to be adjusted to a higher point (2 cm higher). Air flow regulation was the critical parameter in microbead size and was controlled by a Cole Parmer 150 mm correlated flow meter. The air flow settings utilized to generate ~1 mm capsules were 42 (6.75 mL/min) for the control alginates and 40 (6.37 mL/min) for the PFC alginates using 1.6% UP-MVG and 46 (7.56 mL/min) for the control alginates and 43 (6.95 mL/min) for the PFC alginates, utilizing the UP-LVM alginates. Occasionally, slight adjustments had to be made to these values to account for slight variations in viscosity due to cell loading.

7.2.7 Perfluorocarbon Leakage Studies

10mL each of pyromethene labeled perfluorodecalin and perfluorotributylamine were prepared as detailed above (sec. 7.2.8, final concentration ~1 mg/mL). 5 mL of each solution was utilized in the manufacture of 10%v/v PFC emulsions, as detailed above (sec. 7.2.1). These emulsions were used to manufacture 3.3% UP-LVM and 1.6% UP-MVG alginate solutions, as detailed above (sec. 7.2.4). These PFC alginates were then utilized to manufacture 1mm labeled PFC capsules, as detailed above (sec. 7.2.9). Approximately 5 mL of capsules was made for each polymer/perfluorocarbon combination.

Prior to the experiment onset, standard curves of fluorescent dye volume were generated by serial dilution into 500 µL volumes. The initial volume of a 10% emulsion was designated as 50 µL (10% PFC in 500 µL volume) and the serial dilutions were plotted against arbitrary fluorescence units (RFU). These curves were utilized to quantify PFC leakage out of cultured capsules. Cumulative PFC loss over 14 days in culture, the
maximum duration of cell culture experiments, was assessed and these values were used in calculations of modified mass transfer benefit. Studies were performed utilizing both UP-LVM and UP-MVG alginites.

7.2.8 Capsule Stability: Bead Swelling Studies

In order to assess the stability of alginate capsules in culture and to determine the optimal alginate formulation for use in cell encapsulation, swelling studies were performed on empty capsules manufactured with and without 10% PFC emulsions utilizing both UP-LVM and UP-MVG alginites. Empty beads were manufactured as detailed above in section 7.2.8. At the end of manufacture, 10 beads per well in a 6 well non-tissue culture treated plate were placed in 2 mL of cDMEM supplemented with 10% FBS, 1% Penicillin/Streptomycin and 1% L-glutamine. Initial measurements were made using an inverted microscope and scaling software (Zeiss Axiotech v 4.5). All beads were measured. Every day for 14 days, the beads were again measured and the medium changed. Swelling was expressed as mean change in volume compared to the initial volume ± SD.

7.2.9 Diffusion Out Of Alginate Capsules : Glucose and Modeled Insulin

In order to assess mass transfer of relevant metabolites, diffusion experiments were performed based on the methods of Crank (ref), utilizing glucose and 10K FITC Dextran, to represent insulin (MW 6000). Macrocapsules (~3 mm) were manufactured by extruding solutions containing high concentrations of glucose and 10K FITC Dextran fluorescent dye (representing insulin MW 6K) from a syringe into 50 mM barium chloride solution (incubation times as detailed above in sec. 7.2.9) containing identical
metabolite concentrations, to prevent loss of diffusate prior to experimentation. After the gelation period, the beads were stored in osmotically balanced buffer solutions with the same metabolite concentration until use in diffusion experiments, again to prevent diffusate leakage.

Assuming no changes in the mechanical and physical properties of the polymer beads during diffusion, a partition coefficient of approximately unity between the polymer and aqueous solution phase, and assuming no influence of angular position on the diffusate concentration, the time-constrained unsteady-state diffusion in a sphere is described by the following mathematical formulae:

\[
\frac{\partial C}{\partial t} = D \left( \frac{\partial^2 C}{\partial r^2} + \frac{2}{r} \frac{\partial C}{\partial r} \right) \tag{7-2}
\]

subject to the following initial and boundary conditions:

1) The initial internal concentration is \( C_b \)
2) The initial external concentration is 0, \( C_{\text{bulk0}} \)
3) Boundary 1 is zero flux at the center of the bead, \( \frac{\partial C}{\partial r} (r = 0, t) = 0 \)
4) Boundary 2 is a uniform bead(s) surface concentration, \( C(r = R, t) = C_s(t) \)

Here, \( C \) = diffusate concentration in the sphere, \( D \) = effective diffusivity, and \( C_s(t) \) = the surface concentration at time, \( t \).

This relationship can be modified to account for the bulk and bead volumes in the following relationship:

\[
V_b \frac{\partial C_b}{\partial t} = -DA \left( \frac{\partial C}{\partial r} \right)_{r=R} \tag{7-3}
\]
where $V_b$ is the volume of the bulk liquid surrounding the beads, $A_t$ is the total bead surface as expressed by the product of, $N_b$, the total number of beads and the mean surface area of each bead, $4\pi R^2$. This system is subject to an initial condition whereby the initial concentration in the bulk solution is represented by, $C_b(t=0) = C_{b0}$.

The above system of equations and conditions can be solved to determine the diffusate concentration at any time point, $t$, in a finite, well-stirred bulk solution per the analytical solutions of Carslaw and Crank (Carslaw 1947; Crank 1956). The solutions exist for two different conditional cases of interest: first, diffusate moving from the bulk solution into beads that are initially free of the diffusate, the following equation is derived:

$$\frac{C_t}{C_\infty} = \frac{\alpha}{1 + \alpha} \left[ 1 + \sum_{n=1}^{\infty} \frac{6\alpha(\alpha + 1)}{9 + 9\alpha + q_n^2} \exp \left( -D \frac{q_n^2}{r^2} t \right) \right] \tag{7-4}$$

For diffusate moving from the beads into the bulk solution, the following equation is derived:

$$\frac{C_t}{C_\infty} = \frac{1}{1 + \alpha} \left[ 1 - \sum_{n=1}^{\infty} \frac{6\alpha(\alpha + 1)}{9 + 9\alpha + q_n^2} \exp \left( -D \frac{q_n^2}{r^2} t \right) \right] \tag{7-5}$$

Here, $C_t$, represents the concentration at any time, $t$, $C_\infty$, the concentration in the bulk and beads at equilibrium, $r$, the mean radius of the beads, and, $\alpha$, the ratio of the bulk volume to the total bead volume. The variable $q_n$ represents the positive non-zero roots of the equation:

$$\tan(q_n) = \frac{3q_n}{3 + \alpha q_n^2} \tag{7-6}$$
Equation 5 was utilized to determine the effective diffusivities of glucose and 10K FITC dextran (representing insulin, MW 6K) out of the beads into the bulk solution. The concentration was measured over a period of time (2 hours for glucose diffusion and a final infinite point, $t = 16$ hours; 4 hours for 10K FITC dextran and a final infinite point, $t = 16$ hours) and the experimental data were non-linearly fitted using the solutions to determine an effective diffusivity from the least squares best fit. Typically, four iterations were sufficient for accurate determination of the effective diffusion coefficient.

Initial studies were performed comparing control alginate configurations (3.3% UP-LVM vs 1.6% UP-MVG) in order to determine optimal alginate, conferring optimal permeability and adequate stability, for use in cell encapsulation. We assumed that differences with PFC emulsions inclusion, based on the literature, would be negligible. Later studies were performed to assess diffusivity comparing configurations containing PFC emulsions to their relevant controls.

7.2.10 Oxygen Diffusivity: Selected Alginates (UP-MVG)

Calibrated 5 mm diameter oxygen spot sensors (PreSens GmBH, Regensberg Germany) were used for real-time oxygen measurements in alginate solutions (~2 mm height) within a 12 well plate within a standard 37 °C humidified incubator. Oxygen was lowered to 5% by injecting pure nitrogen. One sensor was designated to monitor chamber environment and the others were utilized for oxygen measurement in the individual solutions. In every experiment, several wells were utilized for base solution (HBSS + surfactant at 2% w/v concentration) or physiological salt solution controls to ensure sensor accuracy. At equilibrium, sensor logging was initiated and the entire plate was transferred to a second incubator equilibrated with room air $pO_2$ (142 mmHg). Care was
taken to minimize agitation of the wells and to complete the transfer as quickly as possible, to prevent temperature effects. Oxygen partial pressure was measured (2 sec/data point) until equilibration with room air. Liquid volumes were recorded to document evaporative loss, if any. Diffusion was modeled as that through a single surface of a slab. Transient solutions of concentrations were generated by iterative numeration. The diffusion coefficient was determined using least squares curve fitting of measured oxygen concentration to the theoretical model:

\[
C = C_s + \frac{4}{\pi} (C_0 - C_s) \sum_{n=0}^{\infty} \frac{(-1)^n}{2n+1} \cos \left( \frac{(2n+1)\pi z}{2a} \right) \exp \left( \frac{-D(2n+1)^2 \pi^2 t}{4a^2} \right)
\]  \hspace{1cm} (7-7)

Here, \(C\) is the concentration at time, \(t\), \(C_s\) the max pO\(_2\) measured, \(C_0\) the starting pO\(_2\) utilized, \(z\), is the height of the sensor above the dish bottom and, \(a\), is the liquid height above the sensor. \(D\), is the effective diffusivity coefficient, varied to obtain least squares fitting. Finally, \(n\), is the iteration number for each solution (20 iterations).

