ENGINEERING A PANCREATIC ISLET MICROENVIRONMENT FOR IMPROVED SURVIVAL, FUNCTION, PROTECTION, AND DELIVERY

by

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To Kenny and my family

Your love makes me who I am

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ABSTRACT

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Title: Engineering a Pancreatic Islet Encapsulation Strategy for Improved Survival, Function, Protection, and Delivery.
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It is estimated that 1 in 500 Americans are inflicted with type I diabetes (T1D) with approximately 18,000 children and adolescents diagnosed each year. Islet/β cell replacement with long-lasting glucose-sensing and insulin-releasing functions has the potential to eliminate the need for insulin injections and minimize complications for individuals with T1D. However, limitations remain precluding it from widespread clinical use, including i) limited donor supply, ii) significant loss of functional islet mass upon transplantation, iv) limited functional longevity, and v) need for life-long systemic immunosuppression. To restore glucose-responsive insulin-release back to the patient's body without the need for systemic immunosuppression, our approach involves a subcutaneous injection using a novel fibril-forming biologic, type I oligomeric collagen (Oligomer). Oligomer protects and in situ encapsulates replacement cells beneath the skin by transitioning from a liquid to a stable collagen-fibril scaffold, within seconds, just like those found in the body's tissues. Preclinical validation studies in streptozotocin-induced diabetic mice show that replacement of islets at a dose of 500 or 800, results in a rapid (within 24 hours) reversal of hyperglycemia. All animals receiving syngeneic islets maintained euglycemia for beyond 90 days, while >80% of animals receiving allogeneic or xenogeneic (rat) islets remained euglycemia for at least 50 days. Histopathological analysis of Oligomer-islet implants showed normal morphology with no apparent evidence of a foreign body response and immune cell infiltrate. To our knowledge, this is the first report of an injectable subQ islet transplant strategy that yields rapid lowering and extended glycemic control without systemic immunosuppression.

1. INTRODUCTION

1.1 Type I Diabetes

It is estimated that 1 in 500 or 1.25 million Americans, including both children and adults, are inflicted with type I diabetes (T1D) with approximately 18,000 children and adolescents diagnosed each year.[1] According to the Centers for Disease Control and Prevention (CDC) and the American Diabetes Association (ADA), diabetes imposes an increasing burden on the economy through increased direct medical costs and indirect costs from work-related absenteeism, reduced productivity, reduced labor force participation, and premature mortality.[2] In fact, these costs totaled $245 billion in 2012 with a patient's average medical expenditures being $13,700 per year including hospital inpatient services, physician visits and other outpatient services, medications, insulin, and other diabetic supplies.[1,2]

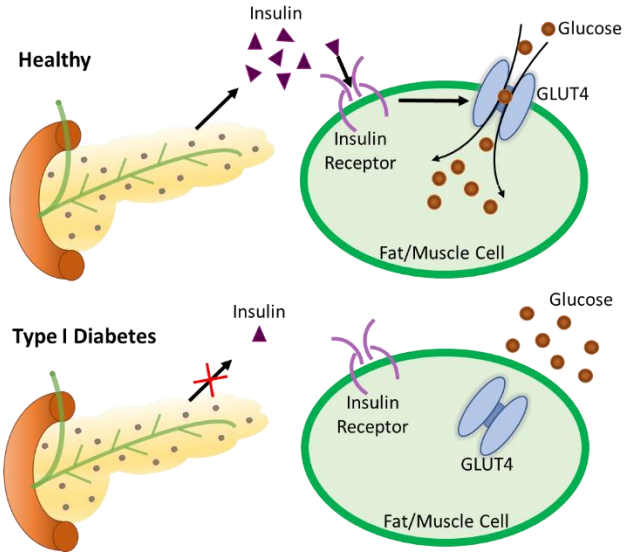

Figure 1-1. Biological effects of T1D.
The effects T1D has on insulin secretion and blood glucose concentration. T1D results in no or very little insulin secretion from the pancreas causing a chronic increase of blood glucose levels and its associated life-threatening complications.

T1D is a metabolic disorder caused by an autoimmune response that is characterized by an insufficient amount of insulin produced by the body. This occurs because the body's immune system attacks and permanently destroys the insulin-producing beta cells located in the islets of Langerhans of the pancreas. Under normal conditions, the pancreas releases insulin in response to an increase in the concentration of blood glucose. Glucose enters the insulin-producing beta cells through a tightly regulated glucose sensing mechanism utilizing glucose transporter (GLUT) proteins, more specifically GLUT2, where it initiates a signaling cascade that triggers insulin secretion.[3] Once insulin is released from the pancreas, it allows glucose, using the GLUT4 protein, to move from the blood into primarily muscle and fat cells where it is converted to energy. During T1D, there is very little or no production of insulin which limits glucose from entering other cells

in the body causing an excess of glucose in the blood (Figure 1-1). Without insulin, the symptoms and complications associated with diabetes are produced including heart disease and stroke, kidney failure, blindness, and loss of lower limbs.

1.2 Standard of care for Type I Diabetes

Traditional means of T1D management involves the regular administration of exogenous insulin via injections or continuous pumps. The insulin either replaces or supplements the patient's own supply and helps maintain a normal blood glucose level. However, this method of treatment requires patients to maintain a strict regimen of diet, exercise, monitoring, and injection, which can be difficult to follow and manage. In fact, according to two recent surveys focused on the United States type I diabetic population, at least one third of patients fail to take their insulin as prescribed, and approximately 20% of adults intentionally skip their doses.[4] This relatively high percentage communicates the patient dissatisfaction of current treatment options and suggests that a large number of people with T1D would embrace a less painful, more convenient, long term solution to insulin management and reduced glucose monitoring. Insulin pumps provide continuous insulin infusion without the need for constant monitoring, but they can be inconvenient and could carry the risk of infection.[5] More recently, continuous glucose monitors and hybrid closed-loop systems have gained recognition for diabetes management. With these systems, subcutaneously attached sensors are able to measure interstitial blood glucose every 5 minutes 24 hours a day, 7 days a week, and in some cases, it automatically adjusts insulin dosage based on data from readings and trends.[6] However, user cost, convenience, and acceptance are limited by the sensor lifetime (3-7 days, depending on the model), the need to calibrate twice a day using a traditional glucose monitor, requirement for a bolus of insulin after meals and physical activity, and the time required for physician and patient education to operate the device.[7] Daily insulin injections are life saving for type I diabetics, however, this standard of care falls short in terms of effective glycemic control, especially when compared to functioning pancreatic β cells. In fact, less than one-third of patients are achieving target blood glucose levels.[8] As a result, individuals with T1D remain at high risk for life-threatening complications, including hypo- and hyper-glycemic episodes, cardiovascular disease, loss of kidney function, and neuropathies. **It is this reality that motivates the search for new therapeutic options that restore normal glucose metabolism to individuals with T1D, allowing them to experience a better quality of life with**

fewer medical complications. Beta cell replacement therapy has the potential to provide long-lasting glucose-sensing and insulin-releasing functions to eliminate the need for insulin injections.

1.3 Pancreatic Islets and Their Microenvironment

In the abdomen, the pancreas is surrounded by the stomach, spleen, and small intestine (Figure 1-2), and can be broken into 3 main parts: (1) the head, the largest part, (2) the body, the middle section, and (3) the tail, the thin end of the pancreas. The pancreas has two main functions, the exocrine function which helps with digestion and the endocrine function which regulates blood sugar.[9] The exocrine pancreas produces digestive enzymes known as pancreatic juices from the acini glandular structured tissue, and secretes it into a network of small tubes known as ducts. The endocrine pancreas consisting of islets of Langerhans located throughout the pancreas secretes hormones such as insulin and glucagon into the bloodstream to regulate blood glucose levels. An islet of Langerhans is an isolated cluster of thousands of individual endocrine cells dispersed among the pancreatic exocrine tissue (Figure 1-2). Each islet contains five different types of cells including alpha, beta, delta, PP and epsilon cells secreting glucagon, insulin, somatostatin, pancreatic polypeptide, and ghrelin, respectively.[10,11] In adult humans, there are approximately 1 million islets with each measuring from 50-250 μm in diameter. Interestingly, the size of islets remains constant between species, but the overall number, composition, and cytoarchitecture varies between species.[10] For example, mouse islets have an inner beta cell core with the other cell types on the periphery whereas human islets have beta cells and the other cell types interspersed within the islet.[11–13]

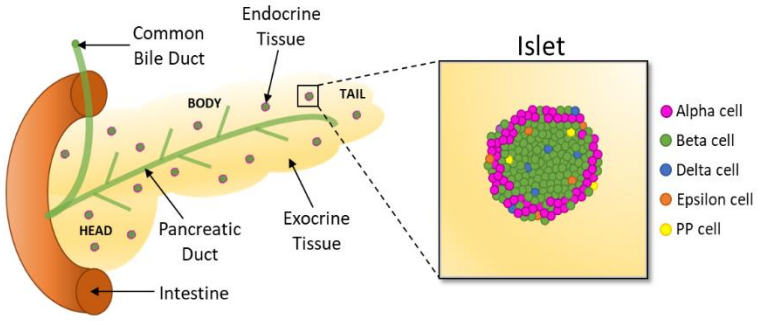

Figure 1-2. The endocrine pancreas.

The pancreas is surrounded by the stomach, spleen, and small intestine and can be divided into 3 main parts: (1) the head, (2) body, and (3) tail. It has two main functions, exocrine and endocrine. The exocrine pancreas is responsible for digesting food while the endocrine pancreas, the Islets of Langerhans, regulates blood glucose levels. A cluster of thousands of cells, an islet contains 5 main cells types that each secrete a different hormone such as insulin and glucagon.

In their native microenvironment, islets are embedded within an extracellular matrix (ECM) microenvironment comprising interstitial type I collagen and basement membrane (BM) proteins (i.e., type IV collagen, laminin).[14] Moreover, within an islet, there is a considerable amount of BM which originates from the pervading microvasculature and not the endocrine cells themselves. Nearly all beta cells within an islet are in direct contact with this perivascular BM.[15] Although not well understood mechanistically, this interface between islet cells and their extracellular microenvironment provides essential biochemical and biophysical cues that guide not only islet morphogenesis during fetal development but also postnatal homeostatic islet function.[10,16,17] In fact, immediately following isolation and conventional culture in suspension, insulin-producing cells undergo multiple cell death processes including apoptosis, anoikis, and necrosis,[18–21] which demonstrates the significance of islet-ECM associations. At present, it is estimated that nearly half the islet mass is lost during donor surgery, preservation, transportation, and isolation.[10,22]

Islets are richly vascularized and receive as much as 15-20% of the pancreatic blood flow even though they comprise only 1-2% of the pancreatic volume.[23,24] The islet capillary network is approximately five times denser than the network in the exocrine tissue ensuring that the islet endocrine cells are adequately in contact with this network.[25] This dense network is critical for glucose homeostasis because of the high oxygen consumption of pancreatic β-cells, timely responses to changes in plasma glucose concentration, and the release of islet hormones into the circulation.[11,26] A synergistic relationship exists between the endocrine islet and its rich capillary network through soluble factor secretion such as vascular endothelial growth factor-A (VEGF-A) by beta cells[11] and a unique and shared basement membrane structure originating from the islet vascular endothelial cells.[27] The loss of islet-vascular interactions upon isolation and poor islet revascularization after transplantation have been identified as major impediments to long-term islet engraftment and function.

1.4 Clinical Islet Transplantation

For clinical islet transplantation (Figure 1-3), a pancreas is procured from a heart-beating, brain-dead donor (allotransplantation) or from the recipient's pancreatectomy (autotransplantation). Once the pancreas has been procured, it is stored in University of Wisconsin solution or histidine-tryptophan-ketoglutarate preservation solution for transportation to the proper facility for islet isolation. Ideally, cold storage is less than 8 hours. Then, the pancreas is

enzymatically digested in a dissociation chamber known as the Ricordi chamber. To avoid overdigestion, the chamber cools and removes dissociated islets, and then purifies islets using a Ficoll density gradient. The purified islets are washed and resuspended, and a small portion is used for further testing and counting. After isolation, in-vitro tests to determine islet viability, yield, and purity are performed. If an acceptable quality and yield is achieved, islets (an average of 800,000 islet equivalents (IE) over 2 infusions derived from 4–6 patients) are injected into the recipient's intra-hepatic portal vein where they become lodged in branches of the portal vein.[28,29] Islets are not injected into the pancreas because of its high sensitivity to injury or damage leading to severe pancreatitis accompanied by pain and further tissue death.[30]

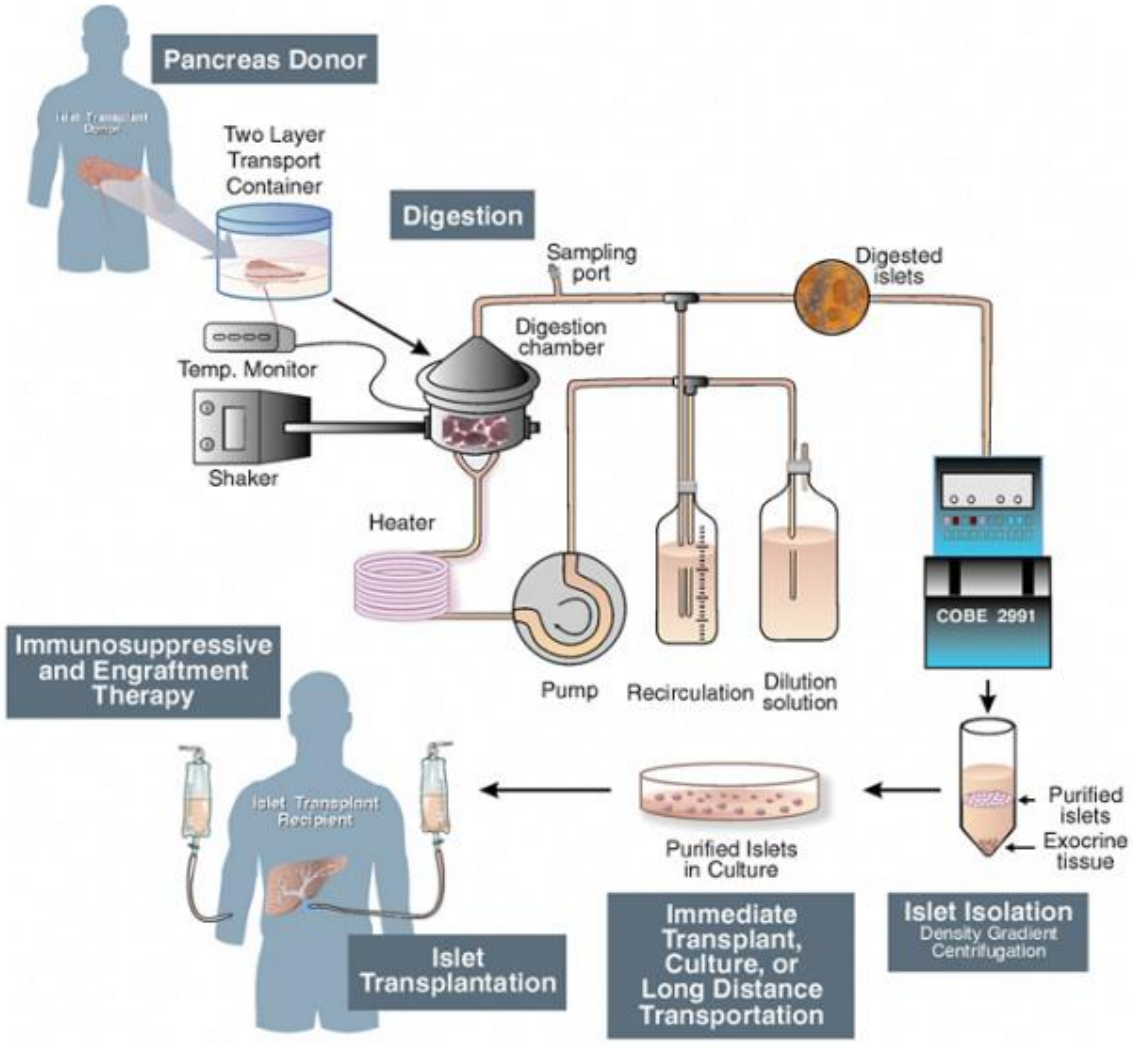

Figure 1-3. Clinical islet transplantation.

Representation of the islet transplantation process from donor to recipient.[40]

Intraportal infusion is the most common method used for clinical islet transplantation in humans because it offers a minimally invasive procedure that is able to regulate glycemic levels through portal insulin delivery.[31,32] However from 1990 to 2000 only 12.4 percent of the islet transplantations performed had resulted in insulin independence for longer than one week, and only 8.2% had resulted in insulin independence for more than a year.[33] In 2000, the Edmonton procedure, incorporating the use of a glucocorticoid-free immunosuppressive regimen, was published resulting in seven out of seven patients achieving insulin independence at 1 year following islet transplantation.[33] Initial successes associated with the Edmonton procedure highlighted the potential and renewed research interests for this cell therapeutic strategy. More recently in an effort to achieve clinical approval in the U.S., a multicenter Phase III clinical allogeneic islet transplantation trial was completed, providing favorable data for use in a Biologics License Agreement submission to the U.S Food and Drug Administration.[34]

Despite these successes, a major shortcoming that needs to be addressed is poor long-term engraftment and function outcomes[35], which effectively increases the number of islets (2-4 donors) required per procedure as well as the number of procedures.[22] It has been estimated that the large majority of transplanted islets (>60%) fail to engraft due to a number of factors, most notably uncontrolled foreign body response (instant blood-mediated inflammatory reaction (IBMIR)), high drug and toxin loads from systemic immunosuppression, insufficient oxygen/nutrient supply, and loss of critical microenvironment cues from surrounding extracellular matrix and vasculature.[36–41] Immediately post-transplantation, a significant amount of transplanted islet mass is damaged primarily as a result of the instant blood-mediated inflammatory reaction (IBMIR). This inflammatory reaction results in platelet adherence, activation, clot formation, and lymphocyte recruitment.[36–39,42,43] In addition to IBMIR, inflammation from the activation of natural killer T-cells has been linked to early islet loss with intraportal islet infusion.[44] Moreover, monitoring of transplanted islets is difficult and often requires additional multiple procedures. As such, recent efforts have been focused on identifying an alternative, optimal site that minimizes exposure of islets to unfavorable conditions and achieves sustained post-transplant insulin independence. For islet transplantation to become a sustainable and widely used treatment, the scarcity of human donors needs to be addressed. Unfortunately, many of the donated pancreases are not suitable for extracting islets for transplants because they do not meet the selection criteria. To address this limitation, there is a push from the JDRF and NIDDK towards developing replenishable and

readily available alternative beta cell sources. These include human pancreatic endocrine cell progenitors and surrogate beta cells from human embryonic stem cells (hESC) and adult cells that have been reprogrammed to pluripotency (iPSC) as well as the use of porcine islets as the most promising alternative sources of beta cells.

