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CELLULAR ENCAPSULATION TECHNIQUES: CAMOUFLAGING ISLET CELLS FROM THE IMMUNE AND INFLAMMATORY RESPONSES ASSOCIATED WITH ISLET TRANSPLANTATION

By

Kristina Kateri Finn

A THESIS

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
the degree of Master of Science

Coral Gables, Florida

December 2008

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FINN, KRISTINA KATERI (M.S., Biomedical Engineering)
Cellular Encapsulation Techniques: Camouflaging Islet Cells from the Immune and Inflammatory Responses Associated with Islet Transplantation. (December 2008)

Abstract of a thesis at the University of Miami.

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Diabetes is a debilitating disease affecting millions of people worldwide. The transplantation of insulin-producing, pancreatic islet cells has been an extensively explored approach for the treatment of Type 1 Diabetes. However, the need for a multi-donor source, the strong host immune responses, and a life-long immunosuppressive therapy regimen limits the widespread applicability of islet transplantation. Encapsulation of islet cells within a semi-permeable biomaterial as a means to mask transplanted cells from the host has been shown to be a viable option for the protection of islets upon transplantation. Recent advancements, incorporating additional knowledge of biomaterials, have revitalized the field of islet encapsulation. This thesis work focused on both micro- and nano-scale encapsulation techniques. Initially, a novel, covalently linked alginate-poly(ethylene glycol) (PEG), termed XAlginate-PEG, microcapsule was evaluated, and was shown to exhibit superior stability over traditional ionically bound alginate microcapsules. The XAlginate-PEG capsules exhibited a 5-fold decrease in osmotic swelling than traditional alginate microcapsules, and remained completely intact upon chelation of ionic interactions. In addition, *in vitro* study of the novel polymer matrix showed high compatibility with mouse insulinoma cell lines, rat and human islets. Furthermore, no disruption in islet function was observed upon encapsulation. The

second study of this thesis work focused on the nano-scale encapsulation of islets with a single layer PEG coating. A PEG polymer was grafted directly on the collagen matrix of the islet capsule to form a stable amide bond. PEGylation of the islet cells was shown to camouflage inflammatory agents, such as tissue factor (TF), present on the surface of the islet, while maintaining islet morphology and function. In summary, PEG dampened coagulation cascade activation, and concealed activated factor X (afX) generation under pro-inflammatory culture conditions. The present findings contribute to the field of cellular encapsulation, both in the fabrication of novel encapsulation techniques and the evaluation of nano-scale coatings. The future potential of this research includes the attenuation of immune responses to transplanted cells, elimination of continuous immunosuppression, and provide flexibility in cell source. Furthermore, the platforms evaluated in this thesis are generalized for all cell types, thereby permitting translation of techniques to alternative cellular therapies.

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CHAPTER I

Introduction

Diabetes is a global disease with immense social and economic impact. Type 1 diabetes, characterized by the destruction of insulin-producing pancreatic β-cells [1], is currently treated with insulin therapy or in the case of severely diabetic patients, pancreatic transplantation. Clinical islet transplantation (CIT) has shown to be a promising, minimally invasive alternative to treat diabetes. Recent advancements made in immunosuppressive regimens over the last few years have improved transplant effectiveness, where recipients independently regulated blood glucose levels for several years [2-6]. However, the detrimental side effects of long-term immunosuppression, including both nephrotoxicity and hypertension [7], and the need for multiple donors to achieve insulin independence are major drawbacks to CIT. Furthermore, the use of multiple donors leads to patient sensitization which impairs their ability for future tissue transplants (islet, kidney or pancreas) [8]. Strong host inflammatory and immunological response to the donor tissue has also resulted in significant loss and impaired function of transplanted islets [9].

The highly inflammatory and immunogenic surface of the islet, due to the expression of highly inflammatory surface proteins and the presence of surface antigens, has lead researchers to attempt to mask this surface through the use of biomaterials, commonly referred to as encapsulation. Through the selection of highly biocompatible and biostable materials, encapsulation serves to mask cell surface proteins, as well as inflammatory extracellular matrix proteins, to minimize instigation of a detrimental host response. Encapsulation methods have been extensively developed and studied on all

scales, from macro, to micro, to nano, where each scale has its own advantages and disadvantages. Current research has shown the tremendous potential of encapsulation in minimizing negative host reactions, where even xenogenic transplantation, the transplantation of cells or tissue between species, is feasible. In this thesis, we have explored the potential of encapsulation both on the micro- and nano-scale as it relates to protecting islets.

CHAPTER II

Thesis Objectives

2.1 General Aims

Prior to the widespread clinical application of islet transplantation, the obstacles of multiple donor need, strong inflammatory and immune response, and life-long immunosuppression must be eliminated. In this thesis, we seek to minimize the detrimental host responses that lead to islet engraftment failure by encapsulating islets in novel biomaterials. The general aim of this thesis was to further cultivate methods of pancreatic islet cell encapsulation for their protection against the host's immune responses to transplantation. The design of effective strategies to encapsulate islets that are capable of providing protection from host attack could significantly improve transplant efficacy by reducing or completely eliminating the need for immunosuppressive therapy. Furthermore, the platforms evaluated in this thesis are generalized for all cell types, thereby permitting translation of techniques to alternative cellular therapies.

2.2 Specific Aims

2.2.1 Paper I: Covalently linked alginate-PEG microcapsules for immunoisolation of pancreatic islets upon transplantation

The specific aim of this study was to evaluate the potential of a novel, covalently linked alginate-PEG microcapsule for the encapsulation of islet cells. We sought to establish covalent linkages between highly biocompatible polymers to improve capsule stability and biocompatibility. The stability, viability, and function of cells encapsulated with the new polymeric material was investigated and compared to traditional ionically bound alginate capsules.

2.2.2 Paper II: Surface modification of pancreatic islets to attenuate the inflammatory response associated with islet transplantation

The specific aim of this study was to assess the ability of PEGylated islet cells to mask the islet surface, while not altering cell viability and function. The ability of PEG to attenuate inflammatory responses instigated by the islet surface was characterized under both pro-inflammatory and standard culture conditions. Results from these experiments will serve as controls for further studies exploring the benefits of multiple nano-scale layering for encapsulation.

Chapter III

Background and Significance

3.1 Diabetes Mellitus *and Islet Transplantation*

Diabetes mellitus is a disease in which the body is unable to maintain normal blood glucose levels with either a decreased production or improper action of insulin. Insulin is a hormone secreted by the pancreatic β cells when stimulated by glucose. Insulin is required for the cellular uptake of glucose, the body's main source of energy. There are two major types of diabetes – type 1 and type 2. In general, Type 1 diabetes is characterized by an autoimmune destruction of β cells resulting in an insufficient supply of insulin; Type 2 diabetes is associated with a decreased sensitivity to insulin resulting in an overproduction of insulin, and eventually requiring exogenous insulin therapy upon β-cell dysfunction.

Currently, diabetes affects 246 million people worldwide and is expected to affect 350 million by 2010 [10]. The social and economic impact has been shown to be extremely debilitating. Total annual economic cost of diabetes for 2007 was an estimated $174 billion, with the medical costs accounting for $116 billion: $27 billion for the direct treatment of diabetes, $58 billion to treat diabetes-related chronic complications, and $31 billion in excess general medical costs [11]. The major complications associated with diabetes, and more often than not the cause of death in diabetic patients, include cardiovascular disease, retinopathy, neuropathy, atherosclerosis and nephropathy [12-14]. Diabetes was expected to cause 3.8 million deaths worldwide in 2007, about 6% of total global mortality [15].

Type 1 Diabetes Mellitus (T1DM) or Insulin Dependent Diabetes Mellitus (IDDM) is considered the most severe classification of diabetes, commonly occurring prior to adulthood. It is postulated that T1DM can be instigated by either genetic or environmental factors, such as infections, although this hypothesis has not been proven [16-18]. This organ-specific, autoimmune disorder results in the targeted destruction of insulin-producing β cells located in the islets of Langerhans in the pancreas, thereby eliminating the body's ability to generate insulin [19].

Insulin functions to signal target cells to increase the uptake and storage of glucose molecules. Furthermore, after the stomach converts sugar, starches and other food to glucose and the glucose enters the bloodstream, properly functioning β cells are needed to supply the appropriate amount of insulin required to transfer glucose into cells. The absence of insulin results in a decreased cellular uptake of glucose, amino acid and potassium absorption, as well as other crucial metabolic functions.

Current treatment of T1DM includes exogenous insulin therapy and organ or cell transplantation. Insulin therapy requires life-long exogenous insulin administration via injection or a catheter-pump system. It has been well demonstrated that maintaining normal blood glucose levels in patients with T1DM dramatically increases the chance of living a complication free life [9]. Recent advancements in pump technologies, including improved insulin formulations, have enabled diabetics to more closely regulate their blood glucose levels; however, there are still some inconsistencies with exogenous insulin administration when compared to the finely tuned glycemic control of a properly functioning pancreas. Unstable blood glucose fluctuations associated with this therapy typically leads to the secondary complications previously mentioned [12-14].

Alternatives to insulin therapy regimens require surgery and involve either a total pancreas transplant or the transplantation of isolated islet cells, known as clinical islet transplantation (CIT). Whole gland replacement has been shown to establish a state of normoglycemia at a success rate of 80% at one year [20]; however, total replacement is not a viable option for most patients with T1DM due to the high risk for surgical complications and the need for a lifelong immunosuppressive therapy regimen [20].

Islet transplantation has been shown to be as effective as pancreas transplantation with a success rate of 82% at one year, while also being a much less invasive alternative [21]. In CIT, islets are obtained from the pancreas of deceased organ donors, or allotransplantation. Since the islet cells constitute approximately 1% of the entire pancreas, the isolation process aims at enriching the endocrine component, while reducing the exocrine pancreatic tissue. A pancreas is digested using collagenase to breakdown connective tissue, allowing for the collection of islets with preserved structural integrity and potency [9]. The islets are then purified and cultured prior to transplantation into the liver via the portal vein, for eventual engraftment into the well perfused liver sinuses of the recipient [9]. The liver is the most commonly used site for islet infusion in the clinical setting. Other engraftment sites such as the spleen, the kidney sub-capsular space, the omentum, the peritoneal cavity, and sub-cutaneous space have been tested in experimental models. Islets are not transplanted back into the pancreas due to safety issues associated with manipulation of this organ, such as severe pancreatitis, even though experimental reports seem to indicate this may be possible to pursue [22].

Numerous advances in islet transplantation have been recorded in recent years. A notable study published by Shiapiro *et al,* in June 2000, demonstrated a successful trial in

which seven out of seven diabetic patients treated with islet transplantation were free from exogenous insulin therapy for 1 year [23]; subsequent clinical trials have confirmed and extended these observations reporting graft function for over five years, although only in a small number of patients. Widespread clinical application is hindered by the need for two to four donor pancreases for each recipient, inadequate long term graft function, and also the requirement of lifelong immunosuppression [9, 24]. The need for a large primary cell source is not supported by the number of donors currently available for transplant, thus limiting the number of transplants that can be performed each year. The use of lifelong immunosuppressive drugs is detrimental to the treatment of diabetes because of their cytotoxicity to islet cells and other possible side effects [25]. Therefore, islet transplantation is generally restricted to patients experiencing severe and recurrent episodes of hypoglycemia [8].

The venues explored to resolve these issues include *in vitro* modification for decreasing tissue immunogenicity, application of encapsulation techniques for immunoisolation, and the exploitation of multiple tissue sources including xenogenic islets and stem cell derived insulin-producing cells [9, 26, 27]. The focus of this thesis was to utilize encapsulation techniques to decrease immunogenicity and minimize inflammatory reaction to transplanted islets, while maintaining cell viability and function.

3.2 IBMIR, Immune response and Sensitization

3.2.1 Instant blood-mediated inflammatory reaction (IBMIR)

The thrombotic reaction generated by the direct contact of islets with ABO-compatible blood was termed by Bennet *et al* as an *instant blood-mediated inflammatory reaction* (IBMIR) [28]. This reaction involves the co-activation of the coagulation and

complement systems as well as the rapid adhesion of activated platelets to the surface of the islet. Furthermore, as islets are transplanted into the bloodstream, leukocytes, including both monocytes and granulocytes, infiltrate the islets resulting in a disruption of islet integrity, and islet loss [28]. Additionally, the degree of tissue factor (TF), a known inflammatory surface protein, expressed by islet cells has been correlated to the intensity of the IBMIR [29]. IBMIR has been shown to occur during CIT in humans as well as in porcine and non-human primate transplantation models [28, 30]. It has been estimated that IBMIR may account for a loss up to 50% of islets within the first day of transplantation [31].

Tissue factor (TF), a transmembrane protein present in subendothelial tissue, platelets, and leukocytes, is the primary instigator of the coagulation cascade. TF operates as a receptor for the cleavage of factor VII to factor VIIa (fVIIa) [32, 33]. TF in combination with fVIIa represents the extrinsic pathway of coagulation in which an inactive protease factor X is converted into the active protease factor Xa [32, 33]. The Xase complex results in a combination of fXa with factor Va, which initiates the prothrombinase complex. The prothrombinase complex is responsible for the rapid conversion of prothrombin to thrombin, see Figure 3-1. In addition, the presence of thrombin activates cofactors V and VIII which form a positive feedback system to continuously generate thrombin [34]. Increased thrombin production leads to the unrestrained formation of a fibrin clot.

TF expression can be induced in cells, such as endocrine cells, when in contact with blood. The cell interaction with various immune complexes, cytokines, or lipopolysaccharides (LPS), present in blood, results in TF production [30]. Cytokines

such as monocyte-derived cytokines interleukin (IL-1β) and tumor necrosis factor α (TNF- α) have been shown to induce TF production in endothelial cells [35-37]. Therefore, cells exposed to stress and/or cytokine storms typically express increased levels of TF on the cell surface.

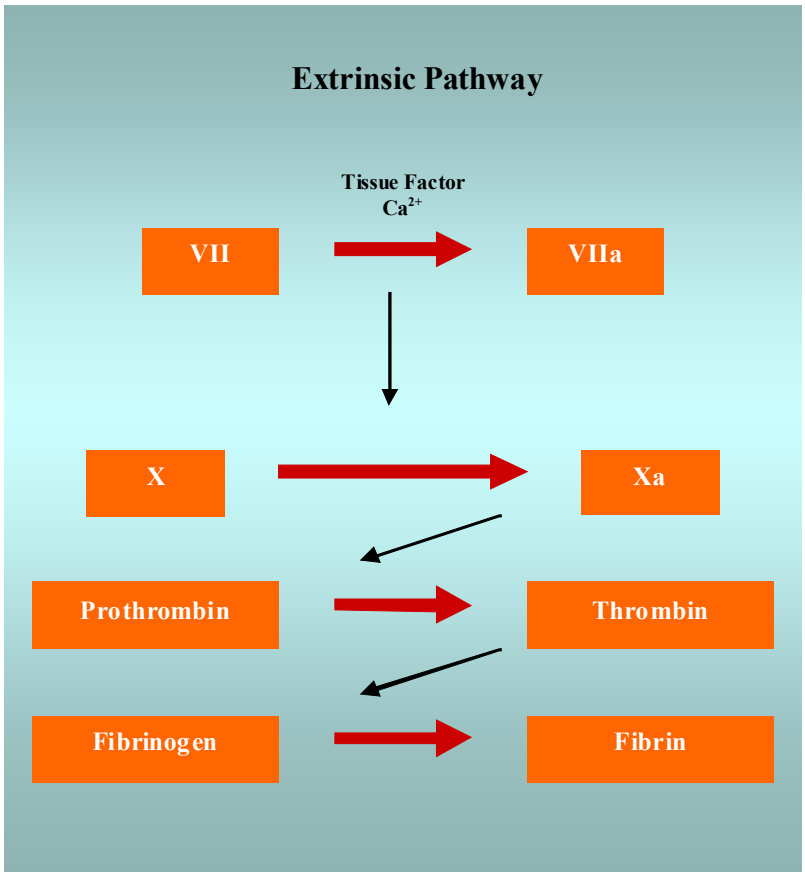


Figure 3-1. Extrinsic Pathway of the Coagulation Cascade. Tissue factor (TF) is a key factor for the initiation of blood coagulation.

A regulator of blood coagulation is the membrane glycoprotein, thrombomodulin (TM). TM binds to thrombin to form a TM-thrombin anticoagulant complex. This complex activates circulating protein C (PC). PC is a vitamin K dependent precursor of serine protease, and activated Protein C (aPC) inactivates the cofactors Va and VIIIa involved in thrombin generation [38-40]. Thus, a negative feedback system, instigated by aPC, is used to regulate thrombin generation.

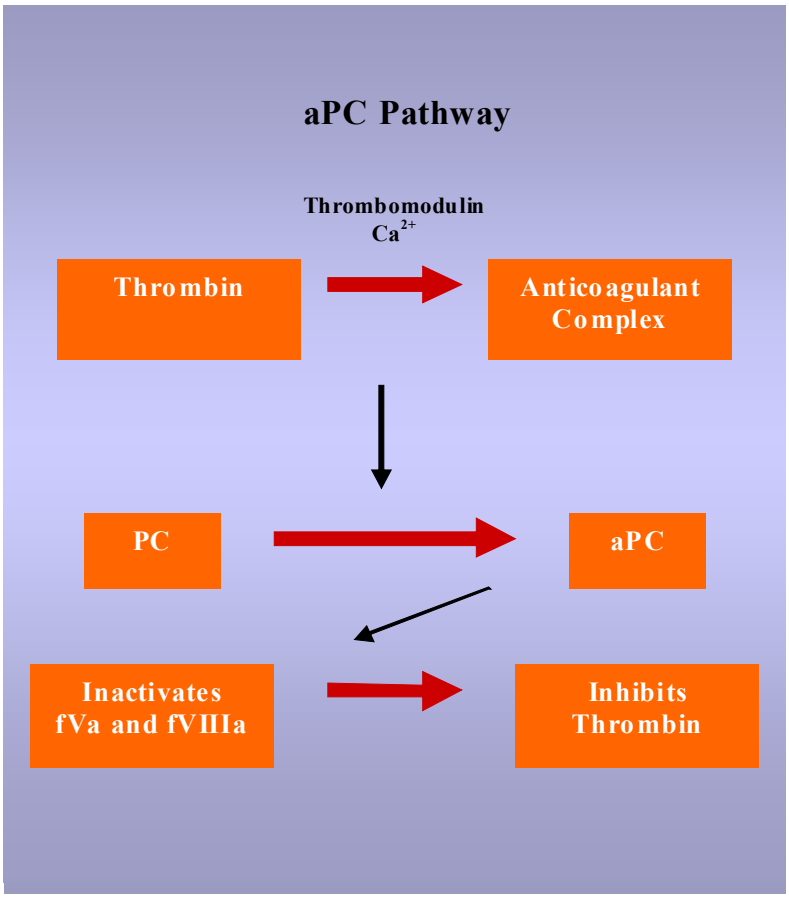


Figure 3-2. APC Pathway of the Anti-coagulation Cascade. Thrombomodulin (TM) is a key factor for the attenuation of blood coagulation.

The balance between TM and TF is a natural mechanism many cells, such as endothelial cells, utilize to alternate between an inflammatory and anti-inflammatory surface. Typically, TF and TM are not expressed by endothelial cells under normal conditions; thus, they are relatively inactive in instigating coagulation [41]. However, the balance is shifted to thrombogenic, with a downregulation of TM and upregulation of TF, when exposed to inflammatory cytokines such as TNF-α, IL-1β or endotoxin [42-45]. The evaluation of TM and TF expression allows for an understanding of the regulatory role of cells in thrombin generation.

3.2.2 Immune Response

When allogenic islets are transplanted into the recipient, the islet allo-antigens instigate an antigen presentation pathway to the host immune cells. The *direct pathway* of T cell activation employs the major histocompatibility complex (MHC), in which antigens are presented to host immune cells by donor antigen producing cells (APCs) [9]. MHC molecules are present on all cells of the body (class I), but only the MHC molecules present on the surface of certain immune cells (class II), including macrophages and immune cells, elicit a host immune response due to the co-stimulation of T cell requirement to be fully active [46]. The *indirect pathway* involves the recipient APCs, where the donor antigens shed from the islet surface are collected, degraded and presented by the host's APCs [9].

Immunosuppressive therapy is currently used to minimize the immunological response instigated by allogenic islet transplantation. These drugs weaken the immune system to prevent graft rejection. Several trials of allogenic islet transplantation have shown a reproducible reversal of diabetes [23, 47, 48]; however, detrimental secondary complications including nephrotoxicity, neurotoxicity, gastrointestinal intolerance and hypertension have resulted in continuous administration of immunosuppressive medications [7]. In addition, drug-induced β-cell toxicity is also a concern of a long-term immunosuppressive regimen application [20].