The boundary conditions were the \(C_s\) value, assumed to remain constant at the upper surface, and the assumption of zero flux at the plate bottom surface. These methods were utilized to assess diffusion through emulsions and alginites post-manufacture. Emulsion base solutions and HBSS were used as controls to test the accuracy of the system based on published literature values. Corrections for solution height differences due to varying solution surface tensions were assumed as the prior measurements of base solutions since they could not be accurately measured in the opaque alginate solutions.
7.2.11 Oxygen Solubility: Selected Alginates (UP-MVG)

Due to bubble formation during measurement and difficulty in removing all air from the system during cap sealing, the viscous alginates did not lend themselves to accurate oxygen solubility measurement as detailed in our prior methods (Chapter 6). In this method, both measured concentration using the Trinder reaction and the measured slope of oxygen consumption are representative of the fold solubility in an emulsion relative to a base solution. From the linear fit of the curves generated by both metrics (fold concentration, fold slope) in serially diluted emulsions, we could accurately extrapolate the solubility of pure perfluorocarbons. From this equation, the fold solubility of any emulsion can be determined using the following formula:

$$Fold_{SO_2} = (1 - \text{wt}\%_{\text{alg}}) \times \text{v/v}\%_{\text{PFC}} \times Fold_{SO_2}(\text{PFC}) + \text{v/v}\%_{\text{base}} \times SO_2_{\text{base}} (7-8)$$

Here, $Fold_{SO_2}$ is the fold oxygen solubility relative to the control solution, $\text{wt}\%_{\text{alg}}$ is the weight percentage of the alginate in solution, $\text{v/v}\%$ is the volume percentages of the respective solutions (PFC and base), $Fold_{SO_2}(\text{PFC})$ is the fold oxygen solubility of the pure PFC (extrapolated) and, $SO_2_{\text{base}}$, is the oxygen solubility of the base solution. For our calculations, an average equation, generated from the mean of the measured concentration by Trinder reaction and the measured fold slope of oxygen consumption, was utilized to generate a standard curve. For perfluorodecalin, the linear equation utilized was:

$$Fold_{SO_2} = 17.484 \times \text{v/v}\%_{\text{PFD}} + 0.95115 \quad (7-9)$$

For perfluorotributylamine, the linear equation utilized was:

$$Fold_{SO_2} = 14.348 \times \text{v/v}\%_{\text{FC-43}} + 1.023 \quad (7-10)$$
7.2.12 Cellular Oxygen Consumption Measurements

Aliquots of 500,000 - 1,000,000 MIN-6 cells (p30-55) were added to a stirred chamber oxygen measurement device (Instech Labs, Plymouth Harbor, PA) in complete medium (DMEM), without bicarbonate, buffered with 25 mM HEPES. The chamber was sealed and pO\textsubscript{2} recorded until the chamber oxygen level reached 0 mmHg. At the conclusion of each reading, the cells were collected and stored in a known volume of DNA extraction buffer (AT-extraction buffer). DNA content was later determined using the pico-green DNA Quantikit. OCR was calculated using the following equation:

\[
OCR = \frac{\Delta pO_2 (\text{mmHg})}{t_{\text{sec}} \times S_{O_2} \left( \frac{\text{mol}}{\text{mmHg}} \right)} \times \frac{m^3}{m^3\text{tissue}}
\]  

Here, \(\Delta pO_2\), is the change in oxygen partial pressure over time within the chamber, \(S_{O_2}\), is the oxygen solubility coefficient in the solution within the chamber and, \(m^3\), represents the tissue volume in cubic meters. The volume of tissue was determined in the following steps: first, the cell number was determined utilizing the measured DNA content and the following conversion value of 6 pg DNA per cell. From this, using the average measured MIN-6 cell diameter of 12-15 µm, the total volume was calculated as:

\[
V_{\text{total}} = N_{\text{cell}} \times V_{\text{cell}}
\]

Average calculated OCR in mol/m\textsuperscript{3} for triplicate runs was utilized in finite element models to determine optimal capsule loading densities. Islet OCR was not performed as the number of islets obtained for encapsulation experiments were not substantial enough to perform oxygen consumption measurements.
7.2.13 Finite Element Modeling

Two dimensional diffusion/reaction finite element modeling of oxygen concentration in 1 mm microcapsules with and without perfluoroemulsions and containing cells was performed using Comsol v. 3.5. Models were designed to simulate static culture of cell containing microcapsules in standard culture conditions (95%RA/5% CO₂, 142 mmHg pO₂) and in physiological oxygen concentrations, ~5% O₂ (40 mmHg pO₂). Considering a specific fixed volume, the concentration of oxygen within that volume must be conserved. In other words, the rate of change of the oxygen concentration within a specific volume must be equal to the net flux of oxygen into that volume plus the rate of production (or consumption, in this case) within the volume. This mass balance relationship together with Fick’s Law can be written mathematically using the governing species conservation diffusion/reaction equation for each geometry, expressed here in the time dependent solver format of the modeling software:

\[
\frac{\partial c}{\partial t} - D \nabla^2 c = R \tag{7-13}
\]

where \( c \) represents concentration, \( D \), is the diffusion coefficient and, \( R \), the reaction rate (oxygen consumption rate). The system is assumed to be in equilibrium, so there is no change of concentration with respect to time and the initial term drops out. Further, it is assumed that there are negligible convective forces acting in this system and therefore, there is no need to include these terms in the governing equation. From steady state, this simplifies the mass balance equation to:

\[
R = -D \nabla^2 c \tag{7-14}
\]

where \( D \), as mentioned above, is the diffusion coefficient of oxygen through the modeled substrate (medium, tissue, polymer), \( \nabla^2 c \), the Laplacian operator (the second
derivative of concentration with respect to one spatial direction, since each geometry is symmetric about a plane, line or point) and $R$, the reaction rate or oxygen consumption rate of the tissue.

$R$, the rate of oxygen consumption per unit volume of tissue, is expressed by a simplified representation of the dependence of oxygen consumption on the concentration of oxygen. This is described by Michaelis-Menten kinetics:

$$R = R_{max} \times \frac{c}{c + K_m}, \quad R=0 \text{ when } c < K_c$$ (7-15)

where $R_{max}$ is the maximal consumption rate per unit volume of tissue determined by our experimental OCR measurements for MIN-6 cells and from published literature values for islet cells, and $K_m$ is the Michaelis-Menten constant: the oxygen concentration where the consumption rate is $\frac{1}{2}$ of $R_{max}$. The $K_m$ values we utilized, 0.4 mmHg for islets and 6.58 mmHg for MIN-6 cells, were derived from the value for mitochondrial oxidative phosphorylation corrected for the solubility dependence on temperature, for islets and from our direct measurements, for MIN-6 cells. These values were also utilized in the determination of anoxia. Tissue with oxygen levels below the $K_m$ value was considered to be anoxic. Additionally, oxygen consumption in islets was assumed to cease at a $pO_2$ of 0.1 mmHg, called the critical $K$ value, $K_c$. Values for $R_{max}$ and diffusive permeability came from oxygen consumption measurements of each cell type and direct measurements of both diffusivity through the alginates and solubility measurements in the base solutions and the PFC emulsions used to dissolve the alginates, respectively.

These equations must be solved subject to two boundary conditions dependent on the particular environmental parameters. Table 7-1, following, details the coefficients utilized for each model set up and the initial conditions. Diffusive permeability values for
alginate and PFC alginate were calculated from measured solubilities in corresponding solvents and effective diffusivities through the alginate formulations:

![Table 7-1](https://example.com/table7_1.png)

Table 7-1: Parameters utilized for finite element modeling of microcapsule culture with MIN-6 cells and IEQs

The two boundary conditions of the modeled system were:

\[ c = c_0 \text{ (incubator pO}_2\text{) } \quad (7-16) \]

for the top air/culture medium interface and insulation/symmetry along the plastic sides and bottom of the culture device (zero flux).

\[ -D \nabla c = 0. \quad (7-17) \]

The time dependent solver rather than steady-state solver was utilized due to the highly non-linear nature of the systems we analyzed. The modeling was still carried out, however, to the time of system equilibrium, depending on the height of culture medium applied. The time to solution in the time dependent solver was estimated by the ratio of the path length of diffusion squared (cm²) to the diffusivity of oxygen through the (cm²/s),

\[ L^2 / D. \quad (7-18) \]
Media height was set at 2 mm, simulating the standard culture conditions of the microcapsules in 6 well plates. A culture density of 50 capsules per well, approximately 21 IEQ/cm² or 110,000 cells/cm² (0.4% surface area coverage, IEQs, 0.2% surface area coverage, MIN-6 cells, 4.1% surface area coverage, capsules) For MIN-6 cells, oxygen consumption was assumed to be uniform throughout the capsules, as the cells were homogenously distributed, and was expressed as a dilution of \( R_{\text{max}} \) based on the v/v ratio of cell to empty polymer. To calculate cell volume, MIN-6 cells were examined under the microscope and measured to be \(~13-15\ \mu m\) in diameter. They were assumed to be spherical for the purpose of modeling.

For the islets, there was no oxygen consumption in the alginate phase, as it was confined to the non-homogenously distributed islet phase. Islets were loaded at a density of 8,000 IEQ/mL in cell experiments, based on prior publications, and this value was utilized to calculate an IEQ/capsule value of 4.2, based on a standard IEQ volume of 1.75E-06 cm³ (mL). To represent this in models, three IEQ of the standard 1.75E-06 mL volume were drawn and then a fourth of a volume 20% larger, or 2.1E-06 mL, was drawn (diameter 158.8 \( \mu m\)). Intra-capsule oxygen gradients were examined in both MIN-6 and IEQ containing capsules and further, in IEQ containing capsules, intra-islet gradients were examined. Loading density in the MIN-6 capsules was determined from the theoretical modeling targeting the \( K_m \) value in the control condition, to limit cell growth and maximize observed benefit in the PFC group. In the islets, tissue below \( K_m \) and below \( K_c \) was quantified. Additionally, models were performed at \textit{in vivo} pO₂ (40 mmHg) to assess the benefit of the PFC emulsions in the transplant setting.
7.2.14 MIN-6 Culture and Encapsulation

As a means of screening and optimizing emulsions as oxygen delivery vehicles in microcapsules, mouse insulinoma line 6 (MIN-6) cells (p30-55) were encapsulated. All cell culture and cell encapsulation was performed utilizing proper sterile technique. Cells were grown from cryopreserved aliquots of p15 gradually passaged from plating in 6-well culture dishes to T25 flasks, T-75 flasks and finally, into T175 flasks. Cells were cultured and fed every 2-3 days with Dulbecco’s Modified Eagles Medium (DMEM) high glucose (4.5 g/L) supplemented with 10% FBS, 1% L-glutamine, 1% Penicillin/Streptomycin and 79 µM 2-mercaptoethanol. Typically, > 80% confluent flasks yielded 2.5-3.0E+07 cells per flask. At the onset of each cell culture experiment, one to two flasks of cells was trypsinized with 6 mL of Trypsin/EDTA (vendor) for three minutes at 37 °C and the cells collected after the enzymatic digestion was stopped by the addition of 10 mL chilled complete medium. Cells were centrifuged, the digestion media aspirated and the cell pellet re-suspended in fresh culture medium of a known volume. Counting was performed on an aliquot of cells stained with trypan blue utilizing an hemocytometer under microscopic examination.

