

Design of Biomimetic Extracellular Matrix to Improve Pancreatic Islet Engraftment

by

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This is to certify that I have examined this copy of a doctoral dissertation by

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*To my family
and to my grandmother Cemile,*

ABSTRACT

Pancreatic islet transplantation has emerged as a promising treatment for type 1 diabetes (T1D) and engraftment of beta cells after transplantation determines the success of clinical setting. However, clinical application of islet transplantation is still limited by life-long use of immunosuppressive drugs and insufficient number of islets to achieve normoglycemia. In this study, we tailored biochemical and biophysical properties of hydrogels to promote insulin secretion function and immunoregulatory potential through incorporation of mesenchymal stem cells (MSCs) and natural extracellular matrix mimetic peptides such as RGDS, IKVAV and insulintropic peptide (GLP-1). Co-encapsulation of islets with MSCs and/or peptides contributed to significant increases in insulin secretion compared to control. Although deleterious effects of cytokines were not completely inhibited, we observed that protection against pro-inflammatory cytokines can be achieved in free and PEG hydrogel encapsulated MIN6-MSC heterospheroids. To tailor biophysical properties of islet microenvironment, we developed unique type of nano-thin coating for insulin secreting beta cell aggregates. These aggregates which were prepared as pseudoislets through hanging drop method were coated with sequential layers of nanogels using physiologically compatible medium without toxic prepolymer solutions. These coated pseudoislets were determined as viable and functional for insulin secretion. We also confirmed *in vivo* biocompatibility of CHPOA nanogels through subcutaneous transplantation into CD1 mouse. This study is promising and offers new opportunities through coating of insulin secreting islets with advanced functional materials under completely physiological conditions and will contribute to longer functional islets for clinical translation of cell transplantation technology and particularly for the treatment of T1D.

ÖZET

Pankreatik adacık nakli, tip 1 diyabet (T1D) için umut verici bir tedavi olarak geliştirilmiştir ve beta hücrelerinin nakil sonrası vücut tarafından kabul edilmesi klinik terapini başarısını belirlemektedir. Fakat adacık nakli klinik uygulanması hala ömür boyu immünosupresif ilaç kullanımı ve normoglisemiye ulaşmak için gerekli olan adacık sayısının yetersiz oluşu ile sınırlıdır. Bu çalışmada, insülin salgılama fonksiyonu ve immüno-regülatör potansiyeli desteklemek için mesenkimal kök hücre (MSC) ve RGDS ile IKVAV gibi doğal hücre dışı ortam taklidi peptitler ve insülinotropik peptit (GLP-1) ekleyerek hidrojellerin biyokimyasal ve biyofiziksel özelliklerini uygun hale getirdik. Adacıkların MSCler ve/veya peptitlerle birlikte enkapsüle edilmesi kontrole kıyasla insülin salgılamada belirgin artışlara katkıda bulunmuştur. Sitokinlerin tüm zararlı etkileri tamamen önlenemese de serbest ve PEG hidrojel içinde olan MIN6-MSC hücre kümelerinde proinflamatuar sitokinlere karşı koruma gözlemledik. Adacık mikro-ortamının biyofiziksel özelliklerini uygun hale getirmek için insülin salgılayan beta hücre kümeleri için özgün nano-kalınlıkta kaplama yöntemi geliştirdik. Sallanan damlacık yöntemi ile yalancı adacık olarak hazırlanan bu hücre kümeleri toksik polimer öncesi solüsyonlar kullanılmadan fizyolojik olarak uyumlu ortam kullanılarak art arda nanojel katmaları ile kaplanmıştır. Kaplanan bu yalancı adacıkların canlı olduğu ve insülin salgılamak için fonksiyonel olduğu belirlenmiştir. Ek olarak CHPOA nanojellerin biyoyumluluğunu CD1 farelerde subkütan nakil ile teyit ettik. Bu çalışma ümit vericidir ve tamamen fizyolojik koşullar altında ileri düzeydeki fonksiyonel malzemeler ile insülin salgılayan adacıkların kaplanmasında yeni olanaklar sunmaktadır ve hücre nakli teknolojisinin klinik uygulaması ve özellikle T1D tedavisi için daha uzun süre fonksiyon gösteren adacıklara katkıda bulunacaktır.

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NOMENCLATURE

Ang-1	Angiopoietin-1
APC	Antigen presenting cell
bFGF	Basic fibroblast growth factor
CCK	Cholecystokinin
CHP	Cholesterol bearing pullulan
CHPOA	Acrylate modified cholesterol bearing pullulan
CNT	Carbon nanotube
CSII	Continuous subcutaneous insulin infusion
DAH	Differential adhesion hypothesis
DITH	Differential interfacial tension hypothesis
DM	Diabetes mellitus
EC	Endothelial cell
ECM	Extracellular matrix
EPAC-2	Exchange protein activated by cAMP-2
FDA	Fluorescein diacetate
FE-SEM	Field-emission scanning electron microscopy
GIP	Glucose-dependent insulintropic peptide
GLP-1	Glucagon like peptide-1
GLUT2	Glucose transporter 2
GPCR	G-protein coupled receptor
GSIS	Glucose stimulated insulin secretion
HGF	Hepatocyte growth factor
HUVEC	Human umbilical vein endothelial cells
IBMIR	Instant blood mediated inflammatory reaction
IDO	Indoleamine 2,3-dioxygenase
IFN- γ	Interferon- γ

IL	Interleukin
IL1- β	Interleukin 1- β
KRB	Krebs Ringer Bicarbonate
LFA-1	Leukocyte function associated antigen-1
MAEC	Mouse aortic endothelial cells
MEK	Mitogen-activated protein kinase kinase
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cell
mTOR	Mechanistic target of rapamycin
NVP	N-vinyl-pyrrolidone
PAMAM	Pol (amidoamine)
PDGF-BB	Platelet-derived growth factor-BB
PEG	Poly(ethylene glycol)
PEGDA	Poly (ethylene glycol) diacrylate
PI	Propidium iodide
PI-3 kinase	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLG	Poly (lactide-co-glycolide)
PVA	Poly(vinyl alcohol)
SIS	Small intestinal submucosa
SMC	Smooth muscle cell
STZ	Streptozotocin
SVA	Succinimidyl valerate ester
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TEA	Triethanolamine
TGF- β	Transforming growth factor- β
TNF- α	Tumor necrosis factor- α
VDCC	Voltage dependent Ca^{2+} channel
VEGF	Vascular endothelial growth factor
VIP	Vasoactive intestinal peptide

Chapter 1

INTRODUCTION

Tissue/organ transplantation is a mainstay in clinics for a severe number of organ failure. Based on Global Observatory on Donation and Transplantation data in 2014, 119 873 organs were transplanted worldwide corresponding to $\leq 10\%$ of global need [1]. However, rejection and tissue/organ donation continue to be major troubles for many patients. For the ones having transplantation, serious immunosuppression is required, but current immunosuppression technologies still need to be improved to address challenges related to serious systemic side effects. Diabetes mellitus (DM) is one of such diseases requiring beta cell transplantation for patients suffering from severe hyperglycemia [2].

DM is a chronic disease of pancreas, the insulin secreting organ of the body. Typically, loss of insulin secretion or function drives occurrence of DM [3]. Currently, number of diabetic patients has reached to 415 million with prevalence of 1 in 11 adults. It was also reported that 5 million patients lost their lives due to diabetes in 2015 corresponding to 1 death in every 6 seconds. In the next few decades, it is estimated that the patient number will increase up to 35% to 642 million and parallel to such devastating change, healthcare expenditure will rise 19% globally [4].

In type 1 diabetes (T1D), autoimmune subgroup of DM, pancreatic insulin secreting beta cell destruction leads to insulin deficiency. Lack of this hormone impairs glucose balance and causes hyperglycemia. To control hyperglycemia, multiple doses of insulin injection or pancreas/pancreatic islet transplantation are applied depending on severity of disease [5]. However, after transplantation, heavy immune attack limits the success of the graft and points out necessity of immune control. Immunosuppressive drugs have been established as an essential therapy to sustain islet graft survival and this often includes sirolimus and tacrolimus [2]. However, these immunosuppressive drugs have serious

adverse effects on transplanted beta cells and whole body. For example, tacrolimus, an inhibitor of calcineurin, seriously affects islet insulin secretion and viability, as adverse events on insulin content and integrity of pancreatic islets were described [6-9]. Sirolimus was designed to bind 12-kDa FK506 binding protein to inhibit mechanistic target of rapamycin (mTOR) for cell cycle arrest. However, it was shown that sirolimus activates autophagy of beta cells in addition to down-regulation of insulin expression [10-12]. Further, side effects such as mouth ulcers, nephrotoxicity, anemia and hypercholesterolemia were induced with sirolimus uptake [13]. As a consequence, donor limitation and islet isolation should be evaluated critically for performance of islet grafts.

To address immunosuppressive drugs and repeated isolations, biomaterial based approaches depend on the idea of formation of a barrier around islets before transplantation to decrease the possibility of host immune system-mediated destruction of pancreatic islets. By this way, it is proposed that a specifically designed barrier will allow oxygen and nutrient uptake and will not interfere with the removal of cellular wastes and with the transfer of hormones (e.g. insulin). This approach also opens the idea of using alternative insulin secreting beta cell sources by minimizing the risk of rejection [14]. With recent advances, macro to nano-coatings are reported for beta cell transplantation [5]. In addition, co-transplantation with other cells such as endothelial cells (EC), mesenchymal stem cells (MSCs), T regulatory cells (Tregs) and sertoli cells is a distinguishing strategy to control beta cell mass for engraftment and co-transplantation has shown to be promising for normoglycemia *in vivo* and *in vitro* [15-19].

To address beta cell source, MIN6 beta cells were described as one of the most promising cell lines as models [20]. For example, when pseudoislets composed of MIN6 beta cells in PEG hydrogels were transplanted into the peritoneal cavity of streptozotocin (STZ)-induced diabetic mouse, normoglycemia was obtained after 4-days and removal of this transplant yielded hyperglycemia [21]. Also, the role of cell-cell interactions [22-24] and effect of different compounds on insulin secretion [22] were investigated on MIN6 beta cells.

Vascularization has also taken great interest in pancreatic islet transplantation for survival and insulin secretion. In a normal individual, pancreas is highly vascularized and, therefore, this network should be re-established after transplantation. This limitation

also controls the number of transplanted islets, as most transplanted islets die within a few days after transplantation due to hypoxia, immune reaction and nutrient deficiency [25].

The aim of this thesis was to develop strategies that will address the challenges of T1D therapy and investigate the potential of these approaches for future research. In this thesis, novel strategies, from macro to nano-coating, extracellular matrix (ECM) derived peptides to insulintropic peptide, MSC to EC have been tested to overcome limitations of immunoprotection, transplanted cell number, repeated isolation and vascularization. The potential of macro and nano-coating with PEG hydrogels and CHPOA nanogels for beta cell replacement were investigated, respectively. To achieve this goal, islets or MIN6 pseudoislets were used as model beta cells. In macro-sized hydrogels, RGDS, IKVAV, glucagon like peptide-1 (GLP-1) or combination have been applied where necessary. Potential of these membranes was analyzed in terms of their capability to sustain viability and insulin secretion function of beta cells. As support cells, MSCs have been examined for immunoprotection against pro-inflammatory cytokines and for control of insulin secretion. EC were also used, to develop models for vascularization research.

A general overview of T1D with current clinical treatment strategies is presented in Chapter 2. Limitations associated with clinics are reviewed and alternative strategies offered in tissue engineering are also explained.

In Chapter 3, PEG hydrogel immunoisolation barriers are introduced and an application of macro-sized PEG hydrogels in beta cell therapy is given to address immunoisolation and donor limitation. This chapter includes the use of ECM derived peptides (RGDS and IKVAV) and insulintropic peptide (GLP-1) with covalent immobilization in PEG hydrogels for co-encapsulation of MSCs and islets.

In following chapter, Chapter 4, development of MIN6 pseudoislets as alternative beta cell spheroids is discussed and investigation of CHPOA nanogel coating as an immunoisolation barrier in the form of nano-thin coating on beta cells is performed.

Chapter 5 is about investigation of PEG hydrogel encapsulated MIN6 pseudoislets in diabetic mouse model.

In Chapter 6, composite system of CHPOA nanogels and PEG hydrogels are developed. It includes characterization and *in vivo* biocompatibility of composite

hydrogels.

Chapter 7 explains the use of MIN6-MSC heterospheroids with macro-sized PEG hydrogels for diabetes research. This chapter is a comprehensive study on MSCs for enhancement of insulin secretion and protection against proinflammatory cytokine induced beta cell death.

In Chapter 8, hanging drop method is presented to form MIN6-HUVEC heterospheroids and to explain behavior of cell within this cluster. Also, possible effects of hetero-clustering on beta cell function and viability are discussed.

Conclusions and future work that this study contributed are summarized in Chapter 9.



Chapter 2

LITERATURE REVIEW

2.1. Diabetes Mellitus (DM)

DM is a chronic disease of pancreas (Figure 2. 1), the insulin secreting organ of the body. Typically, loss of insulin secretion or insulin function drives the occurrence of DM [3]. This organ consists of islets of Langerhans (endocrine tissue), blood vessels and acinar cells (exocrine tissue). They are composed of α , β , δ and PP cells that secrete glucagon, insulin, somatostatin and pancreatic polypeptide, respectively [26, 27]. In these micro-organs, vascularization is 10 times higher than the exocrine part of the pancreas. As a result, even though these cell clusters constitute 1-2% of total pancreas, 5-10% of pancreatic blood flow is delivered to the islets [15, 28].

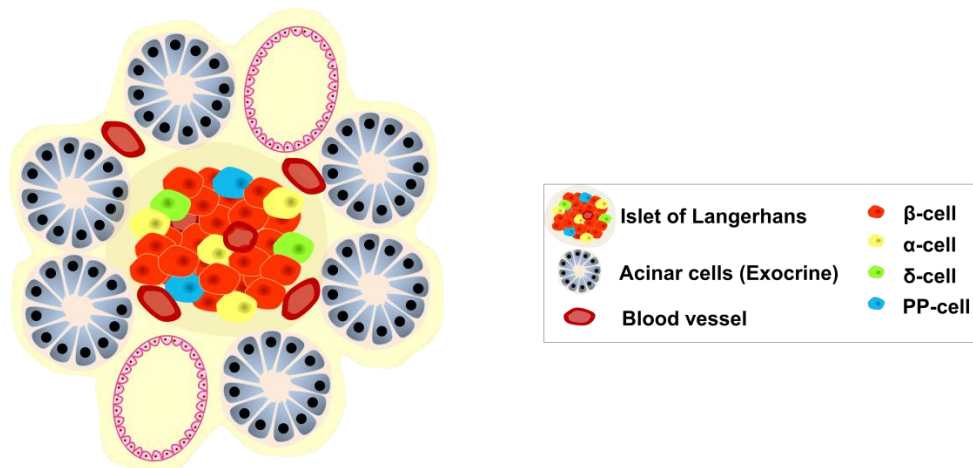


Figure 2. 1: Schematic representation of pancreatic tissue. Adapted from Ref. [26].

The etymology of DM evolved from its symptom of excess sweet urination. “diabetes” comes from Greek word “diabetes” meaning “to go through, siphon” due to excess urination. “mellitus” is derived from Latin and Greek root of “honey” due to sweet urine [29]. The history of diabetes dates back to ancient Egyptians around 1500 BC

with the observation of excess urination and weight loss. Later on, with the studies of Greek physician Aretaeus, it was first discovered that this urine tasted sweet. In the following centuries, Matthew Dobson, an English physician, recognized that sweet taste comes from elevated levels of glucose in the urine [30]. Discovery of islets of Langerhans was by Paul Langerhans in 1869 [27]. In the late 19th century, Joseph von Mering and Oskar Minkowski explored that pancreatectomized dogs had lethal diabetes. Nearly two decades after this discovery, Edward Albert Sharpey-Schafer proposed that diabetes occurred due to lack of a pancreas derived chemical compound which he named as insulin and this was actually proven when Frederick Banting and Charles Best used pancreatic extracts on diabetes induced dogs. James Collip and John Macleod were able to purify bovine insulin. In the following years, insulin therapy was in clinics [30].

Currently, the number of diabetic patients has reached 415 million with prevalence of 1 in 11 adults in 2015. It was reported that five million patients lost their lives due to complications associated with diabetes. In the next few decades, it is estimated that patient number will increase up to 35% to 642 million and parallel to such devastating change, healthcare expenditure will rise 19% globally [4].

In diagnosis of these patients, fasting glucose levels are ≥ 126 mg/dl. In this process, with the symptoms of hyperglycemia, casual plasma glucose level is measured ≥ 200 mg/dl. In oral glucose tolerance test, plasma glucose levels are ≥ 200 mg/dl after 2h. In addition, A1C (glycated hemoglobin) level is $\geq 6.5\%$ [31, 32].

Diabetes has three main classes: Type 1, type 2 and gestational diabetes. In type 1 diabetes (T1D) also known as juvenile-onset diabetes, beta cells are destroyed by immune attack. This form consists of 5-10% of the total diabetic patients. Further differing from other types, insulin supply is essential for survival due to lack of beta cells whereas in others, insulin is required for control.

90-95% of diabetic patients have type 2 diabetes (T2D) which is due to insulin resistance or relative insulin deficiency. T2D is mostly linked to obesity. In addition to changes in lifestyle and diet, treatment of T2D includes use of insulin, metformin to control hepatic glucose production, sulfonylureas and meglitinides to increase insulin secretion, insulin sensitizers, GLP-1 modulators, and α -glucosidase inhibitors to manage intestinal carbohydrate absorption [32-34].

Gestational diabetes is observed during pregnancy and is related to glucose intolerance. Genetic defects in beta cell function or insulin action, endocrine pancreas related diseases, drug/chemical induction, infections such as cytomegalovirus and syndromes such as Down's syndrome can lead to gestational diabetes. Once patients are diagnosed, diet and exercise therapy is applied. In the following stages, insulin, glyburide and metformin are also used [32, 35].

2.2. Insulin

Insulin is a peptide hormone of pancreas secreted from beta cells in the islets of Langerhans. It mainly functions in control of blood glucose levels through regulation of metabolism (carbohydrate, protein, lipid) and in cellular glucose uptake. Insulin receptors are present in all tissues including brain, endothelium, pituitary gland and adipose tissue with distinct functions. For example, in brain, it is suggested to be associated with appetite, memory, olfaction, cognition and satiety. In endothelium, it stimulates nitric oxide production that functions in relaxation. Insulin acts in management of food intake with β -endorphin and secretion of growth hormone in pituitary gland. In adipose tissue, it promotes glucose uptake and lipogenesis [36].

Insulin is transcribed from the short arm of the chromosome 11 in the form of proinsulin. First, pre-proinsulin mRNA is synthesized with ribosomes of rough endoplasmic reticulum. During this synthesis, 100 amino acid-single chain of a signal peptide, B chain, C chain and A chain are formed. Signal peptide is then removed and this modified insulin is called proinsulin. Proinsulin is transferred to golgi apparatus with vesicles where zinc ions are complexed. Proinsulin is converted to insulin and stored in precipitated form until secretion [36].

Insulin has total of 51 amino acids in biologically active form and it consists of 2 chains: Chain A and Chain B (Figure 2. 2). These chains are 21 and 30 amino acids in human, respectively. The presence of disulfide bonds between chains from A7 to B7 and A20 to B19 connect A and B chains. An intradisulfide bond between A6 and A11 also exists [37].

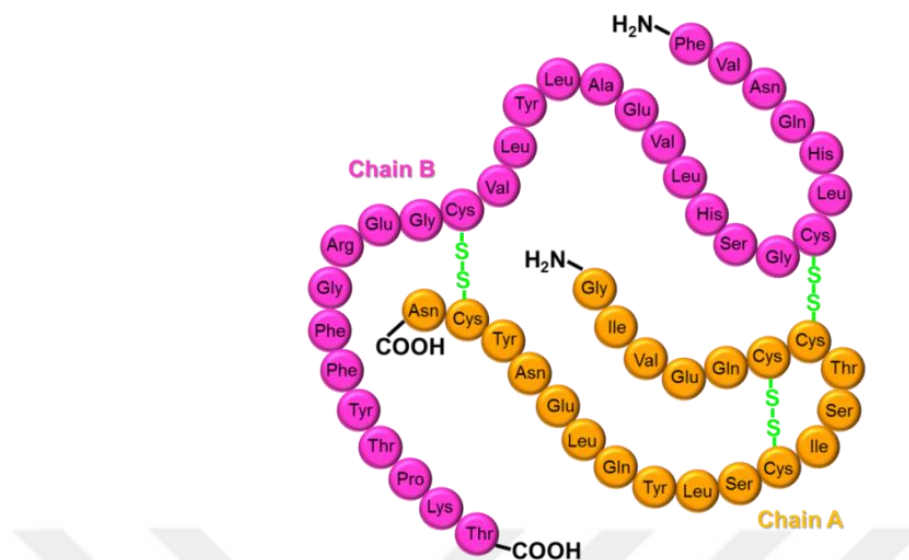


Figure 2. 2: Amino acid sequence and disulfide bonds of insulin A and B chains [38].

Insulin is present at dimer conformation in micromolar concentrations. It assembles into hexamers in the presence of zinc ions. Insulin has 2 conformations. In T conformation, antiparallel C-terminal helix of A chain (A12-A20) is linked to N terminal helix (A1-A8) of the same chain. In the case of B chain, a central helix (B8-B19) with N- and C-terminal strands is formed. In 2 zinc crystal structure, monomers in insulin hexamer are adjusted into this conformation. In other conformation, R conformation, B chain helix extends towards N-terminus. In 4 zinc crystal structure with high chloride concentrations, R3T3 is observed. In phenol containing solutions, monomers are adapted into R conformation [37].

Under fasting conditions, insulin is secreted at a rate of 0.25-1.5 U per hour. Under this condition, venous or arterial insulin is around 3-15 mIU/L (18-90 pmol/L) [36]. Postprandially, the glucose is transported with glucose transporter 2 (GLUT2) on beta cell membrane, goes under glycolysis and aerobic respiration (Figure 2. 3). As a result of these reactions, ATP/ADP ratio increases and ATP dependent K^+ channels are closed. This closure causes membrane depolarization and opens voltage-dependent Ca^{2+} channels (VDCC). Ca^{2+} influx occurs and vesicles carrying insulin hexamers dock and release insulin [39]. During docking, vesicle membrane and target membrane soluble N-ethylmaleimide-sensitive factor attachment protein receptors, v-SNARE and t-SNARE, interactions play an essential role. On the cell membrane, snaposomal associated protein-23/25, syntaxin and synaptotagmin-7 reside and form t-SNARE. On vesicle,

vesicle associated membrane protein-2 (v-SNARE) is located. Syntaxin interacts with Munc18 which prevents vesicle docking. Munc-13 communicates with Munc-18 and interferes with Munc-18 inhibitory function. As a result, a complex of a coiled coil of 4 helices (1 from VAMP, 1 from syntaxin and 2 from SNAP25) is formed. For full zippering, after Ca^{2+} binding to synaptotagmin-7, it removes complexin clamp and complete docking and vesicle content release occur [40-42]. During this process, F-actin based cytoskeleton act as a block and a track for granule movement. When granule release is required, F-actin is re-organized and insulin is secreted [39, 43].

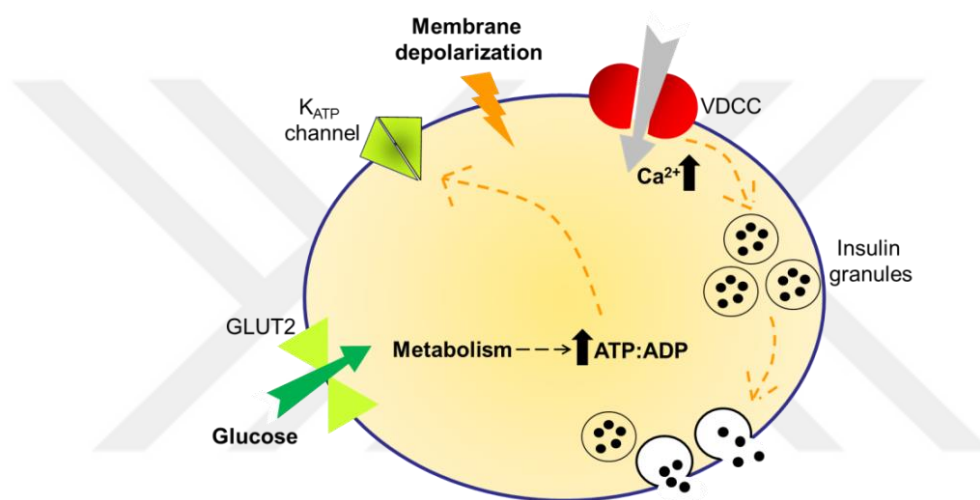


Figure 2. 3: Insulin secretion from beta cells after increase in environmental glucose levels.

In addition to glucose (main inducer of insulin secretion), other physiological stimulators also exist (Figure 2. 4). Ketoisocaproate and amino acids such as leucine act as feeders of the tricarboxylic acid (Krebs) cycle. Through this anaplerotic pathway, intracellular level of ATP is increased and insulin secretion is enhanced. Acetylcholine, cholecystokinin (CCK), ATP and fatty acids act through G-protein coupled receptors (GPCR) (G_q) and increase insulin secretion through elevated levels of Ca^{2+} . Glucagon-like peptide-1 (GLP-1), glucose-dependent insulintropic peptide (GIP), vasoactive intestinal peptide (VIP) and oxyntomodulin also act through GPCR (G_s) and activate kinases pathways including protein kinase A (PKA) and exchange protein activated by cAMP-2 (EPAC2). Increase of cAMP levels in this pathway enhances insulin secretion. In contrast, adrenalin and somatostatin act through G_i and have inhibitor action on insulin secretion [44, 45].

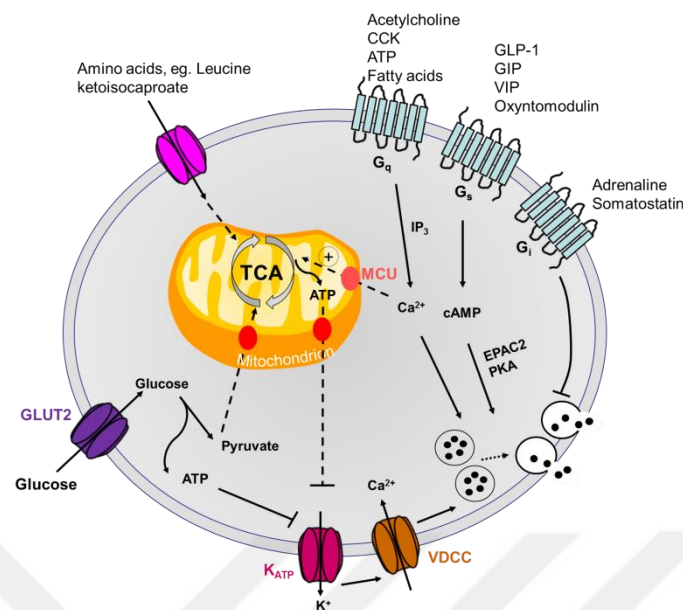


Figure 2. 4: Regulation of insulin secretion. Adapted from [44].