1.5 Encapsulation for Beta Cell Replacement Therapies

Over the past several decades, various microencapsulation, containing one or a few islets, and macroencapsulation, containing a large mass of islets, approaches have been studied in an attempt to create immune-protected beta cells.[45] To overcome the need for life-long immunosuppression, the use of encapsulation has emerged to provide a physical barrier between transplanted β-cells and the recipient. It has been recognized that engineering of the transplant microenvironment is needed to create an environment that enhances islet cell viability, promotes prompt neo-angiogenesis, is biocompatible, and modulates immunity in order to achieve long-term function outcomes.[46,47] Immunoisolation through encapsulation has been extensively researched as a potential method to eliminate the need for immunosuppression and its harmful side effects, but full clinical translation of an encapsulation strategy has not yet been accomplished.[48] Encapsulation involves the creation of a semipermeable environment using biological or synthetic materials that protects islets against host immune response while simultaneously permitting diffusion of essential nutrients such as oxygen, insulin, and glucose.[22,49] However, biocompatibility of the material itself is vital to function and viability of the encapsulated islets. If there is insufficient biocompatibility, there could be a build-up of fibrosis and non-specific protein absorption of the capsules resulting in necrosis and graft failure.[50–54]

1.5.1 Microencapsulation

Microencapsulation approaches involve encapsulation of individual or a small number of islets generally in a sphere-shaped format. Several materials have been used for microencapsulation including alginate, agarose, and polyethylene glycol (PEG). The most researched and published material used for microencapsulation is alginate, an unbranched anionic polysaccharide derived from the cell walls of brown algae. Cations, calcium or barium, are required to initiate crosslinking for the formation of a gel. The use of alginate for microencapsulation has achieved promising results in several animal models including rodents,[55,56]

dogs,[57] and nonhuman primates (NHP).[58,59] Further, a few clinical trials have been performed by Soon-Shiong et al.,[60] Elliott et al.,[61] Calafiore et al.,[62,63] and Tuch et al.[64] Although these clinical trials have reported insulin secretion from transplanted islets, long term correction and control of blood glucose levels was unable to be achieved. Challenges with biocompatibility and incomplete capsule coverage remain that limit these systems from advancing further.

In an effort to enhance its efficacy and biocompatibility, polycations such as poly-L-lysine[65], poly(vinylamine)[66], poly(allylamine)[67], or poly-L-ornithine.[68] However, these poly-amine coatings have been found to promote a greater inflammatory response reducing survival compared to islets that have been encapsulated with alginate alone.[53,69] To compensate for this reactivity, an additional outer thin layer of alginate is added to the capsule creating a 3-layer microcapsule with an alginate core, polycationic second layer, and alginate outer shell.[70] Additionally, other materials including poly(ethylene glycol) (PEG) and poly-L-lysine (PLL), growth factors including VEGF[71–75] and hepatocyte growth factor (HGF)[76], and ECM proteins[77] have been incorporated into alginate to enhance islet viability and function and to reduce hypoxic stress. Another challenge associated with microencapsulation has been the incomplete coverage of islets within capsules leading to protrusion of the cells outside the capsule. This causes significant immune responses to the transplanted microcapsules which ultimately leads to graft failure.[78]

Agarose and PEG have also been used as materials to microencapsulate islets. Iwata et. al. first reported the use of islet-agarose microbeads implanted into the intraperitoneal space in 1988 and later showed reversal of diabetes in nonobese diabetic mice for 80 days with allografts.[79,80] More recently, transplanted syngeneic agarose microencapsulated islets were able to function for >100 days in nonobese diabetic mice while unencapsulated islets lost their function within 3 weeks.[81] Another common material used to microencapsulate islets is PEG hydrogels.[82–84] The use of PEG for encapsulating islets was first introduced by Jeff Hubbell while at the University of Texas. It occurs by an interfacial polymerization which uses a photo-initiator, eosin Y, to crosslink and bind PEG to the surface of the islet.[85,86] This method can achieve much smaller capsules (50-70μm diameter) compared to alginate and agarose capsules resulting in easier diffusion path and greater access to nutrients minimizing the effects of hypoxia. Initial studies encountered challenges when translating to large animal models since larger animals had a much higher reactivity to the PEG coatings.[87] More recently, one group has studied conformal coating encapsulation of islets using PEG as well as a combination of alginate and PEG.[88–90] In one such study, islets ultra-thinly

(10-50 μm) coated with alginate-PEG layers had no detectable difference in viability and function from the controls after 5 days.[90] Another group using the conformal coating technique, incorporated maleimide Matrigel with PEG to enhance viability and function with the presence of islet-like ECM.[91] Allogeneic islets (750-1000 IEQ/mouse) functioned for >100 days when transplanted into the epididymal fat pad of diabetic C57BL/6 mice.[92]

1.5.2 Macroencapsulation

Macroencapsulation is becoming more attractive because it allows for easier retrieval or reloading of islets, if needed, and provides greater control over material parameters than microcapsules.[93] However, oxygen diffusion and nutrient transport could be limited in a macroencapsulation device. For transplantation, these therapies are most commonly placed in the peritoneal cavity, an omental pocket, or a subcutaneous site to accommodate the larger volume of the device. One advantage of macroencapsulation is the ability to easily retrieve the devices with minimal surgery risks in the case of loss of function, adverse effects, or malignancy. Some of the materials that have been used for macroencapsulation include, polytetrafluoroethylene (PTFE), alginate,[94–96] and agarose.[97,98] One strategy developed at the Rogosin Institute (Xenia, OH) encapsulates donor islets in double layered agarose macrobeads.[97,98] In one study, encapsulated porcine islets lowered blood glucose for >6 months in diabetic rats.[98] However, when encapsulated porcine islets were implanted into pancreatectomized dogs there was not complete remission of the diabetic state at high islet doses even though blood glucose was lowered.[99,100]

In order to improve cell oxygenation within the device and long-term graft survival, the βAir device from BetaO2 technologies was created. It is a combinational device composed of 3 main compartments: (1) a 500-600 um thick alginate slab with embedded islets (2) a gas chamber with ports to allow external oxygen supplementation, and (3) a 25 um PTFE membrane surrounding the device to separate transplanted islets from the host tissue.[94,95,101] A recent phase I/II clinical trial in four T1D patients investigating the safety and efficacy of the implanted device containing human islets (1800-4600 IEQs/kg body weight) was completed.[102] After 3-6 months, the device was shown to be safe and prevented rejection of the transplanted islets, but function was limited with no detectable C-peptide levels in the blood and no impact on patient metabolic control.

Another macroencapsulation technique developed by TheraCyte involves the use of the Encaptra® drug delivery system to encapsulate replacement beta cells. This device is comprised

of a bilayer PTFE membrane, the outer layer is fenestrated providing strength and facilitating vascularization while the inner layer is a tighter membrane providing isolation from the immune system. With this device, there was successful reversal of diabetes for 4 weeks to 6 months in rodent models using both macroencapsulated rat islets and neonatal porcine islets.[103,104] However, to achieve success at lower curative doses, devices were preimplanted 3 months prior to islet transplantation in order to achieve sufficient vascularization around the device. Although there was success in rodent models, there has been limited data on reversal of diabetes in larger animal models. When transplanted in nonhuman primates, the cell doses were substantially below curative doses for NHP.[105,106] More recently, ViaCyte has been using modified versions of the device to encapsulate and immunoisolate pancreatic endoderm cell products derived from human embryonic stem cells.[107,108] There are currently versions: PEC-Encap™ (VC-01) and PEC-Direct™ (VC-02). Both versions require preimplantation to promote vascularization prior to cell transplantation to avoid hypoxia of the graft. However, while VC-01 limits access to immune cells, PEC-Direct allows direct vascularization to the encapsulated cells requiring patients with these devices to be on a immunosuppressive regimen.[109] Phase I/II clinical trials in the U.S. and Canada are currently being performed to evaluate the safety and tolerability of both VC-01 and VC-02.

1.6 Type I Oligomeric Collagen for In-Situ Encapsulation

Our solution involves subcutaneously transplanting encapsulated islets using a self-assembling, injectable type I oligomeric collagen (Oligomer) for in-situ islet encapsulation to enhance engraftment and long-term function in-vivo. This strategy is innovative on several fronts, specifically in 1) the application of a novel, self-assembling injectable type I collagen for in-situ islet encapsulation, 2) the transplantation of encapsulated islets into the more accessible subcutaneous space, 3) the potential to provide localized immunosuppression therapy, and 4) the translation of this encapsulation strategy to more readily available cell sources. Oligomers represent an acid-soluble domain of type I collagen, but unlike conventional monomeric varieties (telocollagen and atelocollagen), oligomers retain naturally occurring intermolecular crosslinks as produced in the body. Published work shows that oligomers overcome known limitations of conventional monomers, with its rapid polymerization upon neutralization (<1 minute at 37°C), slow turnover (resistant to proteolytic degradation) in vitro and in vivo, and ability to be custom fabricated with a broad range of architectures and mechanical properties.[110–115] The improved

stability and mechanical integrity exhibited by oligomeric materials effectively eliminates the need for exogenous crosslinking, which is routinely applied to collagen-based biomaterials. Oligomers are ideally suited for therapeutic cell delivery since they i) exhibit rapid suprafibrillar self-assembly yielding highly interconnected collagen-fibril matrices resembling those found in vivo; ii) are standardized based upon their fibril-forming capacity; iii) support cell encapsulation and distribution throughout the construct; and iv) allow customized multi-scale design across the broadest range of tissue architectures and physical properties.[116] Previously published in-vivo studies have shown that the collagen-fibril material persists, inducing neovascularization, innervation, and no significant inflammatory response.[111,112,117] Our macroencapsulation strategy targets the subcutaneous space due to ease of accessibility for transplant administration and removal, amenability for post-transplantation monitoring of islets, and ease of administration via minimally invasive procedures.[31,118,119] Additionally, Oligomer can be tailored in terms of biophysical properties and local delivery of factors to maximize β cell protection, health, and function.

1.7 Note About Content and Thesis Organization

This thesis is addressing the problem that exogeneous insulin therapy is the only effective treatment for T1D patients, and that for 33% of patients this does not provide adequate glycemic control resulting in life-threatening secondary complications. Therefore, there remains a need for a long-term insulin independent solution. Beta cell replacement therapy has the potential to provide long-lasting glucose-sensing and insulin-releasing functions to eliminate the need for insulin injections. However, current methods are limited by the need for life-long systemic immunosuppression and the limited donor availability. Oligomer encapsulation has the potential to be a simple injectable strategy providing immune protection while maintaining islet health and function.

The introductory material above was taken from my Qualifying Literature Assessment (QLA) and Preliminary Exam document. Chapter 2 represents a manuscript that was accepted for publication in the American Journal of Physiology Endocrinology and Metabolism. This chapter details efforts to define 1) how biophysical properties of fibrillar type I collagen (Oligomer) affect overall islet health and function in vitro and 2) the functional efficacy of liquid Oligomers when applied in a simple subQ injectable format for in situ islet encapsulation in chemically induced

diabetic mice. A key takeaway from Chapter 2 is that function was achieved within 24 hours and there was no evidence of a foreign body response to transplanted Oligomer-islet constructs. Chapter 3 represents a second manuscript planned for submission that extends the in vivo studies performed in Chapter 2. This chapter documents the effects of 1) allogeneic vs xenogeneic replacement islets, 2) islet dose, 3) the format of subcutaneous transplantation, and 3) the site of transplantation on efficacy and longevity of function of islets in chemically-induced, immunocompetent diabetic mice. Finally, Chapter 4 outlines proposed next steps for preclinical validation studies that 1) define the efficacy of Oligomer/β-cell replacement therapy in small and large animal autoimmune diabetic settings and 2) combine Oligomer with clinically relevant replenishable β cell sources, namely porcine islets and human SC- βcells.

2. IN-SITU TYPE I OLIGOMERIC COLLAGEN MACROENCAPSULATION PROMOTES ISLET LONGEVITY AND FUNCTION IN VITRO AND IN VIVO

2.1 Introduction

Type 1 diabetes (T1D) is a debilitating and burdensome public health problem affecting an estimated 1.25 million men, women, and children in the U.S. alone.[1] The current standard of care is exogenous insulin with whole pancreas transplantation exercised only in the most difficult-to-treat cases in which there exists severe hypoglycemia unawareness. Although insulin therapy is life-saving, it provides inferior control relative to functional islet cells and does not eliminate chronic complications (i.e., cardiovascular disease, kidney failure, retinopathy, and neuropathy) that contribute to a large portion of medical costs and loss of quality of life.[40] Even with a strict insulin regimen, T1D patients still experience dangerously high or low blood-glucose levels that can potentially be life threatening. In fact, less than one-third of people with T1D in the U.S. consistently achieve target blood-glucose levels.[8]

Although pancreatic islet transplantation is an attractive therapeutic alternative for T1D patients, this approach remains classified as experimental in the U.S. since long-term safety and efficacy have yet to be achieved.[120] At present, clinical islet transplantation involves image-guided infusion of a large number of islets (on average 800,000 islet equivalents (IE) over two infusions derived from 4-6 patients) into the portal vein where they become lodged.[29] Early results associated with the Edmonton procedure highlighted the potential of this cellular therapy.[33] More recently, a multi-center Phase III clinical allogeneic islet transplantation trial was completed, providing favorable data for use in a Biologics License Agreement submission to the U.S Food and Drug Administration.[34] Despite these successes, a number of persistent obstacles preclude it from gaining more widespread use. Specifically, there exists a limited supply of quality-controlled donor islets for transplant and the requirement for long-term immunosuppression has significant side effects.[121] Most importantly, the longevity and function of transplanted islets must be improved for patients to achieve long-term insulin independence. Poor islet engraftment and performance have been attributed to loss of critical microenvironmental cues from surrounding extracellular matrix (ECM) and vasculature as a result of islet isolation as well as the rapid blood mediated inflammatory reaction following islet transplantation.[39,41]

Numerous macro- and micro-encapsulation strategies, involving natural and synthetic biomaterials, have been developed in attempt to create a protective microenvironment and physical barrier that prevents detection and accessibility of transplanted islets by the immune system. Unfortunately, these physical barriers also hinder islet engraftment and associated revascularization and reinnervation, which are known to promote islet health and function. Fibrotic capsule formation, largely owing to poor material biocompatibility, further hinders essential mass transport. Incremental improvement has been achieved by i) transplanting encapsulated islets into highly vascularized alternative sites (e.g., omentum),[122] ii) providing supplemental oxygenation strategies,[123] and iii) co-delivering angiogenic factors, anti-inflammatory factors, and accessory cells;[124,125] however, reliable and reproducible long-term survival and function of encapsulated islets have yet to be achieved.

While less understood mechanistically, the interface between islet cells and their extracellular microenvironment provides essential biochemical and biophysical cues that guide not only islet morphogenesis during fetal development but also post-natal homeostatic islet function.[10,16,17] In-vivo, these islet micro-organs interact with various ECM components, including fibrillar type I collagen which is present within and around islets as well as within perivascular capillaries, although species- and age-dependent differences exist.[126,127] Engagement of specific collagen motifs has been shown to promote islet cell survival, differentiation, and β cell function by triggering downstream signaling pathways, in part, through integrin and discoindin receptors.[128,129] Additional reports indicate that embedment within three-dimensional (3D) collagen matrices, in contrast to 2D surface contact, helps to sustain the necessary cell polarity and organization for prolonged islet function[22,130,131]. Clearly, collagen biophysical properties, including matrix architecture, stiffness, and resistance to proteolytic degradation, are important for guiding proliferation, differentiation, and morphogenesis of various embedded cell types; however, little is known regarding how these parameters influence multicellular functional units, such as islets.

In the present study, we hypothesized that biophysical properties of a rheologically-tunable type I oligomeric collagen (Oligomer) were important determinants of islet viability, cytoarchitecture, and function following macroencapsulation in vitro and in vivo. Unlike monomeric (atelocollagen or telocollagen) collagen formulations, which have been previously employed for islet encapsulation, Oligomers retain natural intermolecular crosslinks, which

support rapid and robust fibril self-assembly upon neutralization, yielding highly-interconnected, D-banded, fibrillar scaffolds.[113,132–134] When compared to monomeric collagens prepared at the same concentration, Oligomer matrices exhibit improved mechanical stability (stiffness and strength) and resistance to proteolytic degradation.[113,132] Initial in-vitro studies applied confocal microscopy, immunostaining, and glucose-stimulated insulin secretion (GSIS) testing to monitor the dependence of islet viability, cytoarchitecture, collagen remodeling, and function following macroencapsulation at various Oligomer concentrations (fibril densities). Oligomer solutions were then utilized for subcutaneous injection and in-situ macroencapsulation of islets within streptozotocin-induced diabetic mice. The subcutaneous space was selected as an alternative transplant site to the portal vein since it offers easy accessibility via injection, potential for monitoring and imaging, as well as retrievability, if necessary, of the transplanted islets. Specifically, short-term (14-day) studies in immunocompromised NOD/SCID mice were used to characterize biocompatibility of Oligomer material and islet engraftment. Follow-up studies evaluated long-term (90-day) survival and function of Oligomer-encapsulated syngeneic islets following subcutaneous delivery in immune competent C57BL/6J mice. Finally, a pilot allogeneic study (60-day) was performed to determine the immunoprotective capacity of Oligomer-encapsulated islets.

2.2 Materials and Methods

2.2.1 Mouse Islets

Mouse pancreatic islets were isolated from 8- to 14-wk old C57BL/6J or CD1 mice (Jackson Laboratory, Bar Harbor, ME) as described.[135] Islet isolations were approved by the Indiana University Institutional Animal Care and Use Committee using AAALAC guidelines. Isolated islets were incubated in RPMI 1640 medium supplemented with 10% fetal bovine serum (HyClone, ThermoFisher Scientific, Waltham, MA), 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma Aldrich, St. Louis, MO) in a humidified environment of 5% CO_2 in air at 37°C prior to experimental use the next day.

2.2.2 Oligomeric Collagen Encapsulation of Mouse Islets for In-Vitro Culture.

Type I oligomeric collagen (Oligomer) was acid-solubilized from the dermis of market-weight pigs and lyophilized for storage as described previously.[132] The Oligomer formulation was

standardized on the basis of molecular composition as well as polymerization capacity according to the voluntary consensus standard ASTM F3089-14.[136] Here the polymerization capacity is defined by the matrix shear storage modulus, G' (in Pa), as a function of oligomer concentration in the polymerization reaction.

Oligomer polymerization kinetics were measured using an AR2000 rheometer (TA Instruments, New Castle, DE) equipped with a stainless-steel 40 mm-diameter parallel plate geometry. Upon lowering the geometry, the Peltier plate was maintained at 4°C for 2 min and then increased to 37°C for 10 min to induce collagen polymerization. Time-dependent changes in shear storage modulus (G′) were measured at 1% controlled oscillatory strain. Polymerization half-time was defined as the time required for G′ to reach half-maximum value (n = 4-5 for each formulation). Oligomer was diluted with 0.01 N HCl and neutralized to final concentrations of 0.5, 1.5, or 3.0 mg/mL, corresponding to polymerized matrices with G' values of 40, 200, and 1000 Pa, respectively. Mouse islets were suspended in the neutralized Oligomer solutions, aliquoted into 96 well-plates (30 islets/100 µL; Cellvis, Sunnyvale, CA), and allowed to polymerize at 37ºC. Oligomer solutions (in the presence or absence of cells) were maintained on ice (4ºC) prior to warming to 37ºC to induce rapid polymerization. Immediately following polymerization, culture medium was added, and the Oligomer-islet constructs were cultured for up to 14 days with medium changes made daily. For comparison purposes, mouse islets were also cultured in a conventional suspension format.

2.2.3 Assessment of Mouse Islet Viability, Cytoarchitecture, and Function Following In-vitro Culture.

Islets cultured in suspension or embedded in Oligomer were treated with Calcein AM and propidium iodide (Molecular Probes, Eugene, OR) for live-dead determinations. Images were collected using laser scanning confocal microscopy on an Olympus IX81 inverted microscope adapted with Olympus Fluoview FV1000 (Olympus, Tokyo, Japan). Image stacks of 40-100 µm thickness with a 3 µm step size were obtained using a 20X air objective, and z-projections were created using Imaris software (Bitplane, Concord, MA).