For encapsulation experiments, cells were suspended in respective polymers (control and perfluoroemulsion containing) at a loading density of 4E+07 cells/mL, based on finite element modeling results. The cells were mixed gently to homogenously distribute them throughout the polymer volume, and then they were encapsulated under sterile conditions, as detailed above in section 7.2.6, with slight changes to the settings due to increased viscosity imparted by the cell content. Microcapsules were cultured in two 6-well non-tissue culture treated plates at a density of 50 capsules per well. 2mL of
complete DMEM was added to each well and plates were cultured in a standard 95% RA/5% CO₂ incubator for 14 days. Culture medium was changed approximately every 24 hours. Three wells were designated for daily medium collection for analysis of glucose consumption and insulin secretion. An additional aliquot of beads was taken for time 0 histological processing. At designated time points, aliquots of capsules were subjected to a series of potency/viability assessments, detailed in the following sections.

Photographs were taken of 10 capsules from each group immediately after encapsulation and the microcapsules were measured using Zeiss Axiovision v4.8 software. These averaged measurements were utilized to normalize all potency/viability assessment and to ensure that initial capsule size was comparable to prevent experimental bias.

7.2.15 MIN-6 MTS (Water Soluble MTT) Cell Viability

The Promega CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay was utilized for determination of cell number per unit volume at predesignated time points. This assay is a homogeneous, colorimetric method for determining the number of viable cells in proliferation, cytotoxicity or chemosensitivity assays. The assay is comprised of solutions of a novel, water-soluble tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine methosulfate) PMS. The MTS dye is reduced by metabolically functional cells into a formazan salt product that, in this case, is soluble in tissue culture medium. The reduction of MTS into the soluble formazan is accomplished by active dehydrogenase enzymes in the mitochondria of viable cells. The quantity of formazan product as measured by the transfer of absorbance at
490nm-absorbance at 620 nm is linearly proportional to the number of living cells in culture, or in our case, within the capsules.

At experimental onset, serial duplicate aliquots of 1E+06, 5E+05, 2.5E+05, 1.25E+05, 6.25E+04 MIN-6 cells were plated in individual wells in a 12-well plate in 500 µL of complete DMEM high glucose for the purpose of generating a standard curve of cell viability as measured by MTS absorbance. To each well, 125 µL of Promega CellTiter 96® AQeuous dye was added. For measurements in capsules, 20 beads were plated per well in triplicate in the same volume as above with three additional wells containing the same volume of culture medium, alone. To account for the large diffusion distances in the capsules, a 3 hour incubation in a standard 37 °C, 95% RA/ 5% CO₂ incubator was implemented. At the end of the incubation period, the plate was placed on a rotating shaker at 600 rpm for two minutes to homogenously distribute the solubilized dye. Duplicate 200 µL aliquots from each well were pipetted into a 96 well plate and absorbance of all wells was measured at 490 nm with a background correction at 620 nm. The transform (490 nm-620 nm) was utilized to generate an initial linear standard curve and then this curve was utilized in time point measurements to calculate cell number normalized against capsule volume (cells/mL). Measurements were performed at d2, 4, 7, and 14, post encapsulation. MTS values were expressed as fold control cell number/volume ± SEM at every time point. To determine significant differences between control and experimental groups, the method detailed in Ch. 5, section 5.2.10 were utilized.
7.2.16 Metabolic Assessment: Glucose Consumption Rate and Insulin Secretion Rate

Daily, 1 mL culture medium samples were taken from 3 designated wells in each culture group and stored at -80°C for later assessment. Glucose concentration in medium samples was measured using an enzymatic absorbance assay in the UV range, which detects the formation of peroxide from glucose and oxygen in the presence of glucose oxidase. Standard curves of known concentration were generated using serial dilutions of the DMEM. 200 µL standards and unknown samples were run in a 96 well plate in duplicate for a kinetic reading period of 1 hour at 37 °C. Absorbance at 340 nm was recorded every 3 minutes and plotted vs. time. The plateau values were plotted for the standard curve and the linear fit of these points utilized to calculate glucose concentration in the unknown samples. Glucose consumption rate (GCR) was calculated and expressed in units of nmol per hour per 10^5 cells using the initial cell loading density. Daily measurements were expressed as mean ± SD.

Insulin secretion was assayed using the Mercodia murine insulin ELISA kit (Mercodia Inc, Uppsala, Sweden). Insulin secretion (ISR) values were calculated and expressed in pmol per hour per 10^5 cells. Daily measurements were again expressed as mean ± SD.

In each experiment (n=3), all experimental raw values were compared against the mean control raw value to calculate the fold control. Then, for each time point (d2, 4,7, and 14), mean fold control ± SEM was calculated. To determine significant differences between control and experimental groups, the method detailed in Ch. 5, section 5.2.10 were utilized.
7.2.17 MIN-6 Capsule Histological Processing

Capsules containing MIN-6 cells were collected at d0, 2, 4, 7 and, 14. A custom formalin lacking phosphate was created by addition of 250 mM CaCl$_2$ to 10% buffered formalin solution. This was done to prevent chelation of the barium and thereby, bead dissolution. The capsules were kept overnight in formalin and then exposed to a gradual dehydration utilizing one hour incubations in 30%, 50%, 70%, 80%, 90%, and finally 100% ETOH. This was followed by two consecutive one hour incubations in xylene and then immersion in two consecutive paraffin stations with agitation and removal of the excess xylene. The embedded capsules were then blocked and sectioned in their entirety on a microtome in 10 $\mu$m slices and collected onto Permafrost (Cardinal Health) slides at a density of 3 sections per slide. The slides were baked overnight at 55 °C to attach the capsule sections firmly to the slide.

After overnight heating, the slides were deparaffinized and rehydrated by sequential washes in xylene (3X), decreasing ethanol concentrations (100% 2X, 95% 2X, 70% 2X, 2 min each), and finally, a 1 minute rinse in BaCl$_2$ to reinforce the beads before rehydration for 2 minutes in water. The slides were then stained in conventional Mayer’s Hematoxylin (10 minutes) another 15 minute incubation in running, de-ionized water followed by a cytoplasmic counter stain using a 1 minute incubation in Eosin. The slides were then sequentially dehydrated (70% ETOH 2X, 95% 2X, 100% 2X, 2 min each) and brought into xylene (Xylene 2X , 2 min each) for sealing and cover slipping.

Ten representative capsular core sections from each group at each time point were photographed and analyzed using Image J quantification software. All images were taken using the same exposure time and microscope settings and analysis was performed using
a set color threshold. Hematoxylin positive staining was gauged and quantified using the following formula:

\[
\frac{P_+}{P_t}
\]  

(7-19)

where, \(P_+\), is the number of Hematoxylin positive pixels as quantified by histogram analysis in Image J, and, \(P_t\), is the total number of pixels in the entire capsule. In this way, the percentage of capsule region positive for viable cells in all three sections per group was determined and a mean viable cell percentage was calculated for each condition. This data was expressed as mean viable cell percentage \(\pm\) SD of the mean. Further, the experimental groups were compared to the control group, and the data expressed as fold control viable cell percentage \(\pm\) SEM. The tests utilized to determine statistical significance between groups are detailed in sec 5.2.10.

7.2.18 Rat Islet Isolation and Culture

After emulsion screening for encapsulation was performed using MIN-6 cells, optimal configurations were tested utilizing isolated Lewis rat islets. All animal procedures were performed at the Translational Research Laboratory of the Cell Transplant Center under protocols approved by the University of Miami Institutional Animal Care Committee (IACUC). Male Lewis rat (Harlan, Indianapolis, IN) donor pancreata (~20-25 per isolation) were digested by a mechanically-enhanced enzymatic process using Liberase (Roche; Indianapolis, IN) followed by separation on discontinuous density gradients (Mediatech; Herndon, VA). Prior to encapsulation, islets were cultured for 36-48 hours in 8.5 cm nontissue-culture Petri dishes (37 °C, 95% RA/
5% CO2) in supplemented CMRL-1066 medium (Gibco-Invitrogen; Carlsbad, CA) at a density of 8,000 IEQ per dish. This was done to allow the islets adequate recovery time prior to the encapsulation procedure.

7.2.19 Islet Encapsulation

On the day of encapsulation, islets were counted. Particles stained with dithizone (Sigma-Aldrich, St. Louis, MO) were scored for diametric range. An algorithm was used to convert these particle counts into enumerated islet equivalents (IEQ). This value was then multiplied by the appropriate dilution factor to estimate the total IEQ obtained. This number was then used to aliquot IEQ into equal groups for baseline potency tests and the encapsulation study.

For encapsulation, IEQ were suspended in respective polymers (control and perfluorooemulsion containing) at a density of 8,000 IEQ/mL, based on previous literature and verified by finite element modeling results (Dionne, Colton et al. 1993; Cotton 1996; Avgoustiniatos and Colton 1997; Duvivier-Kali, Omer et al. 2001). The IEQ were mixed gently to homogenously distribute them throughout the polymer volume, and then they were encapsulated under sterile conditions, as detailed above in section 7.2.6, with slight changes to the settings due to increased viscosity imparted by the cell content. Microcapsules were cultured in two 6-well non-tissue culture treated plates per group at a density of 50 capsules per well. 2 mL of supplemented CMRL-1066 medium was added to each well and plates were cultured in a standard 95% RA/5% CO2 incubator for 7 days. Culture medium was changed approximately every 24 hours. Three wells were designated for daily medium collection for analysis of glucose consumption and insulin secretion. An additional aliquot of beads was taken for time 0 histological processing. At
designated time points, aliquots of capsules were subjected to a series of potency/viability assessments, detailed in the following sections.