Following activation of insulin secretion, two different phases are specified. First phase is 5-10 min long and includes readily releasable pool of pre-docked insulin vesicles. 50-200 of >10,000 granules are released during this phase. Second phase includes reserve pool and during this stage, 5-40 granules per min per beta cell is secreted [39] and each granule has $\geq 300,000$ insulin molecules [44].

2.3. Type 1 Diabetes (T1D)

T1D is the autoimmune version of diabetes in which beta cells of pancreas are destroyed by the immune system. This subclass of DM is a complex disease driven by interplay between genetics and environmental triggers. Expression of human leukocyte antigens, cytotoxic T lymphocyte associated protein-4, protein tyrosine phosphatase non-receptor 22, insulin, interleukin 2 receptor subunit- α are some examples for genetic link of T1D [46]. 30-50% of T1D patients have HLA-DR3/4-DQ2/8 [46]. Also, variable number tandem repeat of insulin gene is known to be important for the development of self-tolerance, as shorter length of repeats is associated with changes in autoimmune regulator binding and low insulin transcription in thymus. These events contribute to the decrease in tolerance, thus, increases the risk of T1D development [47]. In the case of coxsackievirus B infection, C-X-C motif chemokine 10 production in pancreas is enhanced resulting in recruitment of autoreactive T cells. This event assists upcoming stages in T1D [46, 48]. Loss of beta cells induces hyperglycemia (high amount of glucose

in the blood circulation) accompanied by progressive diseases of various organs such as cardiovascular diseases, blindness and kidney failure [29]. The clinical symptoms that lead to T1D and T1D-driven diseases include hyperglycemia, excessive thirst and frequent urination [46].

2.4. Treatment of T1D

Today, although there is no cure for T1D [30], several treatment strategies are used to keep the glucose levels in near normal range. In clinical applications, insulin therapy as injection or insulin pumps is commonly used (Figure 2. 5). Insulin therapy through injection (Figure 2. 5A) is applied several times in a day for a strict control of blood glucose. However, risk of hypoglycemia, day-time based changes in insulin requirement and problems in absorption profiles clearly indicate the need of alternative strategies [5, 49, 50].

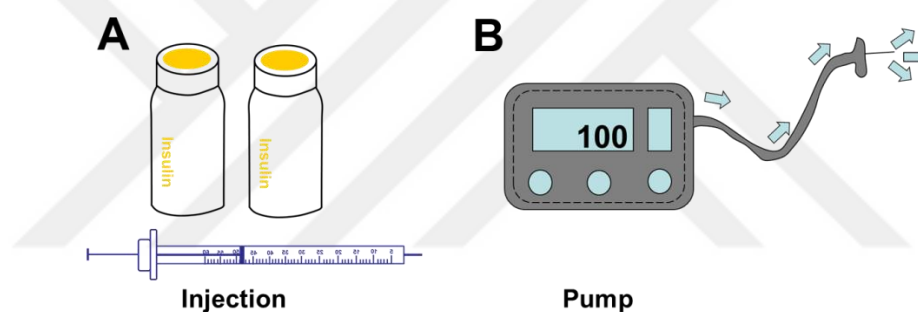


Figure 2. 5: Insulin therapy in the form of A) Injections, B) Pumps. Adapted from Ref. [5].

To deal with such limitations, several advancements such as discovery of new insulin analogs rather than regular insulin, regular control of HbA1c and sensor for time-lapse glucose monitoring are done. In the case of insulin, various insulin analogs from rapid to long-lasting action characteristics have been developed (Table 2. 1) [49]. However, cost-efficiency of this therapy is still debatable, as the cost reaches thousands of dollars for a patient's lifetime [51].

Table 2. 1. Insulin analogs [52-54]

Analog	Modified Chain	Modification
Rapid-acting insulin analogs		
Aspart	B	P28D
Lispro	B	P28K, K29P
Glulisine	B	D3K, K29E
Long-acting insulin analogs		
Glargine	A B	D21G 2 R addition to C-terminus
Detemir	B	D30 deletion, C14 fatty acid addition at B29
Degludec	B	T30 deletion, C16 fatty acid chain addition at K29 through glutamic acid spacer
Premixed insulin analogs		
75% neutral protamine lispro, 25% lispro		
70% protamine aspart, 30% aspart		
50% neutral protamine lispro, 50% lispro		

Insulin analogs are usually subcutaneously injected to the upper arm, abdomen, buttocks or thigh. Intramuscular injection is also performed. In case of injection site, it is suggested to apply into rotating regions on a specific site, as choosing a specific region for all injections can lead to lipoatrophy and lipohypertrophy. Also, injection site can affect the absorption of insulin resulting in profile changes. For example, if the abdomen, the fastest absorption site is preferred, insulin injection in the different parts of the abdomen should be performed [55]. In other cases, insulin pumps (continuous subcutaneous insulin infusion, CSII) are used instead of multiple subcutaneous injections (Figure 2. 5B). In CSII, insulin delivery is performed through pumps compared to pen or syringe based insulin injections. In contrast, several problems exist such as possibility of infection at the insertion site and contact dermatitis [56].

In severe cases, transplantation of pancreas is inevitable (Figure 2. 6A) to reach normoglycemia and elimination of daily multiple insulin doses [5]. Patients having

severe metabolic complications and are unable to prevent acute complications with insulin therapy have transplantation [57]. Also, this transplantation is an alternative for patients with end-stage renal disease where pancreas transplantation with kidney transplantation can improve clinical results [58]. However, donor shortage and immunosuppressive drugs limit the application of pancreas transplantation, and invasiveness and possible complications are other drawbacks of whole organ transplantation [5].

According to International Pancreas Transplant Registry data, 75% of these transplantations was simultaneous kidney pancreas transplantation, 18% was pancreas after kidney transplantation and remaining 7% of transplants was pancreas transplantation alone. With such transplants, patient survival rate was over 95% and 83% after 1 year and 5 years, respectively [59]. Between 2007 and 2011, it was also reported that tacrolimus or sirolimus included therapy for pancreas transplantation alone led 86% and 94% graft survival at post-transplant year 1, 69% and 84% at 3 years, respectively [60]. Nevertheless, the surgery for whole or segment transplantation of pancreas is heavily invasive and can create surgical complications. The donor source (mostly cadavers) and side effects of immunosuppressive drugs after transplantation are other drawbacks of current procedures[5].

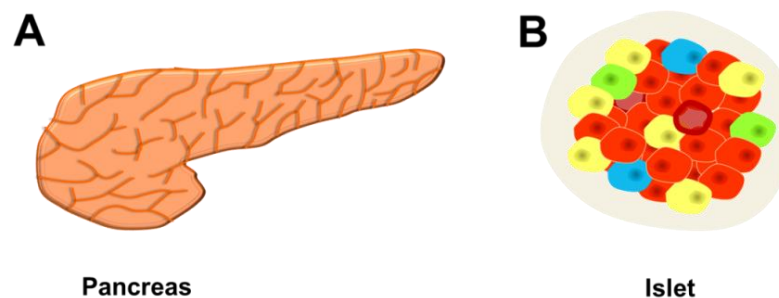


Figure 2. 6: Organ/cell transplantation in the form of A) Pancreas, B) Islets. Adapted from Ref. [5].

The first transplantation of pancreas is reported in 1966 and in the following years, various transplantations were performed [61]. However, islet survival is still a major concern. In early inhibition of islet graft rejection glucocorticoids and azathioprine were used as immunosuppressive agents. Glucocorticoids act on T cells by inhibition of proliferation and induction of apoptosis through inhibition of synthesis of interleukin (IL)-2 and other crucial cytokines [62]. However, severe side effects such as

osteoporosis, myopathy, hyperlipidemia, hypertension, memory deficiency, psychosis, gastritis and gastrointestinal hemorrhage negatively affect the quality of life of the patients [14, 63]. Azathioprine is a purine analog and inhibits DNA synthesis [64]. Similar to glucocorticoids, this inhibitor is highly toxic, as it is associated with hepatotoxic, neurotoxic and gastrointestinal toxic effects [14]. In the case of second generation immunosuppressive drugs, action mechanism of tacrolimus (FK 506) depends on the inhibition of calcineurin. However, this process seriously affects insulin secretion and alters islet viability, as negative effects on insulin content and integrity of pancreatic islets were monitored. These events were accompanied by islet apoptosis in a dose dependent manner in Wistar rats receiving tacrolimus [6-9, 65]. Cyclosporine A, a cyclic undecapeptide interacts with cyclophilin to inhibit calcineurin. However, this immunosuppressant has also direct effects on beta cells, as it impairs insulin secretion and structural integrity of pancreatic islets [66, 67].

Islet transplantation (Figure 2. 6B) is the other option for beta cell transplants. This experimental approach is less invasive, allows pretreatment of islets and use of xenogeneic sources for transplants [5]. In 2000, combination of sirolimus, low-dose tacrolimus and daclizumab, called “Edmonton protocol” was introduced for portal vein transplants of islets [68]. In an international trial, Edmonton group showed that 21 of 36 patients had glycemic control and that of 76% (16) were insulin dependent at 2 years [69]. However, in addition to side effects of sirolimus and tacrolimus, considering that at least 12 000 islets/kg body weight are required for insulin independence [68], current isolation procedures should also be improved, as this procedures lead to loss of 30-50% of islets [70].

2.5. T1D in Tissue Engineering

Tissue engineering approaches focus on immunoisolation, immunotolerance, donor source, functional improvement and vascularization of islets. These different strategies are also combined to alter the potential of designed biomimetic pancreas.

2.5.1. Immunoisolation and Tolerance

Main trouble in all transplantation cases is the induction of host immune reaction due to capacity of immune system to recognize the foreign cells/materials. These non-self tissues are attacked by leukocytes, macrophages and dendritic cells [14]. In a reaction

triggering innate immune system activation, close contact of grafts such as islets with blood promotes instant blood mediated inflammatory response (IBMIR). In this process, both complement and coagulation cascades are activated and this is followed by neutrophil infiltration. IBMIR starts with expression of tissue factor from α and β cells due to interaction with ABO-compatible blood. This event triggers platelet adhesion to collagen and factor VIIa that stimulates coagulation cascade for fibrin clot formation. Activation of thrombotic and coagulation cascades are followed by mainly polymorphnuclear lymphocyte infiltration resulting in islet destruction and loss [71-74].

In the case of acquired immune mediated rejection, antigen presenting cells present antigens to $CD4^+$ T cells through T cell receptors and co-stimulatory signals of CD80 or CD86. First scenario of destruction is mediated by cytotoxic $CD8^+$ T cells resulting in cell lysis. Also, $CD4^+$ T cells have ability to kill target cells through direct contact of FasL-Fas interactions. Molecules secreted from T cells act in two different ways: Cytokines such as interferon- γ and interleukin-4 activate innate immune system cells including macrophages. In another case, factors such as tumor necrosis factor- α , lymphotoxin and nitric oxide can directly kill target tissue. In addition to T-cell mediated destruction, antibody- and complement-mediated mechanisms can also drive graft rejection events [75-77].

2.5.1.1. Material Based Strategies

Biomaterial based approaches mainly depend on the idea of formation of a barrier around islets before transplantation. By this way, it is proposed that specifically designed barrier will allow oxygen, nutrient uptake and will not interfere with removal of cellular wastes and with transfer of hormones (e.g. insulin) (Figure 2. 7). This approach also opens idea of use alternative insulin secreting cell sources decreased risk of immune rejection [14].

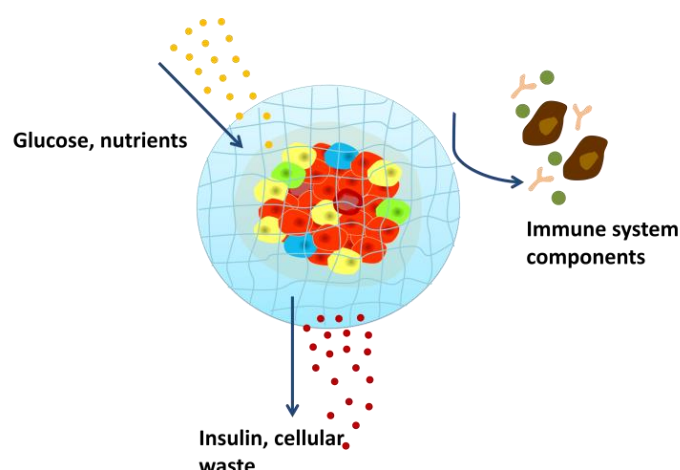


Figure 2. 7: Immunoisolation barrier for pancreatic islets.

These biomaterial coats around islets are intended to be semi-permeable for islet survival and functionality for therapeutics. Further, they need to be biocompatible not to evoke heavy host response [5]. With this technology, pancreatic islets encapsulated in alginate/polyethylene glycol beads, alginate/poly-L-lysine beads and alginate sheets are currently in clinical trials [78]. In addition, cells normally reside in ECM in their natural environment. The interactions between ECM and islets support development, homeostasis and regeneration. So, providing such crucial interactions will significantly improve islet survival and activity [5].

2.5.1.1.1. Materials for Beta Cell Coating

For beta cell coating, the most common natural materials are alginate, chitosan and agarose whereas synthetic ones are poly(ethylene glycol), poly(vinyl alcohol) and poly(lactide-co-glycolide).

Alginate is a polysaccharide of mannuronic and guluronic acid chain units (Figure 2. 8). Divalent cations such as calcium are applied with alginate to form cross-linked hydrogel barriers around islets due to ionic interaction between alginate chains and calcium ions [79]. Further, its capacity to go under gelation at physiological pH and temperature makes it superior over some other materials [80]. Applicability of alginate hydrogels for islet transplantation has been reviewed *in vitro* and *in vivo*. For example, BB rats receiving intraperitoneal transplantation of alginate encapsulated Wistar rat islets gained weight and remained normoglycemic with blood glucose concentration lower than 200 mg/dl for 3 months [81]. Also, capsules derived from this polysaccharide did not

interfere with oxygen consumption of human islets under normoxic and hypoxic conditions compared to naked islets [82]. In a recent study by Veisheh et al., islets were encapsulated in alginate capsules of 0.5 mm and 1.5 mm diameter. The latter one was reported to be promising as percent cure rate of C57BL/6 diabetic mice was 100% for nearly 120 days. In contrast, for smaller diameter, curing was 0% after nearly 40 days. The reason for this failure of smaller sized alginate spheres was associated with fibrosis and immune system infiltration [83]. Male Wistar rat islets encapsulated in alginate microcapsules with pentoxifylline (anti-inflammatory drug controlling IL-2 secretion) induced less IL-2 secretion from lymphocytes in contrast to naked islets and islets encapsulated alginate capsules [84]. In alginate beads, having physically entrapped ECM derived proteins/peptides, human islet viability and insulin secretion was enhanced [85]. However, alginate is also common to the exopolysaccharide of opportunistic bacteria such as *Pseudomonas aeruginosa*, so this material can induce monocytes to deliver cytokines through toll-like receptor 2 and 4 [86].

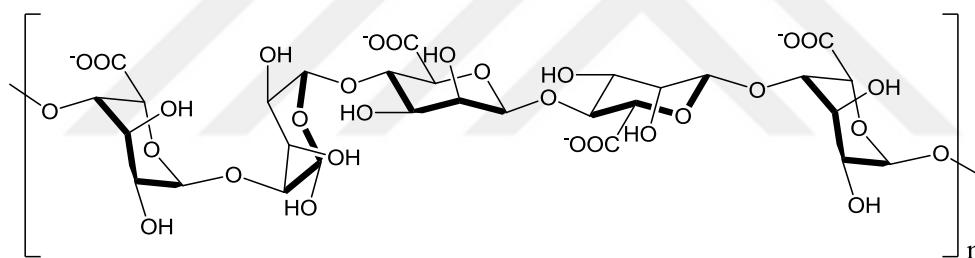


Figure 2. 8: Chemical structure of alginate.

Chitosan is a linear polymer derived from deacetylation of chitin, a biomaterial found in the exoskeleton of some sea animals and insects. This polysaccharide is formed by linkage of glucosamine and N-acetyl glucosamine by β (1–4) glycosidic bonds (Figure 2.9) [87, 88]. For islet immunoisolation, chitosan encapsulated Wistar rat islets were transplanted into renal subcapsular space of STZ-induced diabetic C57BL/6 mice. T-cells and macrophages were not observed in the transplantation area, fasting glucose levels and intraperitoneal glucose tolerance test results demonstrated reduced blood glucose levels [89].

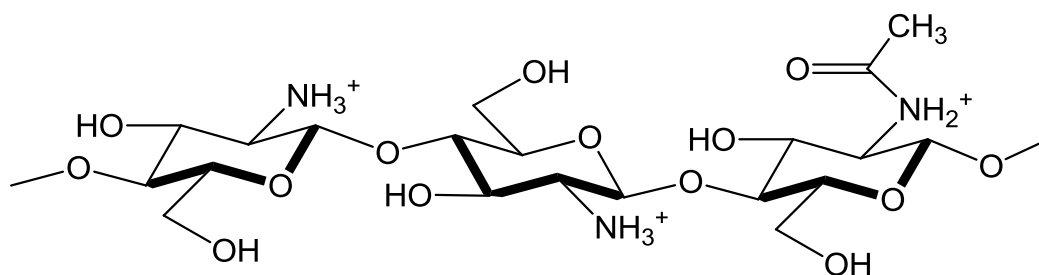


Figure 2. 9: Chemical structure of chitosan.

Agarose is a thermo-sensitive polysaccharide for coating within temperature ranges of 15-30°C (Figure 2. 10) [90]. This polymer is also used for hindering islets from immune system. In one such study, agarose microcapsules within diameter of 100-400 μm were examined and for at least 3 months, no significant mononuclear infiltration was detected. Mice receiving peritoneal or omental transplants showed potential of this system to regulate blood glucose levels [91]. Similarly, auto-transplantation of islets in agarose capsules was reported to be successful for reversal of diabetes in a pancreactomized canine model [92].

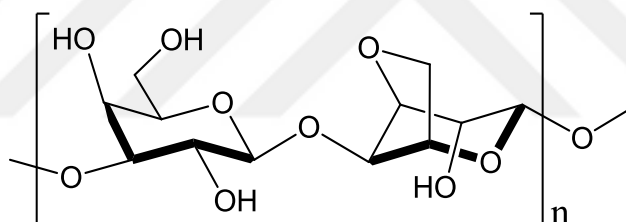


Figure 2. 10: Chemical structure of agarose.

Poly(ethylene glycol) (PEG) is a water soluble, FDA approved polymer with extreme hydrophilicity (Figure 2. 11). This linear, nonionic polymer has an extensive use in tissue engineering and drug delivery due to its biocompatibility, ease of modification and bio-inertness [93-95].

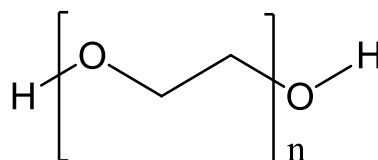


Figure 2. 11: Chemical structure of PEG.

Minimum surface energy of PEG in water provides minimum interfacial energy that is useful in control of non-specific protein adhesion onto PEG incorporated surfaces [96]. These properties make PEG-based systems ideal candidates for immune camouflaging

and functional support of pancreatic islets [97]. For example, covalent incorporation or layer-by-layer assembly of GLP-1 or ECM derived peptides into PEG hydrogel enhances insulin secretion in glucose stimulated insulin secretion test [23, 97-99]. In PEG microcapsules formed with microfluidics, islets viability and insulin secretion was preserved [100]. Similarly, these cross-linked networks can act as affinity molecules to prevent immune system activation. For example, monocyte chemotactic protein-1 affinity ligands derived from its receptor can be bound to PEG network to prevent activation of monocytes against islets [101]. Alternatively, peptide combined PEG hydrogels with a recognition loop of tumor necrosis factor- α (TNF- α) receptor can be used to protect encapsulated cells against TNF- α challenge [102]. Also, anti-Fas monoclonal antibody modified PEG conjugates induce apoptosis in jurkat cells, a model cell line for T cells. In one such experiment, Cheung and Anseth proved that 20% of the jurkat cells were apoptotic when these cells were co-incubated with modified PEG hydrogels [103]. Lee et al. showed that in 3 out of 7 diabetic patients, triply PEGylated islets remained viable for 100 days without immunosuppression whereas uncoated ones were destroyed within 1 week [104]. In m-PEG modified islets, after return to normoglycemia, 6/10 (60%) and 2/24 (8%) islets sustained normoglycemia in modified and naked islet transplantation groups for 100 days in subcapsular kidney space of male C57BL/6 J (H-2^b) mice. Effect of PEGylation was further improved with systemic therapy of anti-LFA-1, as 78% survival was observed [105]. Heparin-PEG nano-coating of islets showed no interference in viability and increases the time required for clot formation [106]. In PEG microcapsules, it was reported that viability and function of CD1 mouse islets were maintained and free-floating debris in medium in uncoated islets were obvious. This debris was trapped in PEG microcapsules and was suggested that this capsule may block movement of immune system components towards islets and prevention of debris movement to outside [107]. PEG hydrogels can also prevent immune system components and do not interfere with insulin secretion profile of islets [108, 109]. However, due to capacity of PEG to resist protein attachment and thus cellular adhesion, in the long-term, PEG hydrogels should be modified to mimic native environment of pancreatic islets [98, 99].

Poly (vinyl alcohol) (PVA) is a water soluble synthetic polymer derived from

hydrolysis of polyvinyl acetate (Figure 2. 12). Similar to PEG, it is also FDA approved and has been commonly used in various biomedical applications [110]. Diabetic Wistar rats receiving PVA encapsulated Sprague-Dawley rat pancreatic islet transplants remained normoglycemic for 12 days [111]. In an allotransplant in PVA hydrogel model, the presence of insulin positive cells could be observed up to 4 weeks after transplantation [112].

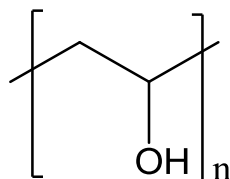


Figure 2. 12: Chemical structure of PVA.

Poly (lactide-co-glycolide) (PLG) is a synthetic FDA approved polymer (Figure 2. 13) that can be used in cell encapsulation to provide immunoisolation and graft vascularization. For example, seeded C57BL/6 mouse islets on this scaffold showed reduced time to return normoglycemia and graft revascularization when transplanted into intraperitoneal fat [113]. Further, seeding of islets on ECM derived protein supplemented PLG and transplantation to the epididymal fat pad of male C57BL/6 mice proved that all mice with collagen IV supplementation could reverse diabetes within 4 days and at post transplantation day 297; histological analysis indicated the presence of insulin positive, highly vascularized islets at implantation sites [114]. However, macrophage, CD4⁺ and CD8⁺ T cells, and dendritic cell infiltration were observed in allotransplant of islets seeded on PLG scaffolds in peritoneal fat pad. In the case of CD4⁺ and CD8⁺ T cells, infusion of donor splenocytes treated with ethylcarbodiimide was able to reduce the number of infiltrating T cells via upregulation of Tregs and inhibition of Teff [115].

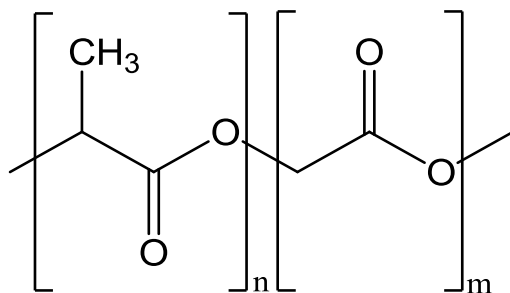


Figure 2. 13: Chemical structure of PLG.

2.5.1.1.2. Forms of Islet Coats

The methods used for islet coating are summarized below and are classified as macroencapsulation and, micro and nano-encapsulation.

Macrocapsules have two major categories: Intravascular and extravascular. Intravascular capsules function as dialysis cartridges. In this strategy, islets are distributed between hollow fibers and an extra tube is used to cover the fibers. These fibers are polyacrylonitrile and polyvinylchloride copolymer [116, 117]. This membrane is then connected to blood vessels of the host vasculature through anastomosis [14]. The risk of thrombosis, infections and the requirement of a major surgery should be taken into account [117]. In contrast, extravascular macrocapsules are usually in the form of tube or disk [14]. These devices offer the advantage of implantation through minor surgery and ease of graft retrieval [117]. Hydrogels made from synthetic or natural materials in macro-size are alternatively suggested for pancreatic islet immunoisolation. These artificial membranes also showed promise *in vivo* and *in vitro* [98, 118, 119].

High surface to volume ratio of microcapsules provides ease of nutrients, oxygen and insulin transport for graft survival. This type of capsules is more durable and mechanical disruption is more difficult compared to macrocapsules [120, 121]. Pre-clinical studies on primates [122], dogs [123] and mouse [124, 125] as well as phase I trials on human patients [126, 127] showed the promise of this technology. It is also applicable to co-encapsulate pancreatic islets with drugs such as curcumin that can effectively function in prevention of rejection of islets by decreasing the expression of fibrosis markers [128]. It has been shown that physically and chemically crosslinked microcapsule of azide modified alginate and phosphine modified PEG around Sprague Dawley rat islets was promising *in vitro* [129]. However, lack of vasculature around transplanted islets may lead to graft failure [130] and difficulty of graft retrieval is another challenge [121].

The main idea of nano-encapsulation is to form a nano-thin layer around islets. Nano-thick membrane also allows faster oxygen and nutrient transfer, water and CO₂ disposal due to short distance. [131]. For example, MIN6-MSC (kidney derived) heterospheroids are coated in phosphorylcholine-modified poly-L-lysine/heparin layers show improved stimulation index [132]. When islets coated with multi-layers of phosphorylcholine-chitosan, alginate and phosphorylcholine-modified chondroitin-4-sulphate were

allogeneically transplanted into kidney capsule of C57B/6 mice, although normoglycemia was achieved, graft rejection was observed in control and 4-layer coated islets for 12 and 15 days, respectively. In case of 8-layer coating, graft survival was prolonged up to 28-37 days [133].