3.2.3 Sensitization

For islet transplantation to achieve insulin independence multiple donors are typically required [9]. The islets used for a particular patient is based on ABO blood compatibility and the number of islet equivalents (IEQs) required for the patient's weight.

The limited donor source prevents distribution based on human leukocyte antigen (HLA) matching. Therefore, recipients of islet transplants may have multiple HLA mismatches, which can lead to the development of antibodies against both donor and nondonor HLA antigens [49]. In addition, the amount of antibodies formed have been shown to increase with a complete discontinuation of immunosuppressive therapy [49]. The broad sensitization of a patient has a negative impact on their ability to undergo future islet, pancreas or kidney transplants, given that it can significantly limit the pool of matched organs for the donor [49].

3.3 Encapsulation

The immunoisolation of transplanted islets is an attractive option for minimizing the host immune destruction of transplanted tissue. This involves encapsulating islets within a semi-permeable polymer biomaterial that allows for small molecule diffusion, such as glucose or insulin, while blocking larger molecules, such as immune cells and antibodies. Thus, islet rejection is prevented by blocking immune cell recognition and damage to the transplanted islets.

The encapsulated systems investigated include intravascular and extravascular macrocapsules, microcapsules and nanocapsules. *Intravascular macrocapsules* involve loading numerous islets within a hollow fibered perifusion chamber in direct contact with blood. The close contact with blood provides efficient nutrient supply to encapsulated islets. These devices have been shown to reverse diabetes in multiple animal models including rats, dogs and monkeys [50, 51]. However, this method requires a strong anticoagulant drug therapy for the prevention of a fatal thrombus generation upon the direct contact of a foreign material with blood. The high risk of an embolism shifted to

the focus to *extravascular macroencapsulation* involving either a disk or tubular biomaterial membrane for islet encapsulation. These devices can be transplanted into the peritoneal cavity, subcutaneous tissue, or under the kidney capsule. Issues associated to these devices include biocompatibility problems as well as insufficient nutrient supply to the encapsulated islets [9]. The most explored immunoisolation approach is *microencapsulation*, in which a few islets are encapsulated within a biomaterial and implanted extravascularly. Microcapsules exhibit superior mass transport properties over conventional macro-devices with a higher surface area per unit volume ratio. However, large void volumes by standard capsules, commonly 400-800μm in diameter, impose significant transport limitations of nutrients, particularly with the supply of oxygen. *Nanoencapsulation* incorporates the direct engraftment of a polymer onto the islet surface to form a molecular immunoprotective coating. The reduced barrier diffusional distance to 20-70μm dramatically improves nutrient supply to the islets as well as ease with intraportal delivery, and has shown promising results in both small and large animal models [52-57]. Nevertheless, the unfounded long-term stability of polymeric coatings limits their widespread applicability.

3.3.1 Microencapsulation

Microencapsulation of cells has been an extensively explored approach to immunoisolate transplanted tissue from the host immune system. The idea behind this approach is to protect foreign cells from the host immune system using an artificial, biocompatible biomaterial. The typical process of microencapsulation involves isolating cells, suspending them within a matrix, such as an alginate solution, and dripping the suspension into a gelation solution, such as divalent cation solution for alginate, to form

porous hydrogel microcapsules encasing the islets. This technique has many potential therapeutic applications including the treatment of chronic pain, Parkinson's disease and diabetes.

Many groups have studied the use of hydrogels for the encapsulation of cells over the past two decades. The biocompatibility of hydrogel membranes has made them a particularly attractive option in the use of microcapsules. The hydrophilic nature of the material reduces the protein absorption and cell adhesion with the absence of interfacial tension between the material and surrounding fluids [58]. Additionally, the pliable nature of hydrogels minimizes any abrasiveness to surrounding tissue [59, 60]. The most frequently used hydrogel materials for cellular encapsulation are alginate [61] and agarose [62].

Alginate has been the most widely used material for microcapsule formation. Alginate is a collective term of a family of polysaccharides primarily derived from brown algae [63]. Alginate molecules are linear binary copolymers of β-D-mannuronic (M) and α-L-guluronic (G) acids with a variation of sequential arrangement and composition depending on the source from which they were isolated. Alginates with a high-G (more than 60% G) composition have been shown to form highly permeable, stable gels when compared to high-M (less than 40% G) content materials [64]. Furthermore, gels of a high-G content maintain improved stability with less swelling and shrinking in ionic solutions [65]. On the other hand, when using a polycation outer coating, such as poly- L-lysine (PLL), the intermediate-G or low-G alginates have been shown to be more stable than high-G alginates [66, 67]. In addition, high-M alginates create less porous beads

with increased biodegradability, although an increased presence of swelling and shrinking is observed during ionic crosslinking [65].

A divalent cation solution, typically containing calcium or barium, is used to form rigid, biocompatible beads from alginate droplets. An ionic cross-link is formed between the carboxylic acid group (i.e. guluronic acids), found on the polymer backbone, and the cation [68]. The gel strength depends on a number of factors including polymer purity, polymer molecular weight, cross-linking ion and ion concentration, and gelation time [64]. Barium, in particular, has been shown to form stronger cross links with alginate, in turn producing stronger gels when compared to calcium [69]. For instance, Calcium-alginate beads were coated with a polycation layer to increase capsule stability in physiological solutions. While successful transplants have been shown with Barium-alginate capsules in the absence of a polycation layer [70]. The type of alginate is also crucial in the effects of the cross-linking cation. For example, stronger gel formation with barium was observed with high-G content alginates and not with high-M alginates [71]. However, most alginate gels, in the absence of additional polycation coatings, are not stable long-term due to the slow leakage of the cations out of the capsule over time [72, 73]. Thus, the alginate composition, polycation outer layering, and divalent cation solution all play a role in tailoring a capsule for a specific application. In addition, the incorporation of covalent linkages within microcapsules has also been explored (see Covalent Crosslinking section for details).

A polycation coating, most commonly PLL, originally applied to provide an immunoprotective outer membrane, has been shown to improve capsule stability [74]. Alginate capsules coated with a polycation layer exhibit reduced swelling and enhanced

long term stability. However, a major drawback of PLL is its possible toxicity to cells, activation of macrophages and complement systems, and induction fibrosis [75-77]. Therefore, while the addition of polycation membranes improves the stability and immunoprotective properties of standard alginate capsules, the use these capsules *in vivo* tend to lead to an accelerated fibrotic response.

Microencapsulated islets have shown much success *in vivo* with an improved graft survival and function over unencapsulated islets. Alginate-based capsules for treatment of diabetes *in vivo* were originally described by Lim and Sun in 1980. This study involved transplanting microencapsulated islets into streptozotocin-induced diabetic rats. The study exhibited a complete correction of the condition for up to 3 weeks, while the morphology and functionality of the cells was maintained for over 15 weeks [61]. Since then numerous studies have showed promising results including the transplantation of PLL-alginate microcapsules exhibiting long-term success in both diabetic dogs [47] and some promise in human patients [48]. Furthermore, Sun et al. showed induced normoglycemia without immunosuppression up to 800 days with the transplantation of encapsulated porcine islets into spontaneously diabetic monkeys [78]. Another report exhibited graft survival up to 7 months of human and rat xenografts transplanted into immunocompetent mice [79]. In addition, Soon-Shiong et al. showed a 9 month graft survival of microencapsulated islets from which established complete normoglycemia of human patients [48].

Currently, two clinical trials involving the transplantation of microencapsulated islets are in progress. A group in New Zealand, called Living Cell Technologies (LCT), is undergoing Phase I/IIA clinical trials in Russia with the xenogenic transplant of alginate

microencapsulated porcine islets with no long-term immunosuppression. Another company, AmCyte, in Santa Monica, CA is performing clinical trials microencapsulated allogenic human islets without long-term immunosuppression. This trial is underway at the Toronto General Hospital (TGH) in Toronto, Canada.

While tremendous success has been shown for islet microencapsulation in small animal models, the translation of these promising results to larger animal models has been limited. The scalability of this design has been a significant hindrance to clinical applicability. Specifically, the volume of the transplanted tissue can increase by as much as 350-fold when transplanting to larger rather than smaller animal models. Another issue surrounding the microencapsulation of islets is the large void volume, for nutrients, particularly oxygen, to travel to reach the islet cells. While much improved over macrocapsules, the typical capsule diameter ranging from 400-800 μm continues to exhibit mass transport limitations [80]. In comparison, the oxygen demand of islets is 10^6 fold higher than chondrocytes and 10^4 fold higher than endothelial cells [81-83]. Additionally, mathematical models have shown oxygen concentrations to decrease radially in cylindrical and spherical devices from islet consumption, with high concentrations on the perimeter [80]. Thus, if oxygen levels are low at the transplant site the cell densities must be reduced as device diameter increases to minimize hypoxia to cells in the center.

Microencapsulation has shown potential for the protection of transplanted islets from the host immune system with a semi-permeable alginate membrane. In addition, microencapsulation cultivates hope for the use of xenogenic and/or nonhuman tissue sources for insulin-producing cells for the treatment of patients with T1DM. Prior to

wide spread application, however, several problems need to be addressed such as biocompatibility, long-term stability, inflammatory responses, indirect immune recognition via indirect antigen pathways, and inadequate oxygen and nutrient transport. This thesis work aims to address the long-term stability and biocompatibility issues by converting traditional alginate ionic bonds to covalent linkages in a highly cell-compatible manner, as well as utilizing only highly biocompatible polymers within the microcapsule.

3.3.2 Nano-scale encapsulation

Nanoencapsulation has been investigated as a means to mask immunological response with barrier fabrication, while avoiding the transport limitations created by standard microcapsules. This technique involves grafting a polymeric material directly onto the cellular surface. Several approaches have been effective in the fabrication of nanoscale layers onto spherical surfaces, including: PEGylation, layer-by-layer self assembly via polyionic or lipid layers, and covalent conjugation, see Figure 3-2. PEGylation is characterized by modifying a surface with a conformal, covalently linked PEG coating. On the other hand, when looking at multilayer coatings, the simplest method of fabricating multiple nano-scale layers is the self-assembly technique, either through polyelectrolyte or membrane-mimetic self assembly. Polyelectrolyte layer-by-layer (PE-LbL) self assembly is based on the principle of electrostatic attraction between opposite charges, initiated by alternating adsorption of anionic and cationic polyelectrolytes [84-86]. An additional method of self-assembly is the coating of lipid

membranes, or membrane-mimetic thin films, onto surfaces. Lastly, covalent conjugation is another alternative for developing multi-layer conformal coatings, and this method offers the ability to crosslink upon each layer fabrication.

Polymers typically used for the fabrication of nano-scale layers are not applicable for direct coating of cell surfaces, due to the use of cytotoxic polymers and solvents. Therefore, a focus of nanoencapsulation techniques for cellular coating is the translation of techniques from a benign surface to application on a viable cell surface. Several researchers have developed techniques for fabricating conformal, polymeric layers directly on to the islet surface. These applications will be discussed further in subsequent sections.

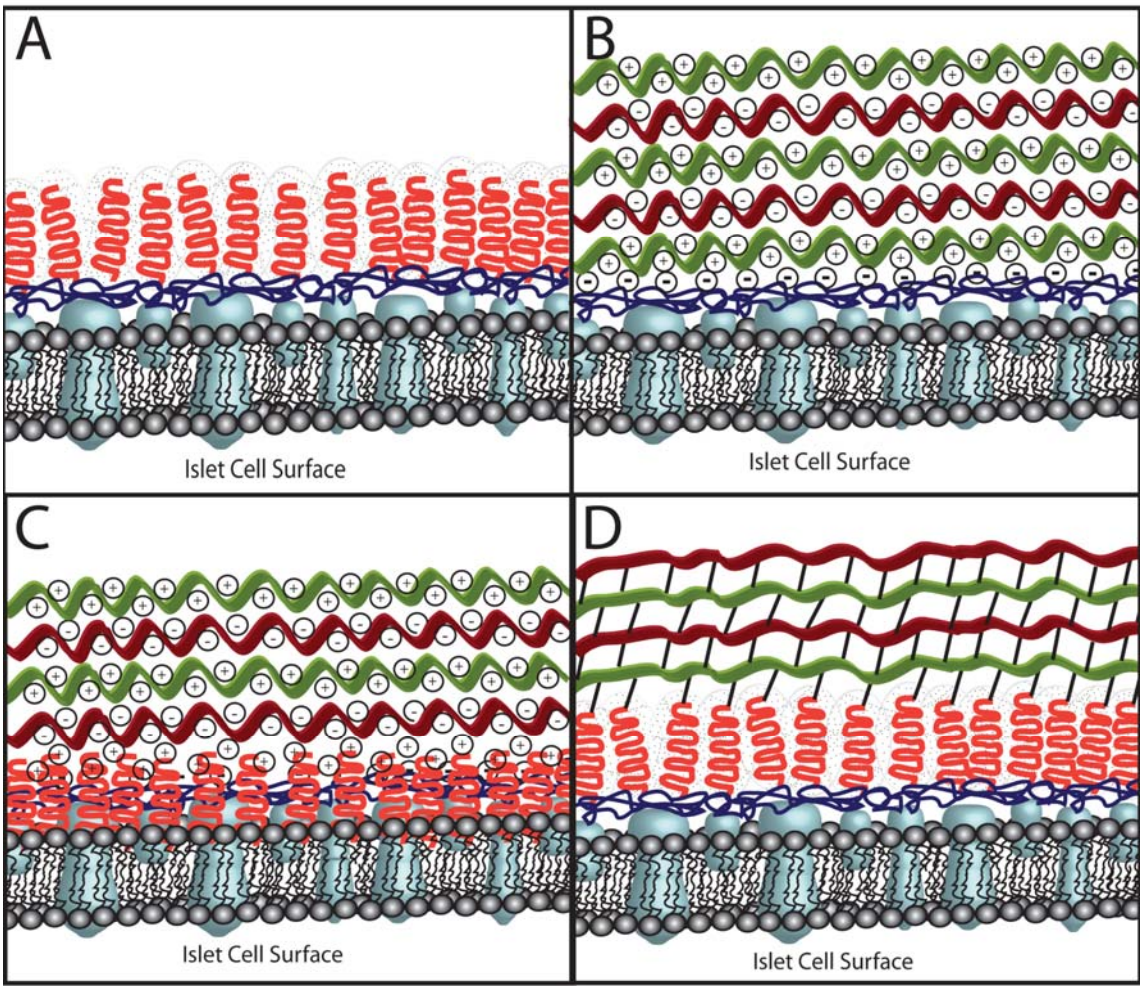


Figure 3-3. Overview of methods used to create nano-layers on cell surfaces: A. PEGylation; B. polyelectrolyte layer-by-layer self assembly; C. lipid based and polyelectrolyte layer-by-layer self assembly; and D. covalent crosslinking.

3.3.2.1 PEGylation

The most widely applied method for nano-scale coating on islets has been the PEGylation of the islet surface through the use of high molecular weight poly(ethylene glycol), PEG, polymers. PEGylation, a term used to describe the chemical conjugation of a PEG polymer to a surface, is commonly used to disguise foreign materials, such as implanted devices, from the host. PEG polymers have been shown to be stable, biocompatible, non-adhesive, and highly soluble molecules within biological

environments [87, 88]. The unique characteristics of PEG include its minimal interfacial free energy with water, high surface mobility, and steric barrier capabilities [89, 90]. The pharmaceutical industry has a well established history of utilizing PEG polymers to increase drug efficacy. For instance, PEG conjugated proteins have been shown to increase protein stability [91, 92], solubility [90, 93-95], and in vivo circulating half-life [96-98]. Additionally, PEG attachment has been shown to protect against enzymatic degradation [93, 99, 100], as well as to reduce the immunogenicity [93-95, 101-103], antigenicity [88, 93, 94, 101] and toxicity of the protein [104]. Protein modification with a PEG of a higher molecular weight and branched structure has also been shown to decrease immunogenecity and antigenicity of proteins as compared to a lower molecular weight and a linear polymer chain [105].

The basic chemical structure of PEG is HO-(CH_2CH_2O)n-CH_2CH_2OH, with functional hydroxyl groups present on both ends of the polymer. In order to tether the PEG to the cell and/or extracellular matrix surface, the hydroxyl group at the terminal end of the PEG is covalently linked to a chemical linker (for example: cyanuric chloride, succinimidyl propionate, benzotriazolylcarbonate, or tresylchloride) capable of covalently binding to functional groups common to the cell or extracellular matrix surface, such as amines. The most common tethering group is succinimidyl propionate, which binds to lysine residues, commonly found on both transmembrane proteins on the cell surface and on many extracellular proteins, such as collagen and elastin [106]. The conjugation of succinimidyl propionate functionalized PEG to free amine groups has no detrimental side effects and forms a generally stable amide bond [107, 108]. Due to

potential reactivity with the PEG polymer, the second terminal hydroxyl group (i.e. the one exposed following linkage to the cell surface) is typically replaced by a methyl group, creating a methoxypoly(ethylene glycol) (mPEG) [106].

The pliable nature of PEG allows for synthesis of numerous forms including a wide range of molecular weights, in addition to the fabrication of single chain and multiple branched polymers. A surrounding hydration zone formulated by the hydrophilic PEG polymer attachment generates a steric hindrance. The zone of hydration has been shown to mask the antigenic factors associated with an antibody-mediated immune activation, but it depends largely on the molecular weight and shape of the polymer [109-111]. Scott et al. demonstrated linear, higher molecular weighted (i.e. greater than 20 kDa) PEG chains provide a larger hydration zone with greater immune protection by more effectively masking antigenic sites. Moreover, branched, highly dense PEG polymer chains provided the most efficient immune protection when compared to linear polymer chains [109-111]. Cell surface modification with PEG polymers has also been proven to inhibit the binding of immunoglobulins and white blood cells (WBC) [109-111]. In addition, PEG attachment has been shown to prohibit the cell-cell interactions necessary for T cell activation, as well as altering cell surface charge [112].

Initial work on cell and tissue PEGylation was focused on the reduction of immune activation by masking the surface. While multiple cell types have been evaluated, initially this technique was applied to red blood cells (RBC) to conceal major and minor surface antigens from host antibodies, with the aim to develop a universal donor RBC as a source for blood transfusions [109-111]. PEG-cyanuric chloride conjugation to RBCs resulted in a significant reduction in agglutination, antigenic

recognition, antibody binding, phagocytosis and immune cell activation [109-111, 113]. Additionally, PEG modification was shown to inhibit human monocyte recognition of both allogeneic and xenogeneic RBC transplants [109-111]. Immune cell PEGylation has also been an area of interest for many years, where T cell activation and proliferation was inhibited following PEGylation of human mononuclear blood cells [110]. In this study, they determined that the polymer coating concealed the CD28-CD80 co-stimulatory pathway [110], a pathway required for T cell activation and proliferation [114-118]. Additionally, a total loss of immunogenicity has also been exhibited with PEG altered, foreign immune cells [119].

Application of PEGylation techniques to the pancreatic islet has emerged in recent reports. Given the tendency of islets to develop free-amine rich extracellular matrix proteins on their surface in culture, the application of succinimidyl propionate functionalized PEG for masking the islet surface commonly results in a high degree of conjugation. Early reports indicated that poly(ethylene) glycol could indeed be tethered in a uniform manner on the islet surface [106, 120, 121]. Extensive research published by Lee et al established optimal parameters for the PEGylation of rat islets such as molecular weight, incubation time, and culture time [120]. In this study, they determined the optimal parameters to be a 1hr incubation time with a 5 kDa PEG. Using this PEG polymer, the integrity of the PEG coating was maintained up to 28 days in culture.

Complete surface engraftment of PEG molecules, via stable amide bonds, on the collagen matrix of pancreatic islets has been shown extensively in vitro to not inhibit cell viability or function [120-122]. For instance, Panza et al. incubated rat islets with a PEG-isocyanate (MW 5000) for 1hr, and a mitochondrial activity assessment (MTT assay)

demonstrated no significant change in viability on post-treatment culture day 1 and 5, and a slight increase in viability on day 2. Islet function was also not significantly altered when assessed by a static glucose challenge. Similarly, Lee et al. incubated rat islets with a 5 kDa mPEG-Succinimidyl propionic acid (mPEG-SPA) for 1hr with no observed change in viability or function [120]. Xie et al. conjugated porcine islets with two PEG derivatives, including monosuccinimidyl PEG (MSPEG, 2 kDa & 5 kDa) and disuccinimidyl PEG (DSPEG, 3 & 6 kDa), for immunoprotection upon xenotransplantation, where treatment with any of the four PEG types did not induce any significant changes in cell viability or function [122].