Photographs were taken of 10 capsules from each group immediately after encapsulation and the microcapsules were measured using Zeiss Axiovision v4.2 software. These averaged measurements were utilized to normalize all potency/viability assessment and to ensure that initial capsule size was comparable to prevent experimental bias.

7.2.20 Islet MTS (Water Soluble MTT) Cell Viability

The Promega CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega) was utilized for determination of IEQ viability (OD) per unit volume at predesignated time points (d2,4, and 7), as detailed above in section 7.2.16. A standard curve was not generated with islets due to IEQ number limitations. Instead, data was expressed as mean OD per unit capsule volume and then as a ratio of experimental to control values ± SEM at each time point. Statistical significance between groups was determined as outlined in section 5.2.10.

7.2.21 Glucose Stimulated Insulin Release-Static Column Method

As an assessment of islet secretory function within polymer matrices, glucose stimulated insulin release was performed at day 2, 4, and 7. The method utilized is detailed in Chapter IV. Briefly, triplicate aliquots of 100 IEQ were loaded in 10mL microchromatography columns suspended in a slurry of Sephadex G10 (GE Healthcare) beads. The IEQ were sequentially incubated in low (2.2 mM), high (16.6 mM) and a second low (2.2 mM) modified Krebs buffer, containing 25 mM Hepes, 26 mM sodium bicarbonate and 0.2% BSA. At the end of each incubation period, 1 mL of eluate was
collected for analysis and quantification of insulin by ELISA (Mercodia Uppsala, Sweden). Mean raw insulin values per 100 IEQ ± SD (based on total capsule volume and loading density of 8,000 IEQ per mL of polymer) were analyzed as were the mean delta insulin (output in high glucose – output in low 1) values ± SD. Delta values were expressed as mean fold control ± SEM.

7.2.22 Islet Capsule Histological Processing

Capsules containing islet cells were collected at day 2, 4, and 7. Fixation, processing and conventional H&E staining were performed as detailed above in section 7.2.18. Quantification was not performed in conventional H&E, but rather, this staining was used for morphological examination. As islets are not uniform particles, ranging in size from 50-450 µm, and do not distribute homogenously throughout every capsule, quantification of viable tissue regions was not feasible.

For immunohistochemical processing, a solution of polyclonal guinea pig anti-insulin antibody was utilized (100X dilution, 1 h incubation) for insulin staining (BioGenex, San Ramon, CA.). Following five serial washes with PBS (5 min each), a solution of fluorochrome-labelled (Alexafluor 488) secondary antibody (200X dilution, 1 h incubation) (Invitrogen Carlsbad, CA) against guinea pig IgG was utilized to visualize the respective hormone containing cells with fluorescence microscopy. After another series of five washes in PBS (5 min each), the sections were incubated (7 minutes) with a nuclear counter-stain, DAPI (4,6-diamidino-2-phenylindole) (Invitrogen, Carlsbad CA.). Slides were mounted and cover-slipped with ProLong Gold Antifade Reagent (Invitrogen, Carlsbad, CA), allowed to cure overnight and then imaged and photographed using an inverted fluorescence microscope with the Zeiss ApoTome slider module.
Negative controls were performed utilizing no primary antibody and processing the remaining steps as on all other sections. This control was performed to determine artefact due to non-specific binding of the secondary antibody. As a positive control, serial sections of fresh rat pancreas were processed as above.

7.2.23 Statistical Analysis

Statistical analysis for experiments with more than two groups was performed as outlined in section 5.2.10. For islet experiments with direct comparison to control (2 groups), non-parametric Mann-Whitney tests were performed assuming non-Gaussian distributions.

7.3 Results

7.3.1 Gravimetric Determination of PFC Content

The determined PFC content for emulsions utilized for alginate manufacture was 9.80 ± 0.82% and 9.56 ± 0.56% for the PFD emulsions and FC-43 emulsions, respectively. The PFC content was not significantly different between the two perfluorocarbon types (p = 0.57) indicating that there was no loss characteristic to either specific PFC compound during the emulsion manufacture.

7.3.2 Initial Particle Size Determination in PFC Alginates

DLS analysis was suitable for analysis of initial particle size in PFC alginates. There was not a significant change in measured particle size comparing emulsion to alginate manufactured from the same emulsion (p = 0.33). however, this only held true with PFD when alginates were dissolved within 2 hours of emulsion manufacture. This is more than likely due to the observed rapid rate of coalescence in PFD emulsions. The
mean particle size in PFD alginates (n=5) was 180.96 ± 15.73 nm and in FC-43 alginates (n=11), the mean particle size was 194.58 ± 17.53 nm. The mean particle sizes were not significantly different (p = 0.15). The measured polydispersity in the PFD alginates was 16.64 ± 1.62% and in the FC-43 alginates, 10.19 ± 3.22%. The FC-43 had significantly lower polydispersity (p < 0.01), in line with the observed temporal particle size stability.

7.3.3 Perfluorocarbon Labelling

Using Exciton Pyromethe 597C, both perfluorocarbons were successfully labeled. Emulsions made with PFD exhibited characteristically higher emission signals than those made with FC-43, in line with the better dye solubilization observed in the PFD. Emulsion fluorescent signal was photo-stable as long as prepared emulsions were stored protected from light. RFU loss over the 14-day measurement period was less than 2% for emulsions made from both PFD and FC-43.

7.3.4 Alginate Microcapsule Formation

Figures 7-2a and 7-2b, following, are representative photomicrographs of beads made with 1.6% UP-MVG alginate with and without 10% v/v perfluoroemulsion, respectively.
Figures 7-2a and 7-2b: Representative control 1.6% UP-MVG alginate microcapsule and PFC emulsion containing 1.6% UP-MVG alginate microcapsule.

Capsules were similar in appearance whether made with PFD or FC-43, or with UP-MVG or UP-LVM. Control capsules were clear while PFC containing capsules had an opaque appearance due to the micellar suspension of surfactant coated PFC nanoparticles.

7.3.5 Perfluorocarbon Leakage Studies

Figures 7-3a and 7-3b, following, display representative standard curves generated for labeled PFD and FC-43, respectively.
Figure 7-3a: Representative fluorescence standard curve for pyrromethene labeled PFD. Best-fit equation: $y = 135089x$, $R^2 = 0.997$

Figure 7-3b: Representative fluorescence standard curve for pyrromethene labeled FC-43. Best-fit equation: $y = 15840x$, $R^2 = 0.998$
Figures 7-4a and 7-4b, following display the measured PFC loss from UP-LVM and UP-MVG microcapsules containing 10% perfluoroemulsions. Results are graphed as calculated volume % remaining in beads based on measured loss.

Figure 7-4a: PFC loss in 3.3% UP-LVM alginate beads over 14 days. Open diamonds represent PFD emulsion containing beads. Open squares represent FC-43 emulsion containing beads.
The loss in UP-LVM was greater in PFD containing microcapsules (1.8% total loss over 14 days) than for those containing FC-43 (0.7% total loss over 14 days). The converse was true in UP-MVG where the FC-43 loss was higher (1.7% total loss over 14 days) than PFD loss in microcapsules (1.13%). PFC loss trended towards a plateau near the end of the 14-day period and microscopic inspection of the beads demonstrated visible loss of opacity around the outer edge of the beads indicating peripheral PFC loss. Loss of 14 days was figured into temporal mass transfer benefit determinations to aid in interpretation of cell encapsulation data.

7.3.6 Capsule Stability: Bead Swelling Studies

Figures 7-5a and 7-5b, following, display the results of the bead swelling studies for microcapsules made from 3.3% UP-LVM and 1.6% UP-MVG with and without
perfluoroemulsions. Results are expressed as percentage volume increase ±SD from Day 0 measurements.

Figure 7-5a: Bead swelling in microcapsules made from 3.3% UP-LVM. Open diamonds represent control 3.3% UP-LVM. Open squares represent 3.3% UP-LVM made with PFD fluoro-emulsions. Open triangles represent 3.3% UP-LVM made with FC-43 fluoro-emulsions.
Figure 7-5b: Bead swelling in microcapsules made from 1.6% UP-MVG. Open diamonds represent control 1.6% UP-MVG. Open squares represent 1.6% UP-MVG made with PFD fluoro-emulsions. Open triangles represent 1.6% UP-MVG made with FC-43 fluoro-emulsions.

Percentage volume increase over the 14 day period was similar when comparing control and perfluoroemulsion microcapsules, and also, when comparing the two alginate formulations, 3.3% UP-LVM and 1.6% UP-MVG. There were no significant differences at any time point in all comparisons (p= N.S. for all time points). Interestingly, it seemed that the UP-LVM seemed to reach a maximal swelling sooner than the UP-MVG and plateaued around day 7, while in the UP-MVG, swelling continued until about day 12.

7.3.7 Diffusion Out Of Alginate Capsules : Glucose and Modeled Insulin

Figures 7-6a and 7-6b, following, detail the measured diffusivities of glucose and 10K FITC-Dextran out of 3.3% UP-LVM and 1.6% UP-MVG alginites.
Figure 7-6a: Effective glucose diffusivity from microcapsules made of 1.6% UP-MVG, dark bar, and 3.3% UP-LVM, light bar, respectively.

Figure 7-6b: Effective 10K FTIC dextran diffusivity from microcapsules made of 1.6% UP-MVG, dark bar, and 3.3% UP-LVM, light bar, respectively.
The effective diffusivities of both compounds were significantly lower in 3.3% UP-LVM alginate than in 1.6% UP-MVG. The mean diffusivity of glucose was 4.48E-06 ± 2.67E-07 cm²/sec in UP-LVM vs. 5.84E-06 ± 1.97E-07 cm²/sec in UP-MVG (p < 0.01). For the 10K FITC-Dextran, the mean diffusivity in 3.3% UP-LVM was 2.28E-07 ± 2.97E-08 cm²/sec and in 1.6% UP-MVG 3.81E-07 ± 1.59E-08 cm²/sec (p = 0.011). As the premise of this work was to improve mass transfer, primarily of oxygen, but to avoid confounding effects of impaired nutrient diffusion, UP-LVM was excluded from further use in encapsulation studies.