Conformal coating of pancreatic islets is other strategy for ultra-thin coating and is performed through electrostatic interaction, covalent bonding or hydrophobic interaction with membrane lipids or proteins. After this attachment, several layers can be deposited through layer-by-layer assembly [134]. The purpose of proposed designs can be functional improvement, elimination of coagulation or inhibition of immune activation. Through this strategy, biologically active molecules such as GLP-1 [97], urokinase [135], thrombomodulin [119], soluble complement receptor 1 and heparin [136, 137] can be coated onto islet surface. For example, Kizilel and her colleagues used biotin-PEG-NHS followed by streptavidin on Lewis rat islet surface. Outer layer of this coat was composed of biotin-PEG-GLP-1 and they demonstrated that layer-by-layer assembly on islet surface did not compromise viability and, improvement of islet insulin secretion in glucose challenge was obtained [97]. In the case of urokinase and thrombomodulin coating, lipid-PEG-maleimide attached polymer can be used to immobilize thiolated protein to the islet surface. By this way, it is possible to inhibit coagulation or degrade fibrin clot without significant destruction in islet function [119].

For covalent bonding, PEG with an activated ester is used to hinder islets through polymeric coating or protein immobilization onto islet surface [118, 138]. For the case of hydrophobic interaction, PEG-lipid [139] or PVA-alkyl groups [135] can surround the target islet. Electrostatic interactions can also be used. For example, Miua et al. used lipid-PEG for the initial coat of Syrian hamster islets and then PLL-alginate was performed for electrostatic interaction driven multilayers. This method did not cause increase in the volume and further, no impairment in insulin secretion was observed [140].

Also, cells such as HEK293 [139], Tregs [18] and sertoli cells [141] have been used to coat the islet surface for immunoprotection through polymeric interactions. For example, biotin-PEG-lipid (provides hydrophobic interaction) was used to attach streptavidin labelled HEK293 and female Syrian hamster islets did not show any necrosis

in HEK293 coats. However, slight decreases in insulin secretion under static incubation was observed *in vitro* [139]. For Tregs coats of human pancreatic islets, researchers demonstrated that this coating did not effect the insulin secretion profile of islets in dynamic perfusion and exposure to Teff induces less amount of IFN- γ production compared to uncoated islets [18]. Biotin-avidin interaction was previously used also in covalent immobilization of leukemia inhibitory factor loaded PLGA particles onto DBA/2 mouse islets. Islets were first treated with EZ-Link Amine-PEG11-Biotin and then avidin attached PLGA particles. When such coated islets were transplanted under kidney capsule of diabetic C57BL/6 (H-2^b) mice, 4 of 7 were kept normoglycemic for more than 100 days [142].

2.5.1.2. Biological Strategies

In addition to material based coating, masking of islets with cells/molecules, inactivation or depletion of T cells, cytokine control and dendritic cell transfer have also been suggested for treatment of T1D. Some of these strategies have been used with materials and are summarized in Section 2.5.1.1.2.

Co-transplantation of cells into diabetic rat and primate models is promising to reach normoglycemia [16, 17]. For example, beta cells can be co-transplanted with cells having regulatory function on immune system cells. B6 (H2^b) mouse hepatic stellate cells co-transplanted with BALB/c (H2^d) mouse islets under kidney subcapsular space showed improved graft survival, as euglycemia was maintained for 15 days for islet only group in contrast to 65 days (until removal of kidney) of co-transplantation group. In histological analysis, it was clearly observed that there was hepatic stellate cell layer around islets, apoptotic T cell number increased and vascularization was initiated [143]. Diabetic recipient animals with taking islet transplantation under kidney capsule plus intravenous CD3 specific antibody and autologous dendritic cell injection showed significantly improved graft survival (19.6 days vs. 77.4 days) [144]. Transplantation of Lewis rat islets with testicular cell aggregates promoted >95 days of normoglycemia showing that these cells also accompanied islet engraftment in Wistar-Furth rat renal subcapsular space. Possible mechanism was suggested to be the use of FasL but probably not limited to this event [145]. It is established that MSC secreting factors such as transforming growth factor- β (TGF- β), hepatocyte growth factor (HGF) and indoleamine 2,3-

dioxygenase (IDO) have regulatory functions and cell-cell interactions including PDL1-PD1, ICAM-LFA1 and VLA4-VCAM have regulatory role on immune system cells [146]. For example, treatment of mice having CB57L/B6 islet transplant under kidney capsule with MSC (BALB/c (H2^d) bone marrow) yielded prolonged islet graft survival. It was further shown that matrix metalloproteinase (MMP)-2 and -9 were responsible for such effect, as they change surface expression of CD25 on T cells, thereby decrease IL-2 response [147]. Co-transplantation of MSCs (murine bone marrow) with BALB/c islets into kidney capsule showed reduced ratio of Th1/Th2 and Tc1/Tc2 with significant decrease in peripheral naive T cells and memory T cells. CD83 (marker of dendritic cells) and CD86 (co-stimulatory) expressions combined with IL-12 secretion decrease were observed [148]. In a cynomolgus monkey trial, increased number of Tregs in the peripheral blood was evident [17]. In addition to immunomodulation, it was reported that MSCs (C57Bl/6 mouse bone marrow) can increase BALB/c islet insulin secretion (stimulation index). As an example, when Davis and her colleagues encapsulated islets with MSC in silk hydrogel, stimulation index was increased and splenocyte proliferation was prevented. Addition of ECM derived proteins, collagen IV and laminin, further improved beta cell function [149]. This study is further supported with the findings that mouse MSCs (C57BL/6J mouse kidney derived) promoted ICR mouse pancreatic islet insulin secretion in co-encapsulation with alginate hydrogels [150]. In another previous study, Yeung and his colleagues indicated that exposure of human pancreatic islets to pro-inflammatory cytokines in the presence of human bone marrow MSCs was protective as shown by TUNEL staining and these islets were still functional after treatment [151]. Transplantation of rat pancreatic islets with rat bone marrow MSCs to the kidney capsule yielded enhanced vascularization in the transplantation area and less number of islets was required to reach normoglycemia [28]. In a previous study by Karaoz and colleagues, co-cultured rat bone marrow MSCs were shown to be protective against STZ-induced rat beta cell destruction in an *in vitro* model [152].

SCs or ECs can be used to form spontaneous coat around islets. Since these cells are adherent, they tend to attach protein-rich basal layer of islets without any modification. Vascularization, reduction in coagulation and immune-protection can be achieved with these cellular coats. For example, in human bone marrow MSC, human dermal

microvascular EC and human islet co-culture, human islets were coated with these cell types and sprouting of EC from islet surface was enhanced [15]. In lymphocyte treatment of MSC and dissociated islet culture, significant decrease in lymphocyte proliferation was recorded. Decreased activation of coagulation cascade was achieved in MSC coated islets [153].

Alternatively, beta cells can be re-aggregated with other cell types for engraftment. Co-culture is related to culture of at least two cell types. Co-aggregation refers to co-culture of cells in formation of a single aggregate (spheroid) where at least two cell types are involved. This method has advantages over co-transplantation as it decreases the risk of washing away of the cells transplanted with islets through bloodstream. Interaction between co-transplanted cells would be interfered and actually, would not be an ideal method. For example, co-aggregation of Sprague-Dawley rat islet cells and human bone marrow derived MSCs revealed that cleaved caspase-3, caspase-3 and caspase-7 expressions were decreased compared to islet only transplanted group. Moreover, insulin and anti-apoptotic protein, Bcl-2 expression and CD31⁺ cell area were increased. In co-transplanted group, SCs were washed away after 7 days of liver transplantation [154]. Sertoli cells in testis form an immunoisolated space and act as a physical barrier for lymphocytes. By making use of such advantages, co-aggregation of islet cells and sertoli cells facilitated intraportal islet transplantation, as mice receiving co-aggregates were normoglycemic for >100 days whereas islet only group became hyperglycemic after ~10 days [19]. In addition to prevention of loss of cells through bloodstream, co-aggregation is useful to decrease cell number. For example, 1-5 Tregs per Teff are required and this approach is not feasible. Instead, co-aggregation can be used to form an immunoprotective area. In intraportal transplantation of CD4⁺CD25⁺ Treg cell (C57BL/6 mouse) and islet cell (BALB/c mouse) co-aggregates into C57BL/6 mice, 6/9 diabetic recipients had blood glucose lower than 250 mg/ml more than 100 days [155]. Improvement of cell-cell contact is also promising in transplantation. MIN6-MSC (kidney derived) heterospheroids were coated in phosphorycholine-modified poly-L-lysine/heparin layers improved stimulation index. In this study, after cytokine (IL-1 β , TNF- α and IFN- γ) challenge, protection against apoptosis was also observed [132]. Mice having EC coated Sprague Dawley rat islets under renal capsule were infused with sertoli

cells and higher vessel density accompanied by decreased number of CD4⁺ and CD8⁺ T cells, IL-2 and IFN- γ were observed compared to control group [156].

As mentioned in section 2.5.1, islet graft rejection is mainly mediated by T cell events. Depending on this information, researchers seek alternative strategies. In one of these, it is possible to coat islets with host antibodies against donor human leukocyte antigen molecules to prolong graft survival up to 200 days by preventing antigen presenting cell (APC) function [157]. Alternatively, host cells such as Tregs can be utilized to coat islets. For example, Marek et al. coated human pancreatic islets with Tregs via biotin-streptavidin interaction, since Tregs function in immune tolerance by preventing excess immune response. Researchers demonstrated that this coating does not affect insulin secretion profile of islets in dynamic perfusion and exposure of coated islets to Teff induced less amount of IFN- γ production compared to uncoated islets [18].

To prevent T cell stimulation/activation, monoclonal antibodies such as anti-leukocyte function associated antigen-1 (LFA-1), anti-CD154, anti-CD45RB and CTLA4-Ig can be used for functional control of T cells. LFA-1 is a beta integrin that acts in events of adhesion and activation of leukocytes, so blockage of LFA-1 interaction can offer advantage for inhibition of activation [158]. CD154 interacts with CD40 and this interaction positively regulates co-stimulatory molecules belonging to APC, IL-2 receptor on T cells and secretion of cytokines acting on proliferation of cytotoxic T cells [158]. Once inhibited, increase in graft survival was reported in a diabetic mouse model [159]. CD45 has a central role in lymphocyte activation and development, so blockage of this molecule was reported to be effective to return to normoglycemia in a mouse model [158]. Fusion protein of CTLA-Ig showed competitive binding to B7 molecules of APCs. By this way, CD28 dependent activation of T cells can be inhibited. For example, in diabetic rat models, it was observed CTLA-Ig expressing islet transplants survived ~1 months whereas within 1 week, transplanted islets were destroyed in control group [160, 161].

Cytokines such as interleukin-10 and TGF- β can be applied as bait for immune regulation. TGF- β activates Treg production and suppresses Teff [146]. Through this mechanism, in a diabetic model, TGF- β gene therapy prolonged graft survival. Complete destruction of the graft occurred in control groups at day 11 whereas 67% of the grafts

were functional at day 50 in TGF- β gene therapy group [162]. Another cytokine, interleukin-10, inhibits pro-inflammatory cytokine production and similar to TGF- β , it can increase graft lifetime [163, 164].

2.5.2. Donor Shortage and Functional Improvement

Adult human pancreas has ~2 million islets that consist of 70-80% beta cells [27]. Assuming that a diabetic patient needs at least 12 000 islets/kg body weight to reach normoglycemia [68], current isolation procedures should also be improved, as harvesting procedures lead to a loss of 30-50% of islets and enzymatic treatment during isolation leads to impairment of islet function and viability [70, 165].

For islet xenografts, organisms such as bovine, porcine, rat, mouse and rabbit were proposed to maintain normoglycemia [5]. Porcine source is the prime islet source for T1D, as its insulin only differs by one amino acid from human insulin [166]. It is inexpensive and has similar physiology with human version [167, 168]. On the other hand, galactose α -1,3 galactose on porcine islets induces CD4⁺ T cell mediated immune rejection [167]. Requirement of sterile husbandry conditions and the risk of zoonotic diseases, especially pig endogenous viruses, limit the use of islets derived from this source [169]. However, Garkavenko and colleagues reported that xenotransplanted patients showed no signs of infection caused by pig endogenous retrovirus, pig cytomegalovirus, pig lymphotropic herpesvirus and pig circovirus type 2 [170].

Engineering cells for obtaining insulin producing cells can also be considered an option. These engineered cells are derived from somatic cells such as fibroblasts, liver cells, epidermal cells and pancreatic ductal cells. SCs stand as other sources to improve the outcome of the clinical diabetes. These include embryonic SCs, MSCs and adult pancreatic SCs. These cells can be programmed to insulin producing cells/islets under specific growth conditions [5]. For example, human embryonic SCs were directed into beta cell lineage and these cells were clustered for intraperitoneal transplantation in alginate microcapsules. It was observed that these clusters in capsule were able to control blood glucose (~90 days of normoglycemia) [171]. However, mutagenesis caused by reprogramming vectors and tumor formation remain an undetermined risk for clinical application [172]. The cost for protein in induction experiments, the possibility of teratoma formation and the degree of differentiation further limit the SC based therapies

in T1D [173].

Instead, immortalized beta cell lines may be solution to address this obstacle due to their insulin secreting nature and ease of proliferation. RINm5F, MIN, INS and HIT are such immortal pancreatic beta cell lines that are currently under investigation for replacement [20, 174, 175]. Among these lines MIN6 and INS-1 are far more promising, since they can response to glucose and express glucokinase [20]. MIN6 beta cells were obtained from a transgenic mice carrying SV40 T antigen after insulin promoter [175]. This cell line is commonly applied in the form of single cells or pseudoislets for control of glucose homeostasis *in vivo* and *in vitro* [132, 176-178]. For example, when pseudoislets composed of MIN6 beta cells in PEG hydrogels were transplanted into the peritoneal cavity of STZ-induced diabetic mouse, normoglycemia was obtained after 4-days and removal of this transplant yielded hyperglycemia [21]. Also, the role of cell-cell interactions [22-24] and the effect of different compounds on insulin secretion [22] were investigated on MIN6 beta cells. However, these cells can loss beta cell phenotype and its sensitivity towards glucose stimulation decreases overtime [179]. Also, they display functional and morphological heterogeneity with increasing number of passages [180, 181].

Rather than monolayers, insulin secreting cells are clustered into aggregates forming pseudoislets. The reason for applying such clusters is that the insulin secreting cells are adhesion dependent and require cell-cell interactions for normal functioning and for keeping the differentiated state. This 3D clustering is also important for beta cells in islets, as islets are in the form of cellular aggregates in the pancreas and dissociation leads to impairments in insulin secretion and response [182]. In this 3D cluster, beta cell potency can also be promoted. For example, pseudoislets composed of insulin secreting cells and sertoli cells/MSCs were applied for cell survival against cytokine activated apoptosis and to increase insulin response in glucose stimulated insulin secretion (GSIS) [19, 132]. Mouse aortic endothelial cells (MAEC) and NIH3T3 fibroblasts were also used as part of co-aggregates with MIN6 beta cells (on PNIPAAm coated PDMS microwells) for improvement in insulin secretion. In this groups, collagen IV expression was increased and probably change in insulin secretion was due to ECM deposition [183]. Similar functional improvement was observed in co-aggregates of D11 beta cells and

human umbilical vein endothelial cells (HUVECs) formed with high aspect rotational vessels [184]. Further, INS-1 beta cells separated by either 100 or 200 μm fibrin gel from HUVECs on RGD coated surface were investigated. In HUVEC incorporated groups, decreasing distance between cells or use of HUVECs improved insulin secretion. HUVEC incorporated groups also displayed higher beta cell number. It was proposed that the ability of the ECs to secrete basement membrane proteins probably contributes to for functional improvement and cell proliferation [185].

In addition to donor source and companion cells, beta cell function and thus, number can also be regulated to prevent repeated islet isolation. In addition to strategies discussed above, beta cell number required for successful transplantation can be decreased by mimicking the native conditions that beta cells face in the body. In the body, cells are supported by a protein and polysaccharide rich network called ECM. It is a highly organized network that allows for cellular communication. Each component in ECM has crucial functions for beta cells. Collagen IV, fibronectin and laminin are the most widely found proteins in islet ECM [186].

Collagen IV gives structural support and plays an important role in cellular processes including adhesion, and migration. Laminin act as a structural component. Fibronectin is another protein required for cell survival and the prevention of apoptosis. These three proteins are also associated with insulin secretion [186-188]. In PEG hydrogels, ~30-day incubation of islets showed that, physically entrapped collagen IV or laminin sustained islet insulin secretion, as 2-fold and 4-fold increases in secretion were measured. Synergistic effects of collagen IV and laminin were also reported. The use of short peptide sequences of IKLLI, IKVAV, LRGDN, PDSGR, and YIGSR, derived from these proteins, provided promising results in decreasing apoptotic cell number and increasing insulin secretion at high glucose levels. However, these peptides were not as potent as the whole proteins [99]. In alginate beads, having physically entrapped collagen IV and ECM derived peptides such as RGD, PDSGR, LRE, human islet viability and insulin secretion were enhanced in culture medium [85]. In another study, encapsulation of MIN6 beta cells in ECM derived peptides of YIGSR, RGD, IKLLI, IKVAV, PDSGR, LRE and/or DGEA immobilized PEG hydrogels showed improvements [23]. Thus, mimicking this environment can simultaneously regulate the function and survival of the beta cells in

islet transplants.

In addition to these peptides/proteins, insulinotropic peptides such as GLP-1 can also be beneficial. For example, in the presence of GLP-1, mouse islet ATP content and insulin secretion can be increased. And this peptide can protect islets against TNF- α induced cell death as reported previously [98].

2.5.3. Vascularization

In the body, blood vessels act as a web to supply crucial compounds such as nutrients, oxygen and other critical signals such as hormones and drugs. This system also removes CO₂ and other waste products from tissues through circulation. Thus, these events sustain tissue survival and functionality for homeostasis. Today, key challenge in successful clinical application of engineered tissue constructs is development of a blood vessel network. So far, biological constructs are limited to tissues of cartilage, bone and cornea, as they overcome the diffusion limitation of aforementioned compounds (100-200 μ m) (Figure 2. 14). However, use of larger constructs or other tissues and handling of such systems as test environments demand for vascular networks for success in clinics. For this purpose, vascularized constructs or strategies for accelerated vascularization are being developed in tissue engineering [189].



Figure 2. 14: Blood vessels. Adapted from [190].

During embryonic development, blood islands consisting of hematopoietic SCs and hemangioblasts are established from mesoderm layer [191]. These EC precursors then develop primitive capillary network. This process is known as vascularization. In angiogenesis, maturation accompanied by remodeling of this network leads to formation of vascular system. When vessels are formed, stabilization occurs through mural cells

that are pericytes and smooth muscle cells (SMCs), and through ECM deposition. This process is regulated by transforming growth factor- β (TGF- β), platelet derived growth factor-BB and angiopoietin-1. Pericytes that cover EC lining of capillaries support and prevent apoptosis of ECs. SMCs around arteries and veins support EC and function in vasocontrol. After formation of a primary network, artery and vein differentiation is determined by genetic (ephrin and notch signaling) and environmental factors (e.g. blood pressure and flow) [192-194]. Even though in the body, angiogenesis occurs in cases of inflammation, ischemia and wound healing, various signals such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and TGF- β , basal membrane degradation through MMPs and adhesion molecules control formation of new blood vessels [193, 194].

Among the factors that control vascularization, VEGF is the most important one with 4 different forms, as it has diverse functions ranging from initiation of angiogenesis and lymphangiogenesis to survival of ECs [195]. Various VEGF forms exist: VEGF-A, VEGF-B, VEGF-C and VEGF-D. VEGFR-2 exerts its effect on EC by VEGF-A binding. This interaction induces MAPK, Akt and Src-p38 pathways for cell proliferation, survival and arrangements in actin cytoskeleton for migration, respectively [196]. Among these, VEGF₁₆₅ is the most predominant form. This isoform is a secreted growth factor, but a fraction remains on the cell surface and the rest can be converted into a diffusible form through the plasmin-mediated cleavage in C terminus located in ECM [195, 197].

bFGF is another mitogen for cellular proliferation and migration. It exerts significant effects on production of enzymes such as collagenase and plasminogen activator [198]. In addition, once ephrinA1 is activated, EC migration and capillary invasion can be obtained [88]. PDGF-BB is expressed on the leading cell of a sprout due to hypoxia, shear stress or other growth factors. This molecule is crucial for vessel stabilization through recruitment of pericytes and serves in anastomoses [199]. Ang-1 is a cytokine acting through Tie2 receptors for blood vessel growth, maintenance and stabilization [198]. TGF- β has two opposing functions in the vascular network assembly. At low doses, this factor enhances production of angiogenic factors and proteinases. However, increasing doses leads to inhibition of EC growth, initiates the destruction of basement membrane and stimulates SMC differentiation [198].

2.5.3.1. Vascularization in Tissue Engineering

Tissue constructs, either prevascularized or *in vitro* vascularized scaffolds can be built by (1) modifying with growth factors or adhesion peptides, (2) seeding with cells or (3) by combining the two (Figure 2. 15). Natural or synthetic materials are used to realize *in vitro* prevascularization or *in vivo/in vitro* vascularization strategies [190].

As for the growth factors (Figure 2. 15Aiii and C), scaffolds modified with angiogenic growth factors are prominent. Among them, VEGF, bFGF, ephrins, PDGF-BB, TGF- β and Ang1 are applied to customize scaffolds for blood vessel formation and when applied *in vivo*, these matrices are used to simply deliver the angiogenic factors to the target site in a cell responsive, hydrolytic or simple diffusion depending manner [199-206]. The reason for the utilization of such specific molecules is due to their potent *in vivo* role. For example, subcutaneous transplantation of VEGF and RGDS immobilized enzyme responsive PEG hydrogels enhanced vessel ingrowth [207]. Furthermore, such modified degradable hydrogels can accelerate the angiogenesis in transplanted pancreatic islets in C57BL/6J mice [208]. In addition to this process, injection of alginate microcapsules of VEGF loaded PLGA can effectively induce angiogenesis *in vivo* [209]. Also, combination of VEGF, Ang-1 and PDGF-BB in alginate hydrogels showed promise in subcutaneous transplantation [200]. It was previously reported that nanoparticle-fibrin system provided a more sustained release of VEGF compared to fibrin only system. Hindlimb ischemia model has shown higher blood pressure and capillary network formation in nanoparticle incorporated group [210].

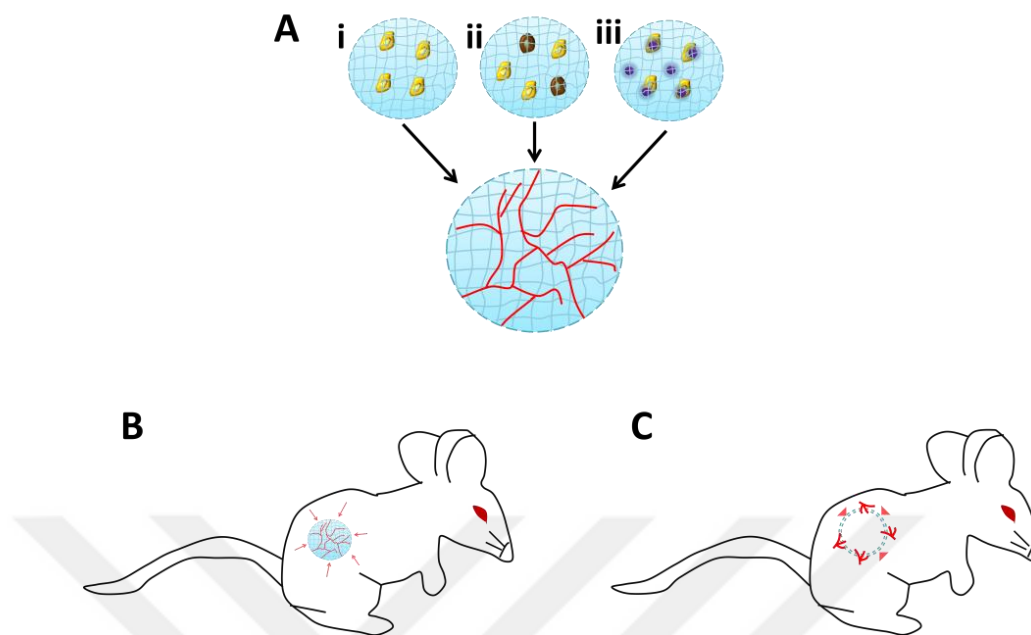


Figure 2. 15: *In vitro* and *in vivo* vascularization strategies. (A) *In vitro* strategies use (i) culture of one type of a cell such as ECs, (ii) co-culture of two different types of cells or (iii) combination of such strategies with adhesion peptides or growth factors. *In vivo* vascularization approaches depend on either (B) transplantation of prevascularized tissues or (C) growth factor delivery/cell infusion to initiate the angiogenesis. Adapted from [190].

In contrast, *in vitro* models utilize co-encapsulation of growth factors or adhesion ligands with 1-2 types of cells into a 3D matrix (Figure 2. 15Ai-iii). As an example, PEG hydrogels modified with VEGF and an adhesion ligand, RGDS, promoted HUVEC migration and development of *in vitro* vascular networks. These networks were then stabilized with co-encapsulated 10T1/2 cells [206]. In collagen and fibrin gels, simultaneous coating of EC and MSC can also induce angiogenesis, as MSCs can behave as pericytes and stabilize newly formed blood vessels [211]. In addition to RGDS, adhesion peptides such as IKVAV and YIGSR can be used to drive vascularization, as these adhesion peptides have specific roles in cell adhesion, migration, cell-cell interactions, capillary branching and tubule formation depending of their integrin on cell surface [212].

Additionally, customized patterned surfaces are applied for construction of blood vessels *in vitro*. For example, Leslie-Barbick and her colleagues used laser scanning lithography to delivery angiogenic signals (RGDS, VEGF) to PEG hydrogel surfaces. Primitive capillary networks of seeded HUVECs were formed on PEG hydrogels and

ECM protein deposition and dependence of tubule assembly on pattern width were observed [213]. Instead, transparency masks can be used to deliver such signals in the form of blank lines to direct the growth of vessels [214]. In patterned collagen hydrogel with PDMS stamps, EC and perivascular cell interaction and changes in states due to inflammatory response were investigated *in vitro* [215]. Carbohydrate glass lattices formed with 3D printing were coated with 10T1/2 cell incorporated fibrin gels. After dissolution of lattice, newly formed channels were used to inject HUVECs. After 9 days, lumens lined by HUVECs were coated with 10T1/2 cells [216]. On PEG hydrogel surfaces, 50-200 μm width stripes having covalently immobilized RGDS were formed with transparency masks and photopolymerization. 50 μm -stripes showed cord-like orientation of HUVECs and ECM deposition whereas in larger width, HUVECs spread only [214].