Immunofluorescence was used to qualitatively assess islet cytoarchitecture and protein expression. Islets cultured in suspension or embedded in Oligomer were fixed in 3% paraformaldehyde (Mallinckrodt, Derbyshire, UK), permeabilized with 0.1% Triton X-100 (Sigma Aldrich), and blocked with 1% bovine serum albumin (Jackson ImmunoResearch, West Grove,

PA). Samples then were treated overnight at 4^{o}C with primary guinea pig anti-insulin (PA1-26938, Invitrogen, Carlsbad, CA) and rabbit anti-glucagon antibodies (ab10988, Abcam, Cambridge, MA). Samples were rinsed and then treated with secondary antibodies (A11073, goat anti-guinea pig Alexa Fluor 488 conjugate and A11035, goat anti-rabbit Alexa Fluor 546 conjugate, Life Technologies, Carlsbad, CA) overnight at 4°C. After rinsing, samples were treated with DRAQ5 (Cell Signaling Technologies, Danvers, MA) to stain nuclei.

Quantitative assessment of islet function was performed via GSIS testing. Islets cultured in suspension or encapsulated in Oligomer were prepared within 24-well Transwell culture inserts (Corning, Kennebunk, ME) for up to 14 days. Basal insulin secretion was stimulated by incubating the samples for 1 hour with 2.8 mM glucose in Krebs Ringer buffer (0.1% BSA, 25 mM HEPES, 115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 1 mM $MgCl_2 \bullet 6H_2O$, 2.5 mM $CaCl_2 \bullet 2H_2O$). Following basal secretion, samples underwent static incubation for 1 hour each, with low (2.8 mM) followed by high (28 mM) glucose concentrations in Krebs Ringer buffer. Insulin secretion was measured using a STELLUX® insulin enzyme-linked immunosorbent assay (ELISA) kit (Alpco, Salem, NH). Stimulation indices (SI), which represents the ratio of insulin secreted with high glucose over insulin secreted with low glucose, were calculated and normalized to values obtained for freshly isolated (day 0) islets.

2.2.4 Subcutaneous Islet Transplantation in Diabetic Mice.

Mouse islet transplantation procedures were approved by the Indiana University Institutional Animal Care and Use Committee using AALAC guidelines. Male 8-wk to 14-wk old NOD.CB17-Prkdc^{scid}/J (immunocompromised model; Jackson Laboratories, Bar Harbor, ME) or C57BL/6J (syngeneic and allogeneic models) recipient mice were injected with low dose streptozotocin (55 mg/kg) for 5 days to chemically induce diabetes prior to islet transplantation.[137] C57BL/6J (immunocompromised and syngeneic models) or CD1 (allogeneic model) islets were mixed with neutralized Oligomer solutions. Oligomer-islet suspensions were then injected subcutaneously through small bore needles (26G) into diabetic mice. Each mouse received 2 injections, one positioned laterally on each side of the back, with 250 islets/500 µL Oligomer/site for a total of 500 islets/mouse. Additional mice received 2 injections of islets suspended in saline (islets only control; 250 islets/500 µL saline/site) or neutralized Oligomer solution (Oligomer only control; 500 µL Oligomer/site) for comparison. For the immunocompromised model, experimental

groups included 1.5 mg/mL Oligomer (n=3), 2.2 mg/mL Oligomer (n=3), 3.0 mg/mL Oligomer (n=3), Oligomer only control (n=3), and islets-only control (n=4). For the syngeneic model, 3.0 mg/mL Oligomer (n=3) and islets only (n=3) were used. For the allogeneic model, 3.0mg/mL Oligomer (n=3) and 4.2 mg/mL Oligomer (n=5) were used. Non-fasting blood glucose was measured 3 times per week after transplantation. Diabetes was classified as two consecutive blood glucose levels above 300 mg/dL. Based on preferences and standards established in the literature, a blood glucose level of 250 mg/dL was selected as the "diabetic threshold".[138,139] At the end of the study, the pancreas was removed and histopathological analysis performed to confirm destruction of endogenous islets.

2.2.5 Intraperitoneal Glucose Tolerance Test.

Intraperitoneal glucose tolerance tests (GTT) were performed at specified times following subcutaneous transplantation to assess islet responsiveness to glucose challenges. Mice were fasted overnight, and then injected intraperitoneally with 2 g/kg of 20% glucose. Blood glucose levels were measured at baseline before injection (time 0) and 15, 30, 60, 90, and 120 minutes following glucose injection.

2.2.6 In-Vivo Histology and Immunofluorescence.

Injection sites and surrounding tissues were removed at specified times and placed in 10% formalin before paraffin embedding and sectioning. Sections were stained with hematoxylin and eosin (H&E) and Masson's trichrome (MTC). For immunofluorescence, sections were deparaffinized, rehydrated, and stained with primary guinea pig anti-insulin (A0564, Dako, Santa Clara, CA) and rabbit anti-glucagon (sc-13091, Santa Cruz Biotechnology, Dallas, TX) at 1:500 dilutions. Sections were then treated with secondary Alexa Fluor 488 goat anti-guinea pig (A11073, Life Technologies) and Alexa Fluor 568 goat anti-rabbit (A11036, Life Technologies) at 1:50 and 1:200 dilutions, respectively. For identification of vascular endothelium, sections were stained with primary mouse anti-CD31/PECAM-1 (BBA7, R&D Systems, Minneapolis, MN) then treated with secondary Alexa Fluor 546 donkey anti-mouse (A10036, Life Technologies). Nuclei were counterstained with DAPI.

2.3 Results

2.3.1 Oligomer fibril architecture affects viability, cytoarchitecture, and function of cultured islets

It has been suggested that loss of critical microenvironmental cues upon islet isolation and culture decreases component cell viability and function, thereby compromising islet health and transplant outcomes.[10,121] To better define how biophysical properties of fibrillar type I collagen affects overall islet health and function, isolated C57BL6/J mouse islets were macroencapsulated in Oligomer ranging from 0.5 to 3 mg/mL and cultured for 14 days. Previous reports show that increasing Oligomer concentration yields matrices of increased stiffness (G') as a result of increased fibril density.[132] Freshly isolated islets and islets maintained in conventional suspension culture served as positive and negative controls, respectively.

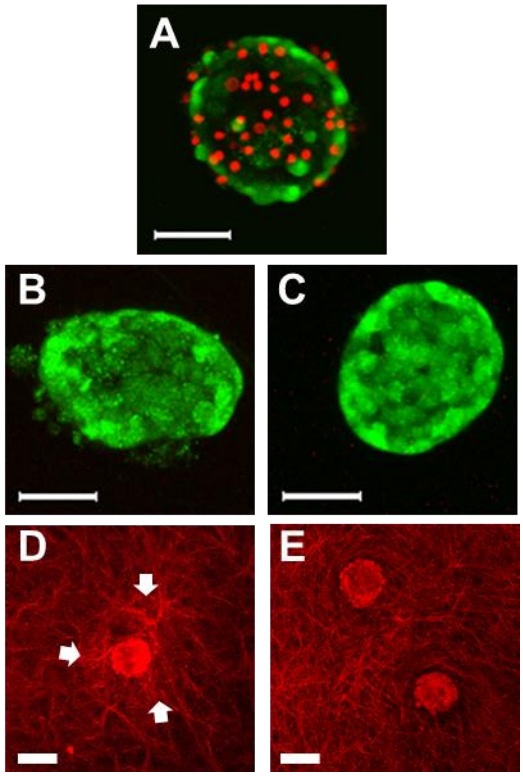

Figure 2-1. In vitro islet viability.

Islets macroencapsulated in Oligomer show sustained viability and fibril density-dependent traction forces following 14 days of in-vitro culture. Representative images of calcein AM (green) and PI (red) stained islets after 14 days of culture in suspension (A), macroencapsulated in Oligomer-1.5 (B), or macroencapsulated in Oligomer-3.0 (C). Confocal reflection images show islet-induced collagen-fibril deformation (arrows) within Oligomer-1.5 (D) but not Oligomer-3.0 (E). Scale bar = 50 μm (A-C) and 100 μm (D,E).

As expected, suspension islets showed a progressive loss of viability over the 14-day culture period (Figure 2-1A), contributing to altered morphology (Figure 2-2A) and significant loss of function (68.2±2.8%), as measured by GSIS (Figure 2B, $p<0.05$). By contrast, Oligomer-encapsulated islets displayed improved viability (Figure 2-1B-E) and morphology (Figure 2-2A), which was dependent upon fibril density. Islet cells actively adhered and exerted contractile forces on the surrounding collagen-fibril matrix, which was evident both macroscopically (construct contraction) and microscopically (islet-collagen interactions). Although the majority (≥66%) of Oligomer-0.5 constructs contracted, hindering further analysis, all Oligomer-1.5 and Oligomer-3.0 constructs retained their volume, displaying

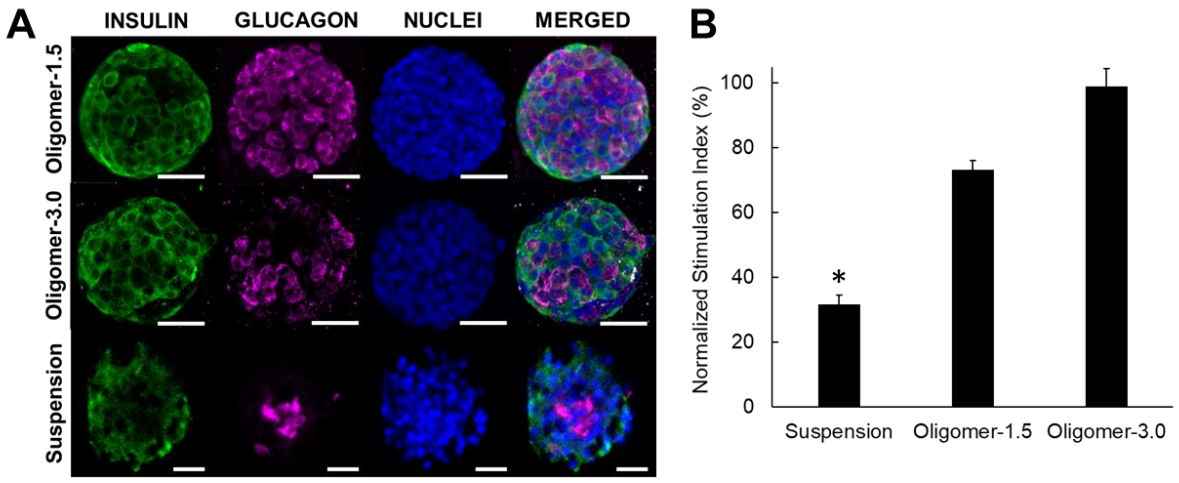

Figure 2-2. In vitro islet morphology and function.

Islets macroencapsulated in Oligomer maintain morphology and function following 14 days of in-vitro culture A. Immunostaining shows islet cytoarchitecture with insulin- (green) and glucagon-positive (purple) cells within Oligomer-1.5 and Oligomer-3.0. Islet cell nuclei (blue) were visualized with DRAQ5. Scale bar = 30 µm. B. 14-day normalized stimulation indices (mean±SD; n=8-12) as measured by glucose-stimulated insulin release. Values were normalized to stimulation indices for freshly isolated (day 0) islets. Asterisk indicates mean SI value for 14-day suspension islets was significantly less than the value for freshly isolated islets ($p<0.05$).

minimal to no islet cell death (Figure 2-1B-C) and progressively less local fibril reorganization with increased concentration (Figure 2-1D,E). Immunostaining confirmed that encapsulated islets stained positively for insulin and glucagon, indicating maintenance of cytoarchitecture and phenotype (Figure 2-2A). Additionally, islets maintained in Oligomer-1.5 and Oligomer-3.0 over the 14-day culture period retained glucose-sensitive insulin release, with normalized SI values of 73.2±3.0% and 98.9±5.6%, respectively, when compared to freshly isolated islets (Figure 2-2B).

2.3.2 Subcutaneous Injection and In-situ Oligomer Macroencapsulation of Islets Supports Rapid Engraftment and Reversal of Diabetes in Immunocompromised Mice.

Since Oligomer-islet macroencapsulation prolonged islet viability and function in-vitro, Oligomer formulations were evaluated for their ability to support subcutaneous delivery and engraftment of islets in chemically-induced diabetic mice. This minimally-invasive transplantation strategy involved mixing islets within Oligomer solutions followed by subcutaneous injection into streptozotocin-induced diabetic mice as shown in Figure 2-3A. Each mouse received 2 injections, one on each side of its back. All mice receiving transplanted islets were injected with a total of 500 islets (250 islets/500 µL/site). Immediately following injection, the Oligomer-islet suspension

self-assembled in situ, forming a stable and continuous collagen-fibril matrix that encapsulated and protected resident islets between the panniculus carnosus muscle and skeletal muscle fascial layer (Figure 2-4). The polymerization half-time for Oligomer at body temperature (37°C) was 20.26±0.12 seconds, 16.28±0.03 seconds, 16.35±0.11 seconds, as measured rheometrically for Oligomer -1.5, -2.2, and -3.0 mg/mL, respectively.

Initial studies involved transplantation of C57BL/6J mouse islets into NOD.CB17-Prkdc^{scid}/J mice, which exhibit acute inflammation but no T- and B-cell mediated immune reactions. The goal was to assess short-term (14-day) morphology and function of transplanted islets as well as Oligomer biocompatibility and tissue response. For these studies, Oligomer solutions at concentrations of 1.5, 2.2, and 3.0 mg/ml (n=3 for each group), which correspond roughly to Oligomer stiffness values 200, 500, and 1000 Pa based upon standardized polymerization capacity, were employed. Based on in-vitro results, these groups were selected to define how Oligomer concentration (collagen fibril density) modulated islet survival, phenotype, and transport properties associated with their glucose-sensitive insulin release. Prior to islet transplant, mean blood glucose values were 533±72 mg/dL, which was the highest amongst the mouse strains used for this work.

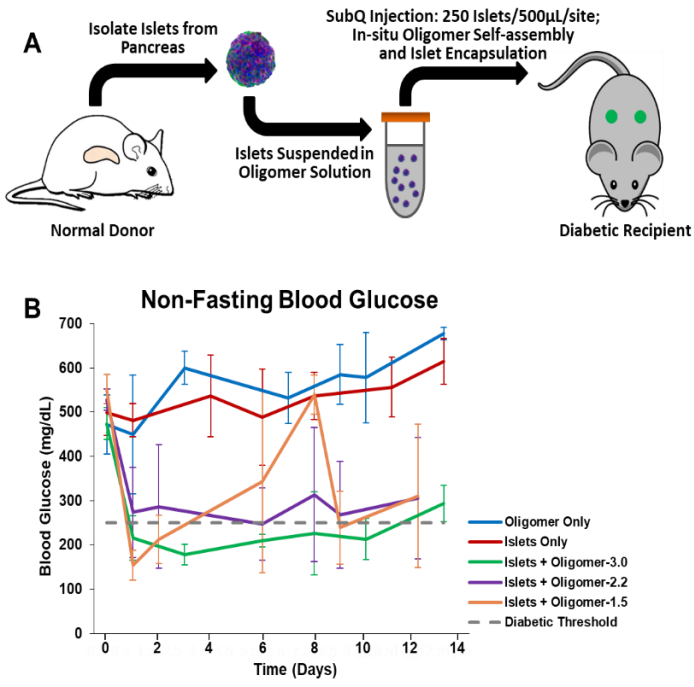

Figure 2-3. Injectable islet implantation into immunocompromised mice.

A. Schematic summarizing in-situ subcutaneous Oligomer-islet macroencapsulation within diabetic recipients. Islets were isolated from normal donor mice, suspended within neutralized Oligomer solutions, and injected subcutaneously into two dorsal sites within streptozocin-induced diabetic mice. Immediately upon injection, the Oligomer-islet suspension self-assembled, transitioning from a liquid to a solid collagen-fibril scaffold and entrapping islets. B. Non-fasting blood glucose levels (mean±SD) following subcutaneous macroencapsulation of C57BL/6J islets in various Oligomer formulations (n=3 for each formulation) into diabetic NOD.SCID mice as compared to subcutaneous injection of islets only (n=4) and Oligomer only (n=3) control groups.

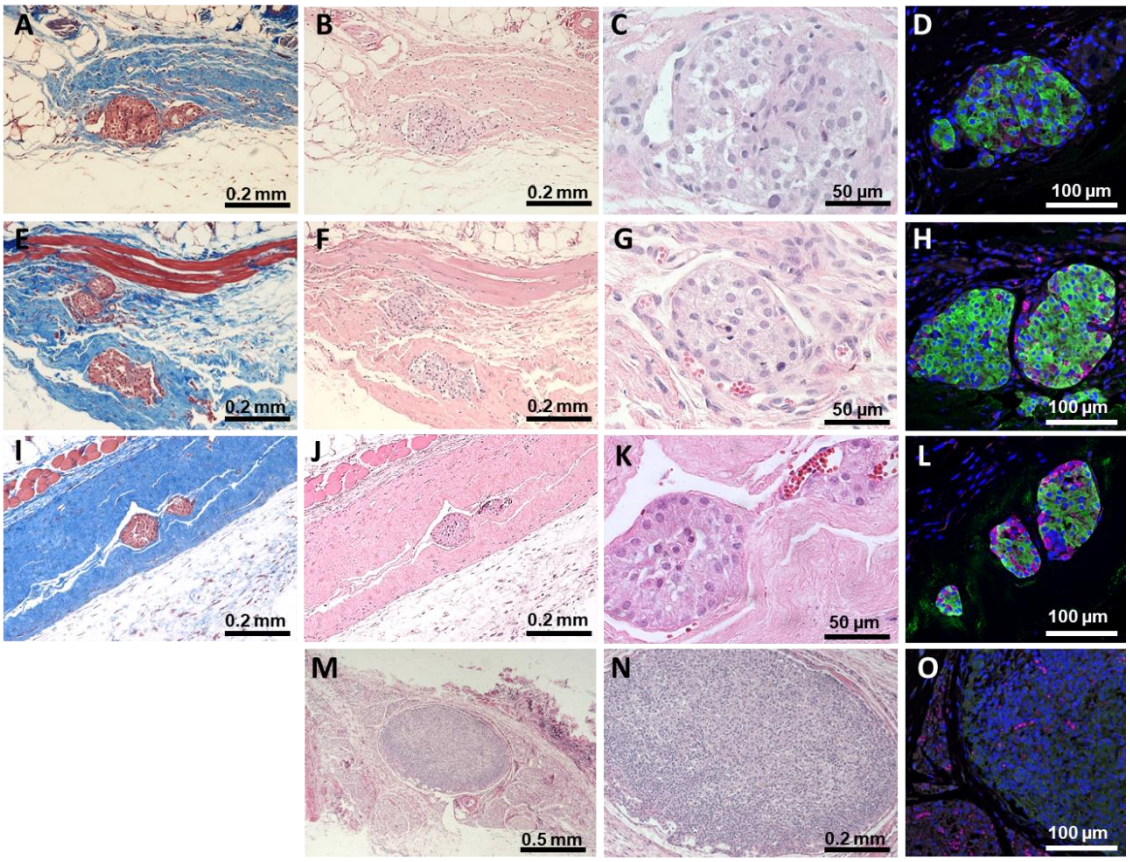

Figure 2-4. Histopathological and immunohistochemical analysis of explants from immunocompromised mice.

Histopathological and immunohistochemical analysis of C57BL/6J islet explants in 1.5 mg/mL (A-D), 2.2mg/mL (E-H), 3 mg/mL (I-L), and saline (M-O) 14 days following subcutaneous transplantation in diabetic immunocompromised NOD.SCID mice. Oligomer-islet constructs were readily identifiable between the panniculus carnosus muscle (PCM) and the skeletal muscle facial layers (SMF). Masson's trichrome stained sections (A,E,I) showed an increase in the amount of fibrillar collagen and more uniform islet encapsulation as Oligomer concentration was increased. Islets maintained multicellular cytoarchitecture with cells staining positively for insulin (green) and glucagon (purple) (D,L,O). Cell nuclei were stained with DAPI. H&E stained sections indicated functional vascularization of islets and no evidence of Oligomer degradation, inflammation, or foreign body response. In contrast, injection of islets only resulted in formation of a large granuloma with loss of normal multi-cellular morphology and protein expression (M-O).