7.3.8 Oxygen Diffusivity: Selected Alginates (UP-MVG)

Figure 7-7, following, displays a representative oxygen trace and the least-squares modeled fit of pO₂ increase in an alginate solution.

Figure 7-7: Representative pO₂ trace and least-squares modeled fit.
Figure 7-8, following, shows the mean calculated effective oxygen diffusivities for the control base solutions and all alginites (control, Pluronic surfactant-PFC, novel PEG surfactant-PFC).

![Figure 7-8: Calculated effective oxygen diffusivities of alginate formulations and relevant control buffer.]

The diffusivity of oxygen through water was used as a reference. The calculated effective diffusivity through the control alginate solution was $2.15 \times 10^{-5} \pm 1.69 \times 10^{-6}$ cm$^2$/s. The calculated effective diffusivity through perfluoro-alginates manufactured with pluronic surfactant (1% F-68/1% F-127) was $1.92 \times 10^{-5} \pm 1.6 \times 10^{-6}$ cm$^2$/s. There was no significant difference in effective oxygen diffusivity between the control alginate and perfluoro-alginates manufactured with pluronic surfactants ($p = 0.09$). However, there was an observable trend towards lower diffusivity through the pluronic based perfluoro-alginates. The calculated diffusivity values were in agreement with published values,
albeit slightly higher, as most measurements were performed on gelated alginate microcapsules with and without cells (Tanaka, Matsumura et al. 1984; Merchant, Margaritis et al. 1987; Kurosawa, Matsumura et al. 1989; Avgoustiniatos and Colton 1997).

7.3.9 Oxygen Solubility: Selected Alginates (UP-MVG)

Utilizing the formulas detailed in section 7.2.6, above, the fold control alginate oxygen solubilities were calculated for every PFC alginate preparation manufactured for cell encapsulation. The formula takes into account the weight percentage of alginate dissolved in the PFC emulsion assuming that oxygen is insoluble in the alginate powder. Therefore, the solubilities are slightly lower (1.6%) than their respective solutions.

For the alginates manufactured from PFD (n = 5), the mean fold derived solubility was $2.60 \pm 0.04$ the control solubility at the relevant culture temperature of 37 °C. For those made from FC-43 (n = 11), the mean fold derived solubility was $2.38 \pm 0.013$. As with the emulsions, the majority of the mass transfer benefit is derived from the increased oxygen solubility in the PFCs.

7.3.10 Cellular Oxygen Consumption Measurements

Figures 7-9 displays triplicate representative oxygen traces from a single MIN-6 cell consumption measurement.
The mean oxygen consumption rate for MIN-6 cells calculated from four separate cell passages and a minimum of three runs per experiment was $3.44\times10^{-02} \pm 4.11\times10^{-03}$ mol/m$^3$ s$^{-1}$. In theoretical modeling, the mean and range of OCR values was utilized (mean ± SD) to determine the range of suitable cell loading densities. The apparent $K_m$, determined from temporal measurements of slope, was $1.74 \pm 0.13$ nmol, or $6.58 \pm 0.48$ mmHg. The islet consumption rate, taken as an average of various published literature values, was $4.0\times10^{-02}$ mol/m$^3$ s$^{-1}$ (Tanaka, Matsumura et al. 1984; Merchant, Margaritis et al. 1987; Avgoustiniatos and Colton 1997; Johnson, O'Sullivan et al. 2011).

7.3.11 Finite Element Modeling: MIN-6 cells

Figures 7-10a and 7-10b, following, display the results of finite element modeling of MIN-6 cells in both microcapsule configurations. The results of the finite element modeling indicated that the ideal cell loading density ranged between $3.4-4.2\times10^7$
cells/mL, dependent on the range of measured oxygen consumption rates, in order to avoid sub-$K_m$ $pO_2$ gradients in the control 1.6% UP-MVG control configuration.

Figure 7-10a: Finite element modeling in control microcapsule configuration containing MIN-6 cells. Mean OCR of $3.44E-02$ mol/m$^3$ s$^{-1}$ was utilized. Optimal cell loading density in this configuration was calculated as $3.7E+07$ cells/mL.
Figure 7-10b: Finite element modeling in PFC microcapsule configuration containing MIN-6 cells. Mean OCR of $3.44 \times 10^{-2} \text{ mol/m}^3 \text{s}^{-1}$ was utilized. $3.7 \times 10^7$ cells/mL was utilized in this model to demonstrate improved oxygen gradients within PFC capsule.

The results of the modeling indicated that the PFC incorporation in the alginate would result in the maintenance of a 1.27 fold increase in cell loading relative to the control configuration.

7.3.12 MIN-6 Culture and Encapsulation

Figure 7-11, following, displays representative MIN-6 microcapsules made with 1.6% UP-MVG alginate, alone, and with perfluoroemulsions containing PFD and FC-43, respectively.
Figure 7-11: Representative microcapsules made from 1.6% UP-MVG, alone (left), with 10% PFD emulsion (middle) and with 10% FC-43 emulsion (right).

The mean diameter of the control 1.6% UP-MVG alginate microcapsules was 1.09 ± 0.07 mm while the mean diameter of PFD and FC-43 emulsions containing capsules was 1.08 ± 0.04 mm and 1.09 ± 0.05 mm, respectively. There was no significant size difference between each of the perfluoro-alginate capsule groups and the control alginate configuration (p = 0.33 and p = 0.83, respectively, vs PFD and FC-43).

7.3.13 MTS (Water Soluble MTT) MIN-6 Cell Viability

Figure 7-12, following, displays the MTS cell viability results from three consecutive MIN-6 encapsulations utilizing control and perfluoro-alginates (PFD and FC-43).
Figure 7-12: MTS viability results from 3 consecutive MIN-6 encapsulations. Results are expressed as fold control calculated cells/mL ± SEM at each time point (d2, 4, 7, and 14). On the left, are comparisons of control versus microcapsules made with PFD emulsion and on the right, are comparisons of control versus microcapsules made with FC-43 emulsions.

Both perfluoro-alginates had significantly (p << 0.05) higher fold viable cell number than the control configuration at several time points, with the exception of the FC-43 alginate at day 2 and the PFD alginate at d2 and d4. There were no significant differences between the two perfluoro-alginates, although there was an observed trend of decreasing viability in the PFD group after day 7 while in the FC-43 group, viability was maintained or improved. It was our belief that this could be due to coalescence of the PFD in the alginate and also due to PFD leakage out of the alginate beads, as we observed in the results of section 7.3.5. The observed fold increase in MTS signal was comparable to the predicted values in the finite element modeling at earlier time points (day 2 and day 4), but the later time points demonstrated higher fold cell viability (1.5 - 1.8 fold) than predicted by finite element modeling. More than likely, this was due to remodeling of the cells within the polymer matrix over time, as observed with cell lines (Stabler, Sambanis et al. 2002).
7.3.14 Metabolic Assessment: Glucose Consumption Rate and Insulin Secretion Rate

Figures 7-13a and 7-13b, following, detail the metabolic assessment of the encapsulated MIN-6 cells examining glucose consumption rate and insulin secretion rate. The results are expressed as fold control ± SEM.

Figure 7-13a: Fold control glucose consumption rate in encapsulated MIN-6 cells supplemented with perfluoroemulsions in polymer utilized for encapsulation. The left panel displays comparisons between control and PFD emulsion containing microcapsules. The right panel displays comparisons between control and FC-43 emulsion containing microcapsules.
Figure 7-13b: Fold control insulin secretion rate in encapsulated MIN-6 cells supplemented with perfluoroemulsions in polymer utilized for encapsulation. The left panel displays comparisons between control and PFD emulsion containing microcapsules. The right panel displays comparisons between control and FC-43 emulsion containing microcapsules.

The glucose consumption rates were not significantly different from the control values (p > 0.05 all time points). Insulin secretion was also not significantly different with the exception of FC-43 emulsion containing capsules at d14. These observations are likely due to the biological variability inherent in passage-to-passage experiments in cell lines and due to noise from sampling and dilution, within each group. Despite non-significant differences, some interesting trends were present with peak increase in GCR and ISR at day 7 in both groups followed by a decrease at day 14. This was similar to the observations in MTS cell viability, perhaps indicative of loss of oxygen transfer benefit due to PFC coalescence within the capsules or PFC loss, as observed in leakage studies. As the alginate is a more viscous matrix than the liquid emulsions, it is likely that the coalescence loss of oxygen transfer theorized to occur in the alginate occurs in a longer time frame than the 4 days observed in emulsions.
7.3.15 MIN-6 Capsule Histological Processing

Figure 7-14 following, display photomicrographs of triplicate MIN-6 microcapsules from each time point and each group, control and experimental.

![Representative H&E photomicrographs of MIN-6 microcapsules from all time points post encapsulation from control and both perfluoroemulsion groups (PFD and FC-43).](image)

It is clear from the histology that at all time points, there appears to be a greater viable cell number in the perfluoroemulsion containing capsules relative to the control capsules, as evident from the more prominent nuclear stain throughout the capsules. At day 2, the PFC capsules have more cells in the interior portion of the capsule than the controls and at later time points, remodeling occurs in all groups, with bands of cells near the capsule periphery and less evidence of viable cells in the capsule core. The bands are thicker in both PFC groups than in the control, evidence of improved oxygen transfer providing metabolic supply for a greater cell loading density.
Figure 7-15, following, displays the results of histological image analysis quantifying the fold control viable pixel percentage (hematoxylin positive).

Figure 7-15: Fold control viable cell pixel % (hematoxylin positive) quantification done using Image J analytical software at all time points (d2, 4, 7, and 14). PFD emulsion containing capsules are displayed in the left panel and FC-43 emulsion containing microcapsules in the right panel. The red bar represents the mean control values (always set to 1).