Currently, prevascularized scaffolds are also investigated for vascular grafts (Figure 2. 15B). This process depends on the idea of establishment of vascularized tissue that is ready for implantation. It is proposed that this *in vitro* or *in vivo* preformed vascular network in a 3D network will accelerate rate of anastomoses with host. Thus, tissue survival and differentiation will be promoted [217]. For example, co-encapsulated HUVEC and fibroblasts in fibrin gels were vascularized *in vitro* and after 7 days, implantation into immunocompromised mice showed enhanced anastomoses accompanied by tissue differentiation and remodeling [218]. This *in vitro* approach benefits from time, as host vessels do not need to go through middle of the implant. However, there will not be a link between the implanted tissue and the host, thus, perfusion will be retarded. In contrast, *in vivo* prevascularization uses cell sheet engineering or arteriovenous loops. It is composed of three main steps that are main limitations of this system: Implantation of a cell-free scaffold, retrieval and implantation of the scaffold to target site [190]. Decellularized small intestinal submucosa (SIS) and heart were also applied as natural scaffolds for vascular network formation. As an example, with the implantation of HA-PLGA-SIS, vascular network assembly was accelerated in chicken embryo chorioallantoic membrane vascular assay and a canine urinary bladder model [219]. Reseeded rat heart after incubating in a perfusion bioreactor was able to show pump function [220].

Rather than engineered materials, bolus administration or direct injection of angiogenic factors were also applied in blood vessel formation. However, this method is not usually the choice, as activity of the factors can be lost and they may be cleared fast. For example, when bFGF was administered intravenously, resulting vessels were fragile and leaky; thus, the risk of regression was increased. Also, the intravenous or intracoronary injection differs. In intravenous administration, bFGF can be lost due to low affinity binding of bFGF to heparin sulfate and this decreased the amount of growth factor reaching ischemic myocardium [221, 222]. This delivery is also poorly controlled resulting in disorganized and transient vessels [223].

In addition to such strategies, direct injection of angiogenic factor expressing cells is also applicable. For example, Yang and his colleagues injected rhVEGF165 transfected MSCs myocardially for ischemia treatment and enhanced angiogenesis at the injection site was observed after 4 weeks [224]. Also, cord blood MSC-HUVEC spheroids were injected to the ischemic hindlimb where MSCs acted as pericytes to stabilize HUVEC laden vessels. With the use of such approach, vascular network formation and thus, decrease in degeneration was observed [225]. Implantation of core cord blood MSC spheroids in HUVEC coat in Matrigel also provided upregulation in angiogenesis in a subcutaneous implantation model [226].

2.5.3.2. Pancreatic Islet Vascularization/Angiogenesis

Vascularization has also taken great interest in pancreatic islet transplantation for survival and insulin secretion. In the body, pancreas is highly vascularized and after transplantation, this network should be re-established [25]. However, blood vessels grow several tenths of micrometers per day [189] and this limitation also controls the number of transplanted islets, as most transplanted islets die within a few days after transplantation due to hypoxia, immune reaction and nutrient deficiency [25].

In cell based strategies, co-transplantation or coating of islets with MSCs and EC was proposed. For example, co-transplantation of MSCs and islets to the renal capsule of NOD-SCID Lewis rats yielded high number of capillaries and showed vWF⁺ and VEGF⁺ MSC compared to poorly vascularized islets. This strategy not only might be considered for vascularization/angiogenesis, but also to decrease the number of islets required to reach normoglycemia [28]. Johansson and her colleagues also coated islets with EC-MSC

in fibrin gels and showed increased vascularization of pancreatic islets by interacting intra-islet vasculature without interfering with dynamic insulin release [15]. Alternatively, expression of angiogenic factors might be useful. For example, when VEGF cDNA transduced mouse islets were transplanted to athymic NIH nude mice, increase in the level of CD31⁺ islets with improved glycemic control was achieved [227].

As an example of how such a strategy can be implemented, a stainless steel cylindrical mesh with a plunger can be implanted subcutaneously. Pileggi and his colleagues proved that after 40 days, implants were vascularized. At the time of islet transfer, plunger was removed and islets were transplanted in these regions. During the next ~3 months, blood glucose was successfully controlled, animals gained weight and vascularization was increased [228].

As an example of biomaterials, VEGF and RGDS functionalized degradable hydrogels were also effective for the re-establishment of vascular network of pancreatic islets transplanted into small bowel mesentery. This strategy had a significant effect on islet functionality, weight gain and engraftment potential compared to islet delivery to hepatic vein [208]. Furthermore, pancreatic islets coated with HBPA-GF (VEGF and FGF) in peptide amphiphile based gel show effective vascularization and number of sprouts emanating from islets was significantly higher than GF untreated groups [229]. Microparticle and growth factor combination were also used to improve islet engraftment. Collagen scaffolds in which heparin, VEGF and microparticles were suspended in through fibrin gels were tested in vascularization of islets in an omentum pouch. Microparticles were composed of polyvinylpyrrolidone/hydrogen peroxide (PVP/H₂O₂) in core and catalase immobilized on surface of poly(D,L-lactide-co-glycolide) (PLGA) in shell. Diffused H₂O₂ was converted into O₂ with catalase and this O₂ was used for oxygenation of islets. VEGF in scaffolds improved islet engraftment. This group was shown to have improved body weight and blood glucose levels [230]. Transplantation of islets with VEGF incorporated PEG hydrogels into epididymal fat pad and into small bowel mesentery as well as subcutaneously improved the percent curing (75%), viability and vascularization of islets [231].

Combination approaches of biomaterials and cells are also reported. As an example, pancreatic islet encapsulated in collagen gels were seeded with EC cells and omental

implantation showed connection of host vessels with vessels around islets. This system was more successful than naked islet transplantation, but only 2/5 animals remain normoglycemic for 2 months [232].

2.6. Cholesterol Bearing Pullulan Nanogels

Nanogels are cross-linked polymer chains that form hydrogel nanoparticles with a size <100 nm. Being used in biomedical applications such as drug delivery and bio-imaging, these 3D networks have capacity to trap various compounds such as drugs, proteins and DNA/RNA [233]. CHP nanogels are nanogels that physically cross-link by self-assembly. CHP molecules are amphiphilic molecules composed of hydrophilic pullulan modified with hydrophobic cholesterol units. In aqueous solutions, interactions between cholesterol groups form cross-links. Addition of polymerizable groups such as acrylate (CHPOA) can be used to immobilize covalently the nanogels in macro-hydrogel structure [234]. CHP nanogels can act as artificial chaperons. In the body, chaperons use hydrophobic interactions to catch denatured proteins and refold them. Thus, they eliminate production of protein aggregations [233]. Similarly, CHP hydrogels use hydrophobic interactions to interact with proteins such as insulin [235] and horseradish peroxidase [236]. When a molecule such as β -cyclodextrin is presented, it interacts with hydrophobic groups of cholesterol and solubilizes these amphiphilic nanogels. By this way, entrapped protein can refold and be released from CHP. Thus, it mimics the function of a molecular chaperon [233, 237]. Also, it can function in thermal stability of entrapped protein. As an example, when chymotrypsin complexed with CHP nanogels was heat treated (92°C), 74% of the original activity of the released enzymes was conserved. However, 7% activity in complexed enzyme and complete denaturation in the free enzyme were obtained [238]. This nanogel has also been used in biomedical applications. As a part of cancer vaccine, they can be used to deliver NY-ESO-1 to evoke and facilitate T-cell response in a clinical trial [239]. In a calvarial defect, CHPOA nanogels releasing FGF18 and BMP2 accelerated osteoprogenitor cell infiltration and thus, healing [240]. CHPOA nanogels crosslinked with PEG-SH units were used to form a macro-size hydrogels and fibronectin coating on these gels showed fibroblast invasion. In subcutaneous model of implantation, vascularization with no severe immune reaction was reported [241].

Chapter 3

Mesenchymal Stem Cells and Ligand Incorporation in Biomimetic Poly(ethylene glycol) Hydrogels Significantly Improve Insulin Secretion from Pancreatic Islets

3.1. Abstract

The main goal of this study was to investigate pancreatic islet function with MSCs in a ligand functionalized PEG hydrogel for the treatment of type 1 diabetes (T1D). Rat bone marrow derived mesenchymal stem cells were encapsulated within synthetic PEG hydrogel, and cell viability and apoptosis within this 3D environment were examined in detail. ATP content and caspase-3 activity of encapsulated MSCs showed that fibronectin-derived RGDS, laminin-derived IKVAV and/or insulinotropic GLP-1 were required to maintain MSC survival. Incorporation of these peptides into hydrogel environment also improved pancreatic islet viability, where combinations of peptides had altered effects on islet survival. GLP-1 alone was the leading stimulator for insulin secretion. Cell adhesion peptides RGDS and IKVAV improved insulin secretion only when they were used in combination, but could not surpass the effect of GLP-1. Further, when pancreatic islets were co-encapsulated with MSCs within synthetic PEG hydrogel, two-fold increase in stimulation index was measured. Synergistic effects of MSCs and peptides were observed with seven-fold increase in stimulation index. The results are promising and suggest that simultaneous incorporation of MSCs and the ECM derived peptides and/or GLP-1 can improve pancreatic islet function in response to altered glucose levels in physiological environment.

3.2. Introduction

T1D is an autoimmune disease and occurs as a result of pancreatic beta cell destruction mediated by T-cells as key players. These events lead to depletion of insulin, which drives hyperglycemia and cause other severe problems mostly at the vascular level [242, 243]. These complications include increased function of endothelin-1 and upregulation of endothelial nitric oxide synthase and generation of more reactive oxygen species resulting in blood flow, vessel abnormalities and DNA damage [242, 244]. Clinical treatment of T1D requires daily control of blood glucose levels and several doses of daily insulin injections. Alternatively, oral/nasal insulin delivery and islet transplantation are used in clinical practice to maintain euglycemia. However, shortage of donor tissue and the requirement for the use of immunosuppressive drugs are some of the main limitations for clinical application of islet transplantation [2, 98]. To date, various strategies have been developed to eliminate immunosuppression, where conformal coatings with living cells (e.g. Treg, HEK293) [18, 134], molecule immobilization (e.g. urokinase) with layer-by-layer assembly on islet surface [97, 134, 245] or encapsulation of pancreatic islets within hydrogel capsules (e.g. PEG, collagen, alginate) have been considered [98, 99, 101, 131, 246]. These previous efforts were successful up to a limited extent, and further investigation is required to achieve long-term islet function in clinical transplantation studies. MSCs have been found to enhance islet graft vascularization and viability, reverse diabetes [28, 247], reduce blood coagulation (compared to controls) and apoptosis when cultured *in vitro* or implanted with pancreatic islets [151, 153]. Recently, Kerby et al. and Davis et al. reported positive influences of MSCs on pancreatic islets within alginate microcapsule or silk hydrogel macrocapsule environment, where approximately 2-fold increase in insulin secretion could be obtained from encapsulated islets compared to control groups [149, 150].

In the present study, we studied the function of encapsulated pancreatic islets and anti-apoptotic properties of MSCs in ligand functionalized PEG hydrogel scaffolds. The synthetic hydrogel matrix includes adhesive peptides (RGDS and IKVAV) and an insulintropic peptide (GLP-1), in combination with MSCs. In the system considered here, PEG hydrogel membrane not only acts as a mechanical barrier, but also as a functional synthetic extracellular matrix with covalently bonded biomolecules for

maintenance of islets and MSCs in a 3D network. The experiments that were performed to investigate this potential is summarized in Figure 3. 1. We observed that MSCs supported encapsulated islets within PEG hydrogel environment and increased insulin secretion upon elevated concentration of glucose exposure. Finally, acrylated RGDS and IKVAV contributed to the viability of the MSCs in 5-day long *in vitro* incubation, and MSCs further supported the function of pancreatic islets within PEG hydrogels.

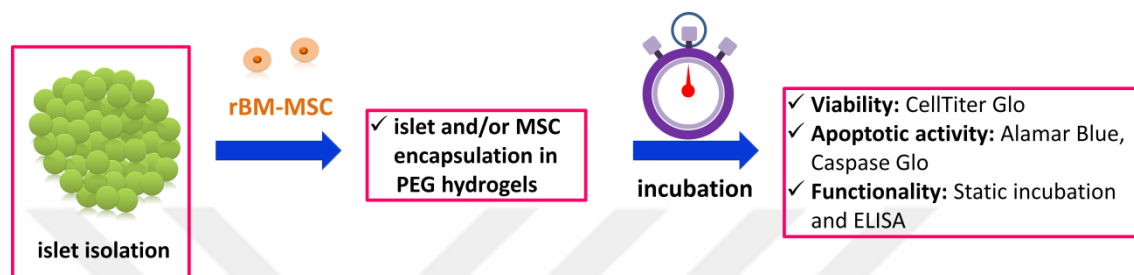


Figure 3. 1: Flow diagram of experiments performed with encapsulated islet and MSCs.

The results presented here suggest that MSCs in combination with ECM derived peptides and GLP-1 can promote the highest GSIS from pancreatic islets compared to the other systems considered in the literature and are ideal candidates for clinical reversal of T1D.

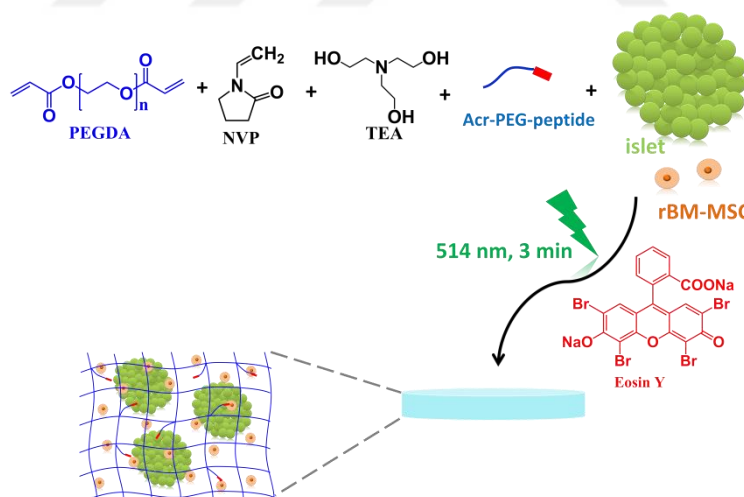


Figure 3. 2: Encapsulation of islets and MSCs in ligand functionalized PEG hydrogel scaffold.

3.3. Materials and Methods

Peptide PEGylation: To increase half-life of the peptides through covalent immobilization and to support cell functionality along with viability, adhesion peptides Arg-Gly-Asp-Ser (RGDS), Ile-Lys-Val-Ala-Val (IKVAV) and GLP-1 (Elim

Biopharmaceuticals) were PEGylated (Figure 3. 3). Briefly, peptides and acrylate-PEG-succinimidyl valerate ester (acr-PEG-SVA, Laysan Bio, 3400 Da) were dissolved in 50 mM sodium bicarbonate buffer (pH 8.2) at acr-PEG-SVA: peptide molar ratios of 1 and 2 for adhesion peptides and GLP-1. After conjugation, dialysis (Pierce, Slide-a-lyzer dialysis cassettes, MWCO 3500 Da) was performed to remove unreacted peptides and esters. Following lyophilization, FTIR (Thermo Scientific iS10 FT-IR) was applied to confirm amide bond formation.

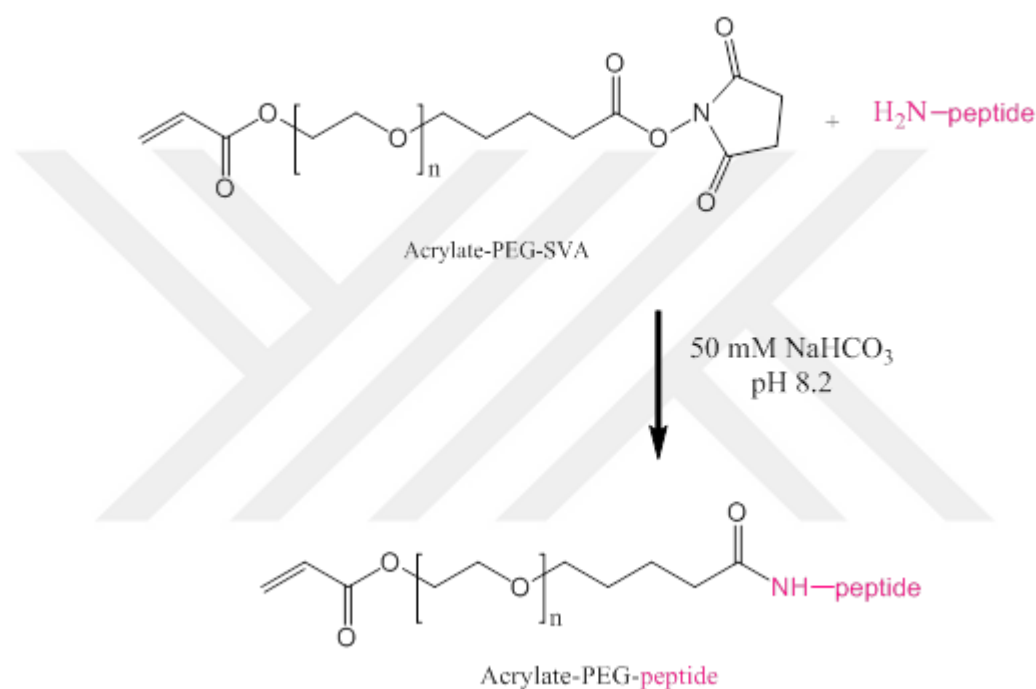


Figure 3. 3: Peptide PEGylation.

MSC Isolation, Culture and Encapsulation: Rat bone marrow MSC isolation was performed as described previously [248]. Following isolation and characterization, MSCs have been cultured in L-DMEM (Gibco, Paisley, UK) supplemented with 10% FBS (heat inactivated, Biological Industries) and 100 IU/ml penicillin-0.1 mg/ml streptomycin (Sigma) at 37°C, 5% CO₂ environment. MSCs were suspended in the prepolymer solution consisting of 10% (w/v) PEG diacrylate (PEGDA, 10 kDa, Laysan Bio, Inc), 225 mM triethanolamine (TEA, Merck), 37 mM N-vinyl pyrrolidone (NVP, Aldrich) and 0.03 mM eosin Y (Sigma Aldrich). For specific samples, 2.5 mM acr-PEG-RGDS, 2.5 mM acr-PEG-IKVAV and/or 20 µM acr-PEG-GLP-1 were included in the solution. Thirty µl of this prepolymer solution with cells 20 000 cells was photopolymerized at 50

mW (514 nm, argon ion laser, Coherent Inc., Santa Clara, CA) for 3 min. After photopolymerization, encapsulated cells were placed into 24-well plates (Costar) and were incubated with the growth medium at 37°C, 5% CO₂. For long term culture, medium was changed every other day.

The prepolymer used in this study has 4 major components (Figure 3. 4). This radical reaction is co-polymerization of PEGDA and NVP. Here, Eosin Y and TEA act as a photoinitiator and as a co-initiator, respectively. NVP is accelerator and PEGDA forms the backbone of crosslinked hydrogel [93].

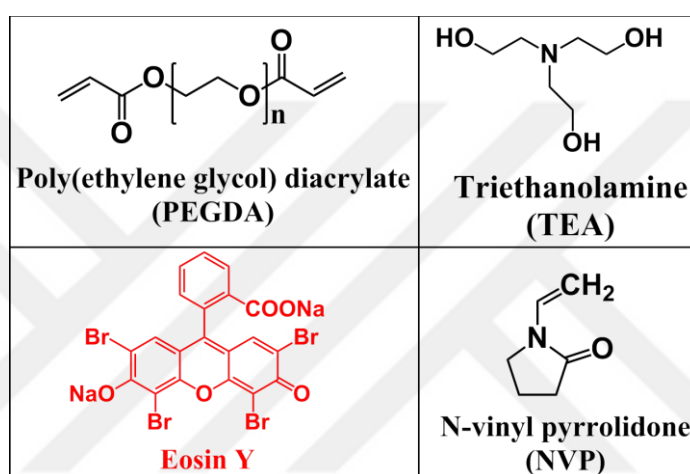


Figure 3. 4: Components of prepolymer solution.

During initiation, eosin Y is excited to its triplet state and an electron is lost from TEA forming TEA cation radical (TEA⁺•). This lost electron is transferred to eosin Y forming eosin Y anion radical. A proton loss from TEA⁺• results in neutral TEA radical (TEA•) (Figure 3. 5A). During this loss, proton is transferred to eosin Y anion radical and converting it to neutral eosin Y radical (Figure 3. 5B). In the following stages, reactions move mostly on double bonds that occur on PEGDA and NVP [93, 249].

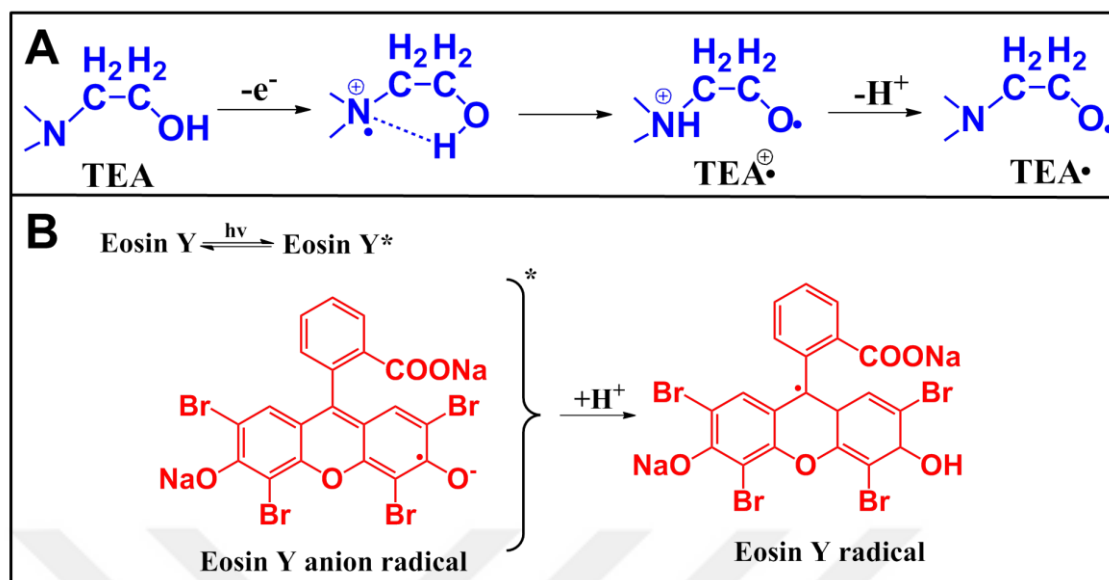


Figure 3. 5: Photoinitiation after exposure to green light ($\lambda=514$ nm). A) TEA $^\bullet$ formation. B) Eosin Y radical formation. Adapted from Ref. [250].

Pancreatic Islet Isolation and Encapsulation: Isolation of pancreatic islets was performed in Prof. Erdal Karaöz's Lab in Kocaeli University. Pancreatic islets were isolated from Wistar rats by enlarging the pancreatic duct with a solution of collagenase P (Roche, Indianapolis, IN). Following digestion at 37°C, histopaque (Sigma) density gradient was applied to purify islets. After handpicking, islets were transferred to RPMI-1640 medium (Gibco) supplemented with 10% FBS (heat inactivated, Biological Industries), 2 mM L-glutamine (Sigma) and 100 IU/ml penicillin-0.1 mg/ml streptomycin (Gibco) at 37°C, 5% CO₂ environment and were cultured overnight [251]. For encapsulation experiments, ~20 islets were suspended in 30 μ l prepolymer solution with/without peptide conjugates and were exposed to laser at 50 mW for 3 min. Cell-laden hydrogels were transferred to 24-well plate (Costar) and cultured for 3 days.

Co-encapsulation of Islets and MSCs: Twenty islets were suspended in 30 μ l prepolymer solution with 20,000 MSCs. Next, this solution was exposed to green light at 50 mW for 3 min. Encapsulated MSCs and islets in hydrogels were cultured for 3 days.

Viability and Caspase Activity: Viability of encapsulated cells was assessed with CellTiter Glo® Luminescent Cell Viability Assay (Promega, Madison WI). Briefly, cell containing hydrogels were incubated with 50% Cell-Titer Glo reagent for 1.5 h at room temperature. ATP standards were used to generate a standard curve. Resulting luminescence was measured with a microplate reader (Synergy H1, Bio-Tek). Measured

ATP amounts were normalized with alamar blue reagent (Invitrogen) for specific hydrogel sample to prevent differences in cell numbers. Briefly, hydrogels were first treated with 10% alamar blue reagent for 3 h and then, measured fluorescence signal (Ex: 550 nm, Em: 590 nm) was used to monitor viable cells [98].

For the caspase activity, encapsulated cells were incubated with 50% Caspase Glo 3/7 Reagent (Promega, Madison WI) for 1.5 h and luminescence was measured. In order to prevent cell number variation between groups, normalization with respect to ATP (pmole) or alamar blue reagent (Invitrogen) for specific hydrogel sample was carried out. *Static Incubation Assay:* Functionality of encapsulated/co-encapsulated islets was tested via static incubation assay. Encapsulated islets were placed into low (2.8 mM) and high glucose (28 mM) buffers for 1 h. After each incubation period, samples were collected and analyzed with rat insulin ELISA (Mercodia AB) to measure insulin content. Insulin concentration at low and high glucose buffers were then used to calculate stimulation index.

Statistical Analysis: All datasets were expressed as mean $\pm$ SD. Statistical analysis was performed with one-way ANOVA with Bonferroni's test. $p < 0.05$ was considered significant.

3.4. Results and Discussion

Since PEG is hydrophilic and resists cellular attachment, PEG hydrogels were covalently modified with adhesion peptides (RGDS and IKVAV) and GLP-1. RGD and its derivative RGDS are ECM derived peptides presented for cell attachment. They are abundant in fibronectin, collagen, laminin, vitronectin and vWF [252]. Thus, they can support various processes including adhesion, proliferation and spreading [253] by interacting with $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$ and $\alpha_{IIb}\beta_3$ integrins [254]. Such peptides are required for viability of many cells including SCs, chondrocytes and SMCs [253, 255]. IKVAV is an adhesion peptide derived from laminin $\alpha 1$ chain that binds to 110 kDa laminin receptor, $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins [23, 256]. It was previously shown that $\beta 1$ integrins on beta cells interact with the basement membrane of islets and control transcription of insulin gene [99, 257]. This peptide can also induce insulin secretion from insulin secreting cells in GSIS [23]. Further, not only it interacts with islets, but also binds to integrins located on SC surface. By this way, it may support long term cell

survival in PEG hydrogels [255]. GLP-1 is a 30-amino acid insulintropic peptide released from L cells in the intestine after meal ingestion, and acts on insulin biosynthesis, protects beta cells by decreasing apoptosis and increasing proliferation [258, 259]. Significant effects of GLP-1 on islet function have been observed in previous studies that involved islet encapsulation within PEG hydrogels [97, 260].