The ability of PEG to mask surface recognition and minimize immune cells activation for islets was evaluated by co-culturing islets and other cell types, including splenocytes (i.e., lymphocytes and macrophages). Lee et al. assessed immunogenecity of mPEG-SPA (MW 5000) conjugated rat islets by co-culturing them with splenocytes for 7 days, and this study demonstrated an absence of splenocyte activation and attachment [107, 123]; however, when the PEGylated islets were cultured with rat recombinant TNF-α for 7 days, an effort to represent an immune cell cytokine (i.e., TNF-α, IL-1 and IFN-γ) release, cell viability was negatively affected. On a positive note, the damage was shown to be dampened by polymer attachment when comparing the unmodified and PEGylated islets [107, 123].

Studies focused on PEGylated islet assessment in vivo have produced only moderate findings. Chen and Scott demonstrated the ability of mPEG engrafted islets to maintain normoglycaemia in a rat allograft model for approximately 9 days [106]. Similarly, Lee et al. assessed in vivo rejection for PEG-SPA coated rat islets in allogeneic

rejection models, where the average allograft survival rate for unmodified and PEGylated islets was 5 and 14 days, respectively [108]. The infiltration of immune cells was shown to be blocked by PEG surface polymers, but an indirect immune response to the cytokine release of the transplanted islets resulted in a congregation of immune cells surrounding the islet capsule along with structural and functional damage to the islet [108]. The xeno-transplant study performed by Xie et al., using PEGylated porcine islets engraftment into immunocompetent diabetic mice, found moderate increases in survival time, where PEGylated islet grafts exhibited positive function for approximately 15 days [122]. These studies demonstrate that PEGylation of islets alone cannot fully protect the islets from immune attack in vivo. Promising findings were recently recorded, however, in a study involving multiple and amplified islet PEGylation. The aim of this study was to reduce immunogenicity by either increasing the amount of PEG present on the islet surface or to intensify PEG presence with the aid of a cationic polymer. The islet allografts in 3 of the 7 diabetic recipients in the multiple PEGylation group survived for 100 days, while still functioning normally [108, 124].

Given the ability of PEGylated cells to assist in dampening immune cell infiltration, the combination of PEGylated islet with additional variables into the system, such as genetic modification or immunosuppressive drugs, may significantly increase functional engraftment time. In one study, the synergistic effect of surface modification with PEG derivatives and genetic modification with Bcl-2, an antiapoptotic gene, demonstrated a significant reduction in islet loss post-transplantation [125]. Another study examining the systemic overexpression of heme oxygenase-1 (HO-1), a cytoprotective agent, in combination with cell surface PEGylation also found positive

effects. The induction of HO-1 prevented the early stage, non-specific inflammatory response initiated by islet transplantation [108]. Furthermore, a combination of HO-1 induction and a low dose immunosuppressive drug [1 mg/kg/day cyclosporine A (CsA)] resulted in an engraftment survival time of more than 100 days [108]. Long-term islet engraftment has been established with a combination of surface PEGylation and a low dose of immunosuppressive drugs, particularly CsA. Lee et al. have shown a rat allograft survival of greater than 100 days with daily injections of 3 mg/kg CsA for PEGylated islets. The low-dose immunosuppressive therapy was suspected to inhibit the indirect activation of immune cells typically caused by cytokine release of transplanted cells, while the PEG was postulated to inhibit host cell migration into the islet cluster [108, 124].

While preliminary studies investigating the efficiency of PEG coatings to prevent immune recognition and dampen inflammatory responses are promising, little is known about the permeability or stability of these layers. Given that cell turnover within the viable islet is expected, it is reasonable to suspect that the PEG coating on the cell surface will be endocytosed or shed after a set period of time.

3.3.3 Covalent Crosslinking

Covalent crosslinking of polymers has been explored, indirectly and directly, to enhance capsule stability, while also implementing polymers to improve capsule biocompatibility and immunogenicity. Indirect methods of covalent bond formation include glutaraldehyde, oxidization, carbodiimide, UV irradiation and elevated temperature [126-130]. Direct methods generate bond formation in situ with alternating layers of polymers containing complimentary reactive functional groups [131-138].

Recent studies used a photopolymerizable group, such as methacrylate group, onto the alginate to form photo-crosslinked microcapsules [139, 140]. A modified approach involved the chemoenzymatic alteration of the alginate block sequence to bind methacrylate groups exclusively on M residues, while maintaining enough G groups for gel formation [141], and used a controlled photoactivated reaction to increase stability. The beads were shown to have high stability, increased mechanical strength and compatibility with human pancreatic islets [142]. In addition, multifunctional hydrogels have been developed via the sequential photopolymerization of PEG hydrogel layers for islet encapsulation [143]. The advantages of UV irradiation, or photopolymerization, are the lack of cytotoxic agents, temperature changes, and large shifts in pH [144]; however, the generation of free radicals to induce polymerization presents a problem when translating to islet encapsulation. Islet cells are particularly susceptible to oxidative stress because of there relatively low anti-oxidant pool. Furthermore, free radicals have been shown to be detrimental to islets when compared to other cell types, and multiple studies have employed free radical scavengers for the protection of islets [145-148]. Furthermore, islet loss has been observed upon application of photoinitiated reactions [80].

On another note, direct methods involving the simultaneous covalent conjugation to form multilayer capsules have been explored to improve upon the interactions of indirect methods. The fabrication of covalent linked multilayers has been applied on several planar and spheroidal surfaces [133, 136, 138]. Additionally, the incorporation of covalent linkages has been used to improve upon microcapsule stability [149-152]. For instance, Chen et al. established a genipin cross-linked alginate-chitosan (GCAC)

microcapsules consisting of an alginate core with a covalently cross-linked chitosan outer membrane [150]. Another group constructed hollow, thin membrane microcapsules with the direct covalent assembly of poly(glycidyl methylacrylate) (PGMA) and poly(allylamine hydrochloride) (PAH) [132]. The techniques used for the polymeric cross-linking within microcapsules may also prove to be translatable to multilayer assembly of nano-scale cellular coatings.

3.3.4 Chemo-selective and biocompatible covalent ligation schemes

Specialized chemo-selective ligation schemes for improved stability of microcapsules and fabrication of nano-scale layers is an advantageous resource for encapsulating islet cells. Chemoselectivity allows for the development of stable layers within a biological environment, in an absence of external catalysts, with the production of only benign by-products. In addition, chemoselectivity is required to permit reactivity within fully supplemented medium, a necessary feature when coating cells with high metabolic rates. Techniques including the Staudinger ligation and Copper-free Click chemistry schemes have been shown to produce highly chemo-selective, efficient, stable, and benign reactions suitable for cellular applications [153, 154]. Another reaction called a Michael-type reaction has also been utilized to form free radical cross-linking reactions of polymers for cellular layer applications upon photoinitiation [155, 156].

The Staudinger reaction, developed by chemist Hermann Staudinger, occurs between an azide, a class of molecules containing three nitrogen atoms, and a phosphine, a molecule containing a phosphorus atom. Two nitrogen atoms are removed from the azide to form an aza-ylide. The appeal of this reaction for applications to cellular engineering was the two molecules do not react with biomolecules and readily react with

each other at room temperature in an aqueous environment [153]. However, the newly formed aza-ylide spontaneously hydrolyzes in water to yield a primary amine and the corresponding phosphine oxide [153]. Thus, although this reaction is highly chemoselective, the instability of the aza-ylide in an aqueous environment minimizes the Staudinger reaction applicability to cells.

In 2000, Carolyn Bertozzi and her student Eliana Saxon introduced the Staudinger ligation, modeled after the Staudinger reaction. The group introduced an ester molecule onto the phosphorus of the phosphine molecule created an electrophilic environment to attract and attach the nucleophilic aza-ylide. A stable amide bond resulted, thus preventing the hydrolysis of the aza-ylide molecule, see Figure 3-3 [160]. The Staudinger ligation has since been applied *in vitro* to living cell cultures, with no physiological damage [157-159]. In addition, successfully applications *in vivo* with modification of cells within living animals, for tagging and tracking disease progression, non-invasive imaging, and the general study of biological processes [160, 161].

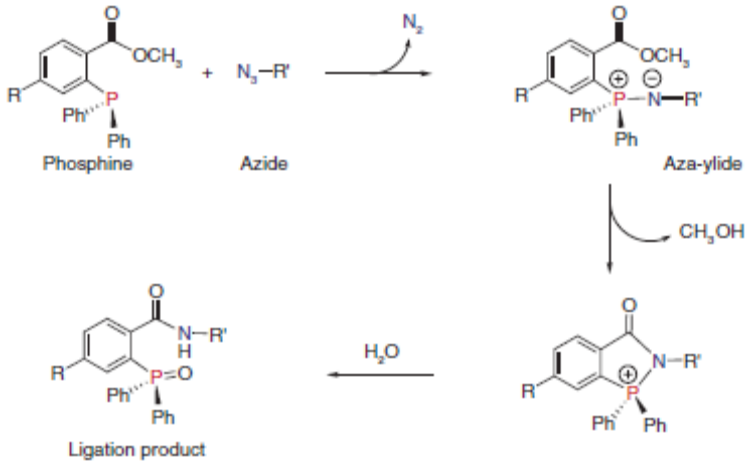


Figure 3-4. The Staudinger Ligation. A triarylphosphine and an azide react to form an aza-ylide intermediate. An electrophillic trap, created by an attached ester molecule, binds the nucleophillic nitrogen atom. The intermediate hydrolyzes in water to form a stable amide bond.

Cellular applications of the Staudinger ligation have shown encouraging results, and its applicability to nano-scale layering or the establishment of stable microcapsules may prove to be extremely promising. Further exploration and application of this highly chemoselective reaction will prove to be beneficial in the arena of cellular encapsulation as well as other tissue engineering applications.

CHAPTER IV

Covalently linked alginate-PEG microcapsules for the immunoisolation of pancreatic islets upon transplantation

Summary

Modified alginate and PEG polymers have been utilized to generate covalently linked alginate-PEG (XAlginate-PEG) microcapsules of high stability. The Staudinger ligation was used to inter-link an azido-functionalized alginate using a diphosphine PEG polymer. Covalent bond formation between the two polymers was verified using Fourier transform infared spectroscopy (FT-IR) and Nuclear Magnetic Resonance (NMR). XAlginate-PEG microcapsules were formed by co-incubation of the two polymers, followed by ionic cross-linking of the alginate using barium ions. The enhanced chemical and mechanical stability of the resulting XAlginate-PEG microbeads were evaluated using ion-free and chelating solutions. Osmotic swelling of XAlginate-PEG microbeads following exposure to ion free water was minimal, 5% increase in bead diameter, compared to traditional Alginate-Barium beads, which exhibited a 24% increase. Chemical stability of covalent bond formation was verified following stability of gels upon the exposure highly concentrated chelating solution. In vitro studies of the novel beads exhibited high compatibility with mouse insulinoma cell lines, as well as rat and human pancreatic islets. A mitochondrial activity assay showed a 219% and 195% increase in cell number on day 2 and a 640% and 510% cell increase on day 7 of encapsulated MIN6 cells in XAlginate-PEG and Alginate-Barium. Viability assessment of XAlginate-PEG and Alginate-Barium encapsulated Lewis rat islets exhibited no significant difference between the two groups. Similarly, mitochondrial evaluation of entrapped human islets showed no significant difference between the covalently linked

beads and traditional alginate capsules. In addition, a functional assessment showed the encapsulated islets' ability to secrete insulin upon a glucose challenge with no disparity between the two groups. The remarkable improved stability, as well as demonstrated cellular compatibility, of the XAlginate-PEG beads makes them an appealing option for a wide variety of applications.

4.1 Overview

Microencapsulation of cells has been an extensively explored approach to achieve immunoisolation of transplanted cells, where the goal is to protect foreign cells from the host immune system using artificial, biocompatible biomaterials. This technique has been applied to treat a wide variety of diseases including the encapsulation of dopamine-producing adrenal chormaffin cells for Parkinson's disease [162] and parathyroid cells for hypocalcemia [163]. However, much attention has been directed to microencapsulation of the insulin-producing islet cells for treatment of Type 1 Diabetes Mellitus (T1DM). Multiple studies have shown temporary reversal of diabetes upon the transplantation of microencapsulated islets [23, 47, 48, 164].

Alginate has been the most widely used material for microcapsule formation. Alginate is a collective term of a family of polysaccharides derived primarily from brown algae [63]. Alginate molecules are linear binary copolymers of β-D-mannuronic (M) and α-L-guluronic (G) acids, with a variation of sequential arrangement and composition depending on their source. The gelation of alginate beads relies upon the presence of a divalent cation (typically Ca^{2+} or Ba^{2+}); however, an ionically bonded alginate hydrogel

lacks the stability and immunoprotective capabilities to withstand the mechanical and chemical strains as well as immune and inflammatory responses associated with transplantation and engraftment [74].

Improvements to long-term stability of alginate microcapsules have been made with the application of a polycation layer on the alginate surface. The polycation layer, most commonly poly-L-lysine (PLL), has been shown to dramatically increase bead stability [74]. The allograft transplantation of PLL-alginate microcapsules has shown long-term success in both diabetic dogs [47] and patients with T1DM [48]. The use of PLL, however, has been shown to be toxic to cells, to activate macrophages and complement systems, and to induce fibrosis [75-77]. Thus, there is a strong need to fabricate biopolymers capable of forming more stable and biocompatible microcapsules without immunogenicity.

A covalently-linked alginate-PEG microcapsule may prove to be an appealing option for the transplantation of pancreatic islet cells. A previously proposed technique, utilizing an indirect method for developing a covalent bond, included grafting a photopolymerizable group, such as methacrylate group, onto the alginate to form photo-crosslinked microcapsules [139, 140]. A modified approach involved the chemoenzymatic alteration of the alginate block sequence to bind methacrylate groups exclusively on M residues, while maintaining enough G groups for gel formation [141], and a controlled photoactivated reaction was applied to increase stability [142]. The beads were shown to have high stability, increased mechanical strength and compatibility with human pancreatic islets [142]; however, the process of photopolymerization has been shown to result in significant islet cell loss [80].

The Staudinger ligation was used to form our covalently linked alginate-PEG polymer capsule. The reacting groups included an azide, a class of molecules containing three nitrogen atoms, and a phosphine, a molecule containing a phosphorus atom. These molecules have been shown to be highly chemoselective, react efficiently with each other, and non-detrimental to cells [153, 161].

In this study, we have characterized XAlginate-PEG microcapsules, covalently cross-linked with the Staudinger Ligation scheme. To generate these cross-links, the carboxylic groups of a linear MVG alginate chain were modified via covalent linkage with an azide using a PEG linker, see Figure 4-1. The complimentary phosphine group was tethered to both terminal end of a poly (ethylene glycol) (PEG) chain, see Figure 4-2. Following incubation of the polymers in solution, the azide groups on the alginate react with the phosphine groups on the PEG chain to form a covalently crosslinked gel, termed XAlginate-PEG. The stability of this XAlginate-PEG gel in the form of microcapsules was assessed, as well as the gels ability to encapsulate cells. The MIN6 β-cell line, an insulinoma cell line derived from a transgenic mouse, was used to observe cell viability and growth within the XAlginate-PEG beads. In addition, both rat and human pancreatic islets were encapsulated and their viability and function were monitored within the novel microbeads.

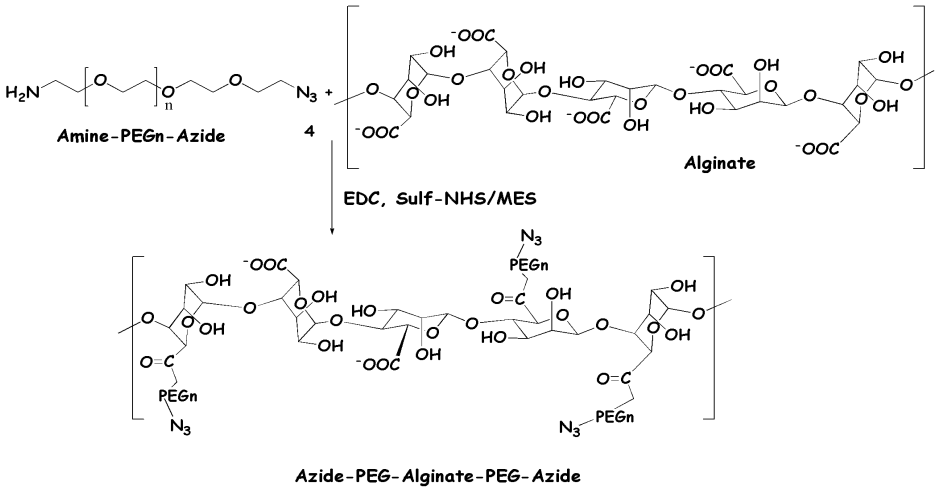


Figure 4-1. Schematic of Alginate-PEG_{326}-N_3, **Azide-Alg [1]** synthesis. The carboxylic groups of a highly purified MVG alginate are activated with ethylene diamino carbo-diamide (EDC). An amine molecule is used a stable amide-linkage between the azide and the alginate chain.

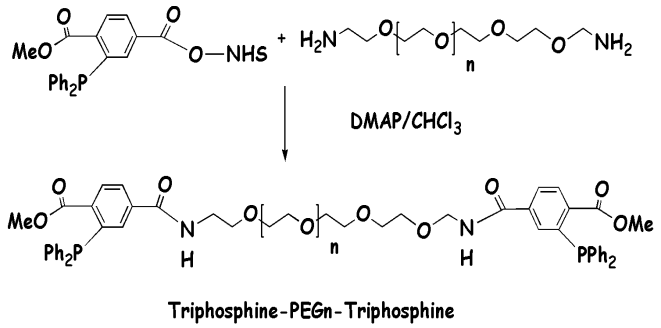


Figure 4-2. Schematic of Poly(ethylene glycol) (PEG) Diphosphine, **2DiP-PEG [5]** synthesis. The terminal hydroxyl groups of a PEG chain were functionalized with amine molecules. Two triphosphine molecules were covalently attached to the PEG with an N-hydroxysuccinimide (NHS) chemical linker.

4.2 Materials and Methods

4.2.1 General Reagents

All chemical reagents were of analytical grade, obtained from commercial suppliers, and used without further purification unless otherwise noted. The solvents and other chemical reagents were purchased from Sigma-Aldrich at the highest purity available.

4.2.2 Polymer Characterization

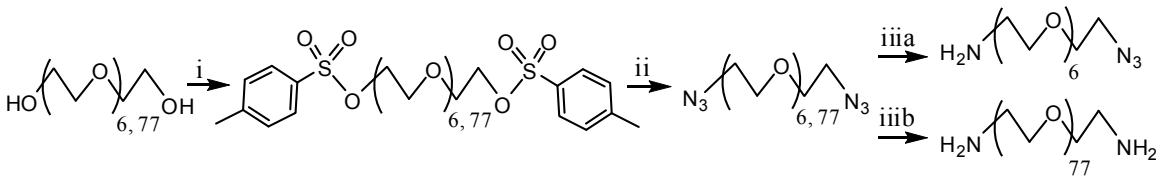
Polymer purity and functionalization was assessed using ATR-FT-IR, mass spectroscopy, and ^{1}H-NMR. ATR-FT-IR spectra were obtained using the PerkinElmer Spectrum 100 FT-IR Spectrometer with the Universal ATR (1 bounce, Di/ZnSe) Sampling Accessory. UV-Vis spectra were obtained using the Molecular Devices SpectraMax M5 Microplate/Cuvette Reader. Samples were submitted to the University of Miami Chemistry Department for mass spectroscopy (VG Trio-2 FAB or Bruker Bioflex IV Maldi-TOF) and ^{1}H-NMR (Bruker, 500 MHz) analysis.

4.2.3 Synthesis and characterization of Alginate-PEG$_{326}$-N$_3$, ***Azide-Alg [1]***

Ultra purified sodium alginate of high molecular weight, high guluronic acid content alginate (UP-MVG) %G, (NovaMatrix, Pronova Biomedical, Norway) was used as the base polymer for azide functionalization and for control studies of standard alginate capsules.