Data is expressed as mean fold control ± SEM. At day 2, there was no significant difference between the control and perfluoroemulsion containing capsules (p = 0.22 and p = 0.26, respectively). However, the PFD emulsion containing capsules had significantly greater viable cell pixel percentages than the control configuration at day 7 and day 14 (p <<0.01), although no significant difference was observed at d4 (p = 0.29). Capsules containing FC-43 emulsion had significantly higher viable cell pixel percentages at day 4, 7, and 14 (p << 0.01 d4 and d7, p = 0.011 d14). Notably, as with the MTS cell viability assay and with the metabolic measurements (glucose consumption and insulin secretion), there was an observed decrease in the fold difference in the PFD group relative to the control, while the FC-43 group maintained the fold difference from day 7 to day 14 of assessment. There was no significant difference between the two perfluoroemulsion
containing groups at day 14 (p = 0.40), as with both the MTS viability assessment and the metabolic measurements. Due to these observed trends and the observed loss of oxygen transfer benefit in PFD emulsions, PFD was eliminated from further use.

7.3.16 Finite Element Modeling: Islets

Figures 7-16a and 7-16b, following show the finite element modeling results for control and PFC microcapsules microcapsules cultured in standard conditions indicating tissue with a pO₂ below the Kₘ, 0.4 mmHg. Figures 7-16c and 7-16d show the same for tissue with a pO₂ below the Kₙ, 0.1 mmHg.
Figures 7-16a and 7-16b: Finite element modeling (FEM) results of islets in standard culture conditions encapsulated in either control (top) or PFC emulsion containing (bottom) microcapsules. White regions are indicative of tissue with a pO$_2$ below the $K_m$ value of 0.4 mmHg.
Figure 7-16c and 7-16d: Finite element modeling (FEM) results of islets in standard culture conditions encapsulated in either control (top) or PFC emulsion containing (bottom) microcapsules. White regions are indicative of tissue with a \( pO_2 \) below the \( K_c \) value of 0.1 mmHg.
Table 7-2, following, details the results of \( v \% \) calculations of tissue below the \( K_m \) and \( K_c \) values in both the control and PFC emulsion containing microcapsule configuration.

<table>
<thead>
<tr>
<th>Group</th>
<th>Environmental ( pO_2 )</th>
<th>% below ( K_m )</th>
<th>% below ( K_c )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>142</td>
<td>28.94%</td>
<td>19.21%</td>
</tr>
<tr>
<td>Control</td>
<td>40</td>
<td>41.92%</td>
<td>29.06%</td>
</tr>
<tr>
<td>PFC</td>
<td>142</td>
<td>19.93%</td>
<td>12.49%</td>
</tr>
<tr>
<td>PFC</td>
<td>40</td>
<td>7.23%</td>
<td>6.26%</td>
</tr>
</tbody>
</table>

Table 7-2: Tissue \( v\% \) below the \( K_m \) and \( K_c \) values in both the control and PFC emulsion containing microcapsules.

Both \textit{in vitro} and \textit{in vivo}, there was an observed benefit in tissue oxygenation in the PFC emulsion containing capsules. In the transplant setting, the control anoxia increased while, surprisingly, the PFC anoxia dropped substantially. It seems that in static culture, where the capsule boundaries are obstructed by impermeable plastic on the basal surface and the static nature of the culture dictates low surface oxygen on the apical surface, the mass transfer benefit afforded by the inclusion of PFC is artificially reduced. When the boundary conditions are equivalent on all sides with no surface \( pO_2 \) gradient, the maximal effect of the PFC is observed with a nearly 5-6 fold reduction in tissue anoxia.

7.3.17 Islet Encapsulation-Pluronic Surfactants

Preliminary screening with MIN-6 cells led us to an optimal perfluoroemulsion formulation utilizing FC-43 as the fluorocarbon moiety and 1.6\% w/w UP-MVG, as the preferred alginate. All studies using isolated rat islets were comparisons between control and FC-43 emulsion alginites. Four consecutive encapsulations were performed using
these configurations. Figure 7-17, following, displays islet microcapsules both control and FC-43 emulsion containing, respectively.

![Image](image.png)

Figure 7-17: Representative control and FC-43 containing islet microcapsules day 0.

The mean diameter of control alginate microcapsules containing islets was 1.01 ± 0.06 mm. For FC-43 containing microcapsules, the mean diameter was 1.00 ± 0.06 mm. There was not a significant size difference between control and FC-43 containing microcapsules (p = 0.49).

7.3.18 MTS (Water Soluble MTT) Islet Viability-Pluronic Surfactants

Figure 7-18, following displays the fold control mean MTS signal of the four consecutive islet encapsulations studied. Although there was a trend toward improved viability observed in the FC-43 group, there were no significant differences at any time point.
In two of the preparations, the MTS viability was lower in the PFC group relative to the control at some time points and these results were unexpected given the substantial increase observed in MIN-6 cells.

7.3.19 Glucose Stimulated Insulin Release-Static Column Method- Pluronic Surfactants

Perhaps the most important metric of islet viability, the glucose stimulated insulin release (GSIR) assay gives important information about the health of the islets as measured by their ability to manufacture and secrete insulin in response to changing glucose levels. As oxygen is a critical component of this metabolic function, we expected that our PFC capsules would perform with improved insulin output in response to
increased glucose. Figures 7-19a and 7-19b, following, display the mean results of the glucose stimulated insulin release from three individual encapsulations.

![Graph showing insulin output with error bars for LOW 1, HIGH, and LOW 2 incubation periods.]

Figure 7-19a: GSIR results from Lewis rat islets encapsulated in control polymer. From left to right in each incubation period, insulin values at day 2, day 4, and day 7, respectively.
The mean insulin output was not significantly different at any time point, but there was an observed lower output in all time points in the PFC group relative to the control. As expected, there was a characteristic decrease in insulin output in both groups as the time post encapsulation increased. Surprisingly, the initial drop in insulin output from day 2 to day 4 was greatest in the PFC group (30 ± 7 ng/mL relative to 22 ± 26 ng/mL in high glucose insulin output). These observations are reflected in the delta values (high insulin output – low1 insulin output) detailed in the figure 7-20, following.
7.3.20 Islet Capsule Histological Processing - Pluronic Surfactants

Figures 7-21a, 7-21b, and 7-21c, following, display photomicrographs of islet containing microcapsules with and without PFC emulsions at d2, d4, and d7 respectively.

Figure 7-20: Fold control delta values (high glucose insulin output - low1 glucose insulin output).
Figure 7-21a: Photomicrographs of islet containing microcapsules at d2 post-encapsulation. Control (left) and PFC emulsion containing (right) microcapsules displayed.

Figure 7-21b: Photomicrographs of islet containing microcapsules at d4 post-encapsulation. Control (left) and PFC emulsion containing (right) microcapsules displayed.

Figure 7-21c: Photomicrographs of islet containing microcapsules at d7 post-encapsulation. Control (left) and PFC emulsion containing (right) microcapsules displayed.
There was not a significant observable morphological difference between the islets in the control and PFC emulsion containing microcapsules. In fact, it appeared as though the PFC emulsion containing microcapsules were able to sustain larger and greater numbers of islets.

7.3.21 Diffusion Revisited: Glucose and Modeled Insulin- Pluronic Surfactants

Based on the observed decrease in oxygen diffusion, MTS viability and insulin secretion, we surmised that there might be decreased diffusive permeability in PFC containing capsules as a result of the low molecular weight Pluronic surfactants. We theorized that this was the result of the amphiphilic Pluronic aggregates that were characteristic of the emulsion nanoparticles. Figures 7-22 and 7-23, following, display the results of diffusion studies utilizing glucose and 10K FITC dextran (modeled insulin) comparing the control alginate formulation to the PFC emulsion containing alginate.
Figure 7-22: Effective glucose diffusivity measurements from alginate capsules: control vs. perfluoroemulsion containing.

Figure 7-23: Effective 10K FITC dextran diffusivity measurements from alginate capsules: control vs. perfluoroemulsion containing.
In both cases, effective diffusivity was significantly hindered by the presence of the PFC emulsion, as with the effective diffusivity of oxygen. Glucose diffusion through the PFC containing beads was approximately 76% of that through the control beads (4.46E-06 ± 5.39E-07 cm²/s, p = 0.02 vs. control) while modeled insulin diffusion was approximately 62% of that through the control beads (2.36E-07 ± 4.99E-08 cm²/s, p = 0.046 versus control).

It was clear from these results that the beneficial effect afforded by improved oxygen mass transfer was countered by the decreased diffusive permeability of glucose and insulin, potentially cancelling any beneficial effect observed from enhanced oxygen. In the case of MIN-6, cells this was not observed more than likely due to the temporal remodeling that occurs in the rapidly growing and dividing cell line. By the 14-day endpoint in the MIN-6 cells, the majority of viable tissue was observed on the periphery of the capsules, where diffusive distances were minimized by the migration of the cells. In the case of islets, where cell replication and remodeling does not occur, the effect of permeability limitations imposed by the emulsion components was the observed decreased cell viability and function.

7.3.22 Polymer Reformulation: Diffusion Revisited- Glucose and Modeled Insulin

As a result of the above permeability studies, we re-investigated surfactants looking for a more hydrophobic composition. In collaboration with the laboratory of Jeffrey Hubbell, we tested a PEG based co-polymer similar to Pluronic F-127 (~MW 11,000). F-127 is a linear molecule with two terminal polyethylene oxides (polyethylene glycols) with an interior polypropylene oxide (PEO-PPO-PEO). The interior PPO is mildly hydrophobic, increasing with increased mass utilized. In the surfactant
composition we obtained from Dr. Hubbell, the interior PPO molecule was replaced by polypropylenesulfide (PPS), which has a much greater hydrophobicity and therefore, a decreased likelihood for forming the aggregate networks that result in impeded permeability.

Emulsions were made from this novel surfactant at the same weight percentage incorporation as the others tested (2% w/v). The emulsions had similar particle size and polydispersity values for those made from the Pluronic surfactants (~195nm ± 6.2nm, ~9.3 ± 2.3%). Additionally, the new surfactant exhibited a temporal particle size stability in emulsions made with FC-43 similar to those made with FC-43 and 2% w/v Pluronic surfactant formulations, reinforcing the dependence of emulsion stability on the PFC used. Liquid alginate (1.6% UP-MVG) manufactured from these PEG-PPS-PEG based emulsions containing FC-43 was utilized for oxygen diffusivity experiments (as outlined in sections 7.2.10).