In this study, MSCs were chosen, as these cells are reported to be immunomodulatory, anti-apoptotic and, have capacity to support insulin secretion and to generate vascular networks [146, 247]. In a previous study by Karaoz and his colleagues, rat bone marrow MSCs were shown to be protective against STZ-induced rat beta cell destruction in an *in vitro* model [152]. Coating of mouse pancreatic islets and bone marrow stromal cells was further shown that insulin secretion (represented as stimulation index) and expression could be improved, and splenocyte proliferation can be prevented in silk hydrogels [149]. This study is further supported with the findings that mouse MSCs promoted mouse pancreatic islet insulin secretion in co-encapsulation in alginate hydrogels and it was shown to be promising for treatment of diabetic mice [150]. In another previous study, Yeung and his colleagues indicated that exposure of human pancreatic islets to pro-inflammatory cytokines in the presence of human bone marrow MSCs was protective as shown by TUNEL staining and these islets were still functional after the treatment [151]. Transplantation of rat pancreatic islets and rat bone marrow MSCs to the kidney capsule yielded enhanced vascularization in the transplantation area and less number of islets are required to reach normoglycemia [28].

3.4.1. FTIR Analysis of PEGylated Peptides

The reason for covalent incorporation of peptides was to enhance their physiological effect on islet survival. For example, insulintropic ligand GLP-1 used here would be cleaved by dipeptidyl-peptidase within nearly 2 minutes [261], when administered intravenously. However, in our system there is no need for intravenous administration of GLP-1, as its covalent incorporation would keep the ligand stationary within the synthetic network and will not involve systemic circulation, when considered for implantation into an *in vivo* model.

After conjugation reaction, FTIR analysis was performed to confirm peptide PEGylation. When acr-PEG-SVA was compared to PEGDA, the presence of C=O

stretching of SVA was observed at 1780 and 1810 cm^{-1} (Figure 3. 6A). After reaction with IKVAV, these peaks were disappeared and instead, at 1540 cm^{-1} (NH_2 scissoring) was obtained proving that amide bond between acrylate-PEG and IKVAV produced. Further, formation of new peaks in conjugate at 1630 cm^{-1} ($\text{C}=\text{O}$ stretching), 3280 cm^{-1} (O-H stretching) coming from structure of IKVAV were observed (Figure 3. 6B). Similarly, when RGDS was conjugated, amide bond was formed and additional peaks at 1630-1650 and 3080-3670 cm^{-1} of $\text{C}=\text{O}$ stretching and O-H stretching were obtained, respectively (Figure 3. 6C). Finally, when GLP-1 was PEGylated, peak belonging to $\text{C}=\text{O}$ stretching of SVA disappeared. Instead, peaks of 1540 cm^{-1} (NH_2 scissoring), 1630-1650 cm^{-1} ($\text{C}=\text{O}$ stretching) and 3090-3650 cm^{-1} (O-H stretching) were obtained indicating that conjugation was successfully performed (Figure 3. 6D).

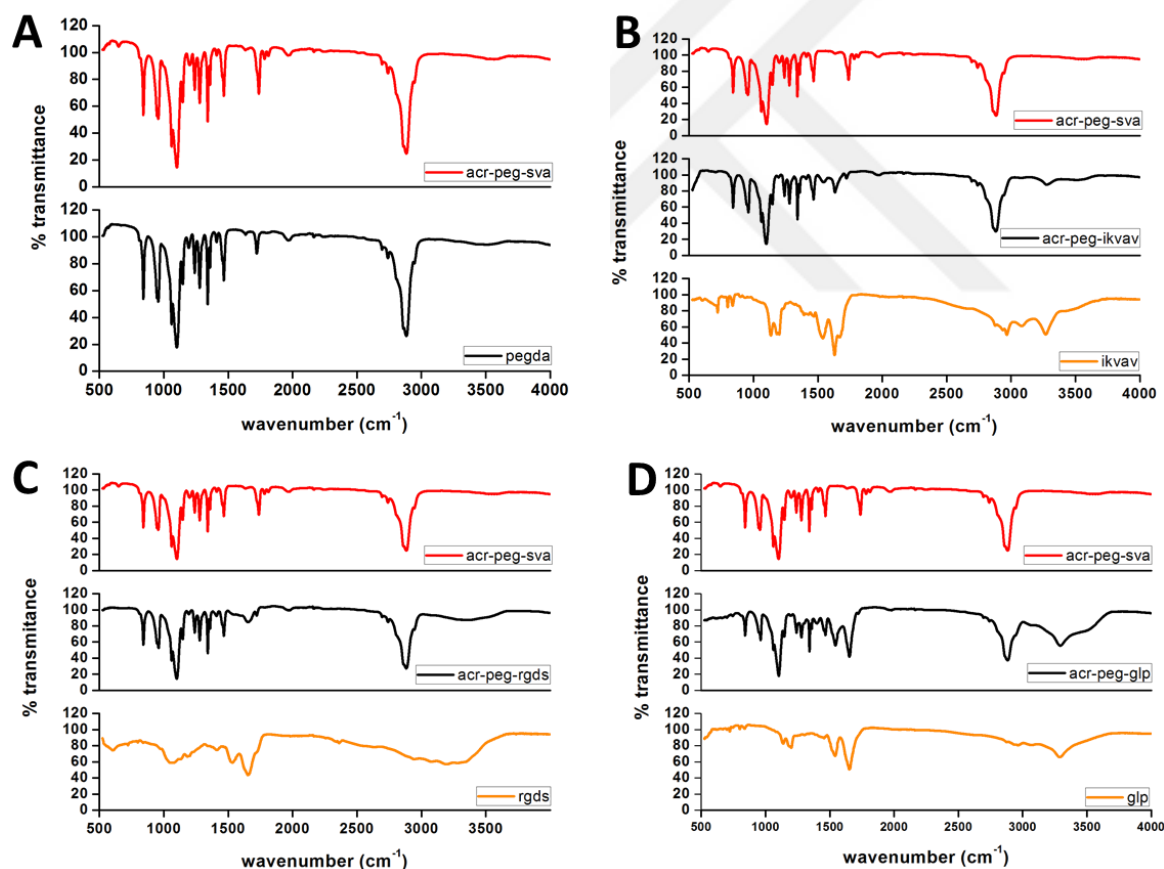


Figure 3. 6: FTIR spectra (transmittance) of PEGylated peptides. FTIR spectra of A) acr-PEG-SVA vs. acr-PEG-acr, B) acr-PEG-SVA, acr-PEG-IKVAV and IKVAV, C) acr-PEG-SVA, acr-PEG-RGDS and RGDS, D) acr-PEG-SVA, acr-PEG-GLP-1 and GLP-1.

3.4.2. Viability and Caspase Activity of MSCs in PEG Hydrogels

Encapsulation of MSCs within cell adhesion peptides and GLP-1-functionalized, covalently crosslinked PEG hydrogels was carried out via visible light photopolymerization. To evaluate the morphology of MSCs upon encapsulation, cells were observed for 5 days. For the conditions studied, the morphology of MSCs remained spherical in PEG hydrogels and no significant changes were observed until day 5 (Figure 3. 7). In contrast, altered survival rates were measured for MSCs encapsulated within functional PEG hydrogel.



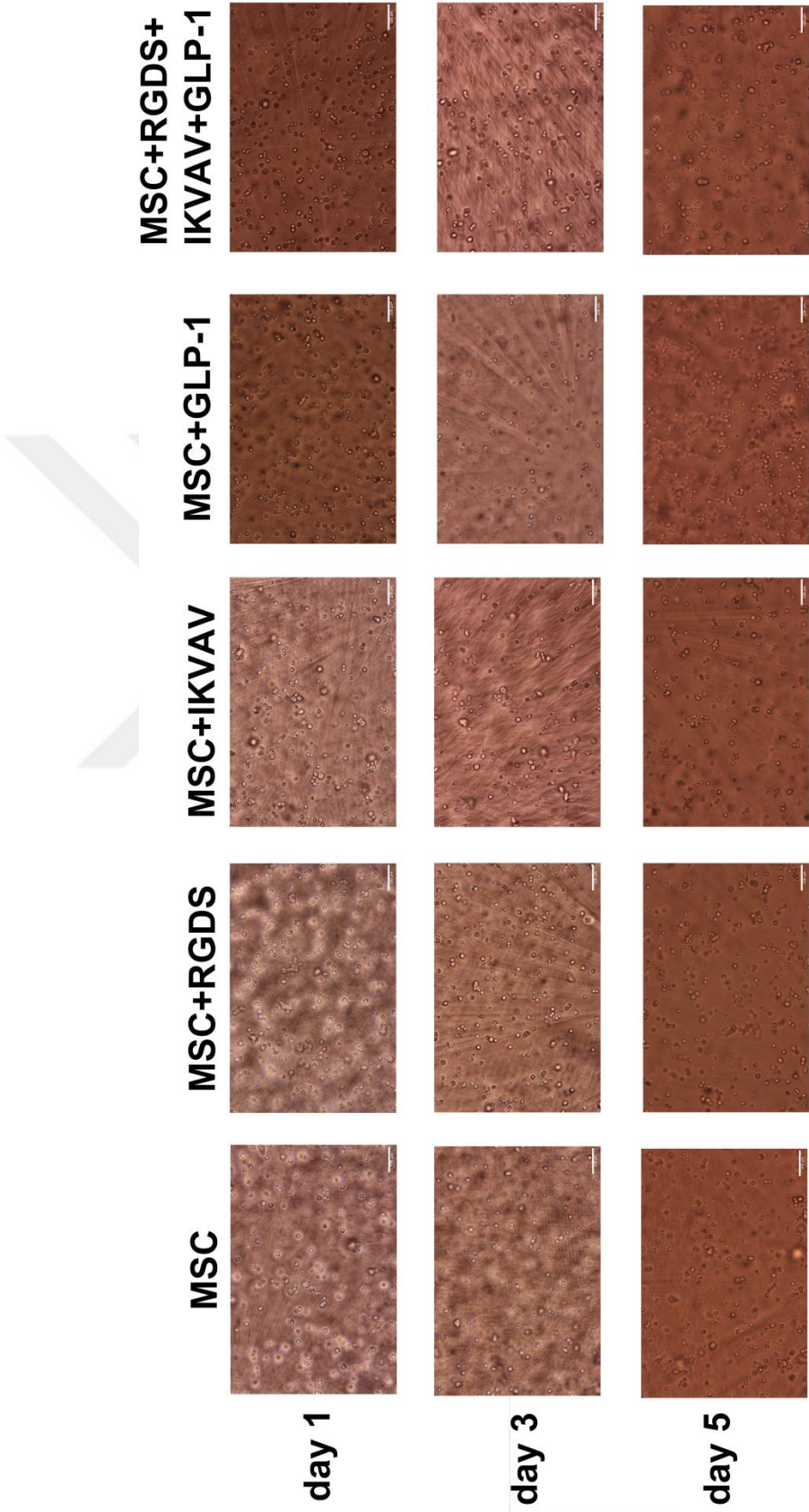


Figure 3. 7: Optical microscope image of MSCs encapsulated in biofunctional PEG hydrogels. Visualization with light microscopy was performed on 1, 3 and 5th days post-encapsulation. Scale bars: 100 μ m.

For MSCs without peptides, ATP amount was 64 ± 7 , 46 ± 7 , 7 ± 0.14 pmole for day 1, 3 and 5, respectively (Figure 3. 8A) decreasing significantly. In the presence of PEGylated peptides, although there has been observed slight reductions in cell viability, cells remained viable. ATP amounts of MSCs with RGDS were 75 ± 3 , 55 ± 2 , 59 ± 19 pmole for day 1, 3 and 5, respectively. These results indicate that $73 \pm 3\%$ and $78 \pm 21\%$ of the encapsulated cells were still viable with fibronectin derived peptide, RGDS. In the case of IKVAV functionalized PEG hydrogels, intracellular ATP was 76 ± 9 , 63 ± 8 and 58 ± 21 pmole for day 1, 3 and 5 with $83 \pm 12\%$ and $77 \pm 24\%$ of the day 1 viability. Incorporation of GLP-1, insulinotropic peptide, also supported cell viability as ATP was 63 ± 5 pmole at day 1, 47 ± 5 pmole at day 3 and 65 ± 1 pmole at day 5 with $74 \pm 9\%$ (day 3) and $103 \pm 7\%$ (day 5) viabilities. Further, when these three peptides were combined, the viability was $113 \pm 17\%$ and $94 \pm 52\%$ of day 1 for day 3 and 5, respectively.

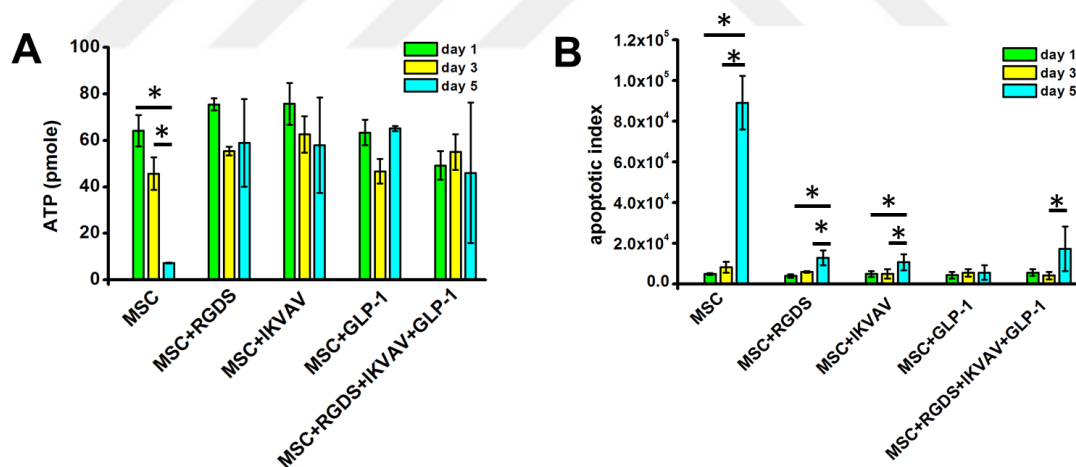


Figure 3. 8: Effects of peptides on viability of MSCs in PEG hydrogels at post-encapsulation days 1-5. (A) ATP content of encapsulated MSCs. Mean $\pm$ SD. n=2 (B) Caspase activity of MSCs within PEG hydrogels represented as apoptotic index. Mean $\pm$ SD. *p<0.05.

The effect of ligand incorporation on MSC viability was also supported by an apoptosis assay, as significant increases in caspase activity in unmodified hydrogels were observed throughout the study. As represented in Figure 3. 8B, in unmodified hydrogels,

apoptotic indices increased from $5\,001 \pm 492$ (day 1) to $8\,358 \pm 2\,751$ (day 3) and $89\,142 \pm 13\,125$ (day 5), although the first day apoptotic indices of unmodified hydrogels were similar to peptide immobilized hydrogels. These indices at day 1 were $4\,007 \pm 903$, $5\,023 \pm 1498$, $4\,411 \pm 1\,717$, $5\,689 \pm 1\,679$ for RGDS, IKVAV, GLP-1 and RGDS+IKVAV+GLP-1, respectively. However, longer incubation periods resulted in slight increases in the caspase activities in all peptide conditions. At the end of 3-day culture period, these indices increased to $6\,012 \pm 388$, $5\,117 \pm 2\,279$, $5\,566 \pm 1\,864$ and $4\,249 \pm 1\,770$ for RGDS, IKVAV, GLP-1 and RGDS+IKVAV+GLP-1, respectively. Moreover, elevated level of caspase activity was observed from all conditions at day 5, but significantly less than unmodified hydrogels.

In the absence of adhesion peptides, due to the lack of cell-matrix signaling related cell attachment, cells begin to increase caspase activity and go under apoptosis, mainly anoikis which occurs as a result of detaching from surrounding ECM [23]. This results in massive cell death after long term cell culture. Our observations demonstrated that 90% of cells lost viability on day 5 which is also consistent with previous findings. For example, Nuttelmann et al. also reported that 85% of MSCs were not viable in unmodified PEG hydrogel scaffolds after 7 days of incubation [262].

Encapsulation of MSCs in the presence of cell adhesion ligands (RGDS, IKVAV) and insulinotropic ligand GLP-1 within PEG hydrogel scaffold, improved MSC survival significantly. The metabolic activity was monitored with ATP measurements, which was conserved for 5 days of incubation. Caspase activity is an indicator of controlled cell death, and this was remained minimal for MSCs encapsulated within functional PEG scaffolds. Caspase activities measured for day 3 and day 5 were similar when RGDS or IKVAV was used as a functional ligand. The reason of capacity of modified PEG hydrogels to maintain viability is that RGDS and IKVAV are reported to be able to support diverse cellular activities ranging from adhesion to spreading [253] by interacting with specific integrins such as $\alpha_5\beta_1$, $\alpha_v\beta_5$ (for RGDS), $\alpha_6\beta_1$ and 110 kDa laminin receptor (for IKVAV) [23, 254].

These peptides are required for the viability of many other cells including MSCs, chondrocytes, smooth muscle cells, and beta cells in synthetic networks [23, 253, 255]. Lee et al. have also reported that RGDS binding integrins, $\alpha_5\beta_1$ and $\alpha_v\beta_5$, are crucial for

the expression of stemness related genes within PEG hydrogel scaffolds [263, 264]. This property is important for MSCs to stay pluripotent, so that they would promote glucose dependent insulin secretion from islets within synthetic 3D encapsulation system.

Interestingly, we observed that when MSCs were encapsulated in GLP-1 functional PEG hydrogel scaffolds, viability and caspase activities were similar to the cases where RGDS or IKVAV was conjugated to polymeric PEG scaffolds. To date, there is no report on the effect of GLP-1 on MSC survival within synthetic networks and it was demonstrated here for the first time that insulintropic ligands such as GLP-1 played an important role for the survival of MSCs within PEG hydrogel scaffolds. This finding here is consistent with the previous study, where Sanz et al. observed that GLP-1 incretin hormone prevented differentiation of MSCs by interfering with the expression of peroxisome proliferator activated receptor- γ , CCAAT-enhancer binding protein and lipoprotein lipase and increased cell proliferation and prevented apoptosis via MEK and PKC pathways *in vitro* in a cell culture medium [265].

Three-day culture period was chosen for the duration of co-encapsulation experiments, as MSCs would eventually differentiate into adipogenic, osteogenic or chondrogenic lineages [266, 267]. The 3-day *in vitro* culture experiments proved that MSCs could still induce islet insulin secretion from islets. Further, in unmodified hydrogels, MSCs survival would be significantly compromised on day 5 and no stimulatory effect would be expected.

3.4.3. Encapsulation of Pancreatic Islets

In the case of pancreatic islets, in unmodified hydrogel system, 0.13 pmole ATP per islet was obtained for pancreatic islets as represented in Figure 3. 9A and this value is similar to previous reports of islet encapsulation in PEG hydrogel system [98]. Incorporation of peptides to the current system yielded ATP up to 0.34 pmole. In all peptide containing conditions, nearly at least 2-fold greater in intracellular ATP level was obtained. Single addition of peptides showed that IKVAV and GLP-1 were more effective than RGDS for cell survival. Similarly, caspase activity was also reduced upon addition of peptides (Figure 3. 9B). Caspase activity for islets encapsulated in unmodified hydrogels was significantly higher than the other remaining groups, where apoptotic activity of 0.99 ± 0.16 was obtained for this control group. Apoptotic activities of islets

encapsulated in the presence of covalently attached peptides were 0.51 ± 0.07 for RGDS, 0.37 ± 0.09 for IKVAV and 0.74 ± 0.04 for GLP-1 containing groups. Simultaneous incorporation of peptides decreased apoptotic activity down to at most 0.27 ± 0.11 -fold of the control group. Caspase activity, and hence apoptotic index was further reduced down to 0.06 ± 0.03 with respect to control group, when all three peptides were conjugated to PEG hydrogel scaffolds.

The effect of establishment of such specific connections on islet viability was noticeable. In addition to *in vivo* actions, these peptides are reported to support survival and functionality of beta cells in PEG hydrogels [23, 98] confirming that IKVAV and GLP-1 are more effective than RGDS. In the absence of peptides, ATP per islet in unmodified hydrogels was lower and apoptotic activity was greater than the modified ones, as expected, due to lack of modification and the capability of PEG to act as a blank state [98].

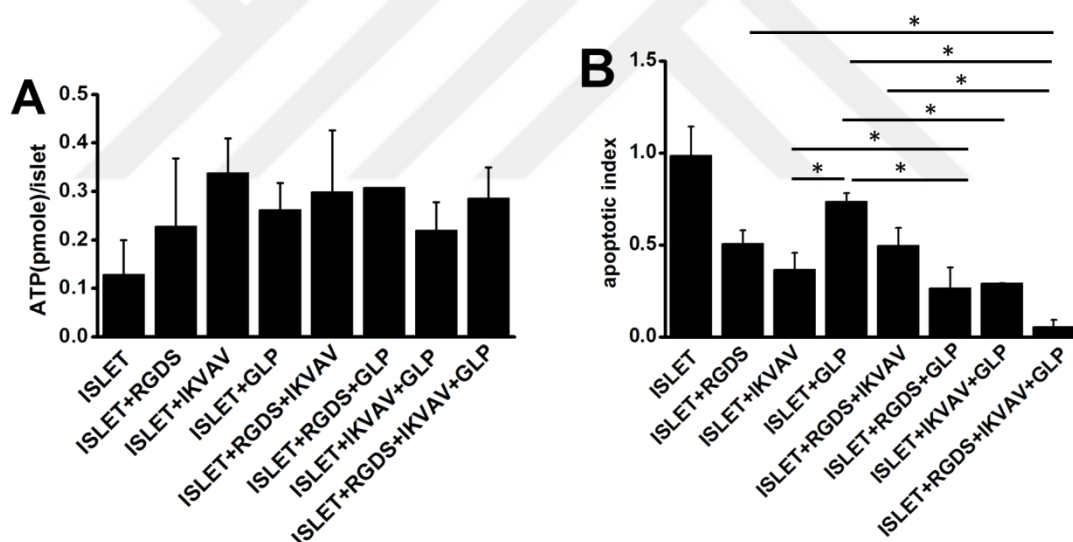


Figure 3. 9: A) ATP amount of islets as an indicator of cell survival in each encapsulation condition at day 3. Mean $\pm$ SD. n=1-3. B) Apoptotic activity of encapsulated islets at day 3. Caspase 3/7 activities during the *in vitro* incubation period were represented as apoptotic activity. Mean $\pm$ SD. n=2-4. *p<0.05. Apoptotic activities of islets differ statistically from all other conditions.

The following events might be activated by peptides to elicit such differences. Cells use 110 kDa laminin receptor and $\alpha_6\beta_1$, $\alpha_3\beta_1$ integrin [23, 256, 268, 269], and GPCR [259] for IKVAV and GLP-1, respectively to interact with the environment. This communication between IKVAV and its receptor/integrin can help stabilization of actin and also can lead to enhanced phosphorylation of focal adhesion kinase resulting in

reorganization of cytoskeleton and arrangements in apoptosis regulators and kinase pathways [23, 270, 271]. Also, binding of GLP-1 to its receptor provokes mitogenesis on islet cells with cAMP induced PKA and phosphoinositide 3-kinase activation [259, 272]. By using PKC signaling pathway, PKC interferes with caspase activation, and with ERK1/2 activation, Bad (a pro-apoptotic protein) is phosphorylated and inactivated [273]. In addition, anti-apoptotic proteins (Bcl-2 and Bcl-xL) expression is enhanced [274] whereas caspase-3 (effector caspase) expression is downregulated [275].

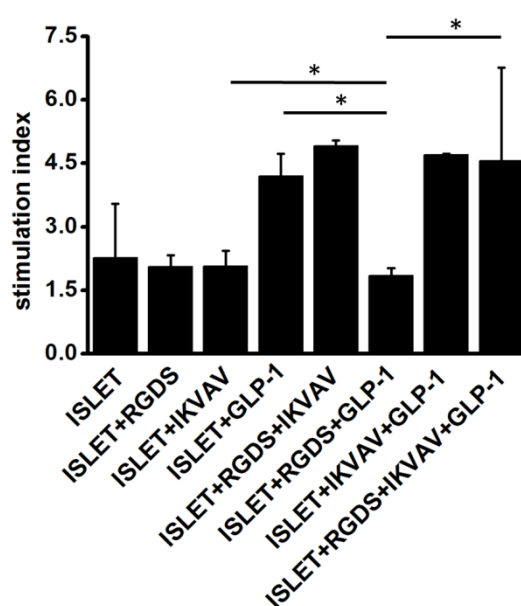


Figure 3. 10: Stimulation index of islets in functionalized PEG hydrogels. Insulin secretion was expressed as stimulation index. Mean±SD. n=2. *p<0.05.

For the functionality assay, encapsulated islets were exposed to glucose challenge with static incubation assay and capability of islets to secrete insulin in response to altered glucose concentrations was expressed as stimulation index (Figure 3. 10). Stimulation index represents the ratio of amount of insulin secreted by islets at high glucose to the amount of insulin secreted at low glucose concentration. This gives more accurate information about GSIS from islets as a clinical release criterion, as in each set, with similar islet numbers the number of cells within each islet might differ, which can be misleading when expressed as insulin amount at low and high glucose concentrations [276]. This was also used in previous recent studies about encapsulated islets to characterize insulin secretion function [149, 176]. Thus, in each case, change of islet

responsiveness against glucose levels under studied conditions would clearly show the role of peptides or MSCs. Stimulation index of islets in unmodified, RGDS and IKVAV modified PEG hydrogels were approximately similar and was around 2. Combination of RGDS and IKVAV has induced islet functionality further by increasing stimulation index up to 4.92 ± 0.13 . Insulinotropic peptide, GLP-1 was shown to be more effective than ECM derived peptides (Figure 3. 10). The addition of IKVAV along with GLP-1 into the encapsulation environment promoted islet function further and this suggests that addition IKVAV and GLP-1 have synergistic effect to promote insulin secretion in response to high glucose. Further incorporation of RGDS into IKVAV+GLP-1 hydrogel was also a trigger for islet function and similar stimulation index value of 4.57 ± 2.19 was measured for the islets encapsulated in this group.