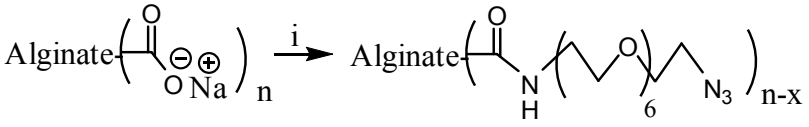
N_3-PEG_{326}-NH_2 **[2]**, the functional group added to **[1]**, was fabricated via Ts-PEG_{326}-Ts **[3]** and N_3-PEG_{326}-N_3 **[4]** polymer precursors. **[3]** was prepared by tosylation (Ts) of both hydroxyl end groups of PEG, $MW_{average}$ of 326 (Sigma). HO-PEG_{326}-OH (2.4g) was dried by stirring under vacuum at 75°C for 1hr. After cooling to room temperature (RT, 21-22 °C), the dry PEG oil was dissolved in 3mL anhydrous dichloromethane (DCM) followed by slow (within 10 min) addition of a freshly prepared solution of 3.94g (20.46mmol) p-toluenesulfonyl chloride and 2.80g (22.69mmol) 4-(dimethylamino)pyridine (DMAP) in 10mL anhydrous DCM, followed by stirring at RT for 3 h. The resulting mixture was washed once with a 30mL mixture of water/ice/1mL HCl (36.8 %) and once with 20mL saturated $NaHCO_3$ solution containing some ice. The

resulting organic layer was dried over anhydrous Na_2SO_4, filtered through a silica gel pad, and the solvent removed under reduced pressure. The resulting polymer, Ts-PEG_{326}-Ts **[3]** (3.90g), was added to NaN_3 (1.27g), and 4mL anhydrous N,N-dimethylformamide (DMF) and reacted for 4h at 80°C under Ar flow for 15 h at RT (21-22°C). Following incubation time, diethyl ether (20mL) was added. Insoluble salts were removed by filtration through a 0.45µm polypropylene filter, diethyl ether was removed under reduced pressure, and the DMF was removed by short-path distillation at 80 °C with help of Ar flow. The residue was dissolved in 20mL diethyl ether and passed through a silica gel pad. The diethyl ether was removed under reduced pressure. The product, N_3-PEG_{326}-N_3 **[4]** was dried first by Ar-flushing and then under reduced pressure. A solution of Ph_3P (1.06g) in 9mL of 50% (v/v) diethyl ether in ethyl acetate was slowly (within 1h) added to a solution of N_3-PEG_{326}-N_3 (1.5g), 50% (v/v) diethyl ether (4.5mL) in ethyl acetate, and 0.5M HCl_{aq} (9mL) under Ar-flow and rapid stirring. Solution was then stirred for 19 h at RT. The aqueous phase was collected and made basic with addition of 6M $NaOH_{aq}$ (1.0mL). The solution was then cooled in ice-water bath for 2h while bubbling Ar, passed through a 0.45µm polypropylene filter, and extracted 7 times with 3mL dichloromethane (DCM). All combined DCM was dried over anhydrous Na_2SO_4, passed through a silica gel pad, and DCM removed under reduced pressure followed by Ar-flushing. Method as outlined resulted in 1.0g of N_3-PEG_{326}-NH_2 **[2]**.



Scheme 1. Synthesis of PEG linker. (i) TsCl, DMAP, DCM; (ii) NaN_3, DMF; (iiia) Ph_3P HCl-H_2O/Et_2O-EtOAc; (iii b) Ph_3P, THF/water

To fabricate alginate-PEG_{326}-N_3 **[1]**, sodium alginate (50mg), N-hydroxysuccinimide (14mg, 0.12mmol), and 2-(N-morpholino)ethanesulfonic acid (62mg, 0.29mmol) were dissolved in 5mL de-ionized water. A solution of N_3-PEG_{326}-NH_2 **[2]** (28mg, 0.08mmol) in 200µL H_2O was added. After stirring for 10min at RT, 1-ethyl-(dimethylaminopropyl)carbodiimide hydrochloride (EDC) was added and stirring continued for 25min at RT. The amount of EDC added varied from 56mg to 110mg, depending on the degree of functionalization desired. For this study, it was found that the addition of 110mg of EDC resulted in the highest degree of N_3-PEG_{326}-NH_2 functionalization of the alginate chain. This alginate, **[1c],** was used for all encapsulation experiments. Following incubation, 55µL of 6 M NaOH was added and stirred for an additional 10 min. Purification was achieved by (at least) 4 days dialysis (10,000 MWCO membrane) against 600mL water changed three times a day. During the first 3 days, 10µL of 6M NaOH was added twice daily to the alginate-containing solution and mixed. During the first 2 days, 400µL 4.26M NaCl was also added twice a day to the alginate solution and mixed. Finally, solution was filtered through a 0.45µm membrane and lyophilized. The yield was 52.8mg of a white, foamy, solid film. The Kaiser test was negative. ATR-FT-IR: 3279, 2924, 2879, 2112, 1658 (shoulder), 1600, 1548 (shoulder), 1408, 1349, 1299, 1124, 1085, 1026 cm^{-1}.

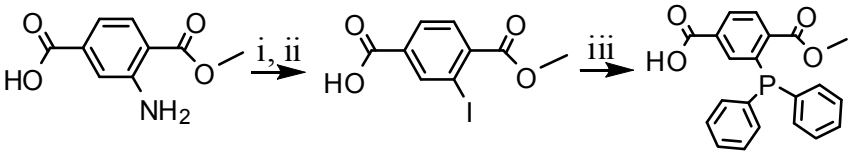


Scheme 2. Functionalization of alginate ***[1]***. (i) EDC.HCl, NHS, NH_2-PEG_{326}-N_3

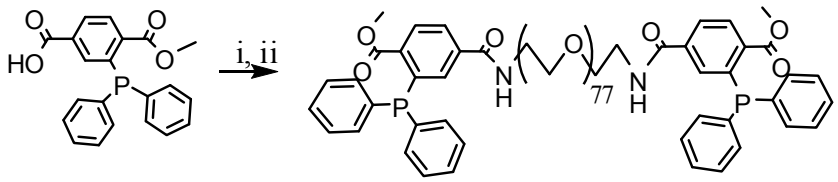
4.2.4 Poly(ethylene glycol) (PEG) Diphosphine Synthesis, ***2DiP-PEG [5]***

Poly(ethylene glycol) (PEG) Diphosphine, called 2DiP-PEG **[5]** was fabricated from the starting PEG polymer of NH_2-PEG_{3419}-NH_2. Functionalization of the end units of the PEG was achieved by first fabricating the phosphine group, or 1-Methyl-2-Diphenylphosphino-Terephthalate (MDT) **[6]**. **[6]** was fabricated by the addition of 1-Methyl-2-aminoterephthalate (1.0g, 5.10mmol) to 10mL of cooled HCl and stirred 10 min. $NaNO_2$ (0.36g, 5.22mmol) in 2.3mL water was injected slowly (within 5min) through a sealed rubber septum into the HCl mixture while stirring (an orange gas formed). After 5min, the mixture was removed from the ice-water bath and stirred 25min at RT. The resulting mixture was passed through glass wool and 0.45µm polypropylene (pp) membrane filter into a stirring solution of (8.6g, 51.7mmol) KI and 14mL water (mixture turns dark red with red-black precipitate). After stirring for 1h at RT, the solution was mixed with 40mL DCM and 35mL of 1.57 M (55.0mmol) Na_2SO_3. The DCM phase was collected and washed once with 35mL of 4.28M NaCl solution. The DCM phase was collect, dried over anhydrous Na2SO4, filtered (0.45µm, pp membrane), and DCM removed under reduced pressure. The yellow solid residue was dissolved in 6mL methanol and filtered (0.45µm, pp membrane). The product was precipitated by adding 4mL water, cooling 1h in ice-water bath, and stirring. It was collected by filtration and freeze-dried overnight to yield 832.0mg of a yellow solid powder of 1-Methyl-2-Iodoterephthalate **[7]**. A flame dried/Ar-flushed Schlenk tube was loaded with **[7]** (750 mg, 2.4506mmol), $Pd(Ac)_2$ (5.5mg,0.0242mmol), 1,3-bis(diphenylphosphino)propane (10.0mg, 0.0242mmol), anhydrous acetonitrile (7.5mL), and N,N'-diisopropylethylamine (900µL, 5.1465mmol). The mixture was stirred until fully dissolved and degassed

(freeze-pump-thaw method). Diphenylphosphine (425µL, 2.4204mmol) was injected (drop-wise) into the acetonitrile solution under Ar flow (solution turned red and clear). The solution was refluxed for 4h under Ar pressure (balloon), cooled to RT (or incubated overnight at RT), and concentrated under reduced pressure. The red sticky residue was dissolved in 5mL DCM and washed once with 50mL water and 25mL 1M HCl. The DCM was removed under reduced pressure. The product was recovered by washing the red-yellow solid residue with 6mL cold methanol, rinsed 5 x 1.5mL cold methanol, and filtration. After drying under reduced pressure, 0.638 g of a yellow solid powder of MDT **[6]** was obtained, MW_{found} = 364.00; $MW_{calculated}$ = 364.33. DiP-PEG **[7]** was formed by the addition of N,N'-Diisopropylcarbodiimide (22.52µL, 0.1440mmol, DIC) to a solution of MDT **[6]** (52.8mg, 0.1449mmol) and N-hydroxysuccinimide (17.2mg, 0.1465mmol) in 1.2mL anhydrous/Ar-flushed DMF. After stirring for 30min at RT under Ar flow, NH_2-PEG_{3419}-NH_2 (200mg, 0.0597mmol) was added, followed by 4-dimethylaminopyridine (14.8mg, 0.1200mmol) after 1hr. After 3hrs, stirring at RT under Ar flow, the product was precipitated with 12mL cold diethyl ether and collected by centrifugation at 0 °C. It was "crystallized" once in 10mL 200 proof ethanol (warm to 37 °C to dissolve, cool in ice-water bath to precipitate, collect precipitate by centrifugation at 0 °C). It was dissolved/precipitated/collected once with 0.5mL DCM/12mL cold diethyl ether/centrifugation at 0°C and dried under reduced pressure. The yield was 212.0mg of a white to light yellow solid powder. The Kaiser test was negative.



Scheme 3. Synthesis of Staudinger ligation phosphine. (i) HCl, $NaNO_2$; (ii) KI; (iii) $Pd(Ac)_2$, Ph_2PH, acetonitrile



Scheme 4. Synthess of bi-functional PEG ***[5]*** for cross-linking alginate via Staudinger ligation. (i) DIC, NHS; (ii) NH_2-PEG_{3419}-NH_2, DMAP

4.2.5 Cell Culture

MIN6 cells were cultured as monolayers in T-flasks and fed every 2-3 days with fresh medium comprised of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Sigma), 1% penicillin-streptomycin (P/S), 1% L-glutamine and 0.001 % (v/v) β-mercapoethanol. Rat pancreatic islets were isolated from male Sprague-Dawley (SD) rats using methods described elsewhere [3]. Human pancreatic islets of Langerhans were obtained from the ICR Consortium. Following isolation, islets were cultured in CMRL 1066 media for 48hrs (rat islets) or MM1 media for 24hrs following arrival (human islets) at 37°C in a humidified atmosphere of 5% CO_2 in air prior to encapsulation. CMRL 1066 and MM1 media was supplemented with 10% fetal bovine serum (FBS; Sigma), 1% penicillin-streptomycin, and 1% L-glutamine.

4.2.6 Microcapsule formation and cellular encapsulation

Standard Alginate-Barium and XAlginate-PEG microbeads were fabricated using a modification of the protocol originally developed by Sun [61]. Alginate-Barium gels were fabricated using 2.5 wt% alginate in PBS solution. Following dissolution of the alginate polymer in the saline, the polymer solution was used for microbead fabrication, as described below. XAlginate-PEG gels consisted of 2.5 wt% Azide-Alg**[1]** and 4.75

wt% 2DiP-PEG**[5]**. The solution of **[1]** and **[5]** were generated by first dissolving 3.1 wt% of **[1]** in saline in one vial and 23.75 wt% of **[5]** in another. Following complete dissolution of the polymers in saline, the solutions were mixed in a 1:1.9 ratio. The mixture of **[1]** and **[5]** were gently mixed for 1hr15mins prior to microbead fabrication (see Appendix Section 7.1 for further details on the selection of pre-incubation time frames and **[1]** to **[5]** ratios). Microbeads were prepared via needle extrusion of the alginate or XAlginate-PEG solution into a gelling basin of 50mM $BaCl_2$ (Sigma Aldrich) and 0.025% (v/v) Tween 20 (Sigma Aldrich) solution. The size of the droplets was controlled by a parallel airflow generator (10kPa pressure of air with a 1in distance from the needle to the gelling solution) and the manual force applied to the syringe. The beads were exposed to the gelation solution for 5min, aspirated, and then rinsed with phosphate-buffered solution (PBS) three times to remove excess barium. The average bead diameter for alginate microbeads was 460±13μm and 520±17μm for Alginate-Barium and XAlginate-PEG microbeads, respectively.

For microcapsules containing cells, MIN6 or islet cells were mixed in either polymer solution immediately prior to needle extrusion. MIN6 cells were harvested from monolayer cultures using trypsin-EDTA (Sigma, St. Louis, MO) and suspended in either polymer solution at a density of 1 x 10^7 cells/ml polymer solution. Viable cell counts were performed using the trypan blue (Sigma, St. Louis, MO) exclusion method. Islet cells were pelleted, media removed, and suspended in either polymer solution at a 1.5% loading density. Islet cell counts were performed prior to encapsulation using the islet equivalence (IEQ) method. Following the homogeneous distribution of the cells within either the Alginate-Barium or XAlginate-PEG solutions, the cell/polymer suspension was

extruded through the microencapsulation generator and microbeads were fabricated. Following a 5min exposure to the barium gelation solution and PBS rinse, the cell-containing microbeads were washed in the appropriate culture media for the cell type. The beads were cultured in non-cell culture treated T75 flasks (Falcon) and rocked on an orbital plate in a humidified 37°C, 5% CO_2 / 95% air incubator for 7-8 days.

4.2.7 Stability

The stability of the barium capsules was tested using a dimensional stability assay. Five Alginate-Barium beads or XAlginate-PEG beads, initially placed in 1mL of saline for at least 1hr prior, were placed in 1mL of ion free water and observed for 70min. The stability of the capsules was monitored against time by an inverted light microscope. Dimensional changes, due to osmotic swelling, were recorded at 10min intervals. To evaluate the chemical stability of the beads, both the covalently linked and ionically linked beads were tested with an EDTA treatment, to leach out the barium ions. Five beads/group were exposed to 2mL of a 200mM EDTA solution.

4.2.8 Growth/viability measurements

The MTT assay has been shown as a means to quantify the survival and proliferation of cells [165]. Thus, encapsulated cell viability and growth was measured by an MTT assay (Promega). Approximately 100 microcapsules from each group were collected, washed twice in DPBS, and conditioned in medium with 0.5mg/ml MTT (Promega) for 1hr15min at 37°C. The capsules were then collected, crushed, the stop/solubilization solution was added, and the beads were incubated overnight. From each well, triplicates of 120μL of solution were transferred to a 96-well plate and the optical densities were measured at 570nm in a microplate reader (Molecular Devices).

The recorded absorbance for each experimental point was then correlated to either cell number (for MIN6 cells) or islet IEQ (for human and rodent cells) by correlating the absorbance to previously validated calibration curves of the respective cell type (see Appendix 7.2).

4.2.9 Measurement of Insulin Secretion

Functional assessment of encapsulated islets was tested via static glucose-stimulated insulin release (GSIR) assay. Ten Alginate-Barium and XAlginate-PEG encapsulated islets were incubated within inserts in triplicates in a 24-well plate with 800μL of a basal glucose solution [Krebs-bicarbonate (KRBB) buffer: 99mM $NaCl_2$, 5mM KCl, 1.2mM KH_2PO_4, 1.2mM $MgSO_4$, 2.6mM $CaCl_2$, 26mM $NaHCO_3$, and 0.2wt% BSA supplemented with 60mg/dL glucose] for 1 hr, following by washing with basal solution. The inserts were then transferred to a new well in the 24-well plate and incubated for 1hr in 800μl of the basal solution, labeled as Basal 1. After a 1hr incubation, the inserts were drained and placed in a stimulated glucose solution (KRBB supplemented with 300mg/dL glucose) for 1hr, labeled as Stimulated 1. After an additional wash step, the islets were then placed in basal glucose solution for an additional hour, labeled as Basal 2. Samples were collected at the end of each incubation period and frozen until ELISA (Mercodia) analysis. Insulin concentration within the samples was quantified by following the manufacture's instructions. GSIR was evaluated as ratio of the level of insulin secretion during Stimulated 1 to the level of insulin secretion during Basal 1.

4.2.10 Confocal microscopy of encapsulated cells

Cells cultured in alginate/PEG-alginate beads were stained with 1-4μM Calcein AM (green dye) and 2μM EthD-1 (red dye) (Live/Dead Invitrogen) for 45min prior to observation using fluorescent confocal microscopy (Zeiss, Germany). The Live/Dead solution was prepared in DPBS. Three-dimensional (3D) images were reconstructed using projection function on LSM image browser (Zeiss, Germany) software.

4.2.11 Statistical Analysis

All statistical analysis performed throughout this study utilized a paired student t-test with significant differences represented by a p-value less than 0.05.

4.3 Results

4.3.1 Capsule stability

The osmotic swelling of the microcapsules was evaluated by observing the dimensional changes of the beads when placed in ion free water. Traditional Alginate-Barium beads were used as controls. The presence of the covalent bonds between the **[1]** and **[5]** polymers were expected to result in a decrease in swelling of the XAlginate-PEG beads when compared to the Alginate-Barium capsules. The swelling of both empty and capsules containing cells was studied. Figure 4-1 exhibits the swelling of empty capsules as an increase in diameter over time. The Alginate-Barium reached a maximum of 24% increase in bead diameter within the first twenty minutes, while the XAlginate-PEG exhibited a maximum 5% increase in bead diameter. The XAlginate-PEG microcapsules exhibited a statistically significant decrease in swelling when compared to the standard Barium-Alginate capsules. Figure 4-2 exhibits the swelling of microbeads containing cells following incubation in ion free water. The bead diameter of both the Alginate-

Barium and XAlginate-PEG samples containing cells increased with time in water. The Alginate-Barium samples showed a maximum 41% increase in bead diameter after thirty minutes, while the XAlginate-PEG beads exhibited a maximum of 26% increase in bead diameter. The cell-filled XAlginate-PEG microcapsules exhibited a similar trend to the cell-free capsules, where a statistically significant decrease in swelling was observed when compared to the standard Barium-Alginate capsules.

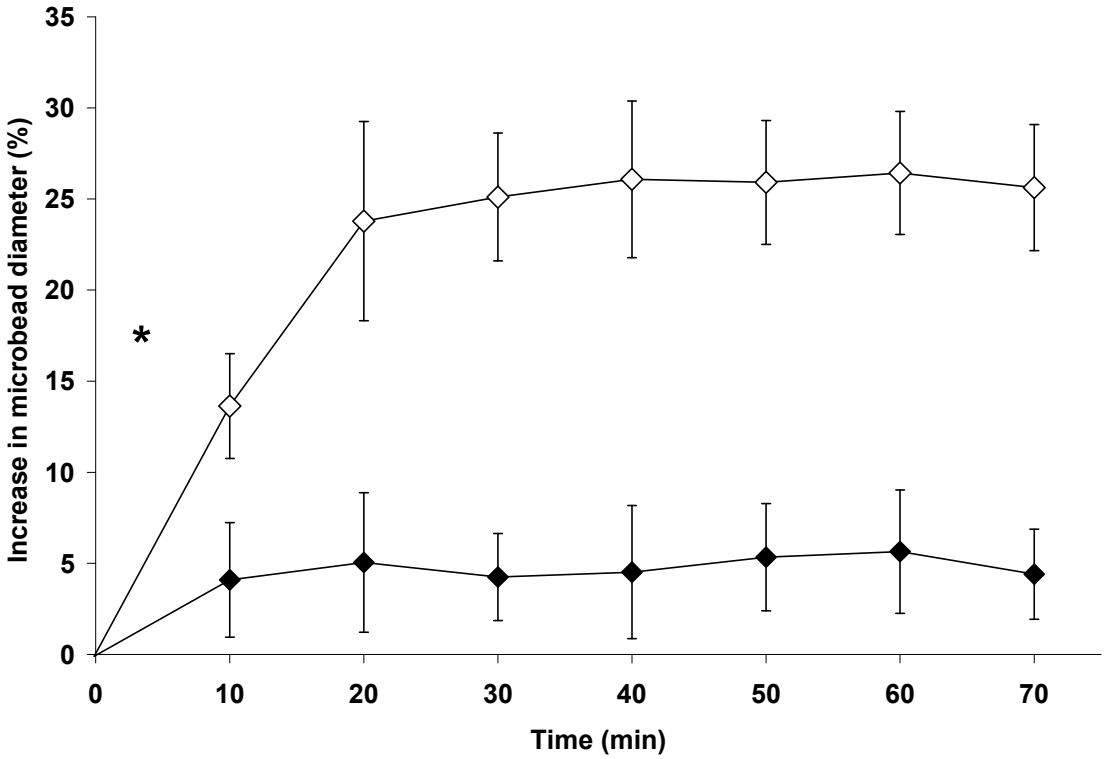


Figure 4-3. Stability and swelling Alginate-Barium (open diamonds) and XAlginate-PEG (solid diamonds) microbeads in ion free water as expressed by percentage increase in microbead diameter as a function of time. Data points ± SD represent the average of five independent measurements. *Significant differences ($p<0.05$) between XAlginate-PEG and Alginate-Barium microbeads.