Figure 7-24, following, displays the results of the effective oxygen diffusion measurements in the new PFC-alginate formulation relative to the control configuration.
Effective oxygen diffusion was greatly improved by the use of the novel surfactant. The calculated value in the PFC-alginate formulation was 2.61E-05 ± 1.48E-06 cm²/s relative to 2.15E-05 ± 1.69E-06 cm²/s measured in the control formulation. The effective diffusivity was highly significantly different between the two groups (p < 0.01). A higher effective oxygen diffusivity, although not observed before the use of this new surfactant formulation, was expected in the PFC containing alginates, as measured oxygen diffusion through pure PFCs is considerably higher (2-5 fold) than through physiological solutions.

Diffusion of 10 kD FITC dextran was assessed out of alginate capsules, as in section 7.2.9, to determine if using the PEG-PPS-PEG surfactant resulted in improvement to the permeability in the polymer matrix. Figure 7-25, following, details the results of
the 10K FITC dextran diffusion experiments comparing control to the novel PFC alginate.

![Graph showing effective diffusivity measurements of 10K FITC dextran from alginate capsules: control vs. novel PFC alginate.](image)

**Figure 7-25**: Effective 10K FITC dextran diffusivity measurements from alginate capsules: control vs. novel PFC alginate.

Effective diffusivity of 10K FITC dextran was substantially improved in PFC alginates utilizing the new surfactant formulation. In fact, the measured value trended better than that measured in the comparable control (4.24E-07 ± 2.27E-08 cm²/s vs. 3.81E-07 ± 1.59E-08 cm²/s) although not significantly different (p = 0.083). Thus, by adjusting the hydrophobicity of the surfactant, we were able to modulate diffusive permeability.

**7.3.23 Islet Encapsulation-PEG-PPS-PEG Surfactant**

With the resolution of the diffusive permeability issues, we initiated new islet encapsulation studies using emulsions made from the PEG-PPS-PEG surfactant and FC-
43. Figures 7-26a and 7-26b, following, display representative islet microcapsules made from control and novel PFC alginate formulations, respectively.

Figures 7-26a (left) and 7-26b (right): *Islet containing microcapsules manufactured with control alginate and novel PFC alginate.*

The mean diameter of control alginate microcapsules containing islets was 0.97 ± 0.03 mm. For PFC containing microcapsules, the mean diameter was 0.98 ± 0.04 mm. There was not a significant size difference between control and FC-43 containing microcapsules (p = 0.14).

7.3.24 ISLET MTS (Water Soluble MTT) Islet Viability-PEG-PPS-PEG Surfactant

Figure 7-27, following, shows the results of fold control MTS viability in three consecutive rat islet encapsulations.
The fold MTS viability was higher than control values at all three time points, and was significantly different at days 2 and 7 (p = 0.015 and p = 0.037). Most importantly, the values were substantially better than those observed in the prior Pluronic surfactant-based PFC microcapsules.

**7.3.25 Glucose Stimulated Insulin Release-Static Column Method-PEG-PPS-PEG Surfactant**

Figures 7-28a and 7-28b display the mean insulin values from the GSIR assay for control and PFC alginate capsules, respectively. Data is expressed as mean insulin output per 100 IEQ ± SD.
Figure 7-28a: GSIR raw data from islets encapsulated in control alginate configuration. From left to right in each incubation period, insulin values at day 2, day 4, and day 7, respectively.

Figure 7-28b: GSIR raw data from islets encapsulated in PFC alginate configuration. From left to right in each incubation period, insulin values at day 2, day 4, and day 7, respectively.
Again, there was a drop off in insulin production temporally in the control group, but conversely, the insulin secretion was maintained in the PFC alginate group, dropping slightly at day 7 and increasing at day 4. Additionally, the insulin production was higher in response to elevated glucose at all three time points in the PFC alginate group.

Figure 7-29 displays the fold control delta value at each time point. The delta is the metric that correlated directly with in-vivo function and an increased delta typically translates to improved time to reversal of hyperglycemia in recipient athymic nude mice (see Chapter 5).

![Figure 7-29: Fold control Delta insulin output (High – Low 1 Insulin Values).]

Notably, the PFC alginate had higher delta values than the control at all time points, but increasingly different at days 4 and 7, when the control insulin output decreased temporally while the PFC alginate maintained increased insulin secretory
output. Day 4 and day 7 had significantly higher fold delta values in the PFC group ($p << 0.01$, both time points).

### 7.3.26 Islet Capsule Histological Processing - PEG-PPS-PEG Surfactant

Figures 7-30a, 7-30b, and 7-30c following, display photomicrographs of control (top) and PFC alginate (bottom) microcapsules containing rat islets at day 2 and day 4, and day 7, respectively.

**Figure 7-30a**: Representative photomicrographs of encapsulated Lewis rat islets at day 2 in control (left) and PFC (right) alginate.

**Figure 7-30b**: Representative photomicrographs of encapsulated Lewis rat islets at day 4 in control (left) and PFC (right) alginate.
The islet morphology and nuclear staining is more consistent with viable tissue in the PFC containing capsules. In the control sections, there is notable central necrosis as indicated by fainter nuclear and cytoplasmic staining and visible regions of tissue disintegration. As the sections photographed were from the same approximate capsule depth and islets were selected of approximately the same size and location within the capsule, it appears that the enhanced oxygen delivery in the PFC alginates better maintains islet structure post encapsulation in addition to preventing the central necrosis that is apparent in the control configuration islets.

7.3.27 Islet Immunohistochemistry: PEG-PPS-PEG Surfactant

Figures 7-31a, 7-31b, and 7-31c, following, display photomicrographs of antibody staining for insulin and a nuclear counterstain, DAPI, at d2, d4 and a negative control from the same staining, respectively.
Figure 7-31a: Immunohistochemistry performed on encapsulated islets in control (left) and PFC (right) microcapsules: Day 2. Green is insulin+ staining and blue is DAPI, nuclear counter-stain. 20X magnification, 150 ms exposure for Alexa 488 and 10 ms for DAPI.

Figure 7-31b: Immunohistochemistry performed on encapsulated islets in control (left) and PFC (right) microcapsules: Day 4. Green is insulin+ staining and blue is DAPI, nuclear counter-stain. 20X magnification, 150 ms exposure for Alexa 488 and 10 ms for DAPI.
Day 7 micrographs were affected by artefact presumably from contaminants during processing. The indication of these representative photomicrographs taken of sections at approximately the same depth in the capsule is that the PFC capsules better maintain insulin secretory capacity, based on the strong signal difference when identical exposure times are utilized for detection. This is in line with our metabolic, MTS and GSIR, observations.

### 7.4 Discussion

Taken together, the data presented indicate that conventional microencapsulation can benefit from the addition of perfluorocarbons in emulsified form. The limitations we encountered were related to 1.) the amount of PFC that could be included (10% v/v maximal) without compromising capsule structural integrity and preventing sterile filtration; 2.) the impaired permeability of important metabolites (oxygen, glucose and insulin) through the perfluoro-alginates due to the aggregation of surfactant components
and 3.) the biological noise inherent in the readouts used to compare the various alginate configurations with encapsulated cells.

While not the dramatic benefit we hoped for, the incremental increase in metabolic function and islet cell survival, our penultimate read out, was significant compared to control formulations, in many instances. Our empirical data often exceeded the predicted benefit from finite element modeling, leading us to question the validity of the model parameters described in the literature and to hypothesize that oxygen, while important, is a component in a cascade of metabolic processes that, when deficient, triggers events that result in far greater loss of cell function and viability than what is predicted by typical “oxygen-only” models. Conversely, an incremental increase in oxygen, afforded by the inclusion of PFC, results in improved maintenance of this cascade of metabolic events, resulting in greater than expected islet viability and function over time.

One observation that was contrary to previously published findings was the measured loss of PFC from capsules. The loss appeared minimal over the 14 day period of measurement and eventually plateaued, but even this slight loss would cause changes in afforded oxygen mass transfer benefit. (Khattak, Chin et al. 2007). We previously (Chapter 6) demonstrated the importance of emulsion stability in the maintenance of oxygen mass transfer benefit and concluded that emulsions made with FC-43 maintained the greatest long-term stability.

We performed some preliminary studies (data not shown) examining particle stability in capsules, as we believed that there was more than likely a similar coalescence in alginate capsules to that we observed in liquid emulsions, only slower. Examination of
microcapsules made with PFD emulsions using confocal microscopy confirmed what we believed with inter-capule particle size increasing over the three days of measurement. Further work needs to be done to confirm this and quantify the rate of coalescence.

Critically, we demonstrated the importance of investigating all parameters of PFC emulsion and alginate formulation in order to maximize enhanced oxygen mass transfer. Historically, PEG co-polymers have been mixed with alginate to tailor diffusion rates from microcapsules used for therapeutic drug delivery. This property is the result of aggregate formation within the alginate matrix by PEG particles, heavily dependent on the w/v% concentration utilized and component hydrophobicity. In drug delivery, this is a desirable property of the PEG networks allowing for controlled delivery. In cellular encapsulation, however, this property is an impediment to the diffusion of essential nutrients into the capsule and waste and secretory products out of the capsule. It was our belief that our observed decrease in islet secretory function and unexpected minimal benefit to islet viability was the direct result of impaired diffusive permeability. Utilizing a surfactant with a more hydrophobic core our work, we significantly improved diffusion of all relevant metabolites (oxygen, glucose, and insulin) resolving the issue of surfactant impediment to permeability. To date, a thorough study of these components has not been performed in the body of literature regarding encapsulation with perfluoro-alginates (Inverardi 1999; Chin, Khattak et al. 2008; Johnson, O'Sullivan et al. 2011). Our work clearly demonstrates that failure to assess oxygen mass transfer components and to optimize perfluoro-alginate configuration results in sub-optimal benefit and, even, detriment to encapsulated cells. We feel that this is the reason for the minimal benefits observed in prior literature (Khattak, Chin et al. 2007; Chin, Khattak et al. 2008; Johnson,
O'Sullivan et al. 2011). Our future work will examine the development of more concentrated PFC emulsions and novel surfactant formulations to maintain particle size stability, optimal permeability of metabolites and even greater enhancement of oxygen mass transfer. Additionally, work will be undertaken to better understand the mechanisms of enhanced viability imparted by the oxygen carrying PFCs on the molecular level.