As shown in Figure 2-3B, islets + Oligomer-3.0 provided the most reproducible reversal of diabetes in all animals (consistent maintenance of blood glucose values below the diabetic threshold) and highly regulated blood glucose levels (small standard deviation bars). Additionally, non-fasting blood glucose decreased below the diabetic level within 24 hours and remained below

the diabetic level for the entire 14-day study period (Figure 2-3B). In contrast, both islet only and Oligomer only control groups remained hyperglycemic, above the diabetic level, for the entire 14-day study (Figure 2-3B). As shown in Figure 2-4, the most uniform encapsulation of individual islets was achieved with Oligomer-3.0, with islets maintaining their normal morphology with evidence of functional revascularization. Islet aggregates were noted within Oligomer-1.5 and -2.2, suggestive of insufficient fibril density for encapsulation and/or inadequate mixing. Immunostaining of Oligomer-islet constructs confirmed a multicellular islet cytoarchitecture with both insulin- and glucagon-producing cells (Figure 2-4D,H,L,O). Surrounding the islets, the self-assembled Oligomer appeared as normal collagenous connective tissue, which increased in density with concentration. A density-dependent fibroblast infiltration was observed with no evidence of inflammation or foreign body response, which is consistent with previous in-vivo studies.[111,112] The fibrillar Oligomer was highly stable and well-integrated within the subcutaneous space for all islet + Oligomer and Oligomer only (n=3) groups (data not shown). In contrast, for the islet only group (n=4), large granulomatous regions were observed with no identifiable islets (Figure 2-4M-O). Such findings are consistent with previous reports that suggest that the subcutaneous space is an inhospitable microenvironment for transplantation of islets alone.[140,141]

2.3.3 Long-term Reversal of Diabetes Following Delivery of Oligomer-encapsulated Syngeneic Islets

To assess long-term survival and function of Oligomer-encapsulated islets following subcutaneous delivery, C57BL/6J mouse islets were injected into C57BL/6J mice (syngeneic transplant). For this study, only Oligomer-3.0 was used. Mean blood glucose levels on the day of transplantation were 344±27 mg/dL. Once transplanted, Oligomer-encapsulated islets showed rapid engraftment, again reversing diabetes within 24 hours of transplantation in all recipients (Figure 2-5A). Additionally, all mice remained below the diabetic level throughout the 90-day study period with small standard deviations, indicative of tight regulation of blood glucose. In contrast, mice transplanted with islets only remained diabetic with blood glucose values over 400 mg/dL (Figure 2-5A). Intraperitoneal glucose tolerance testing (GTT) was performed on day 15 (Figure 2-5B,C) and 90 (Figure 2-5D,E) to further assess glucose responsiveness of transplanted islets. Interestingly, mice transplanted with Oligomer-encapsulated islets achieved normoglycemia 120 min post-glucose injection while blood glucose in islet only mice remained elevated above basal levels (Figure 2-5B,D). Analysis of area under the curve (AUC) indicated that values for

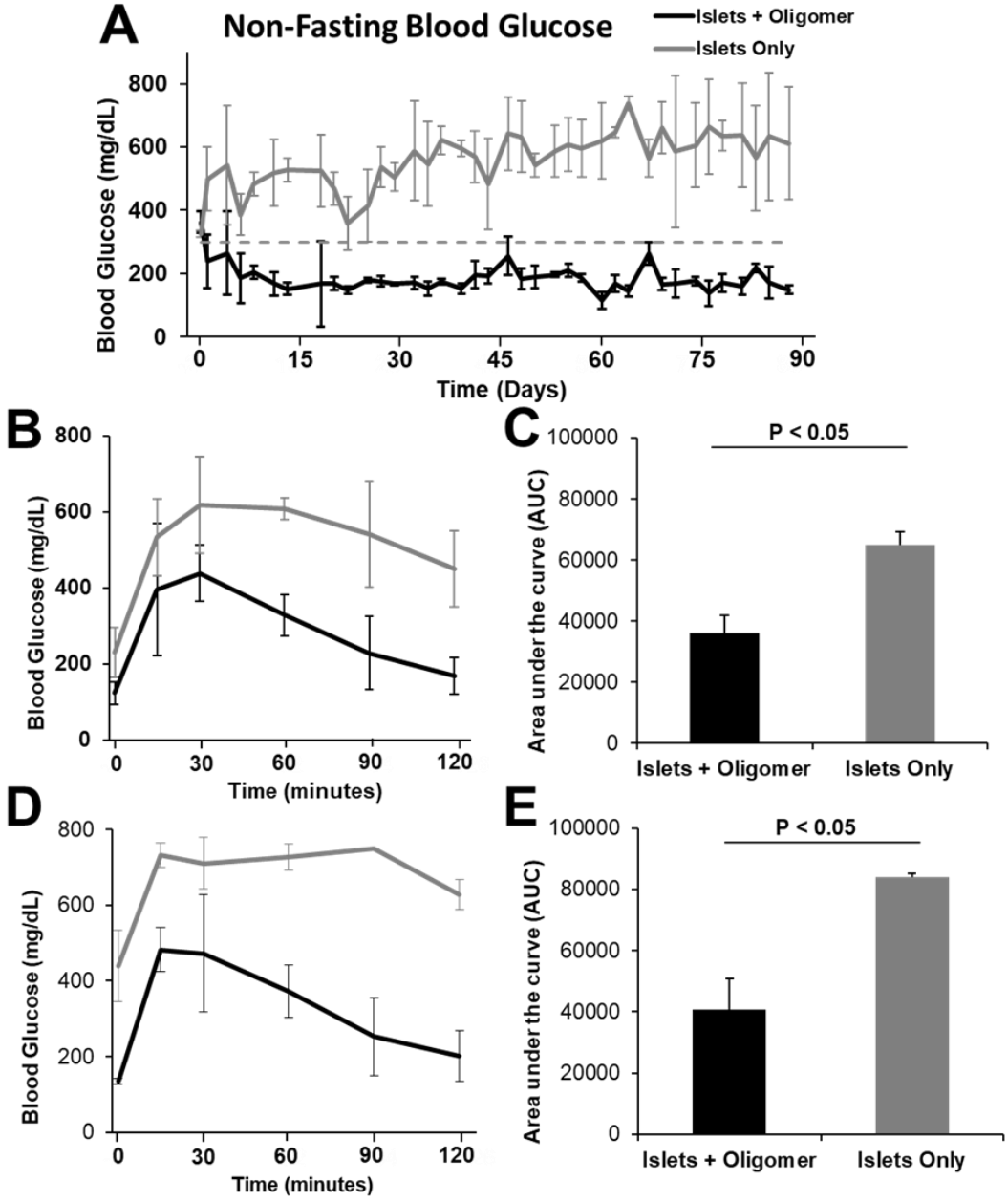

Figure 2-5. Functional outcomes of syngeneic islet implantation studies.

A. Non-fasting blood glucose levels (mean±SD) following subcutaneous in-situ macroencapsulation of syngeneic islets in Oligomer (3 mg/mL; n=3) within diabetic C57BL/6J mice compared to islet only group (n=3). Mice receiving macroencapsulated islets achieved normoglycemia within 24 hours following transplantation. Blood glucose remained below the diabetic threshold (<250 mg/dL) throughout the 90-day study period. The control group remained diabetic throughout the study with widely varying blood glucose values. B-E. GTT (mean±SD) and associated AUC (mean±SD) analysis 15 (B,C) and 90 (D,E) days following transplantation demonstrated the capacity of islets + Oligomer group but not islets-only group to rapidly regulate blood glucose levels following glucose injection. AUC values for islets + Oligomer group were significantly ($p<0.05$) less than those for islet only controls at both time points.

Oligomer-islet mice were significantly lower than those for islet only mice at both 15- and 90-day timepoints ($p<0.05$; Figure 2-5C,E). Histolopathologic and immunostaining analyses showed Oligomer persistence at 90 days with integration into the surrounding subcutaneous tissue compartment and no evidence of chronic inflammatory or foreign body response (Figure 2-6A). Within Oligomer, islets maintained their rounded, multicellular architecture insulin- and glucagon-positive cells (Figure 2-6A-C) and nearby patent vasculature (Figure 2-6B) and CD-31 positive cells (Figure 2-6D,E). As expected, islet only explants showed a robust foreign-body response indicative of graft failure (Figure 2-6F,G).

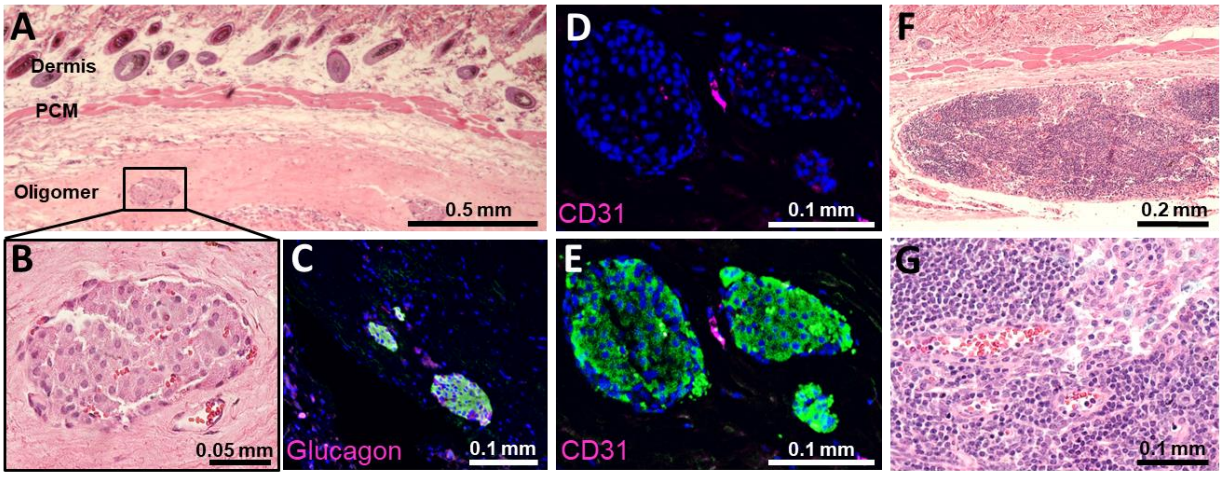

Figure 2-6. Histopathological analysis of syngeneic islet implantation studies.

Histopathological analysis of Oligomer-encapsulated (3mg/mL), syngeneic islets 90 days following subcutaneous transplantation within diabetic mice. A,B. H&E stained cross-section showing Oligomer encapsulated islets within the subcutaneous space below the panniculus carnosus muscle. The self-assembled Oligomer matrix persisted and integrated with surrounding host tissues, with evidence of functional revascularization. C. Encapsulated islets stained positive for insulin (green) and glucagon (purple). D,E. CD31 (purple) staining confirmed the presence of endothelial cells near islets co-stained to visualize insulin (green) and nuclei (blue). F,G. Histopathological analysis of explant 90-days following subcutaneous transplantation of islets only within diabetic mice showed evidence of inflammatory-mediate destruction and necrosis of islets.

2.3.4 Pilot Studies Show Reversal of Diabetes with Immune Modulation Following In-situ Oligomer encapsulation of Allogeneic Islets

To assess function and immunoprotection of Oligomer-encapsulated islets following subcutaneous delivery, pilot allogeneic transplantation studies were performed, where CD1 mouse islets were injected into diabetic C57BL/6J mice. Prior to islet transplant, mean blood glucose

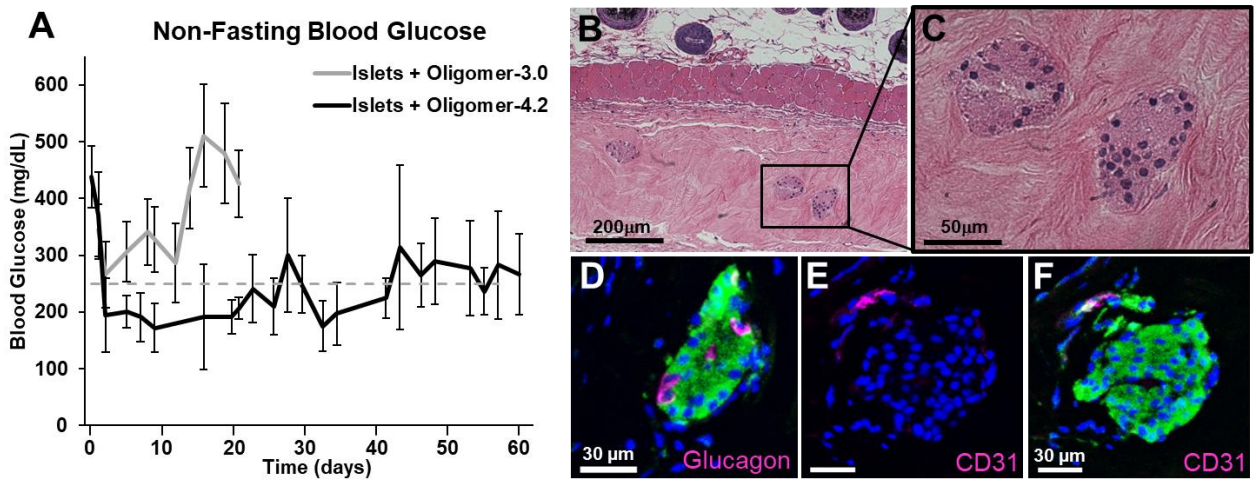

Figure 2-7. Function and histopathological analysis of pilot allogeneic studies.

A. Non-fasting blood glucose levels (mean±SD) following subcutaneous in-situ macroencapsulation of allogeneic CD1 mouse islets in Oligomer concentrations of 3.0 mg/mL (n=3) and 4.2 mg/mL (n=5) within diabetic C57BL/6J mice. B-F. Histopathological analysis of Oligomer-encapsulated (4.2 mg/mL), allogeneic islets 60 days following subcutaneous transplantation. B,C. H&E stained cross-sections showing encapsulated islets within the subcutaneous space below the panniculus carnosus muscle. D. Islets stained positive for insulin (green) and glucagon (purple). E,F. CD31 (purple) staining confirmed the presence of endothelial cells near islets co-stained for insulin (green) and nuclei (blue).

values were 378±74 mg/dL. When Oligomer-3.0 was applied, a blood glucose values declined within the first 24 hours; however, animals remained hyperglycemic (Figure 2-7A). Hypothesizing that the level of immune protection may be related to the amount of Oligomer (or fibril density), follow-up animals were performed using an Oligomer concentration of 4.2 mg/mL, which corresponds roughly to an Oligomer stiffness value of 2000 Pa and polymerization half-time of 16.30±0.02 seconds. In this case, diabetes was reversed within 24 hours with blood glucose values gradually increasing above the diabetic threshold after about 40 days (Figure 2-7A). Histopathologic and immunostaining analyses of 60-day Oligomer-islet explants showed insulin- and glucagon-positive islets surrounded by fibrillar Oligomer (Figure 2-7B-D). Interestingly, a subset of islets showed no evidence of a foreign body response, while others showed very mild inflammatory infiltrate. In some instances, nearby vasculature was evident (Figure 2-7E,F); however, vascularization and inflammation events did not appear to be correlated.

2.4 Discussion

Previous attempts to restore islet-ECM interactions via micro- or macro-encapsulation strategies have yielded variable improvement in islet survival and function, with few studies focusing on the role of material biophysical properties. In this study, we show Oligomer fibril architecture, and its capacity to be precision tuned, is important when developing a macroencapsulation strategy for sustaining functional islet masses in vitro and in vivo. Oligomer rapidly transitions from liquid to a solid scaffold, facilitating in-situ macroencapsulation not only within cultureware but also subcutaneously following simple injection through small bore needles (26G). The higher-order suprafibrillar assembly and improved proteolytic resistance imparted by natural intermolecular crosslinks present within Oligomer molecules [113] addressed a number of shortcomings associated with previous collagen-encapsulation and subcutaneous transplantation strategies.

Upon islet isolation and conventional culture in suspension, insulin-producing β cells undergo multiple cell death processes including apoptosis, anoikis, and necrosis, which has been attributed to the loss of critical cell-ECM interactions, biophysical cues, and vascularization.[18–21] While there is ample evidence that entrapment of islets in conventional monomeric type I collagen enhances islet survival and insulin secretory function in vitro,[130,131,142,143] these constructs are reported to be prone to contraction, poor mechanical stability, and rapid degradation.[143,144] This has prompted the use of collagen in combination with chemical crosslinking agents,[144] nature-derived and synthetic co-polymers,[77,144–146] and microfabricated scaffolds[147] to improve mechanical properties and resistance to proteolytic degradation. For example, Ghahary's group reported that treatment of fibroblast populated collagen matrices with glutaraldehye crosslinking and polyvinyl alcohol (PVA)-borate networks yielded improved in-vitro islet viability and function compared to untreated controls.[144] Alternatively, islets have been cultured on the surface of collagen-containing decellularized tissues, such as intestinal submucosa,[148] liver,[149] lung,[150] and pancreas,[151] which, for the most part, are processed to maintain their native architecture and mechanical properties.

In this report, we show that isolated islets, like a variety of stromal cell populations, actively engage Oligomer fibrils, in attempt to establish tensional homeostasis and a favorable mechanical environment for cell function.[152] As fibril density and associated matrix stiffness increase, islet cells are less able to induce traction-mediated matrix deformation and reorganization. It is

noteworthy that Oligomer matrices possess a higher stiffness-to-fibril density ratio and broader range of tunability compared to their monomeric collagen counterparts,[132] offering greater resistance to cellular traction forces. Decreasing islet-induced matrix remodeling by fine tuning Oligomer stiffness, improved islet survival and cytoarchitecture over the 14-day culture period as well as sustained glucose-responsive β-cell function comparable to freshly isolated islets. The fact that no disintegration or degradation of Oligomer-islet constructs occurred in-vitro can be attributed to native intermolecular crosslinks, which are known to decrease collagen turnover in vivo.[153] While the observed density-dependent phenotypic modulation was likely mediated, in part by, integrin-mediated mechanotransduction pathways, more detailed studies are necessary to elucidate relevant molecular players.

Present-day clinical islet transplantation involves infusion of isolated islets through the portal vein into the liver where they become lodged. Although this procedure is minimally invasive, a number of alternative sites have been evaluated recently.[42,154] Our in-situ macroencapsulation strategy targeted the subcutaneous space, an advocated site for clinical islet delivery since it provides adequate space for implant accommodation, ease of access for transplant administration and removal (if necessary), and amenability for post-transplantation monitoring.[155] Although the subcutaneous space facilitates administration, major challenges for islet transplantation include poor oxygen tension and inadequate vascularization.[156] In fact, islet transplantation, whether alone or encapsulated, into an unmodified subcutaneous site has never reversed diabetes in animals or humans as the microenvironment has been deemed inhospitable to cell survival.[141] It has been suggested that stimulation of angiogenesis is critical to successful subcutaneous islet transplantation,[31,118,157,158] prompting investigation of a number of vascularization strategies. Specifically, vascularization using empty devices or other synthetic materials pre-transplantation of islets,[141,159–163] oxygen generators,[94,123] and co-transplantation of soluble factors (e.g., fibroblast growth factor, hepatocyte growth factor, and vascular endothelial growth factor)[125,164] or cells (e.g., fibroblasts, mesenchymal stem cells, and endothelial cells)[125,165,166] have all been explored with variable success. For most of these strategies, there is a one to four week post-transplantation delay for animals to achieve normoglycemia as islet health and site vascularization and mass transport properties presumably become sufficient for normal glucose homeostasis.