Viability and static incubation results indicated that adhesion peptides can support viability alone, but they should be combined or GLP-1 alone can be utilized for functionality of the islets. For this case, similar to islet viability, the profound effect of IKVAV and GLP-1 arises from their receptor/integrin specific interaction. The superiority of IKVAV over RGDS can arise from initiation of cytoskeletal changes through F-actin and t-SNARE communication for insulin granule docking and activation of nuclear factor κ B pathway that is important for Rab3, GLUT2, UCP-2 and Bcl-2 expression for glucose uptake, thus insulin secretion and oxidative activity of cells [277, 278]. GLP-1 stands as the most dominant effector among the peptides used in this study, as this insulinotropic peptide is a regulator of insulin biosynthesis and release from beta cells through cAMP induced PKA and EPAC pathways [259].

3.4.4. Co-encapsulation of Pancreatic Islets and MSCs

In the case of pancreatic islets as analyzed in this study, the morphology of the encapsulated cells was as represented in Figure 3. 11. Islets were round and intact. Similarly, MSCs were observed to be round.

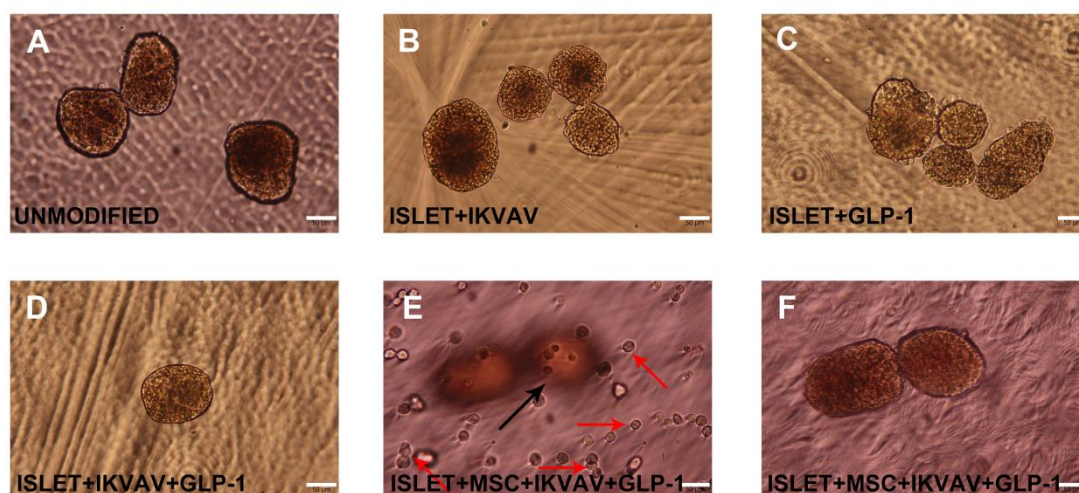


Figure 3. 11: Encapsulated pancreatic islets and MSCs in PEG hydrogels. Black arrow: Pancreatic islet. Red arrow: MSC. Scale bars: 50 μ m.

When MSCs were coupled with the adhesion peptides and GLP-1 into islet encapsulated PEG hydrogels, apoptosis and viability seemed to be unaffected and compared to ISLET+SC, no significant change was observed as represented in Figure 3. 12 with 0.8-1.1 range. To further investigate anti-apoptotic properties in the presence of MSCs, islets were co-encapsulated with MSCs in the presence or absence of covalently bonded ligands within PEG hydrogel scaffolds. The presentation of ECM mimic peptides (RGDS and IKVAV) reduced islet apoptosis (Figure 3. 9), while with co-encapsulated MSCs, islet apoptosis was not significantly different in the presence of ECM derived peptides compared to control (islets+MSCs) (Figure 3. 12). This could be due to the significant apoptosis of MSCs rather than islets. Also, according to Figure 3. 12A, the amount of ATP was not different in co-encapsulated islets and MSCs, with or without presentation of peptides in the scaffold. This might indicate that the majority of the signals were coming from MSCs, and that the presentation of peptides in hydrogel scaffolds did not have a profound effect on apoptosis of the co-encapsulated islets and MSCs. However, several prior studies reported the anti-apoptotic effect of MSCs on islets in cell culture medium, and this was not observed here. This was most probably due to differences in the experimental approaches used here and in other studies. As a result, although there has been several researchers reporting the anti-apoptotic effect of MSCs on islets, viability in terms of ATP content and caspase activity remained nearly same for ISLET+MSC and ISLET+MSC+peptide groups. This was most probably due to the

differences in the experimental approaches used here and other studies. Previous studies focused mainly on reducing the harmful effects of a cytokine (Tumor necrosis factor- α (TNF- α), interleukin-1 β , interferon- γ), or streptozotocin (STZ) and hypoxia induced apoptosis of islets in a co-culture with MSCs *in vivo* and *in vitro* [132, 146, 149, 151-153, 279]. Here, we mainly investigated the protection potential of islets from apoptosis with the anti-apoptotic property of MSCs in the presence or absence of biofunctional signaling molecules [270, 280]. Thus, we could not monitor the anti-apoptotic property of MSCs on islet survival.

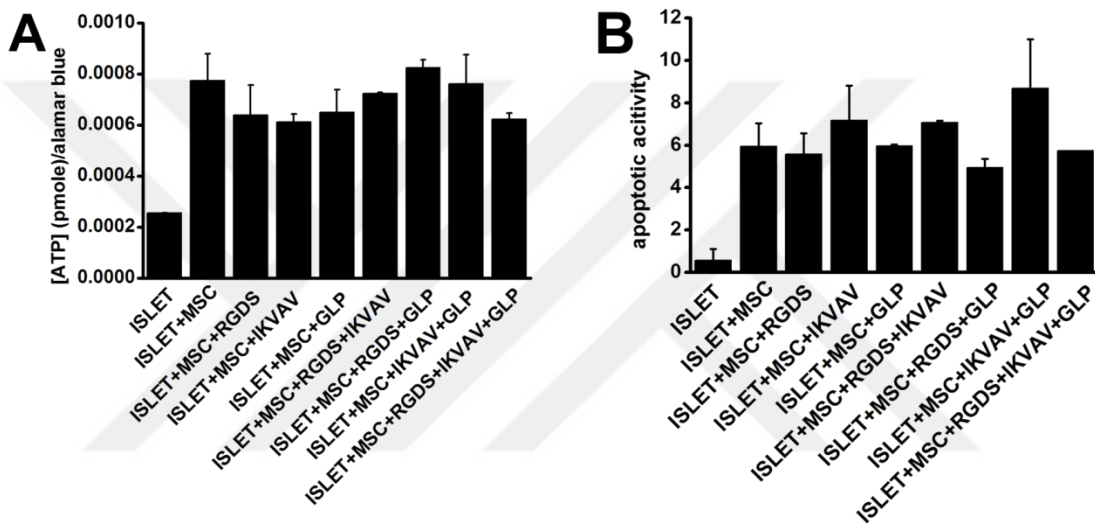


Figure 3. 12: A) ATP amount of islets+MSCs as an indicator of cell survival in each encapsulation condition at day 3. Mean $\pm$ SD. n=2. B) Apoptotic activity of co-encapsulated cells at day 3. Caspase 3/7 activities during *in vitro* incubation period were represented as apoptotic activity. Mean $\pm$ SD. n=2.

Presentation of ligands within 3D PEG hydrogel scaffolds for islets and MSCs provided sufficient cell–matrix interactions; however, cell–cell interaction is also required to prevent apoptotic pathways. In the system considered here, investigation of the influence of cell–cell signaling pathways on islet viability or function was not within the scope of this study and was not considered. As a result, viabilities and caspase activities were observed to be similar and the anti-apoptotic properties of MSCs on islets could not be observed.

Adhesion peptides and GLP-1 had additional effect on pancreatic islet functionality with MSCs (Figure 3. 13). This was the outcome of the effect of peptides on both islets and MSCs. As explained above, these peptides increased islet functionality and supported

SC viability. The maintenance of SC viability might lead to release of tropic factors that also form driving forces of enhanced insulin release, as direct contact between islets and MSCs were not designed. For example, factors such as HGF, TGF- β and IL-6 have positive responses on beta cell function [152, 281]. HGF (through c-Met receptor) and TGF- β activate PI-3K-PKB/Akt pathway [281-284], but the negative effect of TGF- β on beta cells was also observed previously [285]. Although the effect of IL-6 on beta cells has controversial reports [286-288], IL-6 signals through PLC-IP3 are reported to take part in insulin secretion [289]. These events are possible candidates for SC action on beta cells to drive insulin secretion. In addition, RGDS binds to $\alpha_5\beta_1$ and $\alpha_v\beta_3$ [290], and IKVAV can interact with $\alpha_6\beta_1$, $\alpha_3\beta_1$ and 110 kDa laminin receptor for cell survival and functionality [23, 256]. These connections induce changes in cytoskeleton of the cells and through F-actin and t-SNARE interaction, and also NF- κ B activation resulting in insulin release [278, 291]. Also, it is established in the literature that GLP-1 and its receptor interaction drives insulin secretion through PKA and EPAC pathways [259].

Combination of MSCs and presence of ECM peptides and GLP-1 improved islet function significantly. Co-encapsulation of islets and MSCs in unmodified PEG hydrogel scaffolds resulted in a two-fold increase in the stimulation index compared to the islet only condition. We observed substantial improvements when MSCs and islets were encapsulated within ligand incorporating PEG hydrogel scaffolds. The stimulation index further rose to seven-fold of the islet-only group when peptides and MSCs were used in combination.

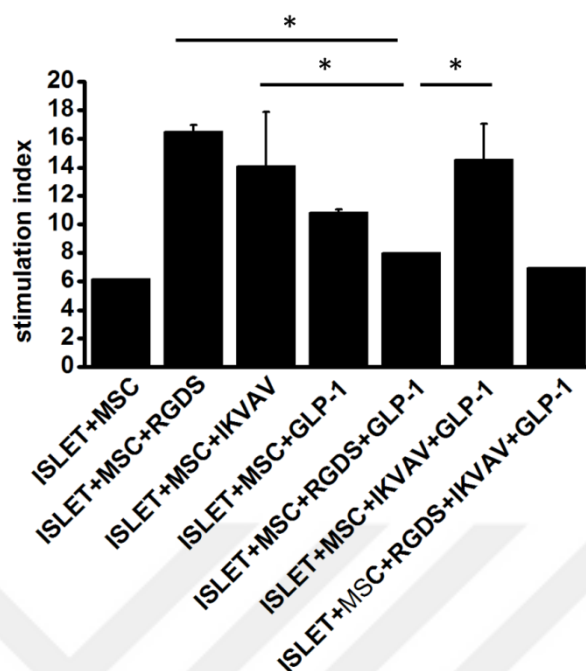


Figure 3. 13: Stimulation index of pancreatic islets in functionalized PEG hydrogels. Insulin secretion was expressed as stimulation index. Mean±SD. n=1-2.*p<0.05.

3.5. Conclusion

The functionality of islets within 3D PEG hydrogels can be significantly improved in the presence of MSCs, ECM derived cell adhesion ligands and GLP-1. Significant improvements in islet functionality was observed on the co-encapsulation of islets with MSCs. Immobilized peptides acted as ECM mimic ligands to stimulate cell survival and insulin secretion pathways. GLP-1, a potent incretin hormone promoted stimulation of insulin gene transcription, islet growth as a result of binding to its receptor on the surface of islet cells, and hence increased insulin secretion in response to high glucose. Further addition of MSCs into the system resulted in significant improvements in functionality; most probably due to soluble factor release from the MSCs. Synergistic effects of cell adhesion peptides on islet functionality were also observed. This study is promising in demonstrating the combination of fundamental parts of a tissue (cell and ECM) and insulintropic ligands for the future design of a bioartificial pancreas and for the treatment of T1D.

Chapter 4

Sequential Coating of Insulin Secreting Beta Cells within Multilayers of Polysaccharide Based Nanogels

4.1. Abstract

Pancreatic islet transplantation has emerged as a promising treatment for type 1 diabetes; however, its clinical application is still limited by life-long use of immunosuppressive drugs, insufficient number of islets to achieve normoglycemia and large transplantation volume. This paper reports on the unique type of nano-thin coating of insulin secreting beta cell aggregates. The coating is based on hydrophobic and covalent interactions of natural acrylate modified cholesterol bearing pullulan nanogels (CHPOA) on MIN6 beta cell aggregates. We demonstrated conformal bilayer coating of nanogels over beta cell aggregate surfaces. Beta cell aggregates, derived from mice, were prepared as spheroids through hanging drop method, which was optimized with respect to hanging drop volume and initial number of beta cells. These aggregates, defined as pseudoislets, were coated with sequential layers of nanogels and were determined as viable and functional for insulin secretion. We carried out coating experiments using physiologically compatible medium, where pseudoislets were not brought in contact with toxic prepolymer solutions which is used in existing approaches. This study is promising and offers new opportunities through coating of insulin secreting islets with advanced functional materials under completely physiological conditions for clinical translation of cell transplantation technology and particularly for the treatment of T1D.

4.2. Introduction

Type 1 diabetes is a chronic autoimmune disease induced by immune destruction of

insulin secreting beta cells in pancreas. Although insulin therapy has been implemented into clinical setting of type 1 diabetes, pancreas/islet transplantation with life-long use of immunosuppressive drugs is an end-point solution for the patients and critical for patient survival. However, repetition of transplantation and isolation of islets from cadavers point out the necessity of a continuous donor source and immunosuppressive drugs [5, 231, 292]. Despite the fact that many immunosuppressive drugs are suggested to be use in islet transplation, side effects on whole body attenuate the proper function of beta cells and decrease the life-quality of patients [14, 165, 293]. Nearly two decades ago, with the development of Edmonton protocol, 21 of 36 patients had glycemic control and that of 76% (16) were insulin dependent at 2 years in an international trial [69]. Yet, prolonging graft survival is still a major concern, as sirolimus and tacrolimus exert significant effects on islet survival and insulin secretion [8, 10, 12].

Considering that at least 12 000 islets/kg body weight are required for insulin independence [68], current isolation procedures should also be improved, as these procedures lead to loss of 30-50% of islets [70]. To compensate this loss, alternative beta cells are established as models for global need and MIN6 beta cells are one of developed beta cells with glucose responsiveness [20]. As insulin secreting cells require adhesion and cell-cell interactions to manage their regular function and maintain their differentiated state, beta cell clustering becomes superior over single cells. Also, in their natural environment, beta cells are a part of 3D cell cluster of pancreatic islets and dissociation of islets negatively affect insulin secretion [182]. For example, stimulation indices for MIN6 pseudoislets and monolayers differed, as it rose 6-fold and 3-fold, respectively. Analysis of underlying reason for such differences yielded changes in expression of proteins in pathways of glucose metabolism, cell interaction, and translational regulation [294]. For this clustering, hanging drop is a promising strategy where droplets remain on the lid due to surface tension and cells concentrated to the liquid-air interface with the effect of gravity form clusters [295]. However, handling of this system is limited to a single study for MIN6 beta cells where MIN6- α TC and INS1- α TC heterospheroids were investigated for surface tension [296] and analysis of MIN6 beta cell clustering has not been studied in detail.

Even though alternative beta cell spheroids were recommended for type 1 diabetes, immunosuppressive drugs are still a challenge to silence heavy immune reaction. To eliminate these drugs, nano-thin coatings are designed that can have notable impact on transplantation for clinical feasibility. The main idea of nano-coating is to form an ultra-thin layer around cells for immunoprotection or functional improvement [131, 297]. Nano-thin membrane allows faster oxygen and nutrient transfer, water and CO₂ disposal due to short distance. Delay in response to glucose is also hindered [131, 298].

Traditional islet surface modification approaches in ultra-thin coating have involved electrostatic interactions, covalent bonding and hydrophobic interactions to achieve nano-thin layers on cell surfaces. In the first approach oppositely charged polymers/ions are employed for electrostatically driven multilayers. For example, MIN6-MSC (kidney derived) heterospheroids were coated in phosphorylcholine-modified poly-L-lysine/heparin layers assembled through electrostatic interactions showed improved insulin secretion and decreased cytokine induced apoptosis [132]. Decreasing void volume while maintaining immunoprotection of islets with a defined thickness of coating surpasses the effect of diffusion limitation and control graft volume. However, defects or incomplete deposition associated with coats can weaken the function of immunoisolation barrier [138]. Despite the fact that the thickness of electrostatically driven multilayers is beneficial, number of layers should be determined delicately. For instance, islet coating with multi-layers of phosphorylcholine-chitosan, alginate and phosphorylcholine-modified chondroitin-4-sulphate in allogeneic transplantation into C57BL/6 mouse kidney capsule, graft rejection was observed in control and 4-layer coated islets after 12 and 15 days, respectively. In case of 8-layer coating, graft survival was prolonged up to 28-37 days [133]. However, use of polycations such as poly-L-lysine and poly(allylamine hydrochloride) can implement significant toxicity to current systems [138].

For covalent bonding, PEG with an activated ester is used to protect islets through polymeric coating or coverage of surface for protein immobilization (e.g. albumin) [118, 138]. Gattás-Asfura et al. have used poly(amidoamine) (PAMAM) functionalized with methyl-2-(diphenylphosphino) terephthalate and hyperbranched alginate modified azide for multilayer coating on islets and no impairment on islets properties was reported for specific groups of sequential layers from islet surface to NHS-PEG-N₃ to 3-layers of

PAMAM 15/20 or 15/40 to alginate. However, long term *in vitro* culture abrogated the stability of covalently-bonded layers, as incomplete coating with PEG layer attenuated homogeneity of deposition [299]. Covalently bonded coatings can also be formed through photopolymerizations, yet deleterious effects on islets due to toxicity of co-initiators and radicals limit the use of this approach [107]. The nature of covalent coating also allows binding of cells different kind where a living barrier in the form of cells can be constructed. By employing this unique assembly of cellular architecture, in our previous work, we coated human pancreatic islets within Tregs via biotin-streptavidin interaction and investigated local immunoprotection potential of Treg coated islets from cytokine attack. Tregs can provide immune tolerance through regulation of T and B cell function and we demonstrated that exposure of Treg-coated islets to Teff induced less amount of IFN- γ production compared to uncoated islets without compromising insulin secretion function of islets [18].

For the case of hydrophobic interactions, PEG-lipid [139] or PVA-alkyl groups [135] can surround the target islet. For example, Miua et al. used lipid-PEG for the initial coat of Syrian hamster islets and then PLL-alginate was applied for electrostatic interaction driven multilayers. This method did not cause increase in volume and further, no deterioration in insulin secretion was observed [140].

In addition to protection as a physical barrier in the form of continuous polymer layer of crosslinked polymer, nanoparticle layer can be formed around beta cell clusters. Immuno-barrier potential of nanoparticles is newly recognized and to date, reports on this area for beta cells are limited. In this design, nanoparticle-based coating can act as a barrier to prevent contact of immune system cells with transplanted cells. Further advantage of nanoparticle as a drug delivery vehicle can contribute to the immunoprotection function. For example, biotin-avidin interaction was previously used in covalent immobilization of PLGA particles onto mouse islets. Islets were first treated with EZ-Link Amine-PEG11-Biotin and then avidin attached PLGA particles. Islet function was conserved in prolonged culture and viability was higher even after 21 days of *in vitro* culture. Leukemia inhibitory factor loaded PLGA particle coated DBA/2 (H-2^d) islets were then transplanted into kidney capsule of diabetic C57BL/6 (H-2^b) recipients and 4 of 7 mice were normoglycemic more than 100 days [142]. Layer-by-

layer coating (mono and 6-layer) with positively and negatively charged silica nanoparticles have also been tested on INS-1 single cells. However, significant decrease of particles on cells was detected for mono and 6-layer coating less than 10 h and stability was improved up to 24 h with trehalose supplement. The reason for this failure was proposed to be internalization of the particles, desorption of the particles from membranes or membrane restructuring [300].

In the present study, we report on a novel coating of acrylate modified cholesterol bearing pullulan (CHPOA) nanogels on MIN6 pseudoislets. MIN6 pseudoislets were formed through hanging drop technique as model beta cell clusters. For this purpose, response of cells in terms of viability to three parameters (initial cell number in the droplets, incubation day and droplet volume) was investigated. After optimization of pseudoislet preparation, sequential layer of coating of pseudoislets was achieved with CHPOA nanogels (Figure 4. 1). This approach utilizes hydrophobic and covalent interactions between nanoparticles and pseudoislets where nanogels were immobilized on pseudoislet surface in a physiological solution without any further treatment. This technique is advantageous compared to traditional layer-by-layer coating strategies as beta cells are not placed in contact with toxic prepolymer solution, which is crucial for islet viability and prolonged insulin secretion function *in vitro* and *in vivo*.

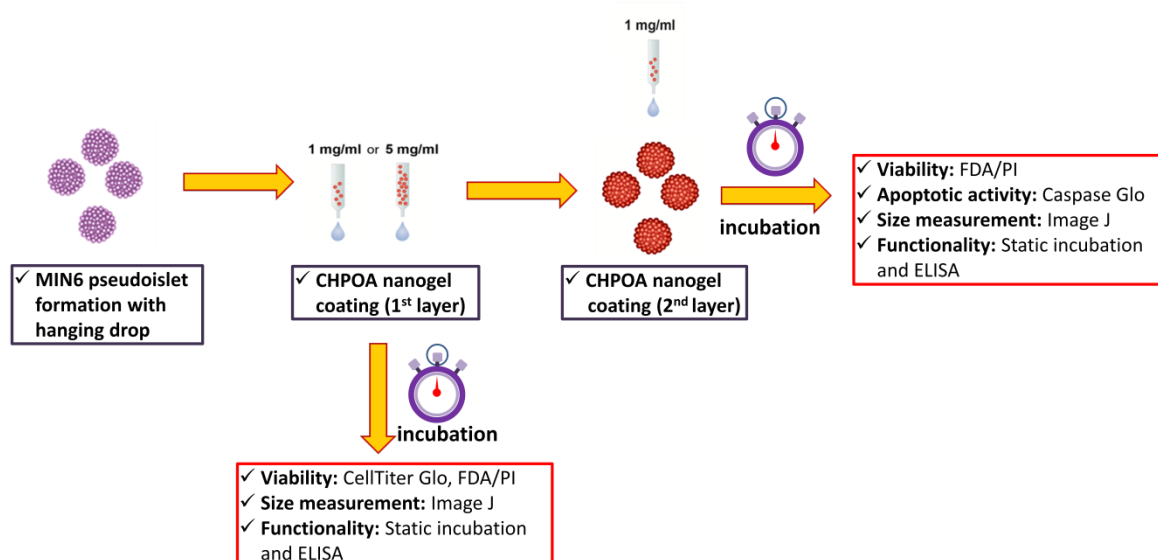


Figure 4. 1: Flow diagram of coating and characterization of MIN6 pseudoislets.

All coated pseudoislets maintained viability and insulin secretion function *in vitro*. The results demonstrated that, this approach can be considered for clinical translation of cell encapsulation research without compromising of cell function and increasing of cell transplantation volume.

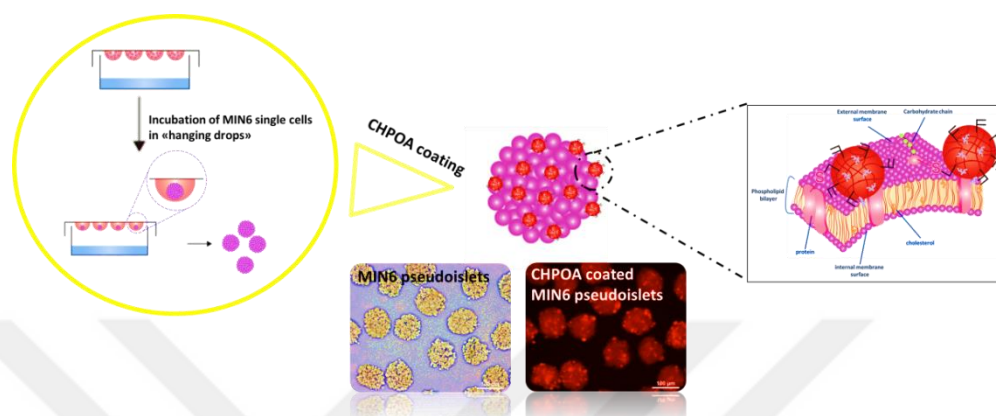


Figure 4. 2: Nano-thin coating for immunoisolation of beta cells.

4.3. Materials and Methods

Culture of MIN6 Beta Cells: MIN6 beta cells are donated by Dr. Donald F. Steiner from University of Chicago, Biochemistry and Molecular Biology with the permission of Professor Jun-ichi Miyazaki from Osaka University, Medical School, Division of Stem Cell Regulation Research. For maintenance, MIN6 beta cells were cultured in DMEM (HG, Gibco), 10% FBS (heat inactivated, Gibco), 100 IU/ml penicillin-0.1 mg/ml streptomycin (Sigma), 2 mM L-glutamine (Sigma), 1 mM sodium pyruvate (Lonza) and 0.10 μ M β -mercaptoethanol (Invitrogen) at 37°C with 5% CO₂ environment.

Formation of MIN6 Pseudoislets through Hanging Drops: MIN6 beta cells (p25-41) grown in 60 mm dishes (Nest) were placed onto the lids of 100 mm cell culture dishes (Corning) in 20/30 μ l drops at a final number of 200, 300, 500 and 1000 per drop. Then, the lids were inverted as in Figure 4. 3, 10 ml 1X PBS (sterile) supplemented with 1% penicillin-streptomycin was placed into the dishes and these cells were cultured in the form of drops at 37°C, 5% CO₂ environment for 1-6 days.

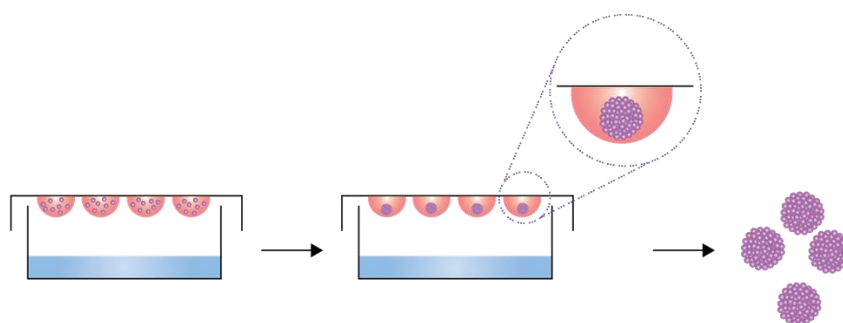


Figure 4. 3: Formation of MIN6 pseudoislets in hanging drops

Microscopic Observation and Live/Dead Staining of MIN6 Pseudoislets: At post-hanging drop formation day 1-6, drops were disrupted and pseudoislets were hand-picked under inverted microscope.