Despite our promising results, the increasing trend in research is moving away from micro-scale encapsulation more towards conformal coatings and nano-scale self-assembling layers for immuno-protecting cells (Desai 2002; Cui, Barr et al. 2004; Teramura, Kaneda et al. 2007; Wilson, Cui et al. 2008; Gattas-Asfura and Stabler 2009; Teramura and Iwata 2009; Kizilel, Scavone et al. 2010). While these technologies have shown some promise, there is a need for better characterization of the completeness of the coatings and the mass transfer properties of these polymer configurations, as several are PEG-based and could be susceptible to the permeability impediments we observed using similar structures. However, this might be less of a concern given the greatly reduced diffusion distances of these coatings. Additionally, there is little data investigating the potential toxicity associated with covalent linkages and their effect on membrane turnover critical to cell signaling and proper function. No matter what configuration is ultimately utilized to immuno-protect islet cells or the generated cell source to treat diabetes, the role of oxygen in maintaining their function and viability in vitro and in vivo is of critical importance. If this cellular therapy is ever to make an impact in the clinical realm, proper oxygenation of these highly metabolically active 3D insulin secreting structures must be taken into careful consideration in every therapeutic device design if scale-up to the human model is ever to become a reality.
CHAPTER 8: CONCLUSIONS AND SUGGESTED FUTURE WORK

8.1 Summary and Concluding Remarks

As of 2009, the annual diabetes-related medical spending stood at 113 billion dollars annually, taking into account the cost of disease treatment and the treatment of underlying secondary complications. One in every ten healthcare dollars spent is applied towards diabetes-related costs. The market for diabetes products is a lucrative one, generating approximately 2.2 billion dollars annually for pharma companies. Given the overall strain of diabetes on the healthcare system and the high rate of morbidity and mortality associated with the disease, there is a strong motivation for research into curative therapies.

One potential treatment option that has surged to the forefront in the last decade is the transplantation of cadaveric donor islets of Langerhans. While promising, many obstacles have been encountered implementing this therapy, detracting from its potential impact in the clinical realm. Perhaps the greatest impediment to the success of islet engraftment is the inadequate oxygenation provided in the transplant setting. Islets, when isolated, are rended from a rich vascular network that is sufficient to maintain their high metabolic demands. Upon isolation, they experience steep intra-islet oxygen gradients that lead to core necrosis and loss of proper secretory function. Upon transplantation, this lack of oxygen is further exacerbated, resulting in sub-optimal function and early graft loss (~70-80%) resulting in further cell death or dysfunction. This early death and dysfunction recruits a strong recipient immune response that without a blanket of strong immunosuppression, leads to early graft failure. One long attempted means of reducing the recipient immune response has been the use of microencapsulation, where islets are
masked in hydrogel matrix essentially rendering them invisible to the immune response. This encapsulation, however, further exacerbates oxygen and nutrient limitations, as diffusive distances increase and further limit cell viability. Clearly, there is a need for improvement of oxygenation to isolated islets both during in-vitro culture and further, in the transplantation/encapsulation setting.

Therefore, the major objective of this dissertation were the following: (1) to enhance oxygen delivery to isolated islets cells in culture targeting more physiological oxygen levels, as islets have been shown to be adversely affected by both higher and lower than physiological pO₂; (2) to develop methods for determining enhanced oxygen delivery and islet potency that would allow us to quantify the benefit afforded by enhanced oxygen delivery; (3) to fully develop and characterize optimal oxygen carrying emulsions of perfluorocarbon moieties for use in islet encapsulation; and (4) to fully develop and characterize optimal alginate microcapsules manufactured from PFC emulsions and quantify their benefit, if any, to encapsulated cells.

In order to achieve these aims, five studies were carried out: (1) the development of a method for the determination of dissolved oxygen content in PFC emulsions, the critical variable to determining their oxygen delivering benefit (Chapter 3); (2) the development of a novel method for the measurement of glucose stimulated insulin release and the demonstration of its highly predictive capacity of islet potency as measured by diabetes reversal time in athymic nude mice (Chapter 4); (3) the development of PFC/PDMS novel islet culture platforms allowing for culture pO₂ tailoring to minimize islet loss and optimize islet function and viability (Chapter 5); (4) the design and thorough characterization of perfluoroemulsions for use in cell encapsulation examining
specifically their oxygen transfer characteristics, (Diffusive Permeability, $P_D$), the product of solubility and effective diffusivity; and, finally, (5) the optimization and implementation of alginate microcapsules supplemented with PFC emulsions and utilized in cell culture experiments with mouse insulinoma, MIN-6, cells and rodent islets. The most important findings of the aforementioned studies are discussed in the following paragraphs.

8.1.1 Method Development

We developed a method to successfully quantify the dissolved oxygen content of manufactured PFC emulsions at relevant temperatures (25 and 37°C). This method allowed us to accurately estimate the oxygen transfer benefit supplied to cells from the emulsions utilized in encapsulation experiments. This method will help others utilizing PFC emulsions to accurately determine diffusive permeability ($P_D$).

Our method of assessing glucose stimulated insulin response in islets of Langerhans proved to be a good metric of islet potency and highly predictive of diabetes reversal time in athymic nude mice transplanted with full mass grafts (2,000 IEQ) of isolated human islets of Langerhans. This metric was useful in our data analysis of culture experiments in enhanced oxygen transfer platforms and in encapsulated islets, helping us distinguish potency gain or loss due to experimental interventions.

8.1.2 PFC-PDMS Platforms to Improve Islet Viability and Function

We created a novel cell culture platform enhancing oxygen transfer to cells by replacing the impermeable plastic bottom surface with a highly oxygen permeable PFC-PDMS membrane. In this way, we were able to supply adequate oxygen to isolated islets
and were able to more closely approximate their in-vivo niche by lowering the environmental incubator pO2. The outcome was improved viability and function as measured by glucose stimulated insulin release, oxygen consumption rate, overnight loss assessment by enumeration, quantitative real time RT-PCR of inflammatory and apoptosis related genes and diabetes reversal time in marginal mass (1,000 IEQ) transplants in athymic mice. We hope that these findings will result in a shift from dogmatic culture methodologies utilizing plastic materials and static culture towards dynamic culture methods that more closely approximate the native environment of the cultured cell type.

8.1.3 Optimization of Perfluoroemulsions for Cell Encapsulation

We optimized the manufacture of perfluorocarbon containing emulsions examining emulsification parameters, surfactant and PFC properties, oxygen diffusivity and dissolved oxygen content. We detailed the components and methods to manufacture nano-scale, sterile filterable and, most importantly, stable, non-coalescing perfluoroemulsions. We described the importance of particle size in the maintenance of oxygen mass transfer benefit. This body of work will allow future investigators to reproducibly manufacture sterile PFC emulsions optimized for use in biomedical applications involving cell culture and transplantation.

8.1.4 Cell Encapsulation with Perfluorocarbon Enhanced Polymer

In this section, we detailed the use of perfluoroemulsions in alginate microcapsules to enhance oxygen delivery to an encapsulated cell line and to metabolically demanding islets of Langerhans. We demonstrated that PFC inclusion has
no effect on capsule stability, when incorporated properly, and that PFC inclusion is beneficial to the viability and function of encapsulated cells. Importantly, we showed that the use of conventional amphiphilic surfactants is detrimental to capsule permeability for several relevant metabolites (oxygen, glucose and insulin) and care should be taken to utilize surfactants with increasingly hydrophobic cores to prevent aggregation of surfactant particles, the cause of impeded diffusion/permeability. We observed a substantial increase in islet viability and function (1.5-2 fold over control) that did not match finite element modeling utilizing literature-based coefficients and our own experimental values (Cotton 1996; Avgoustiniatos and Colton 1997; Khattak, Chin et al. 2007; Johnson, O'Sullivan et al. 2011). Models indicated a modest benefit (1.2-1.3), contradictory to our empirical observations, indicating that oxygen plays a partial role in islet viability and function. Clear from this body of work, oxygen is of utmost importance to the physiological well-being of islets, but the role of factors other than oxygen needs to be better elucidated.

8.2 Recommendations for Future Work

As islet and stem cell therapies advance towards a cure for diabetes mellitus, the integral role of oxygen is becoming increasingly apparent. There is a recent indication in stem cell research that careful modulation of oxygen is critical to the temporal expansion and the differentiation of stem and precursor cells into desired specific phenotypic endpoints (Fraker, Alvarez et al. 2007; Powers, Millman et al. 2008; Fraker, Ricordi et al. 2009; Horton, Millman et al. 2009; Powers, Millman et al. 2010). Additionally, there has been a substantial body of evidence indicating the importance of oxygen delivery and mass transfer limitations in the culture of 3D cell constructs, particularly islets of

Clearly, given this need to better simulate physiological oxygenation to maintain proper cell viability and function in highly metabolic cell aggregates, like islets, there is a strong need for further work into methods for both enhancing oxygen mass transfer and, perhaps, coupling this with novel methods of oxygen generation and controlled release. We feel that systems, such as our PFC/PDMS devices could help researchers optimize their culture methods and might result in improvements to the in vitro outcomes in their experiments.

In the islet transplant realm, enhancing oxygen mass transfer in tissue engineering devices is a necessary outcome in order to achieve realistic device scale-up and implementation in the clinical realm. In future work, we would like to implement our PFC emulsions in tissue engineering constructs in the transplant setting, as we feel the maximal benefit may be observed in this environment. Again, coupled with oxygen generation, we feel that these incremental improvements may help researchers move closer to a clinically applicable device.
REFERENCES