Our approach, which involved subcutaneous injection and in-situ Oligomer-islet macroencapsulation, resulted in rapid lowering, within 24 hours, of blood glucose values below

the diabetic threshold with histological evidence of functional vascularization within 14 days. This uncommon rapid recovery of normoglycemia was observed in immunocompromised, syngeneic, and allogeneic models, suggesting that the natural and stable fibrillar scaffold formed Oligomer acted to sustain the glucose-sensitive insulin secreting function of islets as well as support the necessary molecular diffusion and transport. The mass transport properties of Oligomer, and their dependence on fibril density and molecular size, have been previously defined experimentally and computationally.[167] Following injection and throughout the study duration, Oligomer-islet constructs remained identifiable, facilitating retrievability from their subcutaneous locations. Oligomer material persisted, appearing as normal collagenous connective tissue with density-dependent fibroblast infiltration and no evidence of inflammatory or foreign body response, which is consistent with previous reports where Oligomer has been implanted in other anatomical locations.[111,112] Clearly, the stability and persistence of Oligomer was critical to prolonged islet survival and function, with multiple groups previously attributing failure of collagen-encapsulated islet transplantation to rapid in-vivo degradation.[124,144,168] Also, the volume ratio of fibrillar Oligomer to islets was critical to uniform encapsulation and consistent maintenance of a functional β cell mass, with the highest fibril densities required for allogeneic islet transplants. In the present study, Oligomer contributed to an engraftment response marked by "tissue integration" rather than a fibrotic, foreign-body reaction, which is known to be detrimental to islet health and glucose-insulin transport.[141,169] These results were encouraging since alginate, as well as other nature-derived and synthetic materials, have a long history of biocompatibility issues leading to early graft failure due to fibrosis.[64,170,171] To the best of our knowledge, this is the first report of successful subcutaneous islet transplantation with rapid lowering of blood glucose using an injectable in-situ Oligomer-islet macroencapsulation strategy.

In conclusion, Oligomer fibril architecture was an important determinant of viability, morphology and function of transplanted islets as well as islets cultured in-vitro. While the exact mechanisms underlying the observed improvement in islet function have yet to be determined, we suppose that the ability of oligomers to recreate fibrillar collagen in its natural and stable format contributes in a multifaceted fashion. More specifically, its high in-vivo biocompatibility, with no inflammatory reaction, likely contributes to improved islet health and β cell mass engraftment. The fact that increased survival and function of macroencapsulated islets was observed both in-vitro and in-vivo suggests that physical support provided by the scaffold, together with receptor-

mediated (e.g., integrin) engagement, assists in maintaining essential survival and differentiation signaling pathways. The integration of the oligomer scaffold within the subcutaneous space and associated vascularization supports essential oxygen diffusion and molecular transport while reducing inflammatory cell infiltrate and activation, even in the presence of allograft islets. Additional studies are currently underway to further validate and optimize islet subcutaneous delivery as well as define islet dose-response. The ability of Oligomer to provide important microenvironmental cues that positively impact islet survival while overcoming major shortcomings of conventional encapsulation strategies position it well as a tool for 1) in-vitro maintenance of islet viability and function following isolation as well as 2) the design of functional subcutaneous islet transplant solutions.

3. IMMUNOPROTECTION OF TYPE I OLIGOMERIC COLLAGEN MACROENCAPSULATED ISLETS IN THE SUBCUTANEOUS SPACE

3.1 Introduction

Type 1 diabetes (T1D) is a global epidemic, affecting 1 in 500 or 1.25 million men, women, and children in the United States alone.[1] This autoimmune disease results in complete destruction of pancreatic beta (β) cells leading to insulin deficiency and loss of glycemic control. While daily glucose monitoring and insulin injections are life saving for these individuals, this standard of care falls short in terms of effective glycemic control, especially when compared to functioning pancreatic β cells. As a result, individuals with T1D remain at high risk for life-threatening complications, including hypo- and hyper-glycemic episodes, cardiovascular disease, loss of kidney function, and neuropathies. It is this reality that motivates the search for new therapeutic options for restoring normal glucose metabolism to persons with T1D, allowing them to experience a better quality of life with fewer medical complications.[172]

Replacement islet/β cell therapies with long-lasting glucose-sensing and insulin-releasing functions continue to hold great promise for restoring extended glycemic control to individuals with T1D. Successes achieved with clinical islet transplantation,[33,34] where allogeneic islets are infused via the portal vein into the liver, continue to highlight the potential of this cellular therapy. However, the requirements for large donor islet numbers and risks associated with life-long immunosuppression represent persistent challenges to its more widespread clinical adoption. As an alternative to systemic immunosuppression, physical encapsulation of islets or β cells within a protective semipermeable material have been explored.[48] Ideally, the material should support long-term β cell function by serving as a protective barrier against immune cells and mediators (e.g., antibodies) while, at the same time, facilitating rapid glucose, insulin, and nutrient transport. With these criteria in mind, approaches to date have applied a number of different encapsulation formats applied to different extrahepatic microenvironments. For example, individual or a small number of islets have been coated with thin layers of nature-derived alginate to create microcapsules. Microcapsules are most commonly injected into the peritoneum, which accommodates large volumes and offers relatively high oxygen availability (partial pressure of oxygen). Macroencapsulation strategies have also been explored, where larger islet numbers (roughly a few hundred to several thousand islets) are encapsulated within a single synthetic

material (e.g. PTFE list others) or device. These devices have been implanted into a number of microenvironments (e.g., subcutaneous, intraperitoneal, omentum), with preference given to the subcutaneous space due to ease of implant and retrieval. These devices are often coupled with design elements to improve external vascularization and oxygenation to support islet health and glucose-stimulated insulin release. While a small number of these encapsulation strategies have shown some promise when evaluated preclinically in rodent models and moved into human clinical trials, full clinical translation has yet to be achieved. Despite significant efforts to date, multiple translational barriers have been identified, specifically fibrotic capsule formation owing to poor material biocompatibility and inadequate microenvironment (e.g., oxygenation, vascularization, ECM) to support longevity of islet health and function.

For example, alginate has been the most utilized polymer for microencapsulation strategies with some designs incorporating additional coatings of poly-L-lysine or poly-L-ornithine to increase mechanical stability.[59,173] However, variability of alginate products, alginate impurities, extra coatings may affect biocompatibility and immunogenicity.[51] There have been some successes in rodents and larger animals, but studies in non-human primates and humans have had limited and inconsistent success likely due to aggregation of microcapsules leading to severe hypoxia and ischemia resulting in graft failure.[174,175] More recently, macroencapsulation strategies have been explored to overcome limitations of microencapsulation. One design strategy involves the use of an inner immunoisolating membrane as well as an outer vascularizing membrane.[175] The immunoisolating membrane has pores large enough to allow rapid nutrient and oxygen diffusion while blocking cells from the host immune system. The goal of the vascularizing membrane is to allow vasculature as close as possible to the implant to minimize the formation and effects of a fibrotic capsule. In general, these strategies prevent cell-cell contact and must rely heavily on oxygen diffusion for maintaining islet health and function. Often, this is limited by native oxygen levels at the transplant site, so strategies at enhancing oxygen delivery to implanted devices are being evaluated.[175] In addition to inadequate oxygen supply, islet purity and quality, fibrotic responses to device materials, and inadequate device vascularization continue to be barriers to translation of a widely adopted encapsulated beta cell replacement therapy

Our design strategy involves the use of an injectable novel type I oligomeric collagen (Oligomer) to in situ encapsulate and protect glucose-sensing insulin-releasing replacement beta cells. Oligomer is a patented, highly-purified, acid-soluble collagen exhibiting rapid (<60 seconds)

liquid-to-solid transition at body temperature, forming highly stable collagen-fibril scaffolds with characteristic D-spacing patterns just like those found in the body's tissues.[132,110] Taking advantage of its rapid fibril formation, our macroencapsulation strategy uses a simple injection into the subcutaneous space allowing for ease of accessibility for implant administration and removal, amenability for post-implantation monitoring of islets, and ease of administration via minimally invasive procedures.[118,31,119] Previously, we showed that Oligomer-islet interactions benefited islet health by maintaining islet morphology and function of encapsulated mouse islets in vitro for 14 days.[176] Additionally, streptozotocin (STZ)-induced diabetic mice experienced uncommon rapid, within 24 hours, reversal of hyperglycemia and maintenance of euglycemia for beyond 90 days after receiving in situ encapsulated syngeneic islets.[176] Notably, the natural collagen scaffold induced no foreign body response or fibrosis, overcoming challenges posed by present-day encapsulation strategies. Further, preliminary studies involving delivery of allogeneic islets suggested that islet longevity was somewhat decreased compared to syngeneic islets with functional improvements as the density of encapsulating fibrils was increased.

Here, we wanted to better define the mechanisms underlying oligomer-islet-recipient interactions, with the goal of providing additional preclinical evidence supporting the utility of natural collagen polymers for islet/beta cell replacement strategies. Specifically, we wanted to address how host tissue response and glycemic control is affected by 1) total islet dose, 500 or 800 islets, 2) allogeneic versus xenogeneic (rat) islet sources, 3) injectable versus preformed delivery formats, and 4) intraperitoneal (IP) versus subcutaneous delivery microenvironments.

3.2 Materials and Methods

3.2.1 Isolation of Islets

Pancreatic islets were isolated from 8- to 14-wk old CD1 mice or Sprague Dawley rats (Jackson Laboratory, Bar Harbor, ME) as described.[135] Islet isolations were approved by the Indiana University Institutional Animal Care and Use Committee using AAALAC guidelines. Isolated islets were incubated in RPMI 1640 medium supplemented with 10% fetal bovine serum (HyClone, ThermoFisher Scientific, Waltham, MA), 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma Aldrich, St. Louis, MO) in a humidified environment of 5% CO_2 in air at 37°C prior to experimental use the next day.

3.2.2 Type I Oligomeric Collagen

Type I oligomeric collagen (Oligomer) was acid-solubilized from the dermis of market-weight pigs and lyophilized for storage as described previously.[132] The Oligomer formulation was standardized on the basis of molecular composition as well as polymerization capacity according to the voluntary consensus standard ASTM F3089-14.[136] Here the polymerization capacity is defined by the matrix shear storage modulus, G' (in Pa), as a function of oligomer concentration in the polymerization reaction. For all studies, Oligomer was diluted with 0.01 N HCl and neutralized to a final concentration of approximately 4.2 mg/mL, corresponding to a polymerized delivery matrix with G' value of 2000 Pa.

The microstructure and ultrastructure of Oligomer-fibril constructs were characterized through scanning electron microscopy (SEM). SEM specimens were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, post-fixed in 1% osmium tetroxide, dehydrated in a graded ethanol series, and critical point dried in a Tousimis 931 machine. Dried specimens were coated with platinum in a Cressington 208HR sputter coater. Samples were imaged using an FEI NOVA nanoSEM 200 field emission scanning electron microscope (FEI Company, Hillsboro, OR) using an ET (Everhart-Thornley) detector or the high-resolution through-the lens (TLD) detector operating at an accelerating voltage of 5kV. To characterize the Oligomer-islet interface, transmission electron microscopy (TEM) was used. TEM specimens were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, post-fixed in 1% osmium tetroxide and 0.8% FeCN, dehydrated in a graded ethanol series, and embedded in epoxy resin (EMBED 812, Electron Microscopy Sciences) and polymerized at 60°C. Ultrathin sections were cut at a nominal thickness of 80 nm, picked up on copper grids, and stained with uranyl acetate and lead citrate. Samples were imaged using an FEI Philips CM-100 transmission electron microscope (FEI Company) with a magnification range from 20x to 510,000x, accelerating voltage from 40 to 100kV, and side mounted Orius SC1000 CCD camera (Gatan, Pleasanton, CA).

3.2.3 Subcutaneous Islet Implantation

Islet implantation procedures were approved by the Indiana University Institutional Animal Care and Use Committee using AALAC guidelines. Male 8-wk to 14-wk old C57BL/6J recipient mice were injected with low dose streptozotocin (STZ; 55 mg/kg) for 5 days to chemically induce diabetes prior to islet transplantation.[137] Diabetes following STZ administration was classified as

two consecutive blood glucose levels above 300 mg/dL. CD1 mouse (allogeneic) or Sprague Dawley rat (xenogeneic) islets were mixed with neutralized Oligomer solutions. Oligomer-islet suspensions were then injected subcutaneously through small bore needles (26 gauge) into 2 sites as described previously.[176] The doses were 250 islets/500 µL Oligomer/site or 400 islets/800 µL Oligomer/site for a total of 500 or 800 islets/mouse.

For pre-formed Oligomer implants, Oligomer-islet suspensions were aliquoted into 24-well plates (500 µL/well) and allowed to polymerize at 37°C for 15 minutes to create 2 implants/mouse for a total of 500 islets/mouse. For surgical implantation, a small subcutaneous incision was made midline on the mouse dorsum. The fascia was bluntly dissected to form small pockets just lateral to the incision on both sides. Oligomer-islet constructs were implanted subcutaneously just beneath the cutaneous truncai muscle and the incision site closed with non-absorbable sutures. For intraperitoneal islet transplantation, Oligomer-islet suspensions were injected intraperitoneally through small-bore needles (26 gauge) into diabetic mice. Each mouse received 2 injections, one on each side of the abdomen, with 250 islets/500 µL Oligomer per site for a total of 500 islets/mouse.

Non-fasting blood glucose was measured for the first 3 days after implantation and then 3 times weekly until the end of the study. Based on preferences and standards established in the literature, a blood glucose level of 250 mg/dL was selected as the "diabetic threshold".[138,139] A euglycemic curve was produced with mice being classified hyperglycemic after 3 consecutive readings over 350mg/dL.[177] To confirm implant-dependent euglycemia, Oligomer-islet implants were retrieved after 90 days. Mice were euthanized one week after explant at which time the pancreas was removed, and histopathological analysis was performed to confirm destruction of endogenous islets.

3.2.4 Histopathologic Analysis and Immunostaining

Implantation sites and surrounding tissues were removed at specified times and placed in 10% formalin before paraffin embedding and sectioning. Sections were stained with hematoxylin and eosin (H&E) and Masson's trichrome (MTC). For immunofluorescence, sections were deparaffinized, rehydrated, and stained with primary guinea pig anti-insulin (PA1-26938, Invitrogen, Waltham, MA), rabbit anti-glucagon (2760S, Cell Signaling Technology, Danvers, MA) at 1:50 and 1:75 dilutions, respectively. Sections were then treated with secondary Alexa

Fluor 488 goat anti-guinea pig (A11073, Life Technologies, Waltham, MA), Alexa Fluor 546 goat anti-rabbit (A11035, Life Technologies). For immune cell detection, sections were stained for CD68+ macrophages (MA5-13324, Invitrogen), CD4+ T-helper cells (562891, BD Biosciences, San Jose, CA) and CD8+ cytotoxic T-cells (53-0081-82, Invitrogen) at 1:100, 1:75, and 1:75 dilutions, respectively. Sections were then treated with secondary Alexa Fluor 488 donkey anti-mouse (A21202, Life Technologies) at a 1:100 dilution. For beta cell dedifferentiation, sections were stained with Aldh1a3 (NBP2-15339, Novus Biologicals, Centennial, CO), then treated with secondary Alexa Fluor 546 goat anti-rabbit. Nuclei were counterstained with Draq5

3.2.5 Statistics

Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests were used to analyze the euglycemic curves. The Gehan-Breslow-Wilcoxon test gives more weight to mice that were no longer euglycemic at early time points, while the log-rank test gives equal weight to all time points. ANOVA analysis followed by a post-hoc Tukey-Kramer test will be used to compare average euglycemia times. A p-value less than 0.05 will be used as an indicator of statistical significance.

3.3 Results

3.3.1 Increasing islet dose from 500 to 800 islets does not significantly improve euglycemia time after injectable subQ Oligomer-islet implant

To take advantage of the fibril-forming capacity of liquid Oligomers, we previously used Oligomer in a subQ injectable format for in-situ islet encapsulation.[176] We showed that Oligomer maintained islet health and function in vitro and supported syngeneic islet delivery and function in vivo with uncommon rapid, within 24 hours, reversal of hyperglycemia. Initial allogeneic studies showed a decrease in functional longevity with glycemic control improving as the density or total content of encapsulating fibrils increased.

To extend this work, we wanted to determine the effect of the total islet dose and islet source on functional longevity. 500 or 800 Oligomer-encapsulated allogeneic (CD1 mice) and xenogeneic (Sprague Dawley rat) islets were implanted through subcutaneous injection into STZ-induced diabetic mice. Notably, 80% or more of all mice in each group maintained euglycemia for

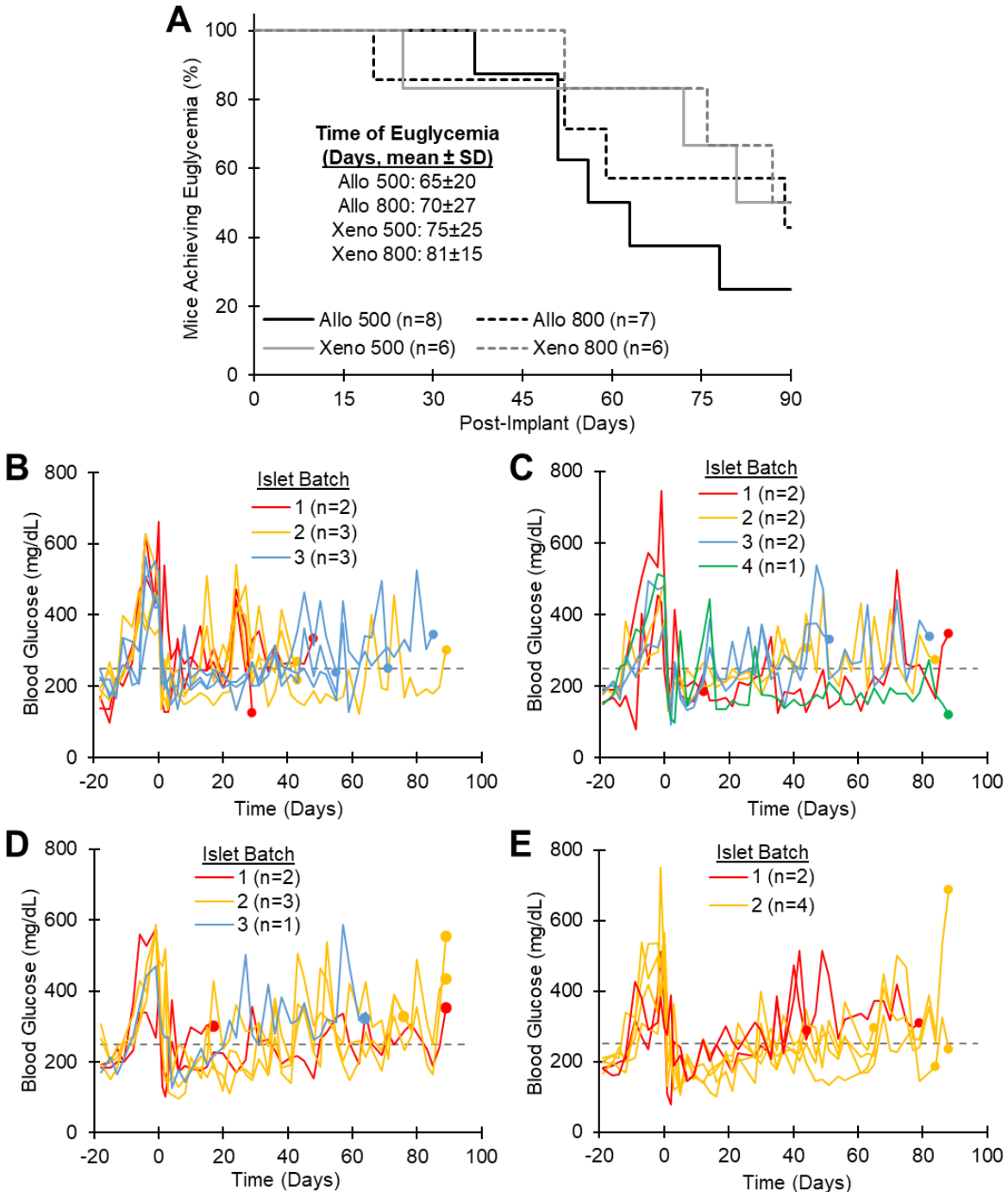

Figure 3-1. Functional efficacy of 500 or 800 Oligomer-encapsulated allogeneic or xenogeneic rat islets.