To observe the effect of initial cell number, droplet volume and incubation time on pseudoislet formation and viability, pseudoislets were stained with fluorescein diacetate (FDA) and propidium iodide (PI). Pseudoislets were treated with a dye solution of 8.5 $\mu\text{g/ml}$ PI and 42.4 ng/ml FDA. After incubation, excess dye solution was removed, pseudoislets were rinsed and observed with fluorescence microscopy (Ex: 450-490 nm, DM: 505 nm, BA: 520 nm and Ex: 510-560 nm, DM: 575 nm, BA: 590 nm). Images were processed with Image J and, red and green areas were used to calculate % viability.

CHPOA and CHPOA-Rh Nanogel Preparation: CHPOA and CHPOA-Rh were synthesized as described previously [241, 301]. CHP with MW 100 000 Da was used to synthesize acrylate and rhodamine B modified forms. Degree of substitution of acrylate and rhodamine was 24.1 and 0.3 per anhydrous 100 glucose units. For nanogel preparation, CHPOA and CHPOA-Rh (10 mg/ml) were dissolved and incubated at 37°C for 5 h. After swelling, it was stirred for 19 h at room temperature and filtered (0.22 μm).

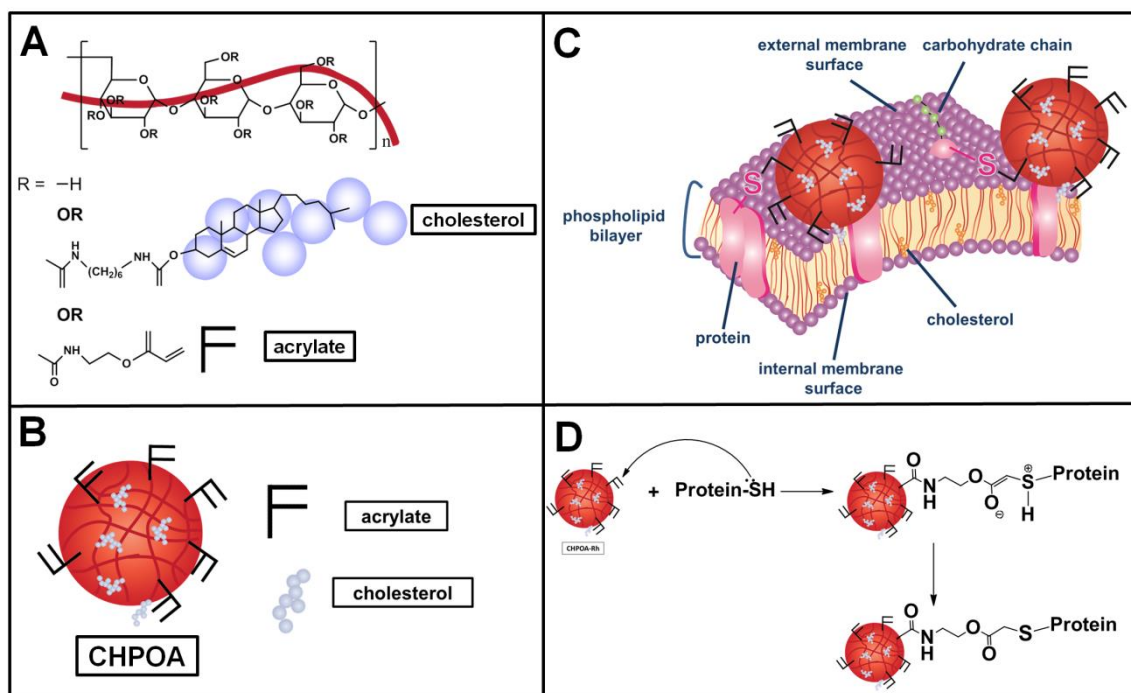


Figure 4. 4: Preparation of CHPOA nanogels and coated MIN6 pseudoislets. A) CHPOA polymer is composed of pullulan chain as backbone, acrylate group and cholesterol group as side chains. B) During self-assembly of CHPOA, hydrophobic cholesterol units collapse and form hydrophobic interactions with each other. As a result of such interactions, CHPOA nanogels are formed. C) During incubation of MIN6 pseudoislets, CHPOA nanogels interact with MIN6 beta cell membrane with hydrophobic interactions and covalent reaction between acrylate group of CHPOA and thiols of proteins present on cell membrane. D) During thiol-acrylate reaction, electron withdrawing capacity of sulfur group drives reaction.

Coating of Pseudoislets with CHPOA/CHPOA-Rh Nanogels: Fifty pseudoislets per 200 μ l of 5 mg/ml, 1 mg/ml or PBS (control) were hand-picked and put into each polypropylene tube (Figure 4. 5A). During 1 h incubation, pseudoislets were tapped/shaken gently for several times. After this period, medium and PBS washes were performed and coating was confirmed with fluorescence microscopy (Ex: 510-560 nm, DM: 575 nm, BA: 590 nm). For double layer coating, control or nanogel coated pseudoislets were incubated for 24 h under standard culture conditions and then were treated with PBS or 1 mg/ml CHPOA-Rh (Figure 4. 5B). For FDA/PI staining, unlabeled CHPOA nanogels were used.

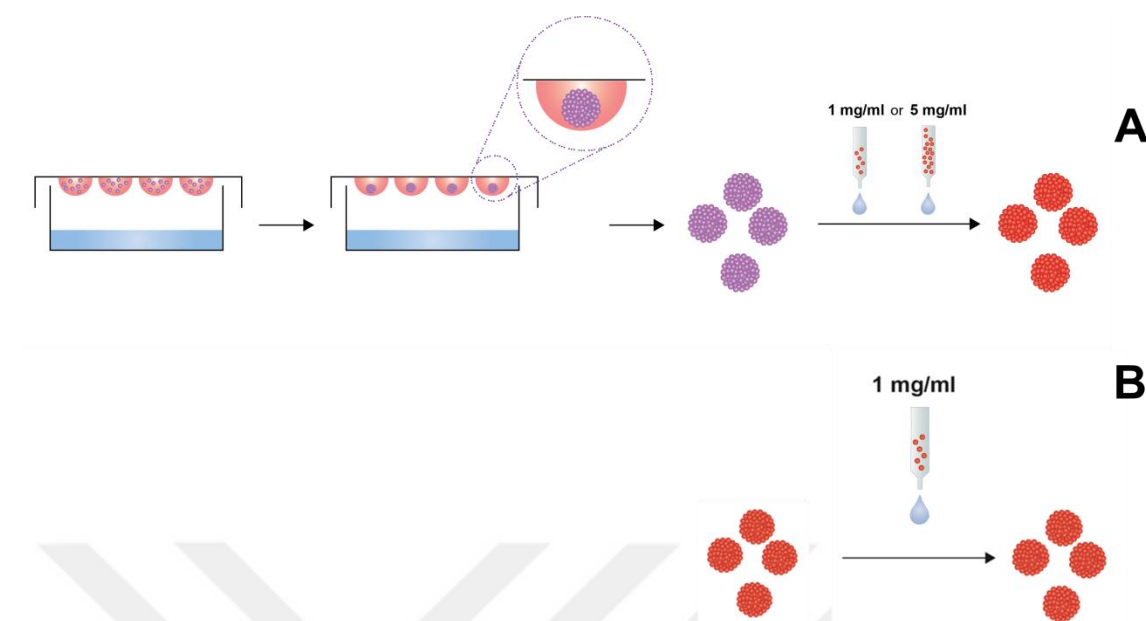


Figure 4. 5: Schematic representation of CHPOA/CHPOA-Rh nanogel coating of MIN6 pseudoislets. A) MIN6 pseudoislets were formed with hanging drops and treated with CHPOA/CHPOA-Rh nanogels. B) Second treatment was performed on coated pseudoislets for double layer coating.

Cellular Activity of Nanogel Coated Pseudoislets: Viability of MIN6 pseudoislets was analyzed with intracellular ATP levels, caspase activity and FDA/PI staining. Ten pseudoislets were treated with 50% CellTiter Glo reagent (Promega) for 15 min or 50% Caspase Glo reagent (Promega) for 1.5 h. For ATP levels, a standard curve of ATP (pmole) was generated for calculation. At the end of incubation periods, luminescence of samples was measured with a plate reader (Synergy-H1 Multi-Mode Plate Reader, Biotek). FDA/PI staining was performed similar to untreated pseudoislets.

MIN6 pseudoislet function was investigated as well. After nanogel treatment, 20 pseudoislets were incubated for 1 h in cell culture inserts (12 μ m) in Kreb's Ringer Bicarbonate (KRB) buffer containing 2.8 mM (low) glucose solution. This low glucose treatment was repeated and KRB buffer containing 28 mM (high) glucose was used as final buffer. These buffers were stored at -20°C until analysis with ELISA (Mercodia). Stimulation index of pseudoislets was calculated as the ratio of insulin secreted at high glucose to low glucose.

Statistical Analysis: Pairwise comparison was performed with Mann Whitney test or t-test. Multiple comparisons were performed with analysis of variance with Tukey's post hoc test. p values less than 0.05 were considered statistically significant.

4.4. Results and Discussion

4.4.1. Aggregation of MIN6 Single Cells into Pseudoislets with Hanging Drop

Previously, hanging drop was used for beta cells for formation of spheroids. This technique was applied to form MIN6- α TC and INS1- α TC cell heterospheroids where surface tension between cells was proposed to be the key factor for aggregate formation [296]. However, viability and function within such aggregates were not analyzed.

In this study, MIN6 beta cells were assembled into spheroids called as pseudoislets to mimic the morphology of naïve islets and protect their insulin secretion function. MIN6 beta cells as single cells were suspended in hanging drops and influence of three parameters (incubation time, initial cell number in each droplet and droplet volume) on pseudoislet formation and viability was investigated in detail. In this method, cell suspensions in either 20 or 30 μ l droplets were placed on the cell culture petri dish lid and when the lid was inverted, droplets remained on the lid due to surface tension where cells concentrated to the liquid-air interface with the effect of gravity and formed clusters [295]. Incubation time was ranged between 1-6 days, where initial cell number in each droplet was chosen between 200-1000 and droplet volumes were chosen as 20 μ l and 30 μ l. On day 1, with an initial cell number of 200 and with both 20 μ l and 30 μ l droplet volume, cells formed aggregates (Figure 4. 6-Figure 4. 9). As incubation period of droplets was prolonged, both cluster size and circular morphology of pseudoislets were promoted. Early forming clusters were first irregularly shaped and spherical morphology occurred after prolonged incubation conditions starting from day 2. Similar results were obtained when initial MIN6 beta cell numbers of 300 and 1000 were used. For an incubation period of 6 days, pseudoislet sizes larger than 100 μ m were obtained. Our finding is also consistent with the previous reports, where researchers used hepatocellular carcinoma cells (HepG2), prostate cancer cells (DU145Luc), human fetal osteoblasts (HFOB) and murine embryonic stem cells (mES-Oct4-GFP), and monitored the formation of spheroids in 15 μ l droplets and observed increasing cluster size and circular morphology with incubation time [302].

Increasing spheroid size with longer incubation time could be explained by aggregation of cells in each droplet into one cluster followed by cell proliferation. Similar behavior of MIN6 beta cells was observed when cells were grown on nonadherent dishes.

Chowdhury and colleagues observed that MIN6 pseudoislets in negatively charged tissue culture plates were irregularly shaped on day 1, transformed into spherical morphology on 2 and were completely spherical on day 4 reaching to 150-250 μm diameter [294].

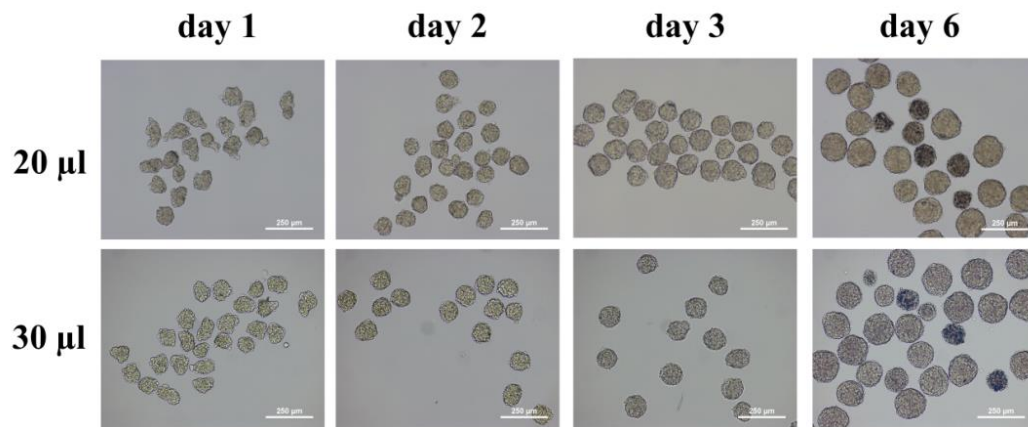


Figure 4. 6: Formation of MIN6 pseudoislets with hanging drops of 20 μl and 30 μl . Initial MIN6 beta cell number is 200. Scale bars: 250 μm .

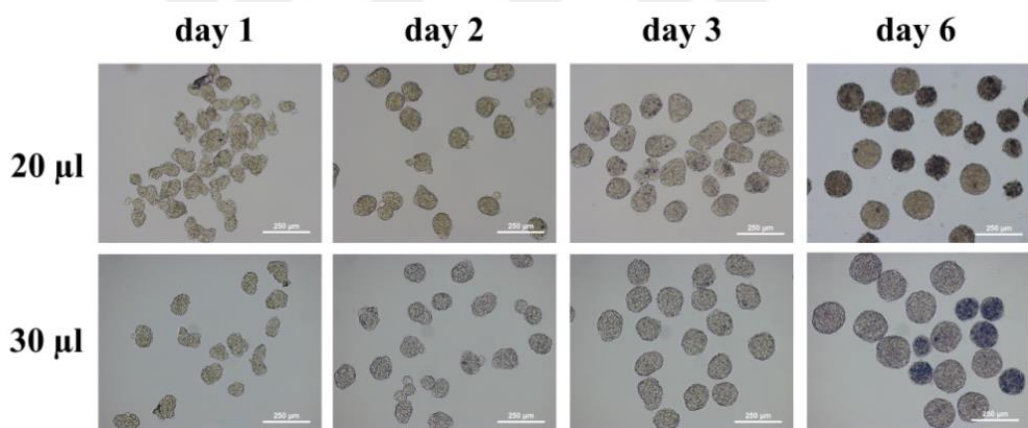


Figure 4. 7: Formation of MIN6 pseudoislets with hanging drops of 20 μl and 30 μl . Initial MIN6 beta cell number is 300. Scale bars: 250 μm .

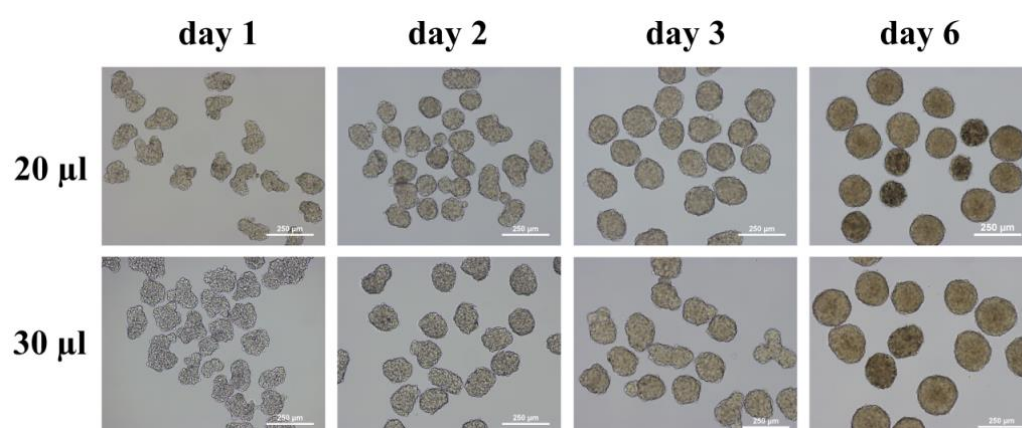


Figure 4. 8: Formation of MIN6 pseudoislets with hanging drops of 20 µl and 30 µl. Initial MIN6 beta cell number is 500. Scale bars: 250 µm.

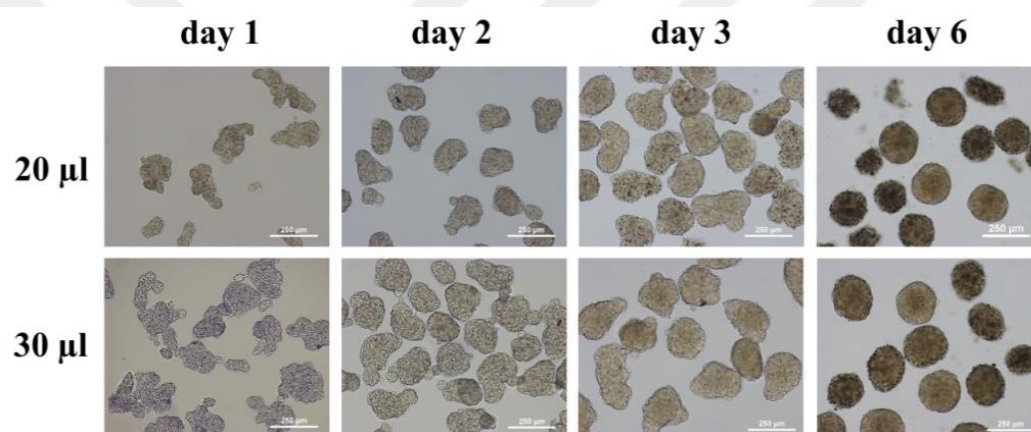


Figure 4. 9: Formation of MIN6 pseudoislets with hanging drops of 20 µl and 30 µl. Initial MIN6 beta cell number is 1000. Scale bars: 250 µm.

Similar approach has been also used for other beta cells previously. Although the number of cells per droplet, beta cell type and/or incubation period were different, beta cells had the ability to form clusters in those studies. For example, rat islets were dissociated into single cells and 200-750 cells per 30 µl were incubated for 5-8 days. Aggregates were formed within 5-6 days, where cluster diameters within 60-120 µm were obtained for the condition of 250 cells/droplet [303]. In another study, 10 000 or 20 000 RIN-5F and RIN-m cells were co-cultured in hanging drops, where static culture or static culture plus shaking plates were used. Spheroids with these two cell lines were formed within 2 days in 50 µl droplets. These three methods were compared and insulin secretion was higher in spheroids formed with hanging drop [304]. In our study, we noticed clustering of cells in 20-30 µl droplets as early as day 1. In addition to formation

of standardized spheroids, the potential of this method was also investigated for the preparation of genetically modified functional islets. Islets can be dissociated into individual cells and transfected for IL-10 expression, followed by re-clustering with 7-day incubation. For initial cell numbers of 125, 250, and 500 cells per 30 μ l droplets, average diameters were measured as 53.3, 56.3 and 74.3 μ m, respectively [305]. Cluster sizes reported in this study are smaller than pseudoislets cluster sizes obtained here. This could be explained by the differences in the proliferation potential of the cells. Since real islet cells do not proliferate *in vitro*, similar initial cell number with islet cells and MIN6 beta cells can result in different clusters sizes [154]. In contrast, MIN6 beta cells have proliferation capacity, and hence increases in cell numbers were observed to contribute pseudoislet size with longer incubation time.

4.4.2. Influence of Initial Cell Number, Droplet Volume and Incubation Time on MIN6 Pseudoislet Viability

To standardize MIN6 pseudoislets, initial MIN6 beta cell number, drop volume and incubation time were considered during experiments and investigation on influence of these variables on MIN6 pseudoislet viability was performed. We observed that with an initial cell number of 200 per droplet, viabilities of pseudoislets in 30 μ l droplets were slightly higher than that of pseudoislets in 20 μ l droplets (98 $\pm$ 2% vs. 90 $\pm$ 8% for day 1, 93 $\pm$ 6% vs. 90 $\pm$ 10% for day 2, 91 $\pm$ 7% vs. 85 $\pm$ 8% for day 3). At day 6, viabilities were around 60 $\pm$ 11% vs. 64 $\pm$ 8% showing significant number of dead cells within pseudoislets (Figure 4. 10).

This demonstrated the presence of significant number of dead cells within pseudoislets, which probably occurred due to the insufficient amount of nutrient and high amount of toxic waste molecules in each droplet. When higher cell numbers per droplet were used, viability was also affected by initial cell number, droplet volume and incubation time.

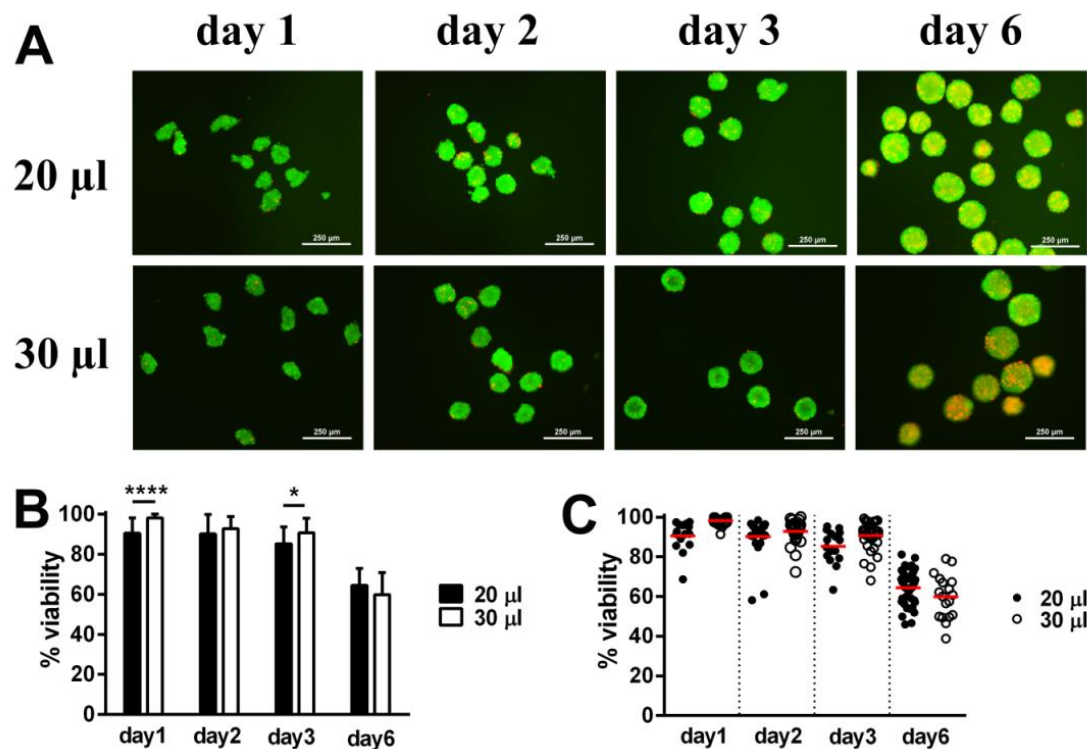


Figure 4. 10: Live/dead staining of MIN6 pseudoislets in different droplet volumes during 1-6 day incubation. Initial MIN6 single cell number is 200. Green: Viable (fluorescein diacetate). Red: Dead (propidium iodide). A) Fluorescent images of MIN6 pseudoislets after live/dead staining. Scale bars: 250 µm. B) Percent viability of MIN6 pseudoislets calculated from fluorescent images. Percent viability represents ratio of green area to total area. These areas were calculated with Image J. Mean±SD. n=15-43. ****p<0.0001. *p<0.05. C) Scatter plot of %viability. Red lines represent mean.

For viability of 300 initial cell number (Figure 4. 11), it was observed that viabilities were $96\pm3\%$ vs. $89\pm9\%$ for day 1, $95\pm4\%$ vs. $90\pm6\%$ for day 2, $85\pm9\%$ vs. $76\pm10\%$ for day 3. At day 6, viabilities decreased to $58\pm10\%$ and $50\pm10\%$ for 30 µl and 20 µl, respectively.

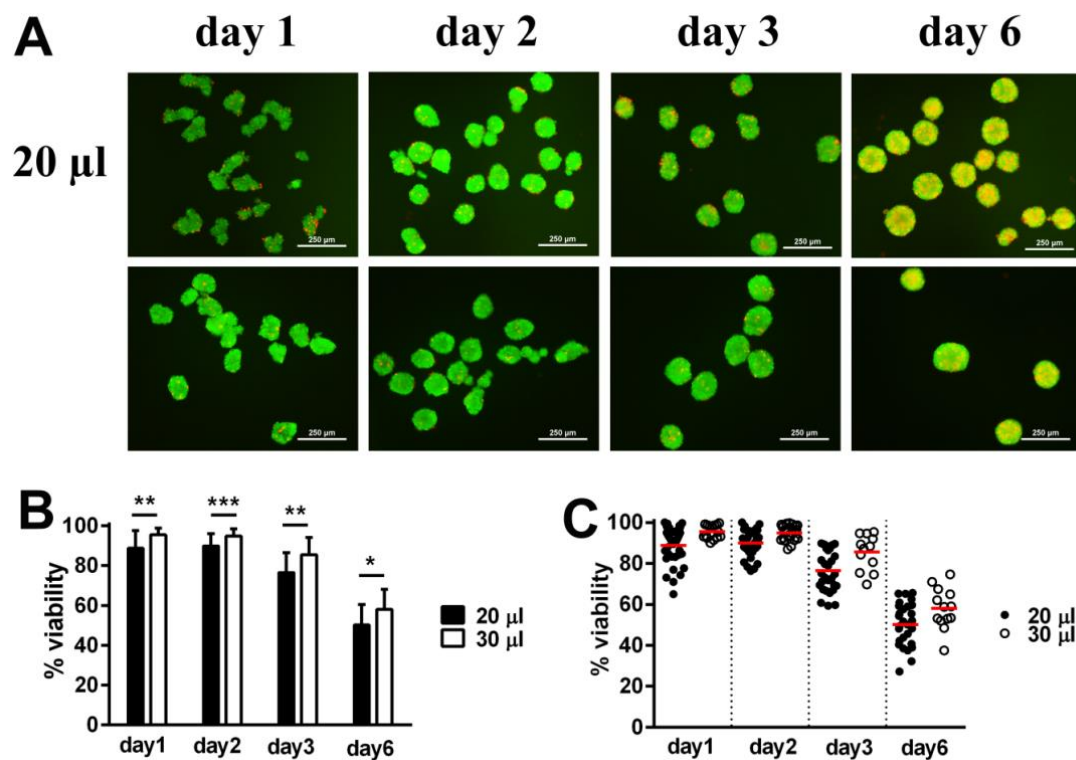


Figure 4. 11: Live/dead staining of MIN6 pseudoislets in different droplet volumes during 1-6 day incubation. Initial MIN6 single cell number is 300. Green: Viable (fluorescein diacetate). Red: Dead (propidium iodide). A) Fluorescent images of MIN6 pseudoislets after live/dead staining. Scale bars: 250 µm. B) Percent viability of MIN6 pseudoislets calculated from fluorescent images. Percent viability represents ratio of green area to total area. These areas were calculated with Image J. Mean±SD. n=12-38.***p<0.001. **p<0.01. *p<0.05. C) Scatter plot of %viability. Red lines represent mean.