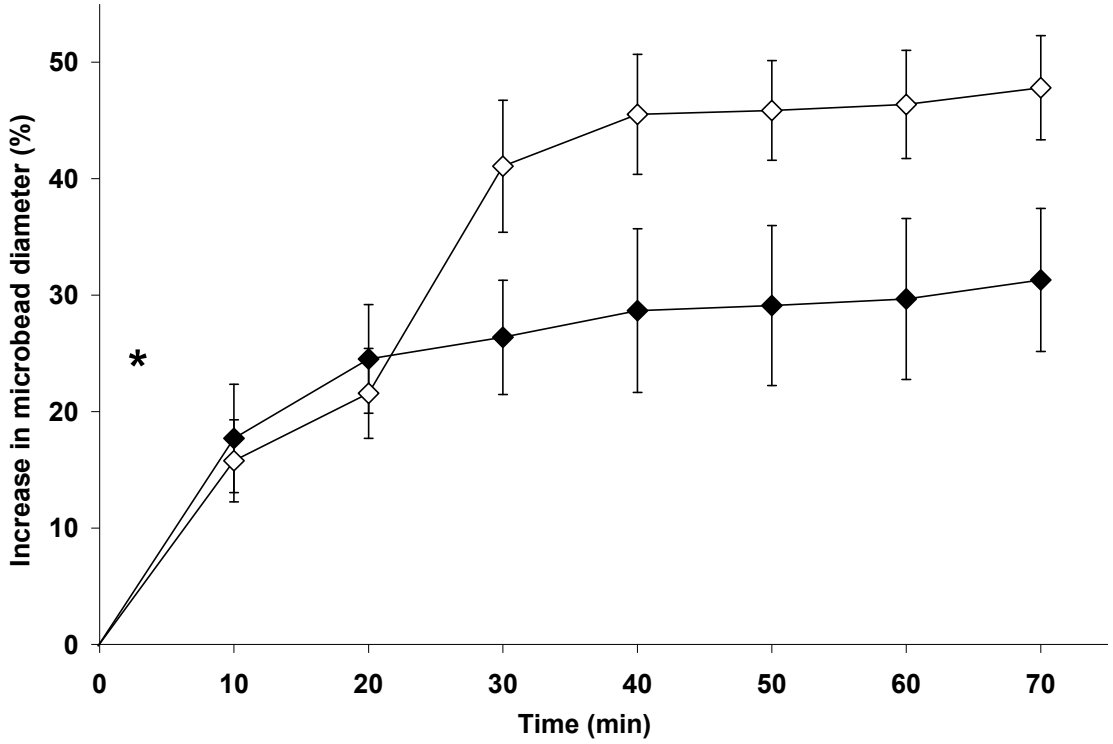


Figure 4-4. Stability and swelling of Alginate-Barium (open diamonds) and XAlginate-PEG (solid diamonds) MIN6 microbeads in ion free water as expressed by percentage increase in microbead diameter as a function of time. Data points ± SD represent the average of five independent measurements. *Significant differences ($p<0.05$) between XAlginate-PEG and Alginate-Barium encapsulated MIN6 cells after 30min.

The covalent bonds between the alginate and the PEG polymers were also evaluated by removal of the barium ions using a concentrated chelating solution. Microbead stability following ion removal was evaluated by observing diameter changes for both empty (Figure 4-3) and cell-filled (Figure 4-4) XAlginate-PEG capsules. Complete dissolution of the Alginate-Barium capsules was observed within 2 min following EDTA exposure, which also verified the complete removal of all ionic interactions. The XAlginate-PEG microcapsules containing cells and those not containing cells exhibited similar swelling behavior in the chelating solution. Additionally, the

swelling observed for the XAlginate-PEG microcapsules following complete removal of the ionic cross-links was similar to the degree of swelling found for standard Barium-Alginate capsules in ion-free water.

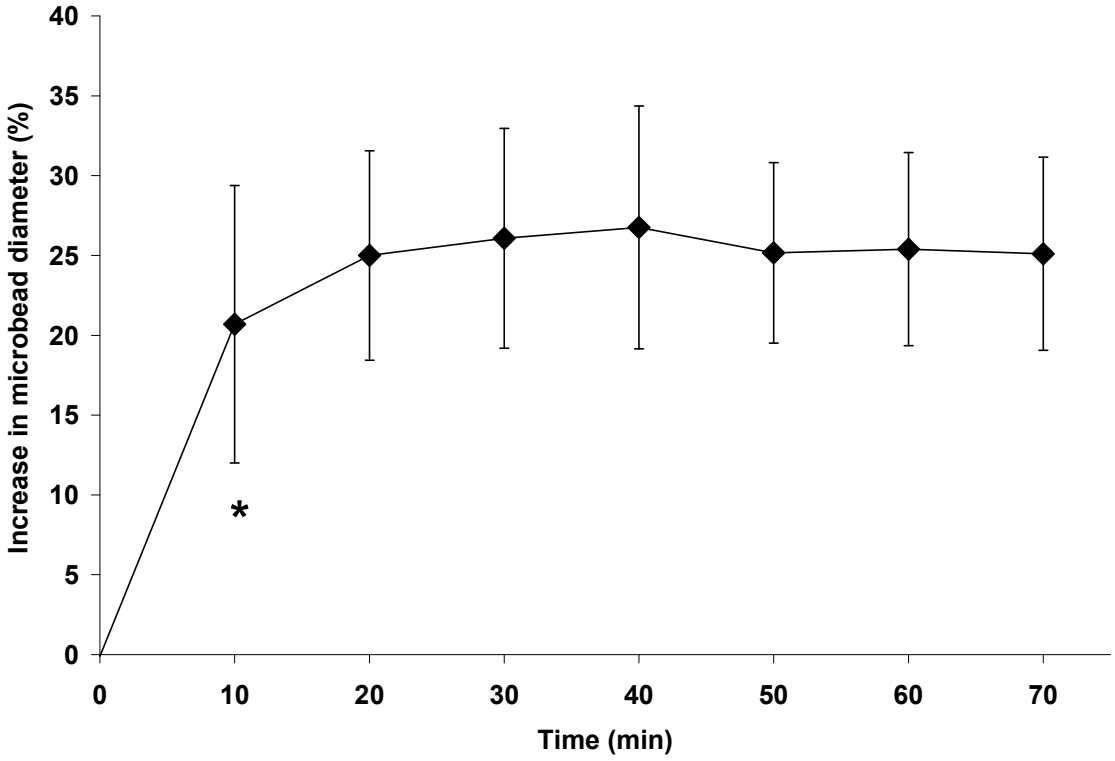


Figure 4-5. Stability of XAlginate-PEG microbeads in a 200mM EDTA solution as expressed by percentage increase in microbead diameter as a function of time. Data points ± SD represent the average of five independent measurements. *Measurement of Alginate-Barium microbeads was not feasible due to dissolution of gel 2 minutes after EDTA addition.

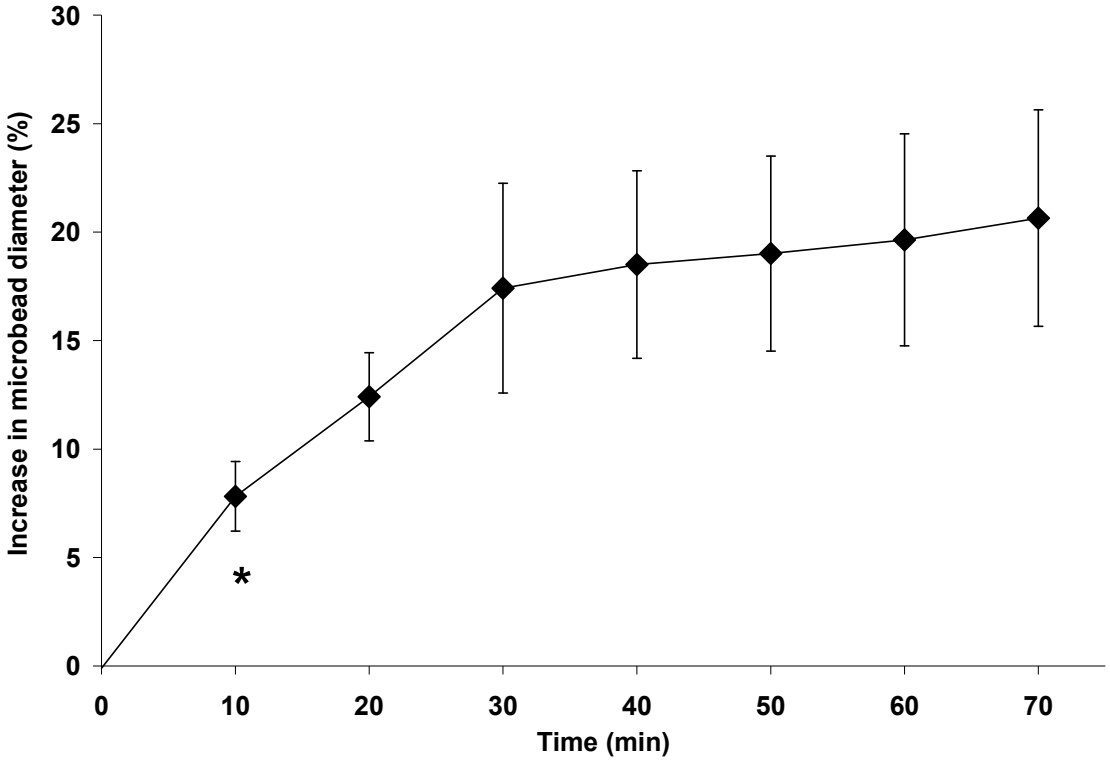


Figure 4-6. Stability of XAlginate-PEG MIN6 microbeads in a 200mM EDTA solution as expressed by percentage increase in microbead diameter as a function of time. Data points ± SD represent the average of five independent measurements. *Measurement of Alginate-Barium microbeads was not feasible due to dissolution of gel 2 minutes after EDTA addition.

4.3.2 Growth/viability of cells in alginate-PEG capsules

The cellular response following alginate-PEG encapsulation was evaluated and compared to Alginate-Barium microbeads. Preliminary experiments evaluated the growth and viability of MIN6 cells within the new XAlginate-PEG beads. The proliferation of the encapsulated MIN6 cells within the XAlginate-PEG and the Alginate-Barium beads was measured as a 219% and 195% cell increase on day 2 and a 640% and 510% cell increase on day 7, respectively (Figure 4-5), illustrating an increase in cell number over the culture period. Confocal microscopy of cross sections of the beads containing cells stained using the live/dead assay, supported the mitochondrial activity findings, where an increase in the number and size of the cell clusters within the microcapsules was

observed for both polymer configurations over time (Figure 4-6). In addition, these images showed a homogeneous distribution of MIN6 cells throughout the capsule.

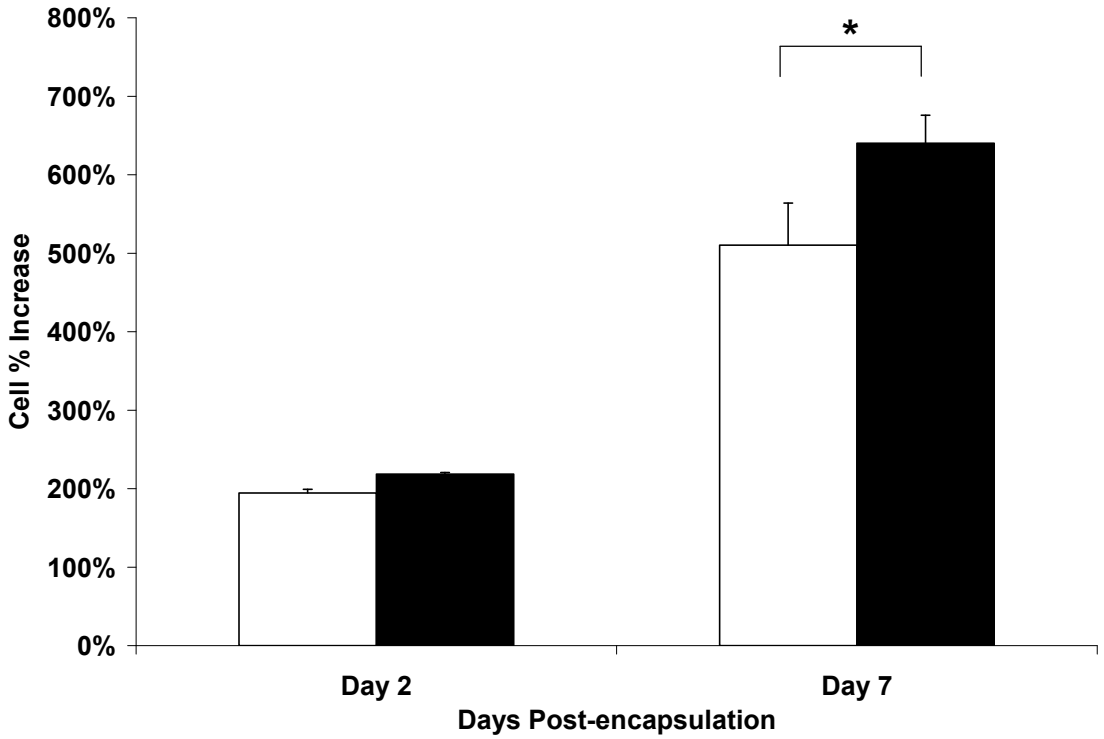


Figure 4-7. Growth/viability of MIN6 cells encapsulated with XAlginate-PEG (solid bars) or Alginate-Barium (open bars), as assessed using MTT. Results are presented as the % increase in cell number as a function of time. Data points represent the mean ± SD, for the MTT assay for 100 beads. *Significant differences ($p<0.05$) between XAlginate-PEG and Alginate-Barium encapsulated MIN6 cells.

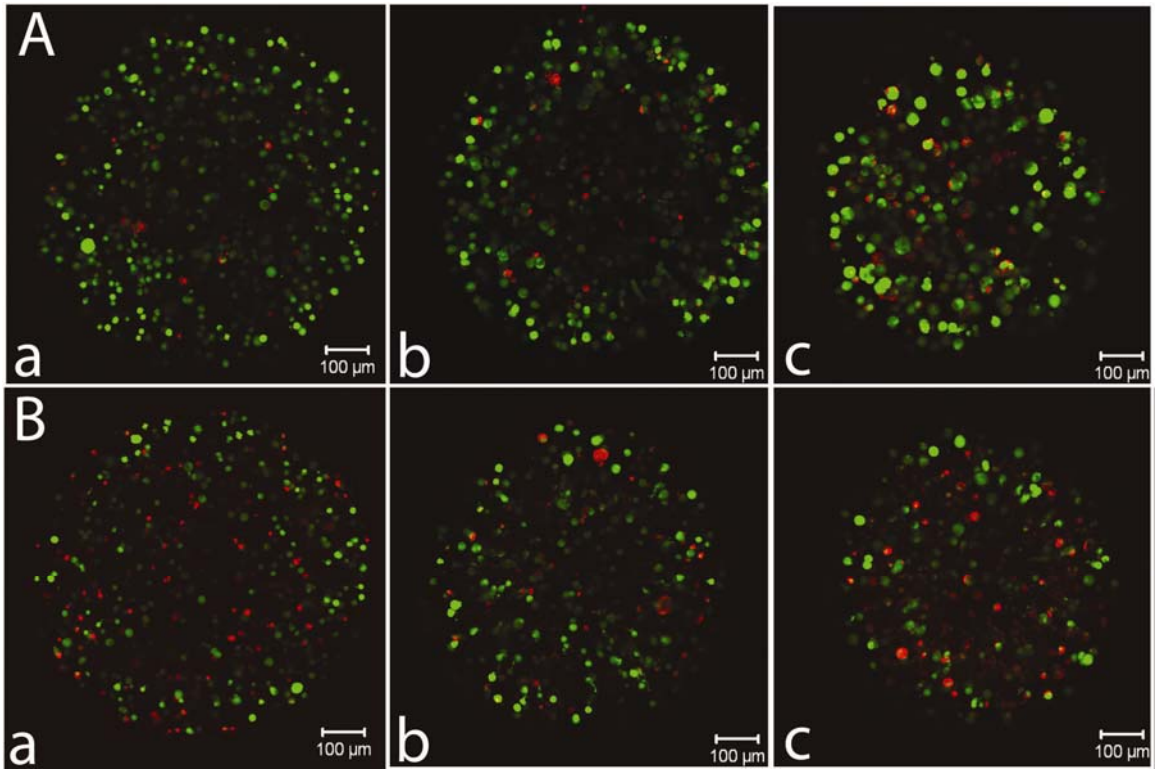


Figure 4-8. Confocal images of encapsulated MIN6 cells stained using Live/Dead fluorescent kit where live cells are labeled green and dead cells are labeled red (see Methods for details). Top row (A): XAlginate-PEG encapsulated MIN6 cells on Day 1 (a); Day 3 (b); and Day 7 (c) post-encapsulation. Bottom row (B): Alginate-Barium encapsulated MIN6 cells on Day 1 (a); Day 3 (b); and Day 7 (c) post-encapsulation.

Lewis rat islets were adequately encapsulated by the two gel solutions with 1-2 islets per bead, as illustrated by the representative confocal images (Figure 4-7). Confocal microscopy three-dimensional reconstructions of live/dead stained cells illustrate high percentage of viable cells for all observed time points and polymeric configurations (Figure 4-7). The XAlginate-PEG and the Alginate-Barium beads exhibited 140 and 128 islets per a hundred beads, respectively, on day 1 and 212 and 260 islets per a hundred beads on day 5 using the previously described MTT assay (Figure 4-8), which is a statistically significant increase in mitochondrial activity between the two time points.

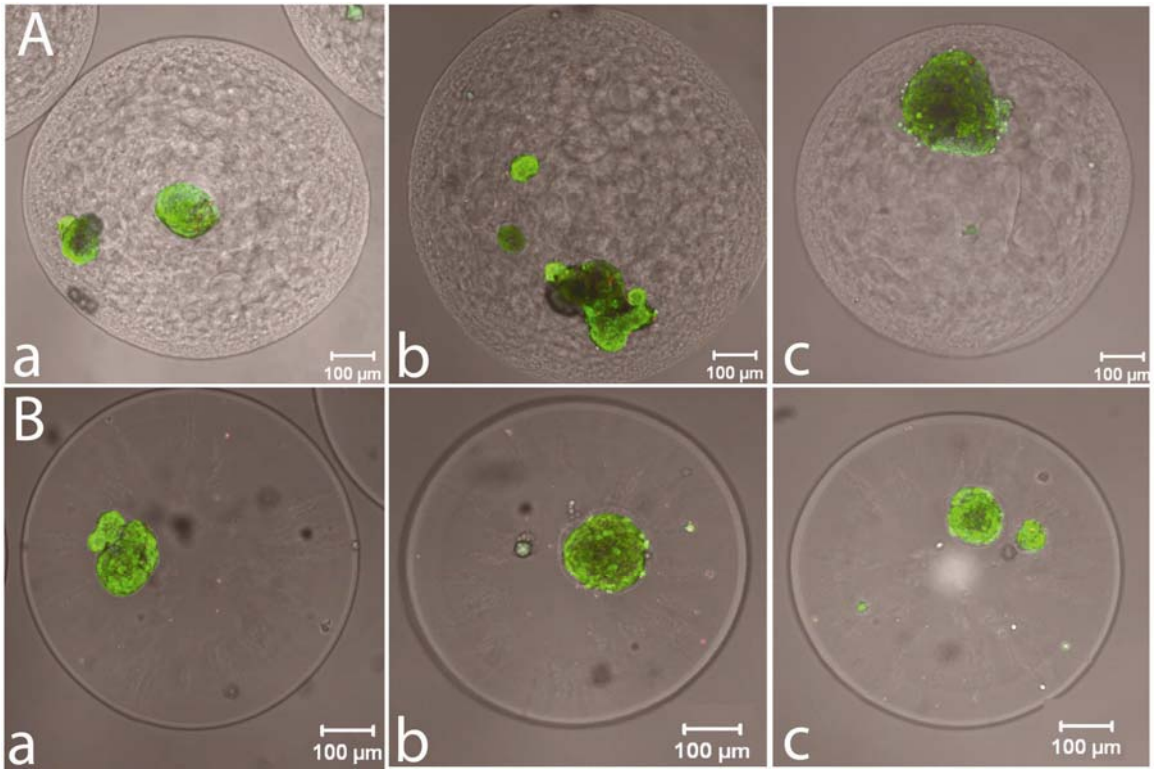


Figure 4-9. Confocal images of encapsulated Lewis rat islets stained using Live/Dead fluorescent kit where live cells are labeled green and dead cells are labeled red (see Methods for details). Top row (A): XAlginate-PEG encapsulated Lewis rat islets on Day 1 (a); Day 5 (b); and Day 8 (c) post-encapsulation. Bottom row (B): Alginate-Barium encapsulated Lewis rat islets on Day 1 (a); Day 5 (b); and Day 8 (c) post-encapsulation.