A. Percentage of STZ-induced diabetic mice maintaining euglycemia following subcutaneous delivery of 500 or 800 Oligomer-encapsulated allogeneic or xenogeneic rat islets. B.-E. Non-fasting blood glucose values for individual mice receiving allogeneic islets (B,C) or xenogeneic islets (D,E) at doses of 500 (B,D) or 800 (C,E). A value of 250 mg/dL was applied as the diabetic threshold (dashed line).

at least 50 days (Figure 3-1A). All animals receiving 500 (Allo 500, n=8) and 800 (Allo 800, n=7) allograft islets were euglycemic until 37 and 20 days, respectively, with 25% and 43% remaining euglycemic after 90 days. Interestingly, all animals receiving 500 (Xeno 500, n=6) and 800 (Xeno 800, n=6) xenograft islets were euglycemic until 25 and 52 days, respectively. At the end of the 90-day study period, 50% of the animals remained euglycemic for both the 500 and 800 xenograft doses. Statistical analysis of euglycemic curves showed no significant differences. Further, we calculated the average time of euglycemia for each group. Allo 500 and Allo 800 mice were euglycemic for 65±20 and 70±27 days, respectively, while Xeno 500 and Xeno 800 had somewhat

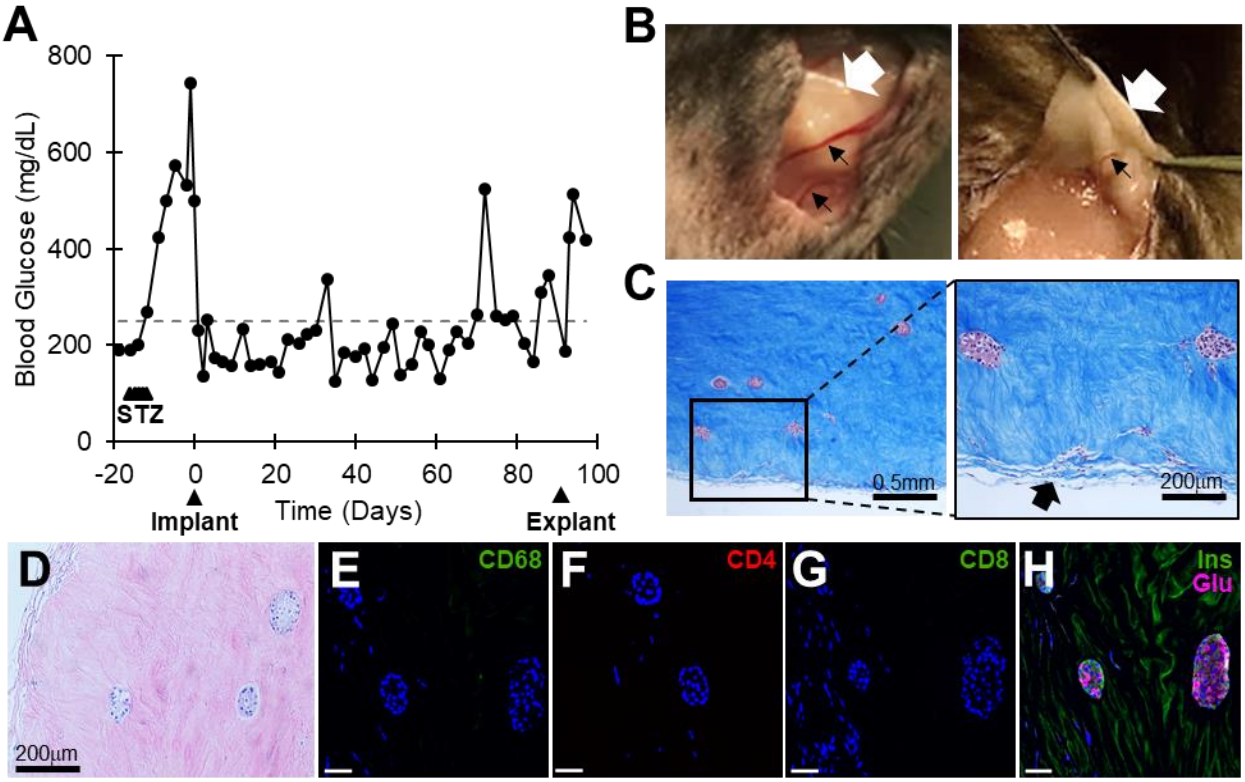

Figure 3-2. Representative STZ-induced diabetic mouse following subcutaneous delivery of 800 Oligomer-encapsulated allogenic islets.

A. Non-fasting blood glucose values for a representative STZ-induced diabetic mouse following subcutaneous delivery of 800 Oligomer-encapsulated allogenic islets (day 0). A value of 250 mg/dL was applied as the diabetic threshold (dashed line). B. Identification and retrieval of the Oligomer-islet implant on day 90 resulted in reversion back to a diabetic state. The external surface of the implant appeared richly vascularized, as evidenced by both macro- and micro-vasculature (black arrows). Masson's trichrome (C), H&E (D), CD68 (E), CD4 (F), and CD8 (G) stained cross-sections of explant documenting the capacity of Oligomer material to prevent immune cell infiltration in absence of a foreign body response. H. Islets maintained multicellular architecture with insulin- (green) and glucagon- (purple) staining cells. Cell nuclei were stained with Draq5. Scale bar is 50 µm for E-H.

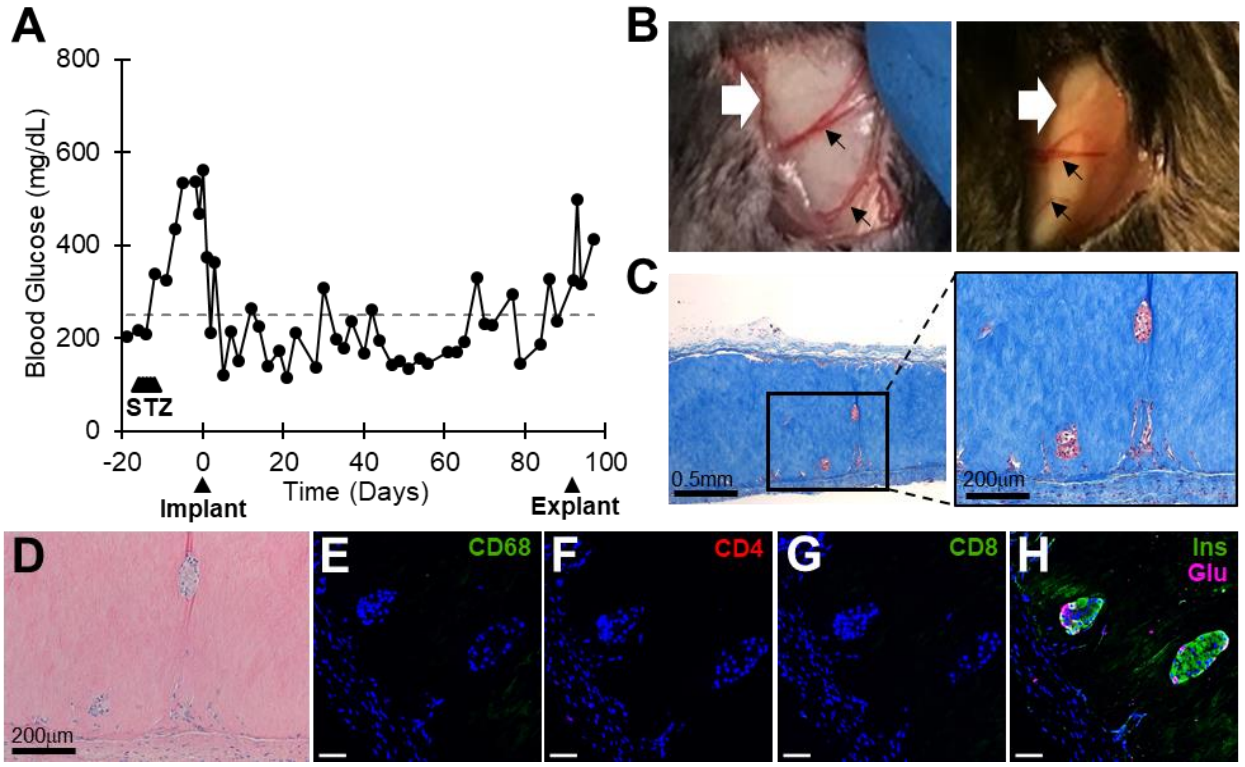

Figure 3-3. Representative STZ-induced diabetic mouse following subcutaneous delivery of 800 Oligomer-encapsulated allogenic islets.

A. Non-fasting blood glucose values for a representative STZ-induced diabetic mouse following subcutaneous delivery of 800 Oligomer-encapsulated xenogeneic rat islets (day 0). A value of 250 mg/dL was applied as the diabetic threshold (dashed line). B. Identification and retrieval of the Oligomer-islet implant on day 90 resulted in reversion back to a diabetic state. The external surface of the implant showed rich vascularization (black arrows). Masson's trichrome (C), H&E (D), CD68 (E), CD4 (F), and CD8 (G) stained cross-sections of explant verifying minimal to no apparent immune response to Oligomer-islet implants. H. Islets maintained multicellular cytoarchitecture with insulin- (green) and glucagon- (purple) staining cells. Nuclei were counterstained with Draq5. Scale bar is 50 μm for E-H.

increased, but not significant average times of euglycemia of 75±25 and 81±15 days, respectively. As seen previously, all groups of Oligomer-encapsulated islets showed rapid engraftment, again reversing diabetes within 24 hours of transplantation in all recipients (Figure 3-1B-E).[176] Alternatively, mice that received islets alone never reversed diabetes and remained in the diabetic state similar to mice that received no cellular therapy (Figure S 1A).

In addition to determining the functional longevity, we wanted to define the immune protection capacity of Oligomer when implanting Oligomer encapsulated allogeneic compared to xenogeneic islets. Oligomer-islet implants were retrieved at 90 days to document that the implant

was the source of glycemic control. Implants were easily identifiable and showed evidence of rich vascularization, macro and micro, on the exterior (Figure 3-2B,Figure **3-3**B). Implants appeared as thin sheets with surface area and thickness ranging from 50-90mm^2 and 100-400µm, respectively (Figure 3-2B,Figure **3-3**B). Upon removal, a rapid increase in blood glucose was observed (Figure 2A,3A), reverting the animals back to a diabetic state. Histolopathologic and immunostaining analyses of 90-day Oligomer-islet explants showed Oligomer persistence with integration into the surrounding subcutaneous tissue compartment and no evidence of chronic inflammatory or foreign body response (Figure 3-2,Figure **3-3** C-G). Islets were readily identifiable within the implant, with no evidence of a foreign-body response at the periphery (Figure 3-2C,D and Figure **3-3**C,D). Within Oligomer, islets maintained their rounded, multicellular architecture with insulin- and glucagon-positive cells (Figure 3-2H, Figure **3-3**H). In both allogeneic and xenogeneic explants, there appeared to be no positive staining for CD68 (macrophages), CD4 (helper T cells), or CD8 (cytotoxic T cells) further suggesting no immune cell infiltration and immune modulation of Oligomer encapsulated islets. Consistent with previous reports, allogeneic and xenogeneic islet only explants showed a robust foreign-body response indicative of graft failure and no identifiable islets (Figure S 1B) due to the inhospitable microenvironment of the subcutaneous space for transplantation of islets alone.[140,141,178]

3.3.2 Use of a more controlled, preformed Oligomer-islet implant does not affect functional longevity

With Oligomer injection and in situ encapsulation there is less control of islet mixing within the collagen and the potential for incomplete encapsulation of implanted islets (Figure S 2). In some cases, we saw evidence of this with the formation of a small granulomatous region and the presence of immune cells on the edge of an Oligomer-islet implant (Figure S 2A,C). However, this did not appear to effect functional longevity of the mouse (Figure S 2B) and the appearance of additional normal insulin and glucagon positive staining islets within the Oligomer-islet implant (Figure S 2D). Therefore, we evaluated if the delivery format, a preformed implant versus an injectable in-situ forming implant, affects functional longevity for both allografted and xenografted islets. Creation of a preformed implant allowed us to have more control and uniformity of the mixing and encapsulation of islets within Oligomer. We hypothesized that the preformed Oligomer-islet implants would perform as well as if not better than the injectable format. However, the surgical procedure for insertion of the implants could result in more inflammation and immune

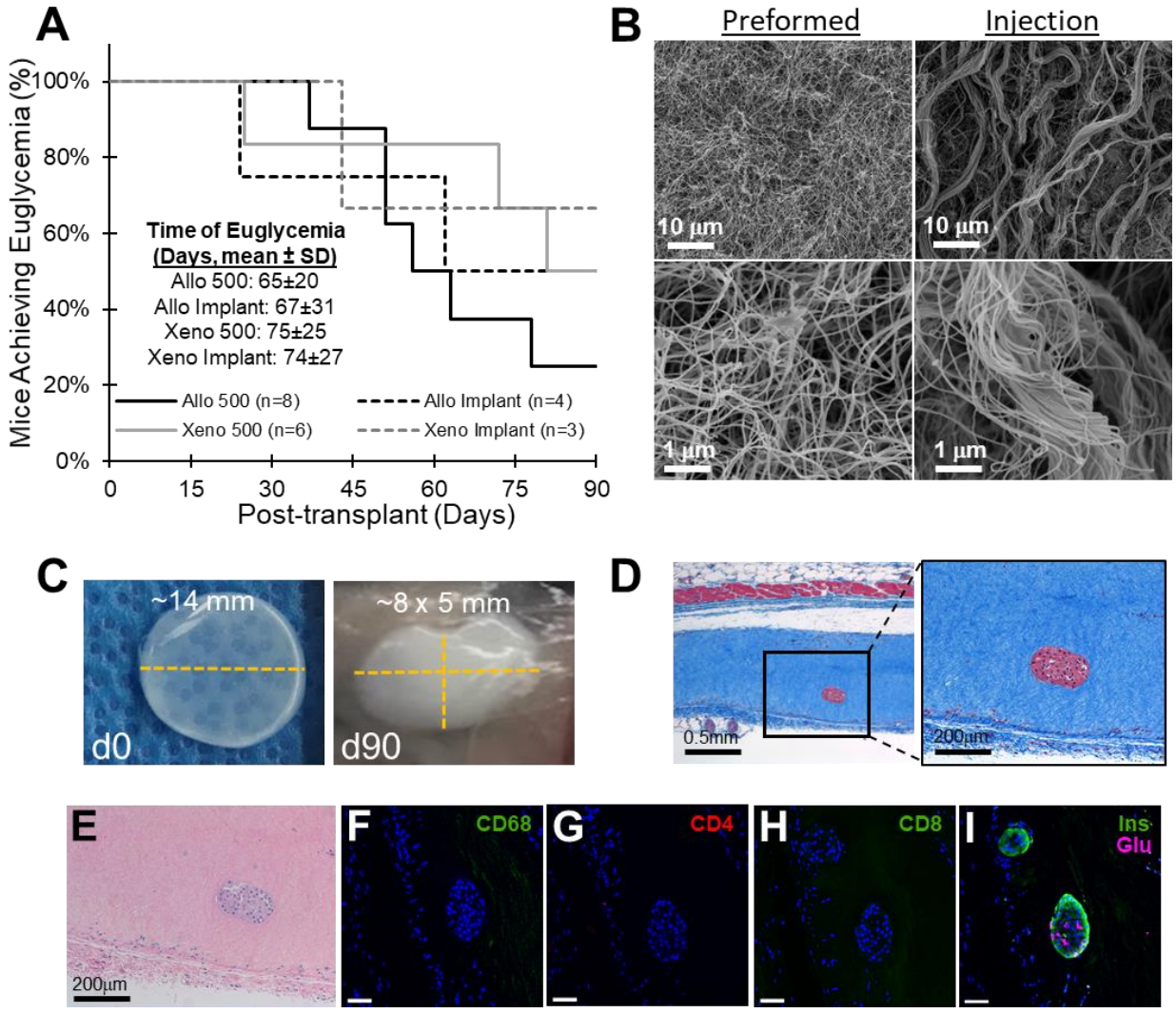

Figure 3-4. Tissue response and functional efficacy of preformed Oligomer-islet implants.

A. Percentage of STZ-induced diabetic mice achieving euglycemia following subcutaneous delivery of Oligomer-encapsulated allogeneic and xenogeneic islets in either pre-formed or injectable formats. B. Representative SEM images of preformed and injected formats at day 0 highlighting differences in fibril arrangement. C. Representative images showing pre-formed implants before surgical implantation and explanted implants after 90 days. Masson's trichrome (D), H&E (E), CD68 (F), CD4 (G), and CD8 (H) stained cross-sections document no significant foreign body reaction or immune cell infiltrate. H. Islets maintained multicellular cytoarchitecture and function with insulin- (green) and glucagon- (purple) staining cells. Nuclei were counterstained with Draq5. Scale bar is 50 mm for F-I.

responses to the implant. Similar to the mice that received injectable implants, mice that received preformed implants had an average euglycemia time of 67±31 (n=4) and 74±27 (n=3) days for allografts and xenografts, respectively. Comparison of the curves for the percentage of euglycemic

mice over time showed no statistical differences between preformed and injectable implants for both allogeneic and xenogeneic islets (Figure 3-4A). At the end of the 90-day study period, 50% (allogeneic) and 67% (xenogeneic) of mice that received pre-formed implants remained euglycemic. Ultrastructure analysis of preformed and injectable implants indicated distinct differences in Oligomer fibril arrangement. Interestingly, preformed implants had uniform and random distribution of fibrils while injected implants appeared to display fibril alignment and formation of fibril bundles likely caused by flow through the syringe (Figure 3-4B).

To create preformed Oligomer-islet implants, we used 24-well plates to allow polymerization then surgically transplanted the implants into the subcutaneous space (Figure 3-4C). After 90 days, Oligomer-islet constructs (Figure 3-4C) were explanted and prepared for histopathological and immunohistochemical analysis. Xenogeneic Oligomer-islet sections showed no apparent foreign body response, identifiable islets in the center and on the periphery of the implants, and normal islet cytoarchitecture with insulin- and glucagon-positive cells (Figure 3-4D-I). Additionally, there appeared to be no infiltration or presence of immune cells when staining for macrophages (CD68) and T cells (CD4 and CD8, Figure 3-4F-I).