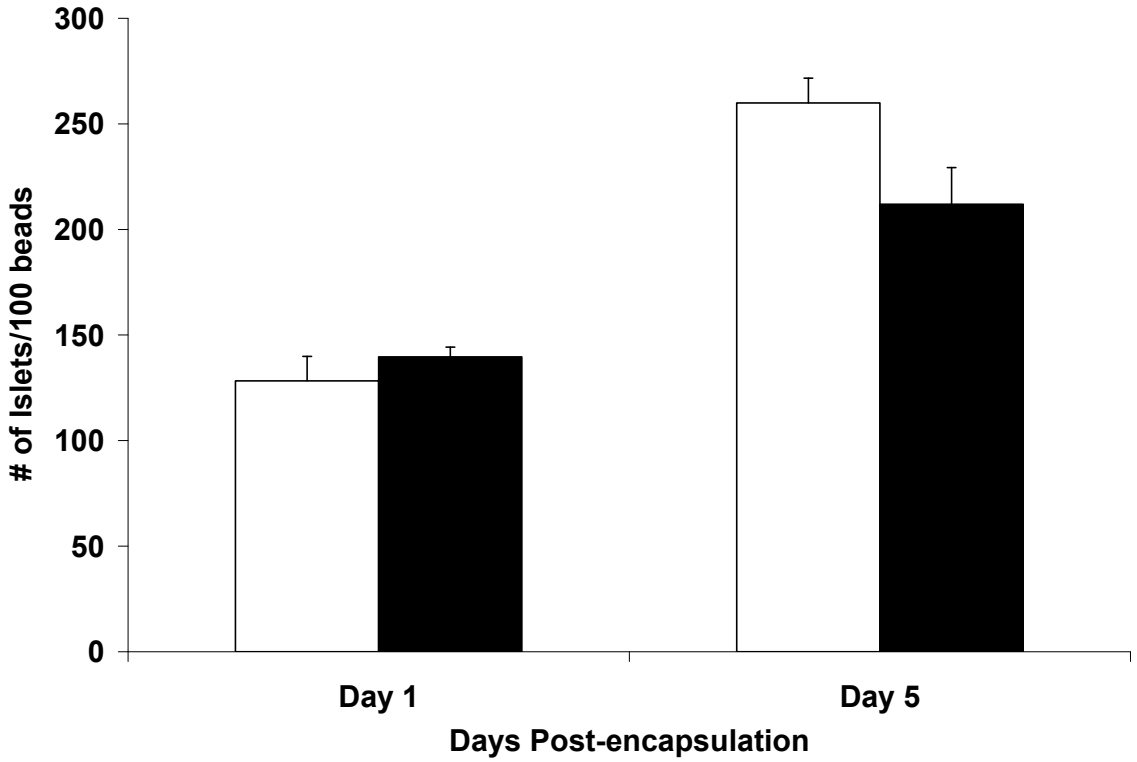


Figure 4-10. Assessment of Lewis rat islet viability following encapsulation with Alginate-Barium (open bars) or XAlginate-PEG (solid bars), as measured using MTT. Values are presented as the number of rat islets per 100 beads. Data points represent the mean ± SD, for the MTT assay for 100 beads.

Human pancreatic islets were adequately encapsulated by the two solutions with 1-2 islets per bead (Figure 4-9). Confocal microscopy of live/dead stained cells exhibited highly viable islets. The cells entrapped by the XAlginate-PEG and Alginate-Barium exhibited 137 and 132 islets per a hundred beads on day 1 and 100 and 159 islets per a hundred beads on day 5, based on MTT (Figure 4-10). While the change from day 1 to day 5 of the MTT readings was not statistically significant within polymer types, human islets within the XAlginate-PEG demonstrated a statistically significant decrease in calculated islet IEQ when compared to Barium-Alginate capsules on day 5.

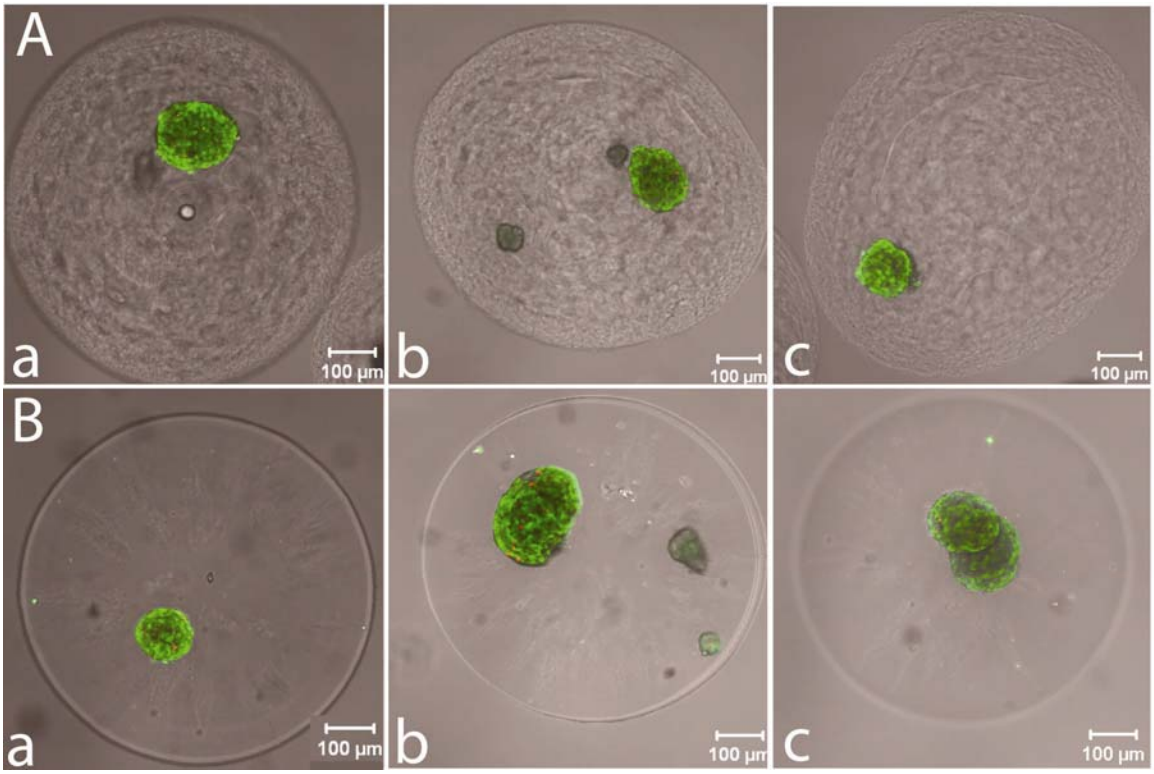


Figure 4-11. Confocal images of encapsulated human islets stained using Live/Dead fluorescent kit where live cells are labeled green and dead cells are labeled red (see Methods for details). Top row (A): XAlginate-PEG encapsulated human islets on Day 1 (a); Day 5 (b); and Day 8 (c) post-encapsulation. Bottom row (B): Alginate-Barium encapsulated human islets on Day 1 (a); Day 5 (b); and Day 8 (c) post-encapsulation.

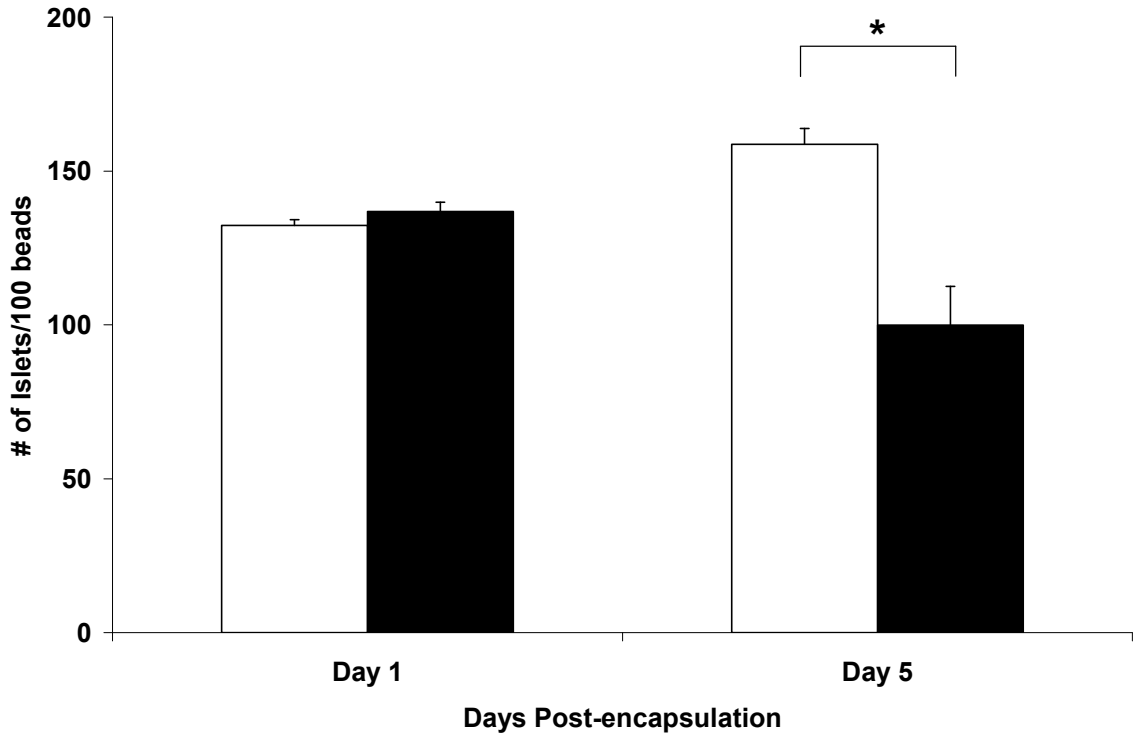


Figure 4-12. Assessment of human islet viability following encapsulation with Alginate-Barium (open bars) or XAlginate-PEG (solid bars), as measured using MTT. Values are presented as the number of rat islets per 100 beads. Data points represent the mean ± SD, for the MTT assay for 100 beads. *Significant differences ($p<0.05$) between XAlginate-PEG and Alginate-Barium encapsulated islets.

4.3.3 Insulin Response to Glucose Challenge

The cellular response to glucose of both rat and human islets was measured following XAlginate-PEG and Alginate-Barium microencapsulation via a glucose-stimulated insulin release (GSIR) assay *in vitro*. This functional assessment was performed day 1 and 5 post-encapsulation. For all experiments, the insulin stimulation from Basal 1 to Stimulated 1 to Basal 2 illustrated the expected response, where Stimulated 1 levels were higher than Basal 1, and Basal 2 levels were approximately the same level as Basal 1 (data not shown). The insulin stimulation index, calculated as the ratio between stimulated over basal insulin output during GSIR, for rat islets was 7.57 and 5.40 for XAlginate-PEG and Alginate-Barium on day 1, and 8.58 and 7.23 for

XAlginate-PEG and Alginate-Barium on day 5, respectively (Figure 4-11). There were no statistical differences observed between these indices for either polymer configuration or time point.

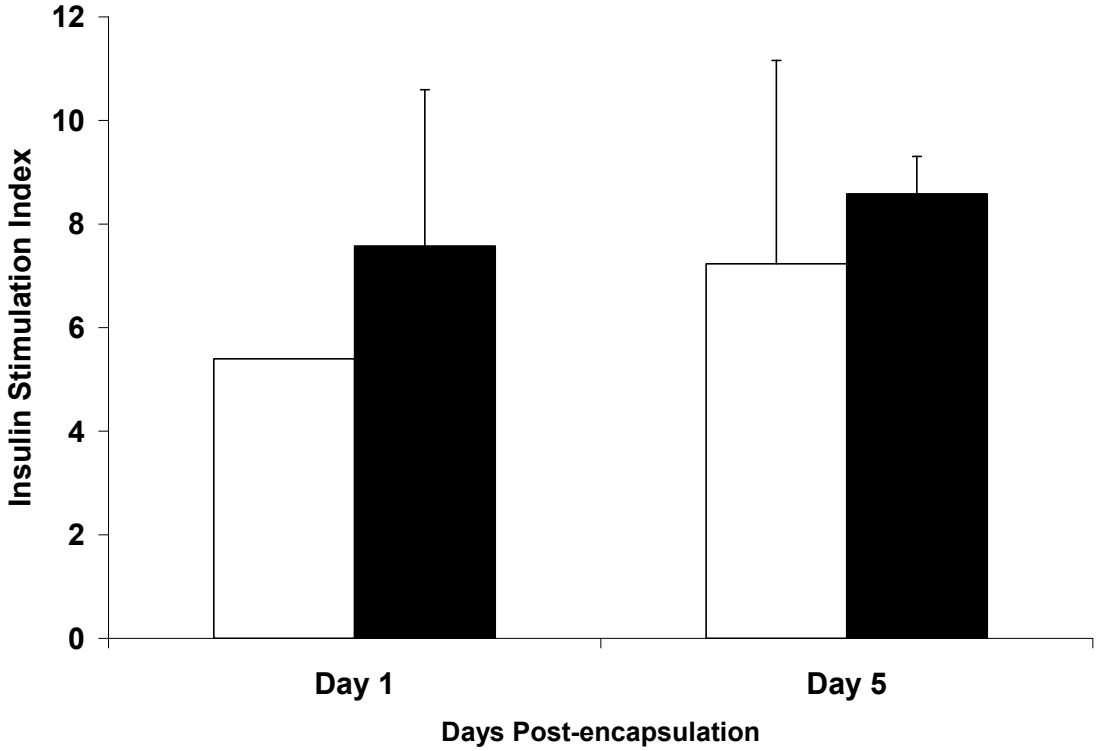


Figure 4-13. Insulin stimulation index of Alginate-Barium encapsulated (open bars) and XAlginate-PEG encapsulated (solid bars) Lewis rat islets. Stimulation indices were calculated, as described in the Methods section. Data points represent the mean ± SD, for a minimum of three independent measurements at each time point (except the Day 1: Alginate-Barium was duplicates).

In addition, the insulin stimulation index for the encapsulated human islets was 2.36 and 2.29 for XAlginate-PEG and Alginate-Barium on day 1, and 4.16 and 4.57 for XAlginate-PEG and Alginate-Barium on day 5, respectively (Figure 4-12). There were no statistical differences observed between the polymeric configurations.

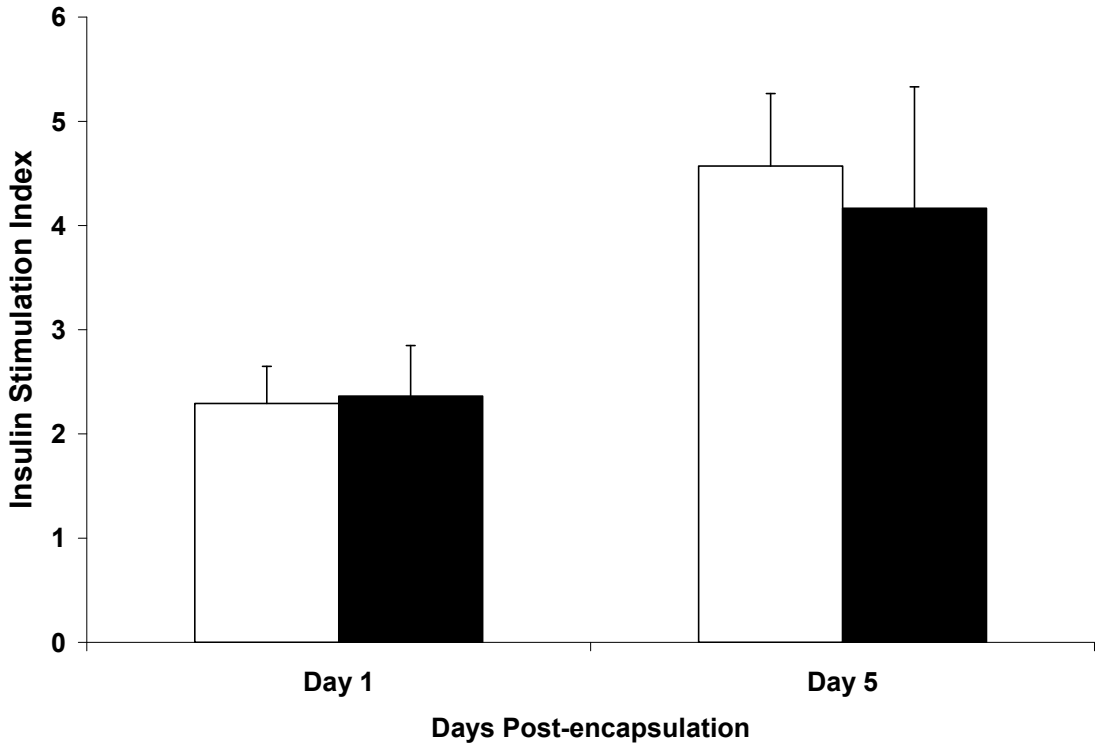


Figure 4-14. Insulin stimulation index of Alginate-Barium encapsulated (open bars) and XAlginate-PEG encapsulated (solid bars) human islets. Stimulation indices are calculated, as described in the Methods section. Data points represent the mean ± SD, for a minimum of three independent measurements at each time point.

4.4 Discussion

In this study, a novel covalently linked Alginate-PEG (XAlginate-PEG) microcapsule was evaluated. Covalent crosslinking within the microcapsule was achieved by modifying the carboxylic groups, residing on the alginate chain, with azides; the azides were bound, with a stable amide linkage, to phosphine-PEG molecules via the Staudinger ligation. Co-incubation of these two polymers **([1]** and **[5]**) resulted in stable gel formation, independent of ionic interactions, within 5 hours (see Appendix 7.1). While this covalent reaction is efficient when compared to alternative, catalyst-free ligation schemes, it is recognized that this delay might not be desirable for cellular encapsulation. To bypass this potential problem, we capitalized on the unique ability of

the functionalized alginate polymer **[1]** to form gels via both ionic and covalent interactions. The superiority of utilizing this method is the ability to instantaneously form a gel using standard ionic methods, followed by the "locking" of the gel form via the formation of covalent linkages between the modified alginate **[1]** and the PEG-diphosphine **[5]** over an extended time course, thereby resulting in a microcapsule of superior stability. Protocol optimization of bead formulations was performed by promoting covalent linkages between alginate and PEG polymers for multiple time periods prior to capsule formation via cationic solution (see Appendix 7.1). The balance of ionic and covalent interactions led to the fabrication of microbeads of exceptional stability and integrity.

The overall stability of the microcapsules was improved with the implementation of covalent linkages. The osmotic swelling of the novel XAlginate-PEG beads was examined by placing the beads in ion-free water, and comparing dimensional changes to traditional alginate beads. Empty, XAlginate-PEG beads exhibited significant less swelling when compared to traditional alginate (Alginate-Barium) beads. In addition, although both the Alginate-Barium and XAlginate-PEG samples containing cells swelled over time, the XAlginate-PEG capsules exhibited significantly less dimensional changes invoked by osmotic swelling. The minimization of swelling may impose less stress on the encapsulated islets, resulting in improved graft function and viability [65].

The incorporation of covalent linkages within the capsule also prevents the slow degradation of capsule integrity upon long-term culture *in vitro* and *in vivo*. The chemical stability of the XAlginate-PEG and Alginate-Barium capsules was examined using a strong chelating solution to simulate conditions of long-term culture, where the cations

slowly leak over time, resulting in a disruption of ionic interactions [166]. The empty, covalently linked capsules remained completely intact, exhibiting exceptional stability, upon exposure to the strong chelating solution, while the ionically bound capsules showed complete dissolution within two minutes of exposure. In addition, the XAlginate-PEG beads exhibited swelling upon exposure to the chelating solution, which was expected due to the removal of the ionic linkages used to initially form the capsule. Similar results were observed with capsules containing cells. Thus, a complete removal of ionic interactions leads to the complete loss of traditional alginate capsules, while the integrity and stability of the newly developed XAlginate-PEG capsules was maintained. These results prove not only the formation of covalent linkages between the **[1]** and **[5]** polymers, but also establish that these linkages are of adequate density to maintain the integrity of the capsule (see Appendix 7.1 for the results of other XAlginate-PEG configurations).

A highly chemoselective ligation scheme was developed for the covalent linkage of the two biocompatible polymers. The azide and phosphine groups readily interacted at a normal physiological pH within an aqueous environment, and exhibited no detrimental side effects on cellular function and viability. Previously developed chemo-ligation schemes, utilizing photopolymerization, have exhibited significant islet loss upon application [80]. The absence of damaging by-products in combination with the use of highly biocompatible polymers makes this reaction ideal for tissue engineering applications.

Detrimental effects on cellular viability or function with the introduction of covalent interactions was not evident. Cells entrapped within the gel matrix retained their

viability and function when in culture. The proliferating MIN6 cells were able to grow within the matrix, as demonstrated by the MTT results. Islets encapsulated in the XAlgiante-PEG beads appeared comparable to the standard microcapsules as seen with confocal images and at most time points in the MTT results. There was a disparity observed between rat and human islet viability on day 5 that poses a question of potential stress seen with long-term culture, and needs to be explored further. However, there was no discrepancy in islet function between the encapsulated rat and human islets.

The incorporation of covalent bonds within a microcapsule could prove to be highly beneficial to improve long-term stability *in vivo*. The shift from ionic to covalent linkages was shown to dramatically improve upon bead stability coupled with minimal degradation over time. This application alone may be shown to improve bead efficacy upon transplantation. In addition, the use of only highly biocompatible polymers should minimize the immune response to transplanted capsules.

The main drawback of this covalently linked microcapsule is the complexity of the polymer fabrication, and its potential difficulties for translation to large scale applications. However, superior bead stability along with highly biocompatible polymers offers significant advantage over traditional alginate capsules. Further exploration of the novel covalently linked capsule will include examining bead permeability, long-term stability, as well as a mechanical assessment and determination of *in vivo* efficacy.

CHAPTER V

Surface modification of pancreatic islets to attenuate the inflammatory response associated with islet transplantation

Summary

Transplanted islets prompt a strong inflammatory response where the cells are recognized by host immune cells as antigens, instigating detrimental cytokine secretion and immune cell proliferation leading to rejection. To prevent immune cell recognition as well as the associated inflammatory response at the time of implant, a polyethylene glycol (PEG) coating was applied directly onto the surface of human pancreatic islets. PEG was shown to dampen the activation of the coagulation cascade upon islet-blood contact *in vitro* using a prothrombin time test (PTT). To further evaluate the efficacy of a PEG coating, PEGylated islets were exposed to a proinflammatory environment. The proinflammatory environment was created by adding a common macrophagic cytokine, tumor necrosis factor (TNF-α), to the culture media. Islet response was initially monitored by reverse transcription polymerase chain reaction (RT-PCR) to observe the tissue factor (TF) and thrombomodulin (THB) activity of human islet cells upon cytokine exposure. Results showed a general trend in upregulation of both TF and TM. Two functional assays were developed to further explore the inflammatory response, where the TF expressed on the islet's surface was used for a generation of activated factor X (afX), and TM was used to generate activated Protein C (aPC). Uncoated islets showed a 5-fold increase in afX generation when exposed to a pro-inflammatory environment via TNF-α exposure, while TNF-α exposure to PEGylated islets exhibited no statistically significant increases. The present results show promise in the ability of PEG to dampen coagulation

and mask inflammatory mediators exposed on the surface of islets, which may be of assistance in reducing islet cell immunogenicity and favor engraftment *in vivo*.

5.1 Overview

Clinical islet transplantation (CIT) has been shown to be a promising cellular therapy for the treatment of T1DM. However, significant islet loss and impaired function results from the strong host inflammatory and immunological response to the transplant [9]. Activated immune cells secrete various cytokines and cytotoxic molecules, inducing structural and functional damage to the islets [167-169]. Interleukin (IL)-1 activates T cells, which has been shown to increase IL-2 and its receptor production, contributing to the amplification of the immune response leading to rejection [170, 171]. Tumor necrosis factor (TNF-α) has been shown to contribute to graft rejection by increasing the molecule adhesion onto grafted tissue [172]. In addition, co-activation of the coagulation and complement systems is instigated by tissue factor and extracellular matrix presence on the islet surface, defined as an *instant blood-mediated inflammatory reaction*, or IBMIR [30].

IBMIR was initially described by Bennet et al. as the co-activation of the coagulation and complement cascades as well as the rapid adhesion of activated platelets to the surface of the islet following direct contact with ABO blood [28]. Tissue factor (TF), a transmembrane protein present in subendothelial tissue, platelets, and leukocytes, is the primary instigator of coagulation cascade. TF operates as a receptor for the cleavage of factor VII to factor VIIa (fVIIa) [32, 33]. TF and fVIIa represent the extrinsic pathway of coagulation in which an inactive protease factor X is converted into the active protease factor Xa [32, 33]. The Xase complex results in a combination of fXa with

factor Va which initiates the prothrombinase complex. The prothrombinase complex is responsible for the rapid conversion of prothrombin to thrombin. In addition, the presence of thrombin activates cofactors V and VIII which form a positive feedback system to continuously generate thrombin [34]. Increased thrombin production leads to the unrestrained formation of a fibrin clot.

An integral component of the anti-coagulant pathway is thrombomodulin (TM), a membrane glycoprotein. TM binds to thrombin to form a TM-thrombin anticoagulant complex. This complex activates circulating protein C (PC). PC is a vitamin K dependent precursor of serine protease, and activated Protein C (aPC) inactivates the cofactors Va and VIIIa involved in thrombin generation [38-40]. Thus, a negative feedback system, instigated by aPC, is used to regulate thrombin generation.

Surface modification of pancreatic islets using poly(ethylene glycol) (PEG) has been investigated as a means of camouflaging transplanted islets from the immune system. PEG polymers have been shown to be stable, biocompatible, non-adhesive, and highly soluble molecules within biological environments [87, 88]. The direct attachment of the polymer to the surface of transplanted tissue can possibly mask surface antigens and adhesive ligands on the islet surface to prevent surface thrombosis. Furthermore, a thin nanoscale layering on the surface allows for improved diffusivity of nutrients, and the reduced volume of the immunoprotective barrier makes this method a viable option for portal vein infusion [120].

The technique, termed PEGylation, involves tethering of a biocompatible PEG polymer to the collagen matrix of the islets surface resulting in a conformal coating of the cell. The terminal hydroxyl group of PEG is connected to a chemical linker, such as a

succinimidyl propionate. The hydroxyl group is oxidized with succinic anhydride followed by activation with N-hydroxysuccinimide (NHS) [9]. The activated PEG can then be conjugated to the amines of the cell surface to form a conformal coating. This method originated with applications to red blood cells [109-111], and it has since been applied to immune cells [110].

In this study, we sought to characterize PEG coatings on the islet surface, as well as evaluate the ability of PEGylation to dampen the overall inflammatory response of islets. To accomplish this objective, we evaluated the effects of molecular weight on PEG coating and the effect of the PEG coating on islet viability and function. To assess the inflammatory profile of PEGylated islets, inflammatory assays were used to evaluate the capacity of the islet surface to instigate both inflammatory and anti-inflammatory pathways. Finally, the PEGylated islets' inflammatory response to both standard and pro-inflammatory culture conditions was evaluated.

5.2 Materials and Methods

5.2.1 Cell Culture

Human pancreatic islets of Langerhans were obtained via the Islet Consortium Network (NIH ICR). Following isolation, islets were cultured MM1 media supplemented with 10% fetal bovine serum (FBS; Sigma), 1% penicillin-streptomycin, and 1% L-glutamine for 24hrs following arrival at 37°C in a humidified atmosphere of 5% CO_2 in air prior to encapsulation.

5.2.2 PEG-grafting

To modify the collagen capsule of the islet with PEG, the islets were washed twice with PBS, followed by suspension in a 11mM glucose solution containing 4 mM

N-hydroxysuccinimidyl-poly (ethylene glycol)-fluorescein isothiocyanate (NHS-PEG-FITC) (Carbomer, Inc. MW 5000 or MW 600). The suspension was incubated at 37°C in humidified air with 5% CO_2 for 1h. The PEGylated islets were washed once with PBS and once with culture medium, followed by a 1 day culture.

5.2.3 Confocal microscopy of PEGylated islets

A confocal microscope (Zeiss, Germany) was used to observe coating uniformity of PEGylated islets tagged with a FITC label. Three-dimensional (3D) images were reconstructed using projection function on LSM image browser (Zeiss, Germany) software.

5.2.4 Measurement of Insulin Secretion

Functional assessment of PEGylated islets was tested via a glucose-stimulated insulin release (GSIR) assay performed using a static column method. Approximately 100 IEQs of PEGylated islets were incubated within microchromatography columns (Biorad) in triplicates with 0.3 mL, final volume, basal glucose solution (Krebs buffer: 99 mM $NaCl_2$, 5 mM KCl, 1.2 mM KH_2PO_4,1.2 mM $MgSO_4$, 2.6 mM $CaCl_2$, supplemented with 60mg/dL glucose) for 1hr, followed by washing with 4mL of basal solution. The columns were then incubated for 1hr in the basal solution, and samples were then collected (Basal 1). After collection, a stimulated glucose solution (KRBB supplemented with 300mg/dL glucose), was added and the samples were incubated for 1hr. High glucose samples were collected (Stimulated 1) by adding 1mL of basal glucose solution to each column. An additional 4mL of basal solution was added to each column, eluted to 0.3mL final volume, and incubated for 1hr. The final samples (Basal 2) were collected. All insulin samples were frozen in -80°C until ELISA (Mercodia) analysis.

Insulin concentration within the samples was quantified by following the manufacture's instructions. GSIR was evaluated as ratio of the level of insulin secretion during Stimulated 1 to the level of insulin secretion during Basal 1.

5.2.5 Prothrombin Time Test (PTT)

The general inflammatory response of human islets was monitored using a prothrombin time test (PTT). This test measured the time it takes for thrombin to cleave fibrinogen to form a fibrin clot, and is monitored with a shift in absorbance over time. Islet cells were spin washed with PBS two times, and aliquoted in 250 IEQs per group. The islets were re-suspended in human plasma supplemented with sodium citrate (Diagnostica Stago), pentuplicates (50 IEQ) were placed in a 96 well plate, 50µL of 25 mM $CaCl_2$ was added to each well, and the optical densities were measured every 10min for 60min at 405nm in a microplate reader (Molecular Devices). Islet cell counts were performed prior to encapsulation using the islet equivalence (IEQ) method.

5.2.6 Tumor Necrosis factor (TNF-α) Exposure

TNF-α (Chemicon International) was initially used at 20U and 10U of activity for 2-4hrs to determine appropriate concentrations and exposure time for response induction without cell death. Final parameters for evaluation of PEGylated and control islets were 10U of activity of TNF-α at a 3hr exposure time.

5.2.7 Activated factor X (afX) and activated Protein C (aPC) assay

PEGylated and naked islets of the experimental group (exposed to TNF-α) and control group were washed twice with PBS. Islet cluster cell counts were performed using the islet equivalence (IEQ) method. Triplicates of approximately 100IEQ for afX (or 500IEQ for aPC) were placed in a 24 well plate with final concentration of 10 nM

fVIIa (or 1 nM α-thrombin for aPC; Haematologic Technologies) and 5 mM of $CaCl_2$ in a Tris-BSA buffered solution (final 20mM Tris-HCl, 100nM NaCl, 0.1 wt% BSA, 5mM $CaCl_2$, and 0.02% NaN_3), and incubated at 37°C in humidified air with 5% CO_2 for 10 min. A final concentration of 160nM fX (or 1μM PC; Haematologic Technologies) was then added to each well and incubated at 37°C in humidified air with 5% CO_2 for 1h. A stop solution (18μL EDTA for afX assay or antithrombin III for aPC assay; American Diagnostica) was then added to each well and incubated for 5min. The well contents were collected and spun down. Triplicates of 30μL of supernatant were placed in a 96 well plate. 270μL of 2.5μM afX chromogenic substrate solution (or 5μM aPC chromogenic substrate solution; American Diagnostica) was added to each well. The optical densities were measured at 405nm in a microplate reader (Molecular Devices).

5.2.8 Statistical Analysis

All statistical analysis performed throughout this study utilized a paired student t-test with significant differences represented by a p-value less than 0.05.

5.3 Results

5.3.1 Analysis of PEGylated islets

The attachment of PEG to the collagen matrix of the islet surface was trialed with two different polymer lengths, a short (0.6 kDa) and a long (5 kDa) PEG chain, both labeled with FITC on one terminal end. The short PEG was shown to infiltrate into the islet and disrupt islet morphology (Figure 5-1a). On the other hand, the long PEG was grafted directly onto the islet capsule in a uniform coating, while maintaining surface morphology similar to that of free islets (Figure 5-1b).

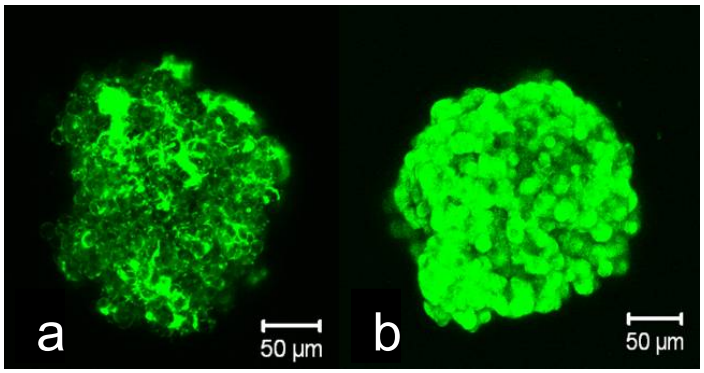


Figure 5-1. Confocal images of PEGylated human islets with multiple PEG lengths: (a) 0.6 kDa NHS-PEG-FITC and (b) 5 kDa NHS-PEG-FITC (see Methods for details).

The insulin secretion by PEGylated islets was assessed after static incubation. Islet stimulation index values were not significantly different between cultured and short or long PEGylated islets (Figure 5-2).

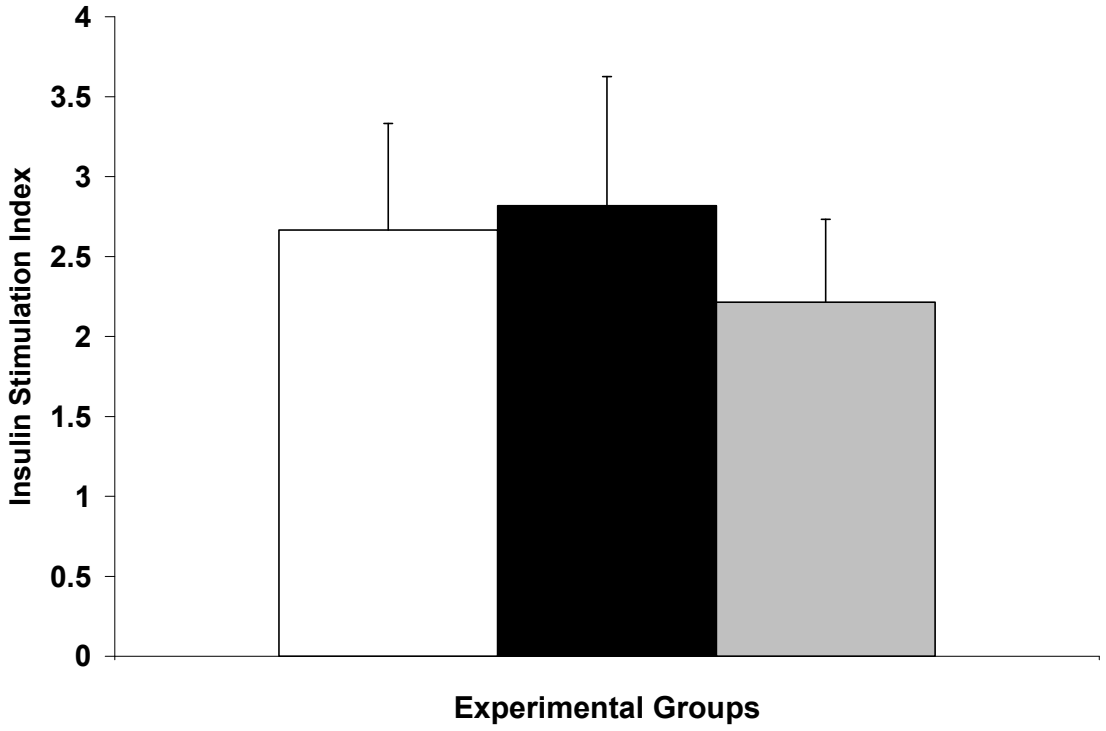


Figure 5-2. Insulin stimulation index of cultured (open bar), long PEGylated (solid bar) and short PEGylated (gray bar) human islets. Stimulation assays are conducted and indices are calculated as described in the Methods section. Data points represent the mean ± SD, for a minimum of three independent measurements at each time point.

5.3.2 PEG capabilities to inflammatory response

After treating islets with a 5 kDa PEG coating, the overall inflammatory response was evaluated using a prothrombin time test (PTT). The ½ max time (defined as clot time) was 7.5 and 9.5 minutes for unmodified and PEGylated islets (Figure 5-3), respectively, showing a significant difference between the coagulation time of the two groups.

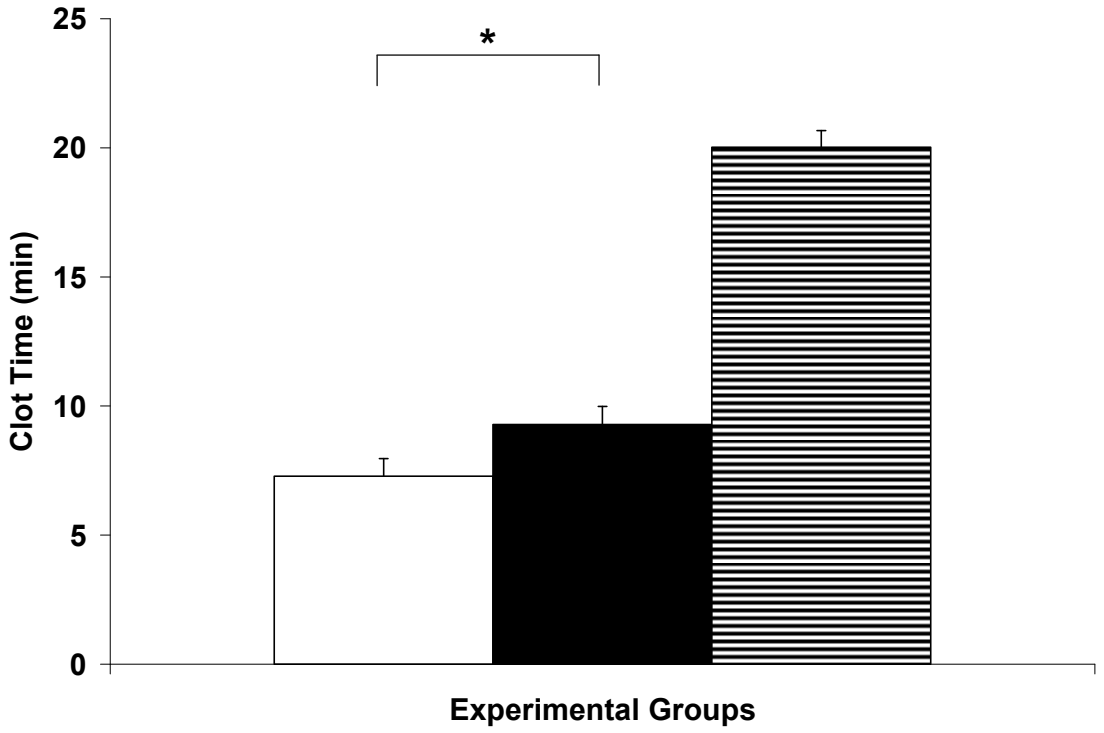


Figure 5-3. ½ Max Time, defined as Clot Time, of human plasma (striped bar), cultured (open bar) and PEGylated (solid bar) human islets. Data points represent the mean ± SD, for a minimum of three independent measurements at each time point. *Significant differences ($p<0.05$) between PEGylated and unmodified islets.

A proinflammatory environment was developed *in vitro* by using the cytokine tumor necrosis factor (TNF-α). To establish appropriate conditions to induce an inflammatory response while preventing cell apoptosis, two levels of TNF-α, 20U and 10U of activity, were cultured with naked islets for 2h or 4h. A PTT was used to observe the overall

inflammatory response to these culture conditions. The results showed a significant decrease in the time to clot with islets exposed to both 10U and 20U of TNF-α over a 4h time period (Figure 5-4). However, cell loss was observed with a 4h exposure time. Thus, the final parameters used in the remainder of the experiments were 10U of activity at a 3hr exposure time.