3.3.3 Injectable Oligomer-islet implant in the intraperitoneal space reduces euglycemia time with allogeneic islets but not xenogeneic islets

For encapsulated islets, the peritoneal cavity has been most often used as the transplantation site. This location allows for portal insulin delivery via the rich mesenteric blood supply and offers higher oxygen levels when compared to the subQ space.[118,175] For this reason, we wanted to determine the effect of delivery site, IP versus subQ, microenvironment on function. We hypothesized that similar or improved maintenance of a functional β cell mass and longevity of glycemic control would be achieved with IP administration. Interestingly, when allogeneic islets were used there was a substantial, although not statistically significant, reduction in average euglycemia time from 65±20 days in the subQ space to 35±30 (n=6) days in the IP space (Figure 3-5A). However, euglycemic curves were significantly different when using the Gehan-Breslow-Wilcoxon test, statistical analysis that gives more weight to early time points, but not the log-rank test. For the IP site, 100% of the allografted mice were euglycemic for only 14 days with only 1 out of 6 mice (17%) remaining euglycemic until the end of the 90-day study period. Alternatively, there were no significant differences when using xenogeneic islets with average euglycemia time being 75±25 and 75±15 (n=6) days for the subQ and IP space, respectively (Figure 3-5A). 100%

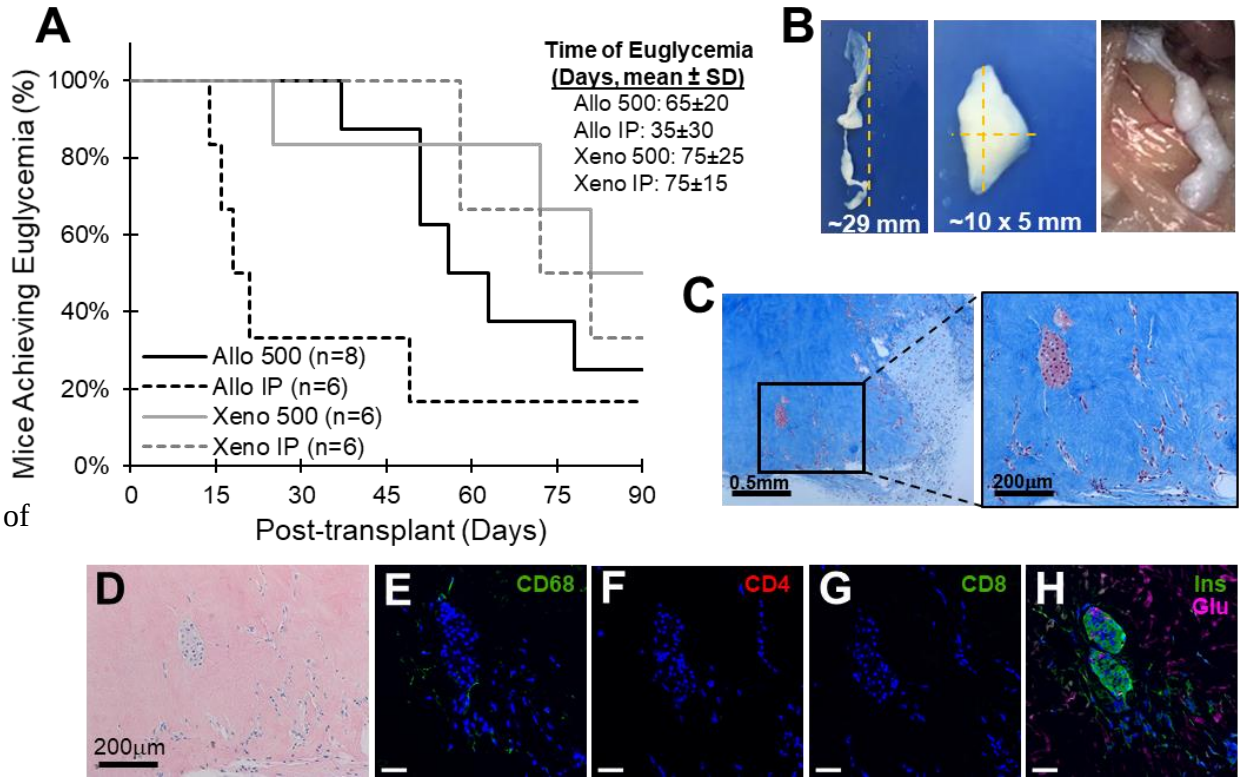

Figure 3-5. Tissue response and functional efficacy of Oligomer-islet implants in the IP space.

A. Percentage of STZ-induced diabetic mice achieving euglycemia following treatment with preformed or injectable Oligomer-islet implants for both allografts and xenografts. B. Gross images of explanted IP administered Oligomer-islet implants showing geometric irregularities. Masson's trichrome (C), H&E (D), and CD68 (E) stained cross-sections showed normal islet morphology with some cell infiltration. Further analysis of immune cell infiltration showed no apparent evidence of CD4 (F) or CD8 (G) T cells. H. Islets maintained cytoarchitecture with insulin (green) and glucagon (purple) positive stained cells. Cell nuclei were stained with Draq5. Scale bar is 50 µm for E-H.

of the xenografted mice remained euglycemic for 58 days versus 25 days; however, 33% versus 50% remained euglycemic by the end of the 90-day study when comparing the IP to the subQ site, respectively.

When explanting IP administered Oligomer-islet implants, the shapes and sizes of the IP constructs varied widely compared to subQ administered implants. In some instances, implants were very thin and elongated, string-like constructs while other times they were somewhat thicker, irregularly-shaped discs (Figure 3-5B). This could be due to the larger and less confined area of the IP space compared to the subQ space allowing for irregular and more spread out fibril-formation of the collagen as it was being injected into the larger IP space. Histopathological

analysis of 90-day explants showed normal islet morphology with some cell infiltrate. (Figure 3-5C-H). Immunohistochemical analysis showed the presence of macrophages, but no evidence of CD4 or CD8 T cells (Figure 3-5E-G).

3.3.4 Beta cell dedifferentiation as a potential mechanism of Oligomer-islet graft failure

Aldehyde dehydrogenase 1a3 (Aldh1a3) is a recently discovered marker of beta cell failure indicating that the cell is no longer a mature beta cell.[179] For implants that fail before or shortly after 50 days, we found evidence of Aldh1a3-positive stained cells within an islet while implants that functioned for the entire 90-day study period showed no evidence of Aldh1a3-positive stained cells (Figure 3-6). Interestingly, this marker was seen only in allogeneic implants and was not found in xenogeneic (rat) implants indicating that perhaps failure in these implants is experienced through an alternative mechanism. Future studies will include RNA sequencing to identify and quantify the presence of other beta cell health markers in failing Oligomer-islet implants.

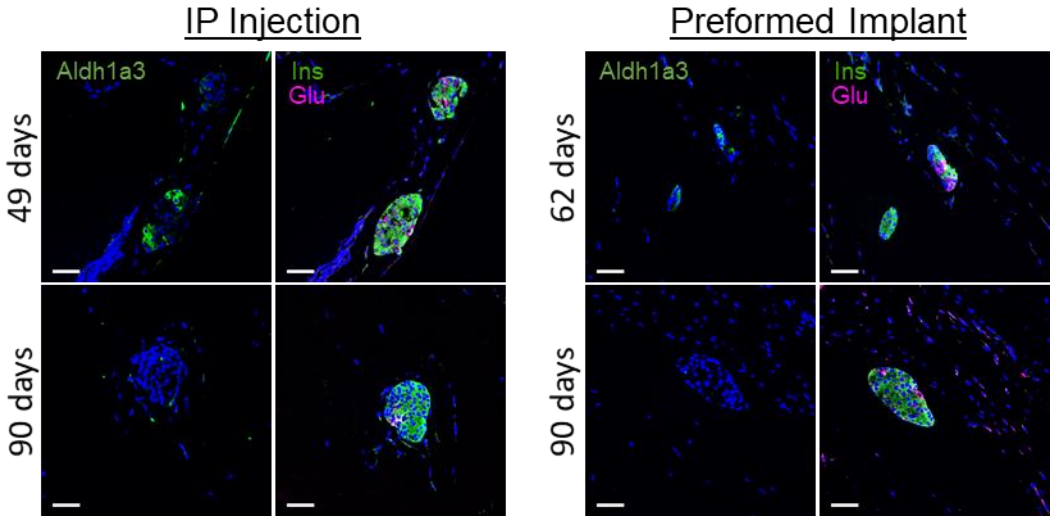

Figure 3-6. Aldh1a3 in failing Oligomer-islet explants.

Beta cell dedifferentiation as a potential mechanism of Oligomer-islet graft failure. Allogeneic Oligomer-islet explants from mice that were no longer euglycemic before 90 days showed Aldh1a3-positive (green) stained cells within islets (Insulin-green, glucagon-purple). Conversely, explants from mice that were euglycemic for the entire 90-day study period showed no evidence of Aldh1a3 within islets. Scale bar is 50 µm.

3.4 Discussion

Previously, we showed that Oligomer-islet interactions benefit islet health by maintaining islet morphology and function of in vitro encapsulated mouse islets for 14 days and syngeneic islets that encapsulated in situ following subcutaneous delivery. Specifically, our in situ macroencapsulation strategy showed uncommon rapid reversal of diabetes in STZ-induced diabetic mice with all animals (3/3) that recieved 500 syngeneic islets maintaining euglycemia for beyond 90 days.[176] Further, pilot studies involving delivery of allogeneic islets suggested that islet longevity was somewhat decreased compared to syngeneic islets. Our collagen-fibril scaffolds restored necessary mechanochemical signaling to support islet morphology and function within an unmodified subcutaneous space. The objective of the present study was to better define the mechanisms underlying oligomer-islet-recipient interactions, with the goal of providing additional preclinical evidence supporting the utility of natural collagen polymers for islet/β cell replacement strategies. Specific questions that we addressed include: How is the tissue response and glycemic control of the recipient affected by 1) total islet dose, 2) allogeneic or xenogeneic islet source, 3) injectable or preformed delivery format, and 4) intraperitoneal (IP) or subcutaneous delivery microenvironment.

From our previous pilot study, we observed a decrease in glycemic control when using 500 allogeneic islets compared to syngeneic islets. We expected a significant increase in functional longevity by almost doubling the total islet dose to 800 islets. In addition to varying the islet dose, we wanted to determine Oligomer immunoprotection capacity as well as glycemic control when using an allogeneic or xenogeneic islet source. Here, we used rat islets as the xenogeneic source due to insulin potency differences between mouse and porcine requiring a significantly more amount of porcine islets to achieve euglycemia in mice.[180] Interestingly, variations in the islet dose nor the islet source significantly affected glycemic control. Notably, we again observed a rapid, within 24 hours, reversal of hyperglycemia for both allogeneic and xenogeneic models highlighting Oligomer's ability to support implanted islet glucose-sensing and insulin-secreting functions and essential nutrient diffusion and transport. Throughout this study, subtle increases in glycemic control for xenografted mice were noticed with more significant improvement in glycemic control being observed when xenogeneic implants were injected into the intraperitoneal space. This difference in glycemic control between implanted mouse and rat islets could be a result of differences in secretory responses to glucose stimulation observed for rat and mouse islets.[181]

Specifically, rat islets have a larger rise during the second-phase of the insulin secretory response resulting in a 25- to 50-fold increase in release rates above basal levels. Alternatively, mouse islet responses are flat and only modestly elevated above basal levels.[181,182] Further, rat islets have also been shown to maintain lower levels of glycemia in STZ-induced diabetic mice.[183] We observed Aldh1a3 expression of islets in failing allogeneic Oligomer-islet implants suggesting beta cell dedifferentiation as a potential mechanism of failure for those implants; although, expression was not observed in failing xenogeneic islets. Further studies incorporating RNA sequencing analysis for the presence of additional beta cell dedifferentiation as well as beta cell stress markers are being performed to provide an in-depth characterization of the mechanisms of Oligomer-islet implant failure.

Inadequate mixing of islets within the encapsulation material has the potential to cause grafted islets to be more susceptible to host immune attack leading to graft failure.[78] Although function was not affected, some evidence of incomplete islet encapsulation was observed. In an attempt to provide more control of islet mixing within Oligomer, we evaluated the use of surgically implanted preformed Oligomer-islet implants. However, this added control did not affect tissue response and functional longevity after 90 days. The IP site has been more commonly used as the transplant site for encapsulated islets due to its larger volume, higher oxygen levels, and portal insulin delivery.[118,175] For this reason, we hypothesized that similar or improved maintenance of a functional β cell mass and longevity of glycemic control is expected with the rich mesenteric blood supply. However, upon injecting Oligomer-islet implants into the IP space, glycemic control was significantly decreased for allogeneic grafts and somewhat decreased for xenogeneic grafts. The IP space is a less confined area compared to the subQ space affording less control of fibril density and uniformity of polymerization resulting in the formation of irregular implant geometries that impacted the functional efficacy.

Oligomer for encapsulation of beta cell replacement therapies is different from other encapsulation design strategies in that it focuses on a regenerative medicine approach to provide immunoprotection and allow glucose-sensing and insulin-releasing functions. Oligomer provides important design criteria including rapid diffusion of nutrients, mechanochemical signaling, promotion of vascularization near the implant, a physical protective barrier from infiltrating immune cells, and inherent immunomodulatory signaling capable of suppressing activation of immune cells (Figure 3-7A). Oligomer is a natural tissue-derived tunable material that forms a

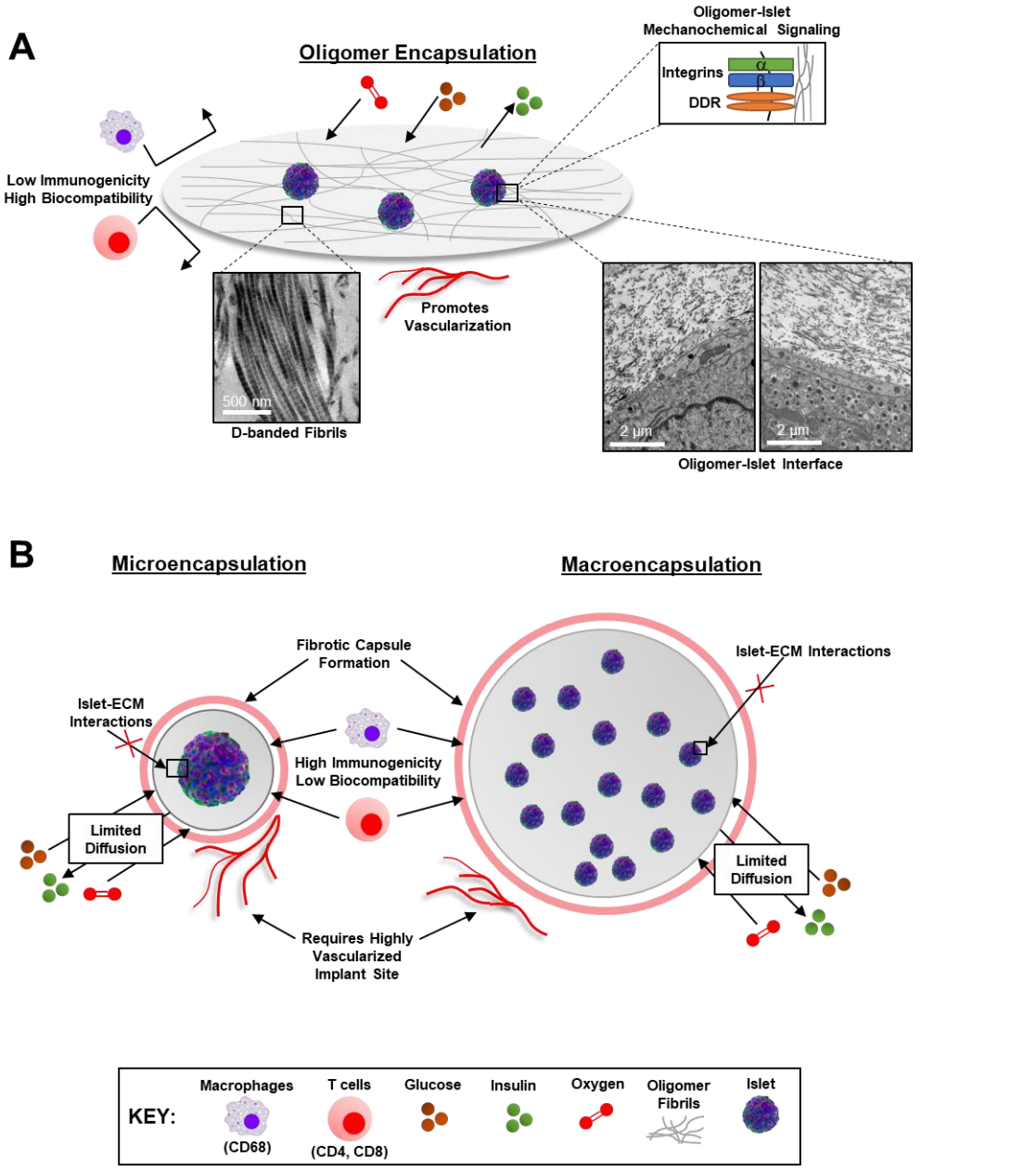

Figure 3-7. Schematic highlighting the unique features of Oligomer encapsulation of islets. A. Oligomer provides important design criteria including rapid diffusion of nutrients, mechanochemical signaling, promotion of vascularization near the implant, a physical protective barrier from infiltrating immune cells, and inherent immunomodulatory signaling capable of suppressing activation of immune cells. B. Conventional micro- and macro-encapsulation strategies with synthetic or nature-derived polymers have been associated with foreign body response from poor material biocompatibility and the formation of a fibrotic capsule limiting glucose-sensing and insulin transport which results in eventual failure of the implant.

scaffold of highly interconnected fibrils with characteristic D-spacing patterns replicating hierarchical organization. Oligomer restores critical mechanochemical signaling lost during islet isolation through integrins and discoidin domain receptors (DDR) important for supporting islet health and function.[184] In fact, TEM analysis of the Oligomer-islet interface showed alignment of Oligomer fibrils around the edges of islets (Figure 3-7A). Conventional micro- and macro-encapsulation strategies with synthetic or nature-derived materials have been associated with foreign body responses and formation of a fibrotic capsule due to relatively poor material biocompatibility, glucose-sensing, and insulin transport, resulting in eventual failure of the implant (Figure 3-7B). Although less understood mechanistically, Oligomer has high biocompatibility and low immunogenicity from the inherent immunomodulatory signaling of collagen.[185] In addition to inherent immunomodulatory signaling, Oligomer provides a physical protective barrier against host immune cell infiltration and activation through the tunability of fibril density. Interestingly, others have shown that higher density collagen matrices reduced T cell activity in vitro.[186] Further studies characterizing the mechanisms of Oligomer to suppress immune cell activation are ongoing.

To our knowledge, this is the first report of an injectable subQ islet transplant strategy that yields rapid lowering and extended glycemic control without systemic immunosuppression, overcoming several obstacles posed by present-day encapsulation strategies. These studies provide successful preclinical validation for Oligomer as an injectable in situ macroencapsulation strategy for beta cell replacement therapy. The next steps for translation of the strategy include 1) defining efficacy in small and large animal autoimmune diabetic settings and 2) combining Oligomer with clinically-relevant replenishable β cell sources, namely porcine islets and human SC-β cells.

4. FUTURE WORK AND CONCLUSIONS

4.1 Proposed Next Steps

For T1D, the most sobering fact is that insulin therapy remains the only effective treatment, but less than one-third of patients are consistently achieving target blood glucose levels resulting in life threatening hypo- and hyperglycemic episodes. Therefore, there remains a need for a long-term insulin independent solution for type I diabetics. β cell replacement therapy has the potential to eliminate the need for exogeneous insulin, vastly improving the quality of life and minimizing secondary complications for patients with T1D.[34] Unfortunately, persistent risks and challenges preclude this procedure from widespread clinical use, including i) limited donor islet supply, ii) cumbersome administration via portal vein infusion, iii) significant loss of functional islet mass upon transplantation, iv) limited functional longevity of islet/β cell grafts, and v) need for life-long systemic immunosuppression.[187] With the ultimate goal of restoring extended glycemic control in absence of systemic immunosuppression, we have been working, with the help of collaborators, to develop and translate the first natural collagen polymer for delivery and encapsulation of replacement β cells. Our solution involves a novel type I oligomeric collagen polymer, Oligomer, for subcutaneous beta cell replacement therapy using an injectable format. Upon injection, Oligomer rapidly transitions, <60 seconds, from a liquid to a solid allowing for in situ encapsulation of replacement cells. The above published and unpublished studies suggest that macroencapsulation of islets within a natural collagen polymer, Oligomer, provides a nurturing and protective microenvironment that i) prolongs beta cell viability and function in vitro and ii) provides rapid and extended glycemic control to STZ-induced diabetic mice. Further, Oligomer can be formulated as an injectable solution that exhibits in-situ islet macroencapsulation, restoring biophysical signals that are critical to islet health and phenotype while thwarting innate and adaptive immune responses with immunomodulatory signaling inherent to natural collagen fibrils. In summary, these results provide strong support for next-level preclinical validation studies that 1) define efficacy of the proposed Oligomer/β-cell replacement therapy in small and large animal autoimmune diabetic settings and 2) combine Oligomer with clinically-relevant replenishable β cell sources, namely porcine islets and human SC-β cells.