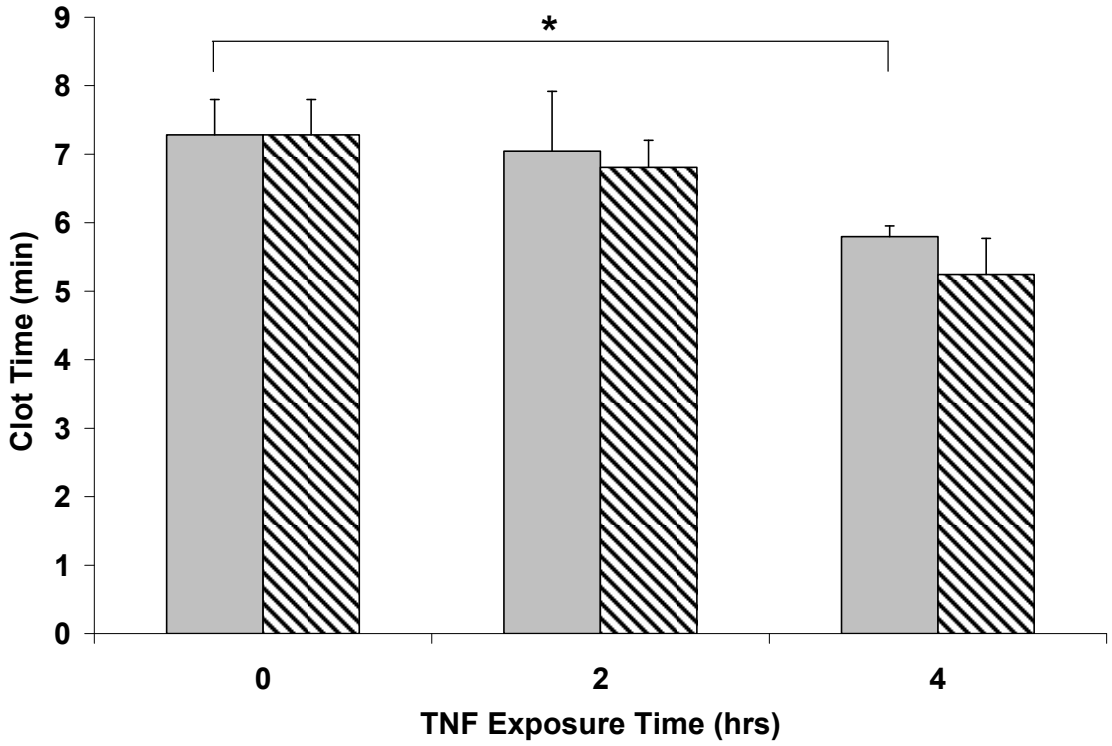


Figure 5-4. ½ Max Time, defined as Clot Time, of cultured human islets exposed to 10U (gray bars) and 20U (open bars) of TNF-α. Data points represent the mean ± SD, for a minimum of three independent measurements at each time point. *Significant differences ($p<0.05$) between unexposed and 10U TNF-α for 4hrs exposed islets.

Islet culture with TNF-α induced the expression of tissue factor (TF), a major component of coagulation, and thrombomodulin (TM), an anticoagulant controller, and was initially monitored via RT-PCR. The general trends observed showed an upregulation of both TF and TM upon TNF-α exposure (Data not shown). For a more concrete evaluation, two functional assays were tailored for monitoring coagulation and

anticoagulation activity of islets exposed to TNF-α. An activated factor X (afX) assay utilized the TF expressed on the islet surface to generate production of afX. Both PEGylated and control, or unmodified, islets were evaluated (Figure 5-5). Unmodified islets exhibited a 5-fold increase in afX generation when exposed to TNF-α. PEGylated islets were found to result in no statistical increase in afX generation when exposed to TNF-α. Comparing TNF-α treated PEGylated to non-PEGylated islets found a 2-fold increase in afX induction with the non-PEGylated islets.

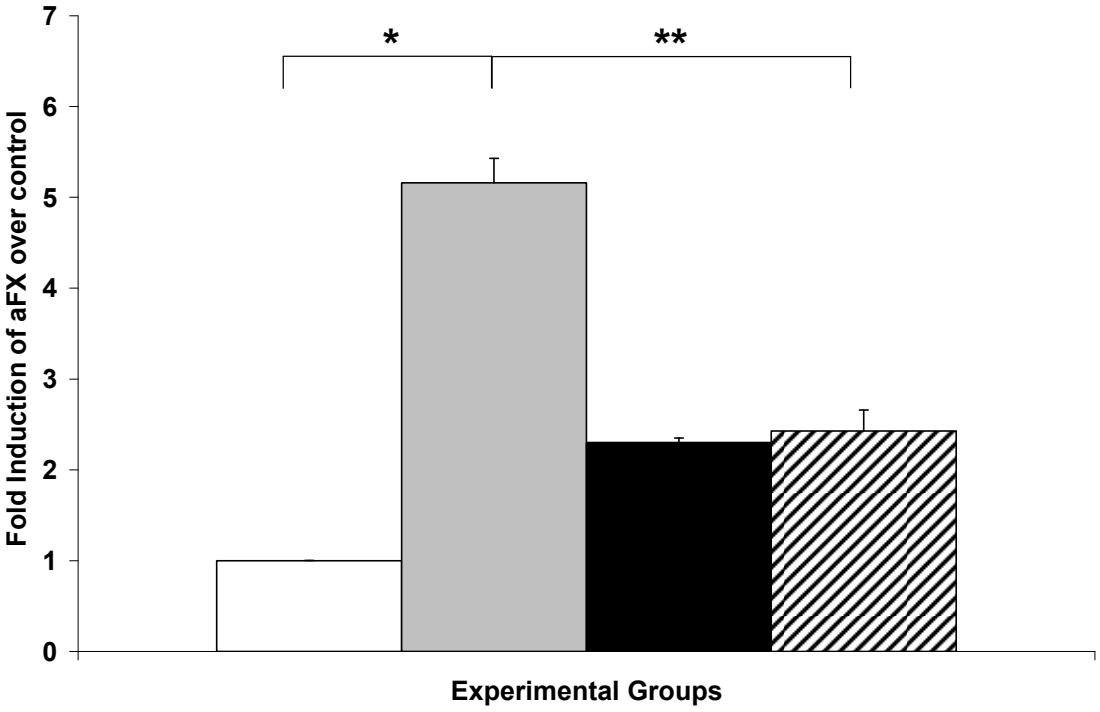


Figure 5-5. Fold induction of afX generation over control, or cultured human islets (HI) (open bar), of cultured HI treated with TNF-α (gray bar), PEGylated HI (solid bar), and PEGylated HI treated with TNF-α (striped bar). Data points represent the mean ± SD, for a minimum of three independent measurements at each time point. *Significant differences ($p<0.05$) between cultured and PEGylated islets.

An activated Protein C (aPC) assay evaluated the ability of TM expressed on the islet surface to generate production of aPC. Preliminary experiments showed a significant

increase in aPC generation of islets cultured with TNF- α at 2, 3, and 4hrs over control islets (Figure 5-6). PEGylated islets were not yet evaluated using this assay.

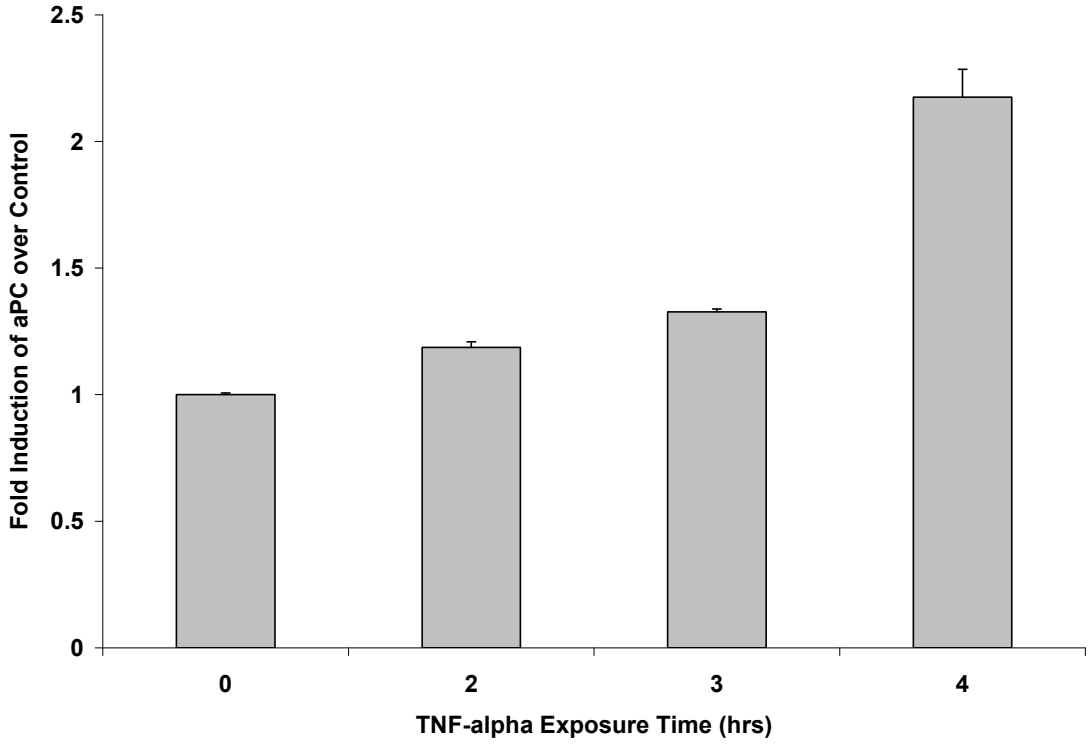


Figure 5-6. Fold induction of aPC generation over control, or cultured human islets (HI) at 0h TNF-α, at 2, 3 and 4h TNF-α exposure times. Data points represent the mean ± SD, for a minimum of three independent measurements at each time point. *Significant differences ($p<0.05$) between cultured and TNF-α treated islets (2, 3 and 4h).

5.4 Discussion

In this study, a polyethylene glycol (PEG) polymer was covalently bound to the amine groups present on the collagen capsule of islets with a stable amide linkage. The intention of applying the polymer coating was to establish controls for future nano-scale applications, which would contain additional layers of polymers over the basic single PEG layer. In addition, we sought to evaluate the ability of the single PEG layer to attenuate the inflammatory response commonly associated with islet transplantation. The length of the PEG chain was shown to be a significant factor when evaluating coating

efficacy. A shorter chain (0.6kD) infiltrated the islet capsule and exhibited a disruption in islet morphology, while a longer chain (5kD) PEG attached directly to the islet capsule to form a uniform polymer coating with minimal change to islet morphology. Notable, application of both a short and long PEG did not exhibit an impairment of islets' ability to function. Due to the uniformity of the long PEG coating on the outer surface of the islet, it was the only polymer length used for subsequent testing.

Ideally, the PEG coating would completely mask the inflammatory agents present on the islet surface, which in turn would reduce islet immunogenicity in the host. A delay or total prevention of coagulation may prove to be beneficial in enhancing islet engraftment. Tissue factor (TF) [173], the initiator of the extrinsic coagulation cascade, and thrombomodulin (TM) [174], a natural anticoagulant, have been shown to be expressed by both islet endocrine cells and vascular endothelial cells within islet tissue. In this study, PEGylation of islets was shown to dampen the activation of the coagulation cascade when compared to unmodified islets.

Additionally, an evaluation of islet response to proinflammatory conditions by culturing the islets at a low-dose, controlled TNF-α exposure was performed. Initially, exposure parameters were established to generate an inflammatory response, while minimizing the induction of cell apoptosis. It has been demonstrated that endothelial cell exposure to proinflammatory agents, such as TNF-α, upregulates TF and downregulates TM. Interestingly, preliminary data indicates that islets exposed to TNF-α exhibited an upregulation of both TF and TM (data not shown).

A closer evaluation of TF and TM presence on the islet surface was evaluated with two functional assays, newly tailored for islets. They measured the amount of

activated factor X (afX) and activated Protein C (aPC) generation instigated by the TF and TM present on the surface of the islet. An increased afX and aPC generation was observed upon exposing cultured islets to TNF-α. The ability of a PEG coating to mask this presence of TF on the islet surface was evaluated. PEG was shown to block the surface expression of TF with a decreased generation of afX. Due to limitations in human islet availability, aPC assessment of PEGylated islets was not included in this study, and will be explored in the future.

In summary, our preliminary data demonstrated the feasibility of establishing PEG conformal coatings and will allow for an easier transition into future nano-scale multilayering applications. Additionally, surface modification with a single layer, PEG polymer was shown to dampen activation of coagulation and minimize the generation of afX by masking the TF expressed on the surface of the islet. Further study involving the long-term assessment of PEG in culture as well as functional assay assessment upon immune cell co-incubation will improve our understanding of PEG coating efficacy.

CHAPTER VI

Thesis Discussion

Encapsulation of insulin-producing islet cells in semi-permeable polymers has the potential to improving existing outcomes for the treatment of T1DM using cell based therapies. The primary focus of this thesis was to investigate different encapsulation techniques for the immunoisolation of pancreatic islet cells to be used for transplantation. Two distinct studies were performed. The first study examined a novel, covalently linked microcapsule, and the second study evaluated a nano-scale encapsulation technique and its efficacy in attenuating an inflammatory response. The common theme within this thesis is the incorporation of covalent linkages within encapsulation applications.

The covalently linked alginate-PEG microcapsule showed superior stability over traditional alginate capsules. This was demonstrated through decreased swelling and the bead's ability to remain intact upon removal of ionic bonds. Islet clusters were shown to be adequately encapsulated and homogenously distributed throughout the capsule. In addition, proliferating cells were able to grow within the gel matrix, while quiescent cells remained undisturbed and viable. Both rat and human islets were shown to function *in vitro* during a glucose challenge. Thus, this newly developed microcapsule fosters much potential for future cellular encapsulation applications. In addition, the establishment of a highly selective chemoligation scheme of two highly biocompatible polymers may be translated to a variety of cellular applications, including nano-scale multilayering encapsulation techniques.

The second study focused on tethering a PEG polymer directly to the surface of the islet to establish a nano-scale protective barrier. The length of the polymer chain was

shown to be of great importance, where a short chained PEG infiltrated the islet resulting in a disruption of islet morphology. Conversely, treatment with a longer chained PEG resulted in a uniform polymer coating on the surface of the islet with minimal disruption in islet morphology. In addition, the PEGylated islets exhibited no functional deviations when compared to unmodified islets. These findings will serve as baseline controls and set the stage for future nano-scale applications. The evaluation of PEG's ability to mask the inflammatory response of islets was also investigated. Our findings showed a single layer of a PEG polymer has the capacity to dampen blood coagulation and minimize the generation of afX by masking the TF expressed on the surface of the islet.

In summary, this thesis worked to cultivate novel encapsulation techniques as well as establish new approaches for assessing the inflammatory response of islets. These techniques may significantly improve transplant efficacy by reducing or completely eliminating the need for immunosuppressive therapy, for allogenic and/or xenogenic cellular grafts. Furthermore, the technologies evaluated in this thesis are generalized for all cell types, thereby permitting translation of techniques to alternative therapies.

Appendix

Preliminary assessment of gel formation

Table A-1. Pre-incubation time frames and ratios of XAlginate-PEG gel formation.

Ratio Azide-Alg[1] to 2DiP-PEG[5]	Incubation Time	Results
1:1.9	24h	Strongly Gelled (> 1:1.7)
	5h	Gelled (> 1:1.7)
	4h	Partially Gelled
	2h	Crosslinking, Viscous Solution, Unable to form microcapsules
	1h15min	Crosslinking, Pliable Solution, Able to form microcapsules
1:1.7	24h	Strongly Gelled
	5h	Gelled
	4h	Partially Gelled
	2h	Crosslinking, Viscous Solution, Unable to form microcapsules
	1h15min	Crosslinking, Pliable Solution Able to form microcapsules

Table A-2. Alginate comparisons based on varying the degree of modification and studies performed.

Alginate Type	Degree of Modification 1-5 (5 is highly modified)	Results	Studies	Notes
K1A94 K1B101 K1B103 K1A105 K1B106	5	**2.5% 1:1.9 Microcapsules** • MIN6 Cells • Rat Islets • Human Islets • Empty **Macrocapsules Spaghetti**	**MIN6 Cells** • Live/Dead – Day 1, 5, 8 • MTT – Day 1, 5 • ddH_2O Swelling • EDTA Exposure **Lewis Rat islets** • Live/Dead – Day 1, 5, 8 • MTT – Day 1, 5 • ISR – Day 1, 5 **Human islets** • Live/Dead – Day 1, 5, 8 • MTT – Day 1, 5 • ISR – Day 1, 5 **Empty Capsules** • ddH_2O Swelling • EDTA Exposure	Solution thickens upon mixing
K1B94	4	**2.5% 1:1.9 Spaghetti** • MIN6 Cells	**MIN6** • Live/Dead – Day 1 • ddH_2O Swelling • EDTA Exposure	Increased swelling observed with lower modifica-tion
K1A55 K1A96 K1A98 K1B98	3	**2.5% 1:1.9 Macrocapsules** • MIN6 • Empty	**MIN6** • Live/Dead – Day 1, 5, 8 • ddH_2O Swelling • EDTA Exposure **Empty Capsules** • ddH_2O Swelling • EDTA Exposure	Increased swelling observed with lower modifica-tion
K1A95	2	Not used	N/A	N/A

MTT MIN6 and Islet Standard Curves

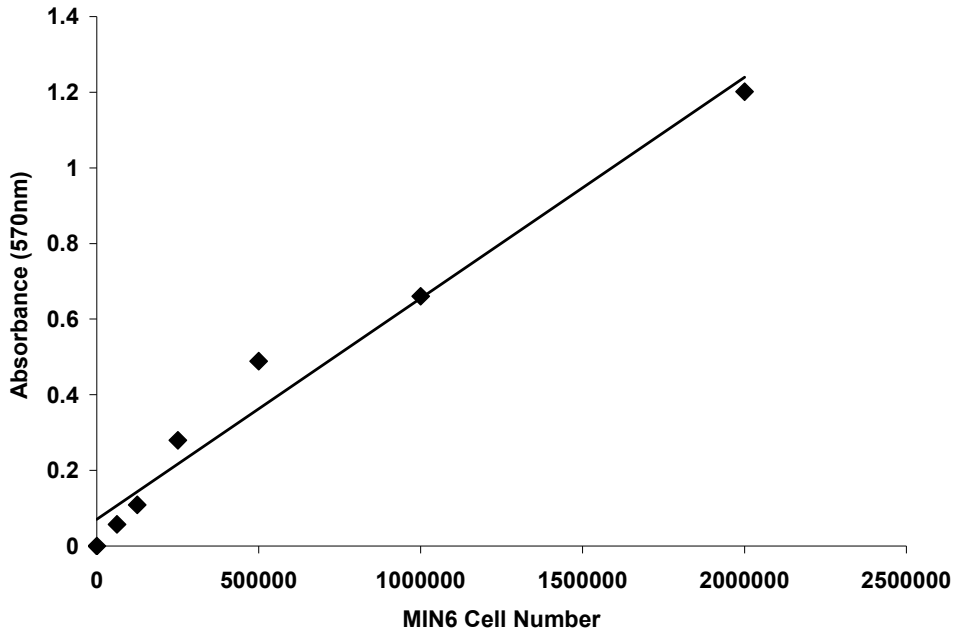


Figure A-1. MTT Standard curve of cultured MIN6 cells.

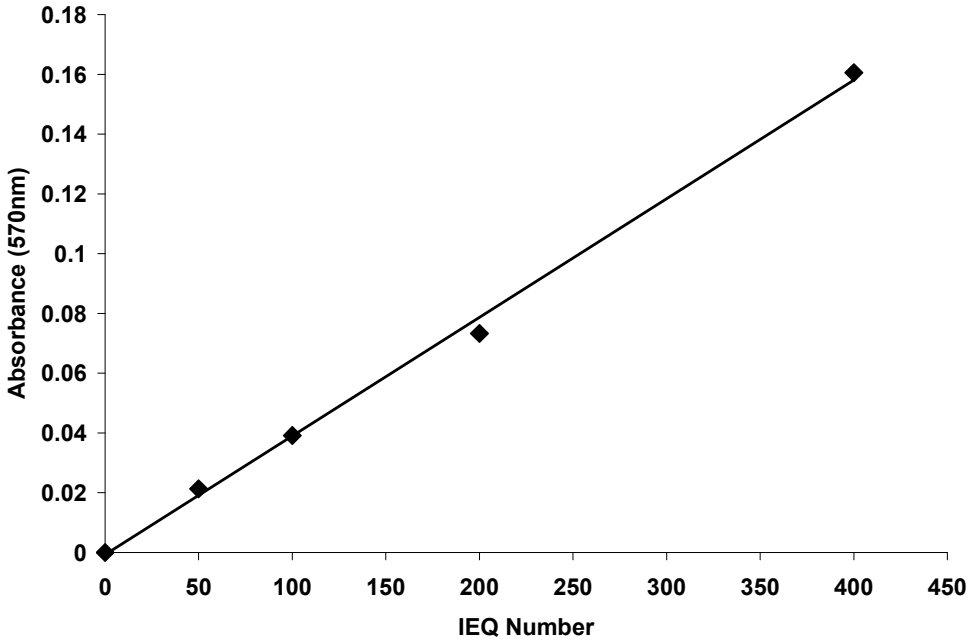


Figure A-2. MTT Standard curve of cultured Lewis rat islets.

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