4.1.1 Define efficacy in small and large autoimmune diabetic animal models

T1D represents an autoimmune disease that results in the destruction of insulin-producing β cells. To extend preclinical testing, the autoimmune non-obese diabetic (NOD) mouse will be used to further define efficacy of our Oligomer/β-cell replacement therapy.[188,189] The NOD mouse is a well-established model that is known to spontaneously develop autoimmune diabetes and has pathophysiologic relevance to human autoimmune diabetes. Using this animal model, two main questions will be addressed: 1) Is there a difference in the longevity of glycemic control and immune response achieved with subQ injectable delivery of allogeneic (CD1 mice) and xenogeneic (rat) islets in autoimmune diabetic NOD mice? and 2) What underlying mechanisms determine islet health, function, and longevity?

Similar to our previous studies, the first question will focus on defining the glycemic control and immune protection achieved following subQ Oligomer delivery of allo- and xenograft rat islets as well as the mechanisms underlying collagen-islet signaling and critical determinants of β cell functional longevity. Studies addressing the second question will explore how modulation of specific microenvironment parameters can be used to maximize β-cell health, potency, and longevity following delivery. The effect of specific microenvironment parameters on islet health, potency, and longevity will be determined, including administration site (subQ vs. IP), implant format (injectable vs. preformed) and addition of targeted, localized modulators of islet health and adaptive immunity. In addition to standard outcome measures from our previous studies, we will incorporate a more detailed analysis of the immune response to implants using a cytokine array analysis and RNA sequencing. Based on our preliminary data, we expect that Oligomer when used in a subQ injectable format, will be able to effectively maintain euglycemia in >50% of autoimmune NOD mice for beyond 50 days following delivery of allogeneic and xenogeneic islets. From our data in STZ-induced diabetic mice similar glycemic control and tissue/immune response outcomes are expected with Oligomer delivery of allogeneic and xenogeneic islets in diabetic NOD mice. Finally, we expect to advance our mechanistic understanding and definition of specific microenvironmental parameters (e.g., inflammatory cytokines, hypoxia, oxidative stress, de-differentiation) that determine β-cell health and functional longevity following Oligomer encapsulation. From these studies, we hope to identify a minimized set of prioritized microenvironment parameters for further studies and scalability to large animals.

Although a number of replacement β cell strategies have yielded promising results in rodent models, successful translation to large animals and humans has yet to be achieved.[45,48] Indeed, it is evident that use of large animals for validating scalability, safety, and efficacy of novel therapies represents an important step in predicting potential pitfalls and successes in humans. Future studies will select a promising Oligomer/β-cell formulation to be scaled and tested in dogs with naturally-occurring diabetes mellitus. Naturally-occurring diabetic dogs represent a valuable translational model for human T1D, with their numerous similarities to humans, including pancreas anatomy and islet morphology, disease pathophysiology involving loss of islets and lack of insulin, and disease-related clinical signs (weight loss, polyuria, and polydipsia).[190,191] Given the long history of development and use of porcine islets for large animal and human studies and that human SC-β cells are in early stages of development, we anticipate that an Oligomer/porcine islet formulation will be selected for evaluation in the pilot clinical diabetic dog study. For porcine islets, an initial dose of 5000IEQ/kg will be used with consideration given to values previously reported in the literature[57,99,192] as well as recommended islet masses for initial and repeat therapies.[193] Published studies document that pancreatectomized dogs respond favorably to porcine islets delivered using conventional encapsulation strategies (IP delivery of agarose-islet macrobeads), with animals becoming insulin-free for approximately 2 weeks following first injection and significantly longer following the second treatment. We anticipate similar results in spontaneously diabetic dogs, with Oligomer supporting longer durations of glycemic control, largely owing to its inherent biosignaling and immunomodulatory properties. Although challenges may arise on scale-up, especially related to identification of sufficient islet doses, the use of a subQ injectable format supports repeat dosing as needed on a patient-specific basis. Key outcome measures will include clinical signs, relevant urine and blood parameters, and owner- and veterinary-reported quality of life.[194]

4.1.2 Evaluate Oligomer for delivery of clinically-relevant, replenishable β cell sources.

Clinical islet transplantation is currently limited by donor scarcity and the need for multiple donors for sufficient β cell mass to achieve insulin independence. For novel β-cell replacement therapies to achieve their full clinical potential, they need to be compatible with plentiful sources of β cells that are safe, reliable, and cost-effective to procure. Xenogeneic porcine islet and human stem cell-derived beta (SC-β) cells have been identified as high-potential replenishable sources for

replacement β cells. At present, tremendous advancements have been made in the manufacturing and quality control of porcine islets and human SC-β cells to address this need. Our initial in-vitro studies will be conducted to evaluate and optimize Oligomer formulations for prolonged in-vitro maintenance of porcine islet and human SC-β cell viability, phenotype, and function, with comparisons to fresh cells and standard culture controls. Follow-up studies will evaluate Oligomer for delivery and protection of porcine islets and SC-β cells in autoimmune diabetic NOD mice, compared to islet only or SC-β cell only controls.

Given that rats, mice, and pigs have similar islet cytoarchitecture and β cell composition,[126] we expect islets from these species will exhibit similar responses to Oligomer encapsulation. As such, we anticipate that the Oligomer formulation currently used will be well suited for maximizing porcine islet viability, phenotype, and function in vitro or in vivo. On the other hand, it is anticipated that specific tuning of Oligomer will be necessary to achieve desired outcomes with SC-β cells, given that these individual β cells are derived from pluripotent progenitor populations and used to form islet-like clusters. Insights gleaned from embryonic development and β cell differentiation and regeneration will be applied when creating tuned Oligomer microenvironments optimized for SC-β macroencapsulation and delivery.

4.2 Conclusions

This thesis provides results that support preclinical validation of Oligomer encapsulation in vitro and in vivo. We showed the importance of Oligomer-islet interactions in maintaining islet morphology and function of in vitro encapsulated mouse islets. and syngeneic islets that encapsulated in situ following subcutaneous delivery. We showed that replacement of islets at a dose of 500 or 800 results in a rapid (within 24 hours) reversal of hyperglycemia in streptozotocin-induced diabetic mice. All animals that received syngeneic islets maintained euglycemia for beyond 90 days, while >80% of animals that received allogeneic or xenogeneic (rat) islets remained euglycemic for at least 50 days. Oligomer encapsulation of islets provides the necessary support to maintain islet health and function with extended glycemic control. Additionally, Oligomer acts as a protective barrier from host innate and adaptive immune responses through inherent immunomodulatory signaling of natural collagen. To our knowledge, this is the first report of an injectable subQ islet transplant strategy that yields rapid lowering and extended glycemic control without systemic immunosuppression.

Our encapsulation therapy, which is designed for islets or SC-β cells, is administered as a simple subcutaneous injection, which supports ease of administration, patient-specific dosing, and removal (if necessary). Successful completion of this work has the potential to yield a minimum-viable product, comprising a subcutaneous injectable Oligomer-islet transplant therapy that, through effective modulation of tissue integration, inflammation, and adaptive immunity, brings long-term insulin independence to veterinary and human T1D patients.

APPENDIX

CHAPTER 3 SUPPLEMENTAL INFORMATION

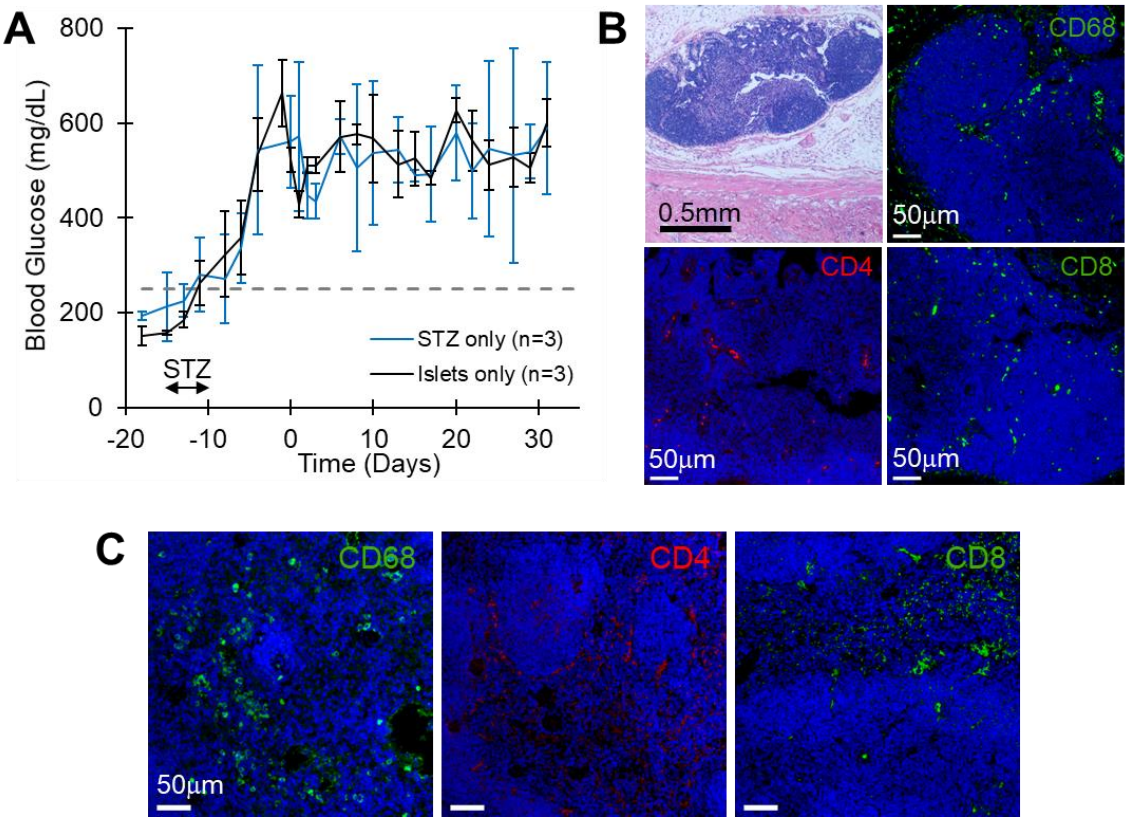

Figure S 1. Experimental controls.

A. Non-fasting blood glucose for control mice that were followed for 30 days after diabetes confirmation to document maintenance of the diabetic state (STZ only, n=3) and mice that received allogeneic islets only (n=3). A value of 250 mg/dL was applied as the diabetic threshold (dashed line). B. Histopathological analysis of explant following subcutaneous implantation of allogeneic islets only within diabetic mice. H&E stained cross-sections showed evidence of inflammatory-mediated destruction and necrosis of islets. CD68, CD4, and CD8 positive-stained cells verified the presence of immune cells. C. Representative images showing normal spleen sections used for positive staining controls for CD68, CD4, and CD8.

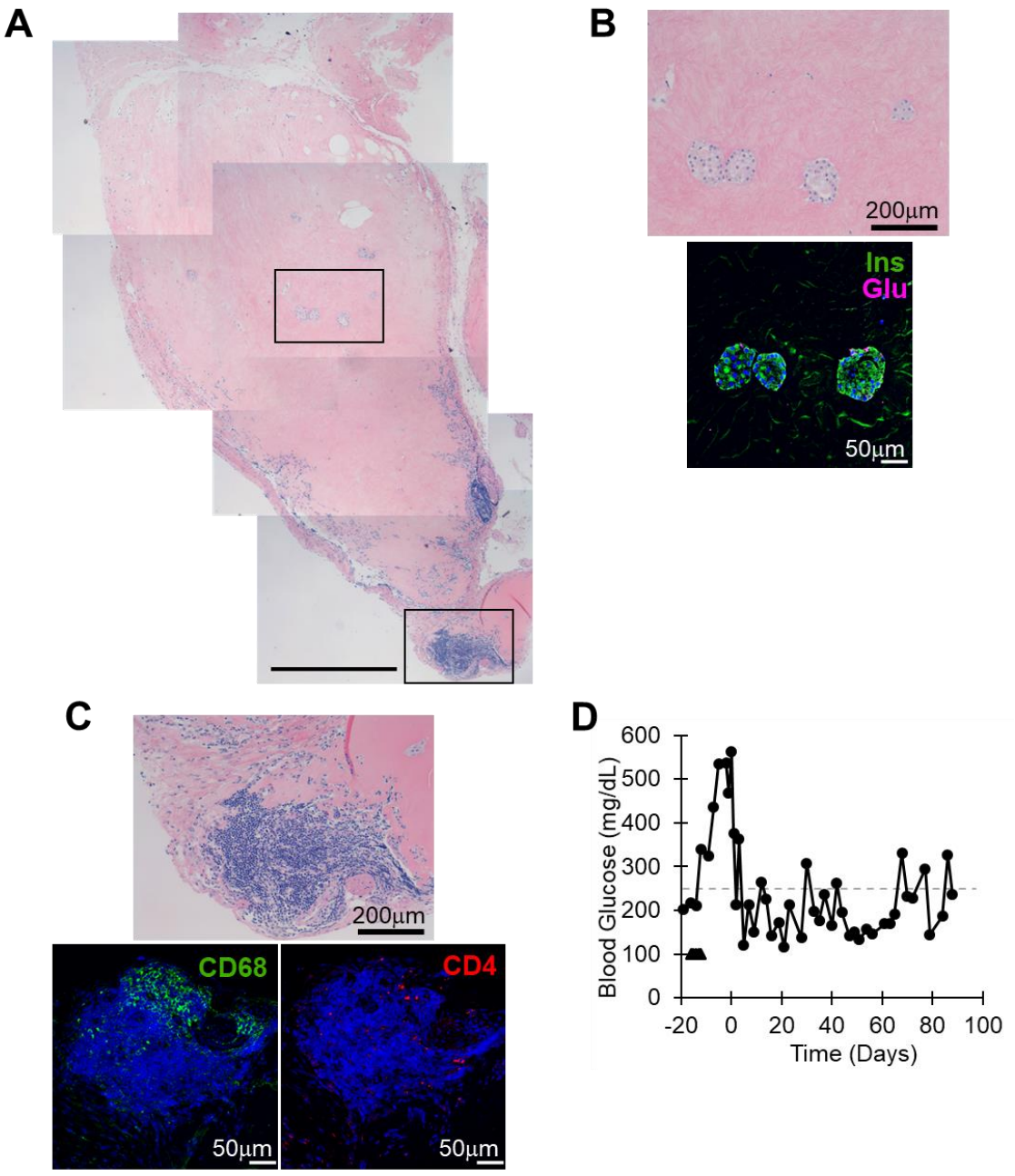

Figure S 2. The role of mixing in islet health and longevity.

H&E stained cross-section showing an overview (A) of an Oligomer-islet implant with regions of normal islets (B) stained positive for both insulin and glucagon. However, there was formation of a small granulomatous region (B) on the edge of the implant where islets may not have been completely encapsulated and were subjected to immune attack by CD68 macrophages and CD4 T cells. Non-fasting blood glucose (D) for this mouse showed maintenance of euglycemia for the entire 90-day study period. A value of 250 mg/dL was applied as the diabetic threshold (dashed line).

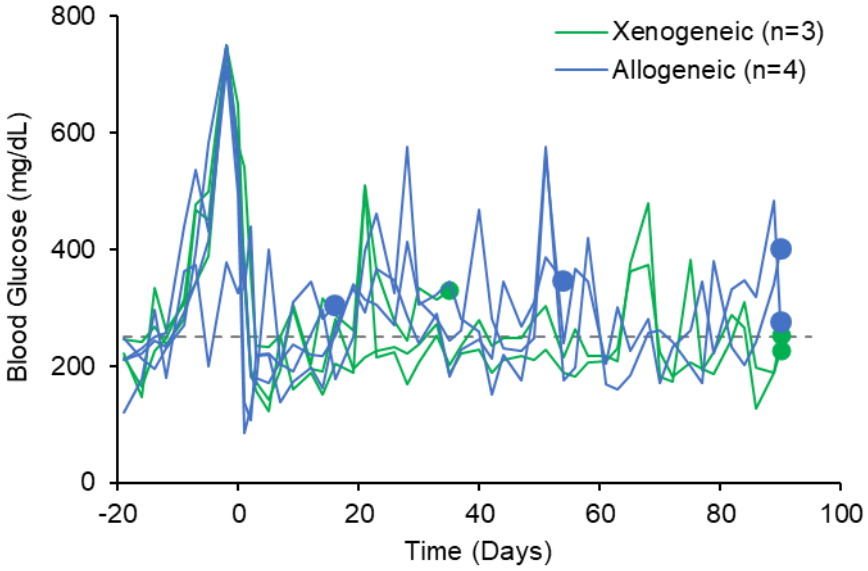

Figure S 3. Non-fasting blood glucose of preformed implants.

Non-fasting blood glucose values for mice receiving preformed Oligomer islet implants with allogeneic or xenogeneic islets. A value of 250 mg/dL was applied as the diabetic threshold (dashed line).

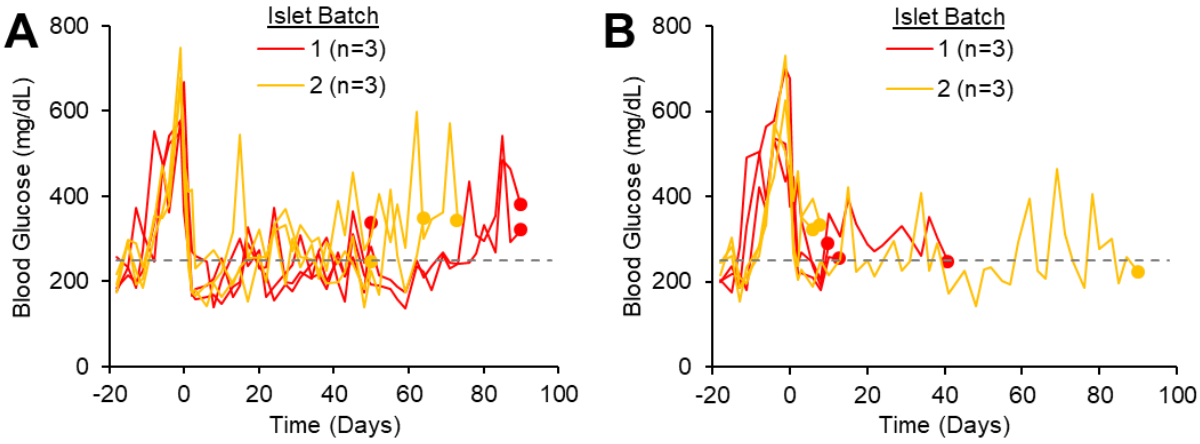

Figure S 4. Non-fasting blood glucose of implants in the intraperitoneal space.

Non-fasting blood glucose values for mice receiving injectable Oligomer-islet implants with xenogeneic (A) or allogeneic (B) islets into the intraperitoneal area. A value of 250 mg/dL was applied as the diabetic threshold (dashed line).

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