For 500 initial cell number case (Figure 4. 12), it was observed that for the first 3-days, 30 µl droplets had slightly higher viability than 20 µl (94±5% vs. 93±6% for day 1, 91±6% vs. 89±11% for day 2, 90±7% vs. 83±9% for day 3). At day 6, viabilities decreased to 56±11% and 52±7% for 30 µl and 20 µl, respectively.

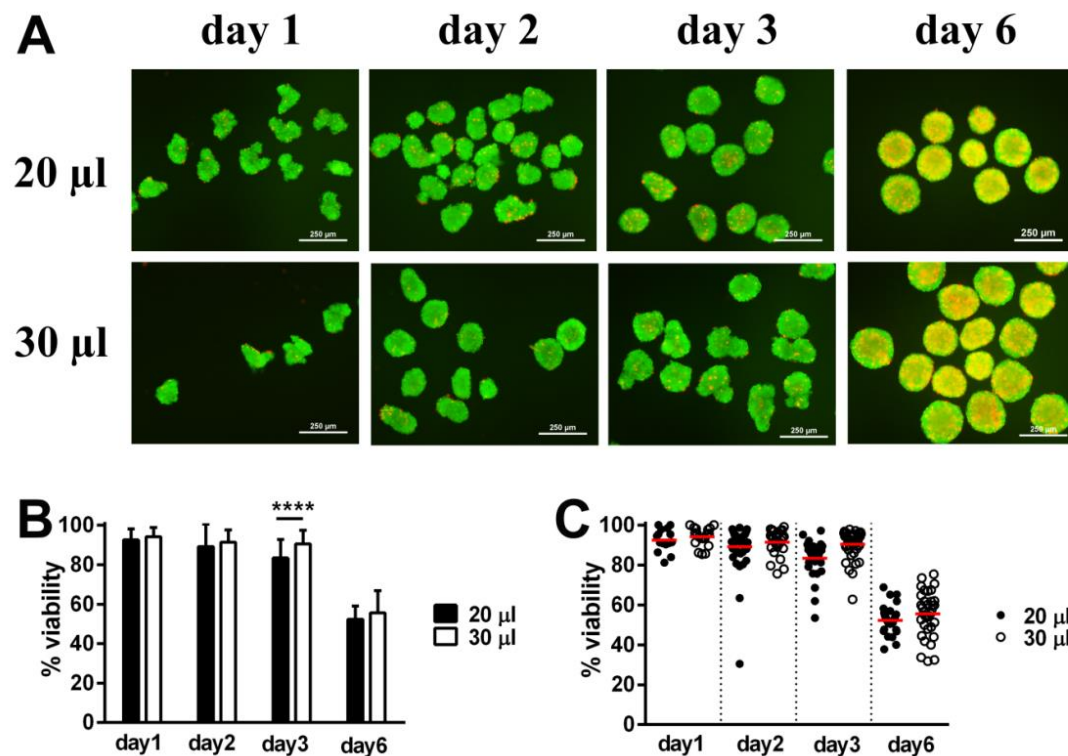


Figure 4. 12: Live/dead staining of MIN6 pseudoislets in different droplet volumes during 1-6 day incubation. Initial MIN6 single cell number is 500. Green: Viable (fluorescein diacetate). Red: Dead (propidium iodide). A) Fluorescent images of MIN6 pseudoislets after live/dead staining. Scale bars: 250 μ m. B) Percent viability of MIN6 pseudoislets calculated from fluorescent images. Percent viability represents ratio of green area to total area. These areas were calculated with Image J. Mean $\pm$ SD. n=15-43. ****p<0.0001. C) Scatter plot of %viability. Red lines represent mean.

Finally, in 1000 initial cell number (Figure 4. 13), significantly higher viabilities were observed in 30 μ l drops for 3 days and viabilities were found to be 89 $\pm$ 5% vs. 95 $\pm$ 4% for day 1, 86 $\pm$ 7% vs. 94 $\pm$ 4% for day 2, 62 $\pm$ 16% vs. 79 $\pm$ 10% for day 3 in 20 μ l and 30 μ l drops. At day 6, viabilities decreased to 44 $\pm$ 6% and 44 $\pm$ 8% for 20 μ l and 30 μ l.

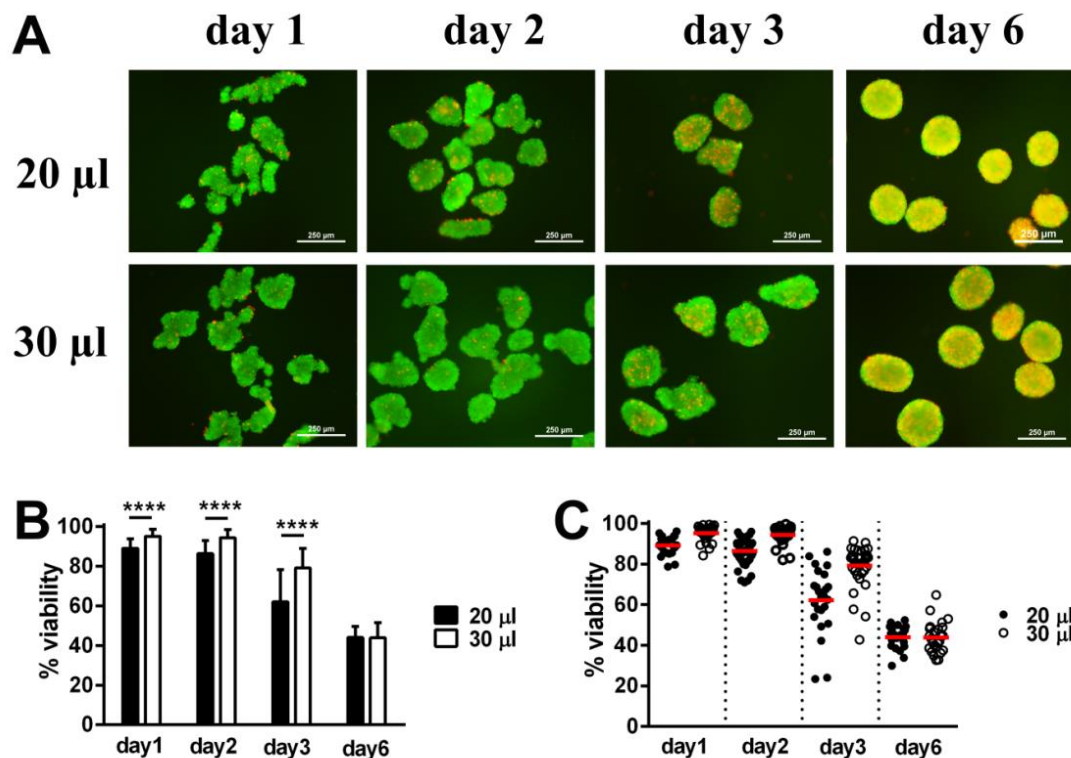


Figure 4. 13: Live/dead staining of MIN6 pseudoislets in different droplet volumes during 1-6 day incubation. Initial MIN6 single cell number is 1000. Green: Viable (fluorescein diacetate). Red: Dead (propidium iodide). A) Fluorescent images of MIN6 pseudoislets after live/dead staining. Scale bars: 250 μ m. B) Percent viability of MIN6 pseudoislets calculated from fluorescent images. Percent viability represents ratio of green area to total area. These areas were calculated with Image J. Mean $\pm$ SD. n=23-42. ****p<0.0001. C) Scatter plot of %viability. Red lines represent mean.

Percent pseudoislet survivals were measured above 80% for the initial cell number range of 200-1000 cells per 20 μ l droplet during the first two days of incubation (Figure 4. 14A). On day 3, viabilities started to differ and higher pseudoislet survival was measured for the lower initial cell numbers in the droplets. On day 6, significant cell death occurred in all groups. The viabilities measured on day 6 were 64 $\pm$ 8%, 50 $\pm$ 10%, 52 $\pm$ 7% and 44 $\pm$ 6% for 200, 300, 500 and 1000 cells per droplets, respectively (Figure 4. 14A). We also tested the survival potential of pseudoislets in a larger droplet volume, where pseudoislets were prepared in 30 μ l droplet volume for an initial cell number per droplet range of 200-1000. For all groups studied, pseudoislets had viabilities above 90% during the first two days droplet incubation time. However, on day 3, the pseudoislet survival was slightly affected by initial cell number and on day 6 pseudoislet survival

was measured between 60%-44% for the initial MIN6 beta cell number range of 200-1000 per droplet (Figure 4. 14B).

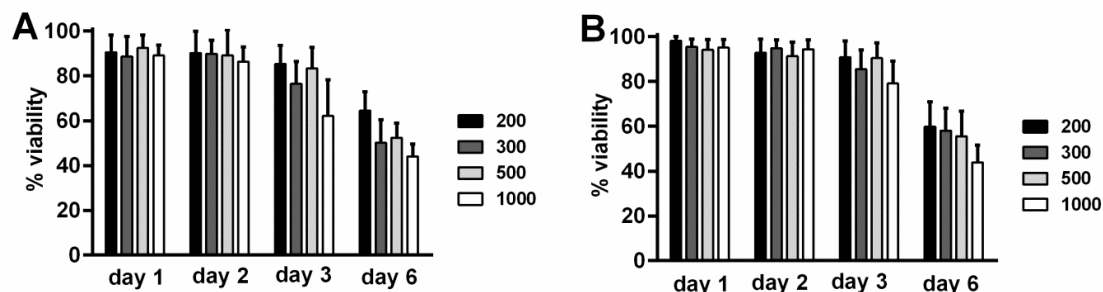


Figure 4. 14: Percent viabilities in A) 20 µl droplets, B) 30 µl droplets calculated from fluorescence images. Incubation time for pseudoislets was in the range of day 1-6 with initial MIN6 single cell numbers of 200-1000 per droplet. Mean±SD. n=12-43.

In general, we observed higher viabilities for shorter droplet incubation times. When we compare the droplet volumes at constant initial cell number, viabilities were higher in 30 µl droplets compared to that of 20 µl droplets during the first 1-3 days of incubation. For both droplet volumes studied, significant cell death occurred as incubation time was increased, where higher decreases in viability were observed with increasing initial cell number in the droplet. Higher initial cell numbers had also negative influence on the viability of pseudoislets at constant droplet volume. The cell death observed for pseudoislets at high cell numbers and prolonged droplet incubation times are due to depletion of nutrients and accumulation of cellular waste products. As cells arrange and continue to grow, limitations for oxygen transfer and waste accumulation eventually leads to cell death.

4.4.3. CHPOA Nanogel Coating of MIN6 Pseudoislets

Coating of beta cells within nano-thin polymers/particles is promising; however, only limited number of reports exists that involve insulin secreting islets. These studies used either electrostatic or covalent interactions for conjugation of polymers/nanoparticles on islet basal membrane [142, 300]. However, these approaches have limitations about the stability of coating on islet surface and multiple steps of islet surface treatment [142, 300].

Our approach has been to make use of hydrophilic pullulan modified with hydrophobic cholesterol units of CHPOA nanogels to coat MIN6 pseudoislets (Figure 4.

4A and B). In a physiologically compatible aqueous solution, cholesterol groups in the CHPOA nanogel structure interact with pseudoislet cell membrane, and form cross-links [234]. Previously, these nanogels have been used as a part of cancer vaccine, where they were used to deliver NY-ESO-1 to evoke and facilitate T-cell response in a clinical trial [239]. In a separate study, CHPOA nanogels were used to deliver FGF-18 and BMP-2, where accelerated osteoprogenitor cell infiltration and thus, healing was observed in a calvarial defect [240]. In this study, we are expanding the use of these natural nanogels to coat insulin secreting beta cells, which has a potential to establish a new paradigm for creating tolerable grafts for diabetes, and also other chronic diseases such as anemia, cancer, CNS diseases.

The coating process of MIN6 pseudoislets within CHPOA nanogels simply requires a single step incubation of MIN6 pseudoislets in CHPOA nanogel solution, where self-assembly of nanogels on pseudoislet surface occurs in a non-toxic aqueous buffer. To confirm the coating around MIN6 pseudoislets, rhodamine B-labelled nanogels were used (Figure 4. 15). For all conditions studied, red fluorescence was observed around pseudoislets as an indicator of deposition of nanogels on beta cell surface. The main driving force for coating was hydrophobic interactions between the cholesterol groups of nanogels and lipid bilayer of MIN6 pseudoislets. Immobilization of nanogels on pseudoislet surface was also promoted by the reaction of acrylate groups in the nanogel structure and thiol groups of proteins on MIN6 beta cell membrane through Michael addition (Figure 4. 4C and D).

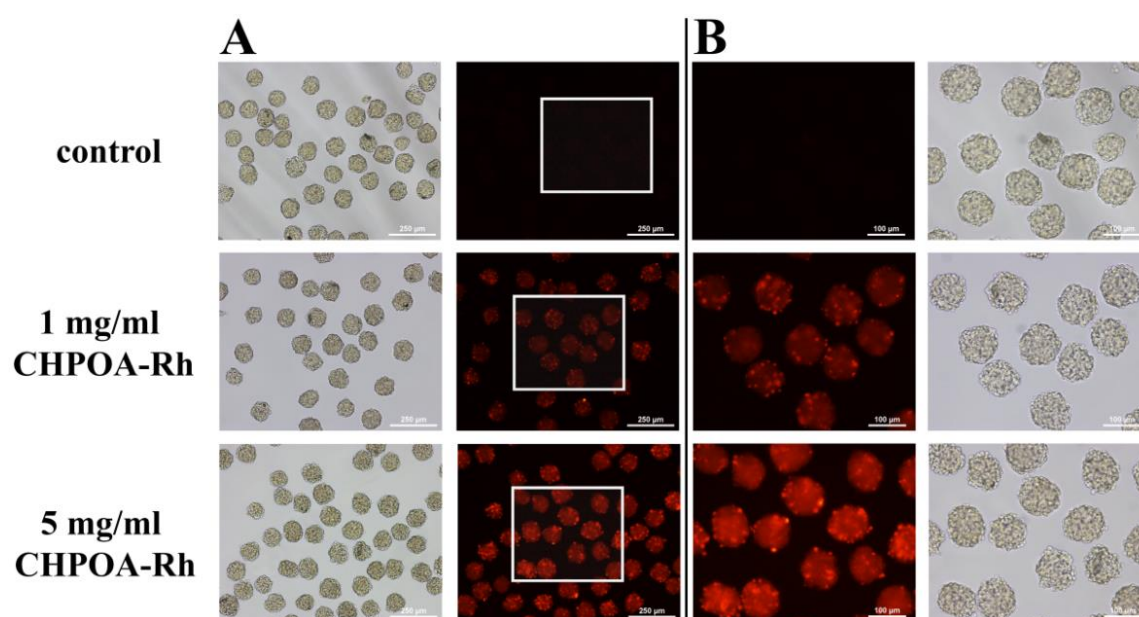


Figure 4. 15: CHPOA coating of pseudoislets with varying nanogel concentration. Initial MIN6 beta cell number is 200 for each droplet and droplet volume is 30 μ l. White boxes in Figure A show the areas represented in high magnification in Figure B. A) Scale bars: 250 μ m. B) Scale bars: 100 μ m.

4.4.4. Analysis of Cellular Activity of CHPOA Nanogel Coated MIN6 Pseudoislets

We next evaluated alterations in the pseudoislet size, intracellular ATP levels and stimulation index after the first incubation of MIN6 pseudoislets in nanogel solution (Figure 4. 16). The diameters were measured as $98.7 \pm 6.9 \mu\text{m}$, $102.3 \pm 7.7 \mu\text{m}$ and $103.5 \pm 7.0 \mu\text{m}$ for control, 1 mg/ml and 5 mg/ml nanogel treated groups, respectively (Figure 4. 16A and B). Intracellular ATP levels were around 1 pmole for all groups (Figure 4. 16C). Stimulation indices represent insulin secretion capability of beta cells in response to glucose challenge and these were measured as 1.80 ± 1.12 , 1.54 ± 0.90 and 1.47 ± 0.37 for control islets, 1 mg/ml CHPOA nanogel coated and 5 mg/ml CHPOA nanogel coated pseudoislets, respectively (Figure 4. 16D). These measurements demonstrated that nanogel treatment did not adversely affect insulin secretion function of MIN6 pseudoislets. In a previous study, Dong et al. showed no significant alterations in glucose sensing and insulin secretion capability of mouse islets after PLGA particle coating [142]. Multi-layer coating of phosphorylcholine-chitosan, alginate and phosphorylcholine-modified chondroitin-4-sulphate of MIN6 pseudoislets also did not compromise insulin secretion function of beta cells [306]. In general, similar trends in insulin secretion behavior and viability were observed for pseudoislets after incubation of

cells in different concentrations of nanogel buffers.

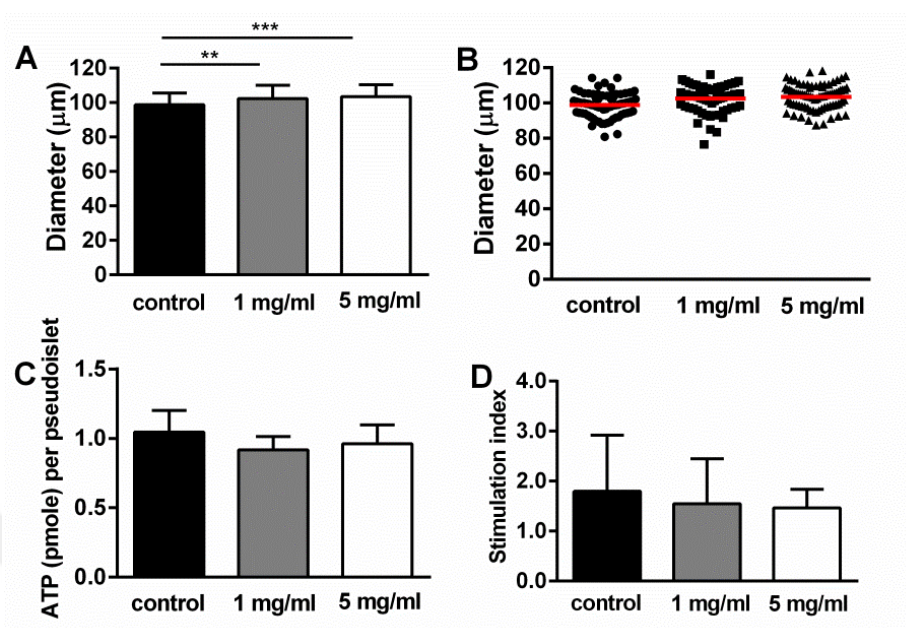


Figure 4. 16: Cellular activity of CHPOA nanogel coated MIN6 pseudoislets. Initial MIN6 beta cell number is 200 for each droplet and droplet volume is 30 μ l. A) Diameter (μ m), Mean $\pm$ SD. n=64-79. B) Scatter plot of diameter (μ m). n=64-79. ***p<0.001. **p<0.01. Mean $\pm$ SD. C) ATP (pmole) per pseudoislet. Mean $\pm$ SD. n=2-3. D) Stimulation index of nanogel coated pseudoislets. Stimulation indices were calculated from insulin secreted in high glucose buffer divided by insulin secreted in low glucose buffer. Mean $\pm$ SD.n=3.

We also explored MIN6 pseudoislet morphology on the same day and 1-day after coating. For both days, nanogels remained attached on pseudoislet surfaces for both concentrations studied (Figure 4. 17). Optical images of control group and coated pseudoislets demonstrated that spheroids were intact throughout the experiment, and that spherical morphology of coated pseudoislets was retained after coating. In addition, regular incubation of coated pseudoislets did not alter spheroid sizes and coating was stable during this incubation period (Figure 4. 18 and Figure 4. 19A, B). In contrast, in a previous report about silica nanoparticle treatment of INS-1 cells, coating was stable only up to a few hours around the cells [300]. ATP levels for all groups were similar for the control and experimental groups and increased from 1 pmole to about 2 pmole after 24 h (1-day) incubation period (Figure 4. 19C).

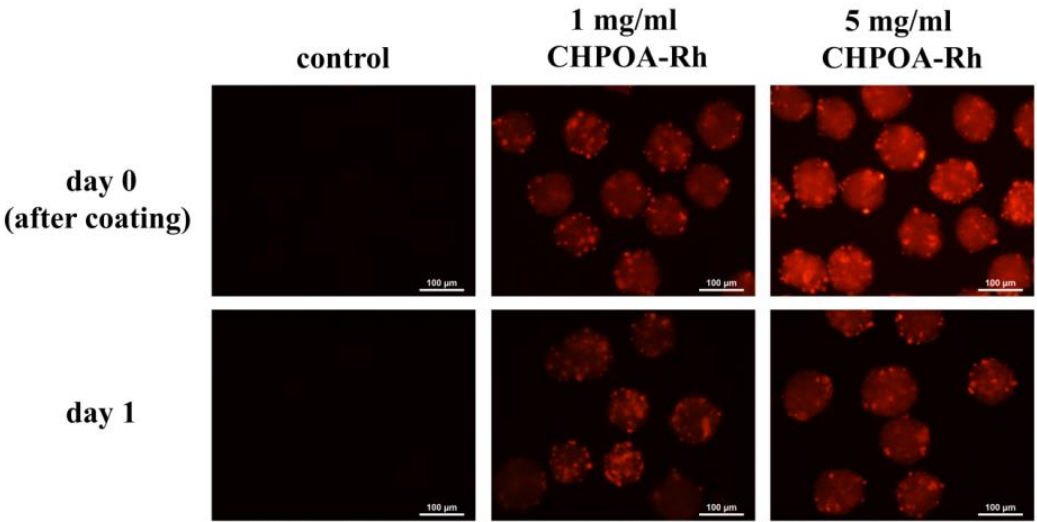


Figure 4. 17: CHPOA coating of pseudoislets with varying nanogel concentration. Initial MIN6 beta cell number is 200 for each droplet and droplet volume is 30 µl. Scale bars: 100 µm.

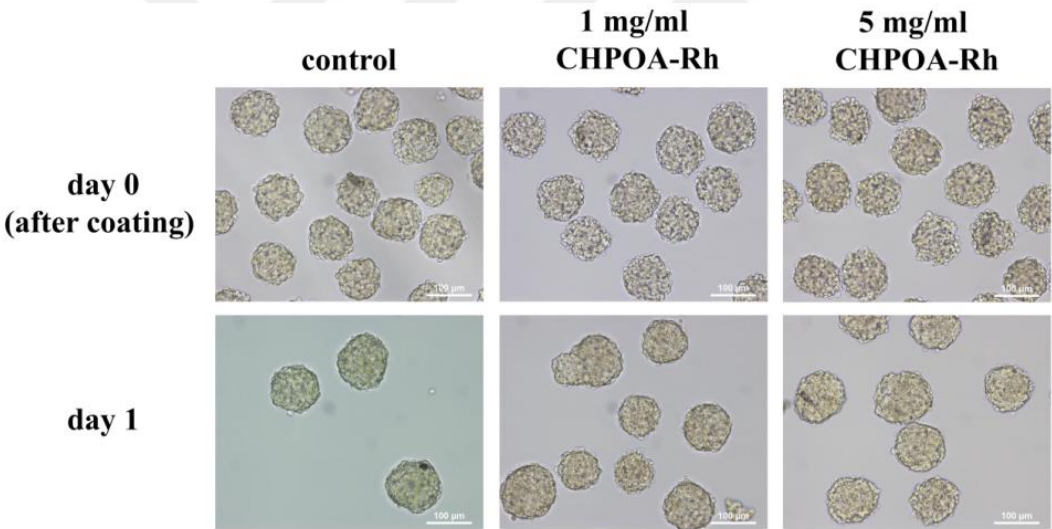


Figure 4. 18: Optical images of CHPOA coated pseudoislets with varying nanogel concentration after coating and 24 h. Initial MIN6 beta cell number is 200 for each droplet and droplet volume is 30 µl. Scale bars: 100 µm.

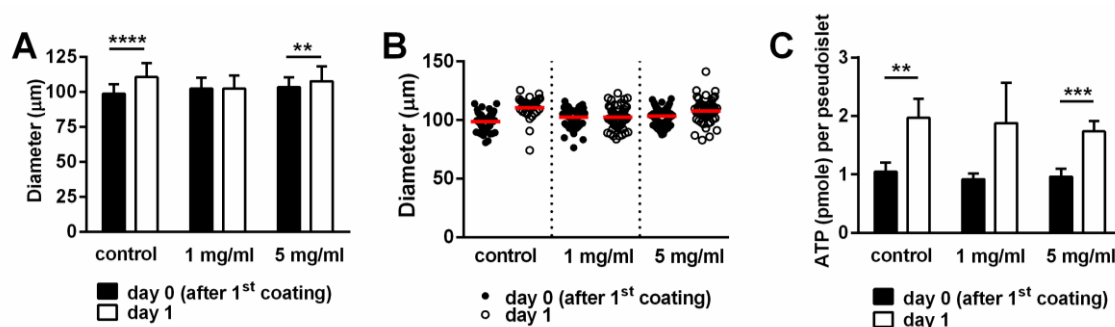


Figure 4. 19: Changes in size and intracellular ATP levels in coated pseudoislets during incubation. A) Diameters after coating and 24 h after coating. Mean±SD. n=29-79. B) Scatter plot of diameter (μm). n=29-79. C) ATP levels of MIN6 pseudoislets immediately after coating and 24 h after coating. n=2-6. ****p<0.0001. ***p<0.001. **p<0.01. Mean±SD.

4.4.5. Bilayer CHPOA Nanogel Coating of MIN6 Pseudoislets

Once pseudoislets were coated with CHPOA nanogels, we tested the possibility for a sequential coating step to achieve bilayer coating of MIN6 pseudoislets. Multiple layers of coating islets would be significant to eliminate any protrusions or sites that might expose islets to immune cells and will ensure complete coverage of pseudoislets. After coating pseudoislets with altered concentrations of nanogel, pseudoislets were treated with 1 mg/ml CHPOA-Rh. For the pseudoislets in control group, cells were only treated with PBS. After the second CHPOA nanogel treatment, fluorescence around pseudoislets was characterized. We observed that intensity of fluorescence was elevated after both first and second treatment of pseudoislets with nanogels, which suggested immobilization of nanogels to pseudoislet surface (Figure 4. 20). We also observed that integrity of pseudoislets was maintained after both steps of nanogel treatment.

We also checked fluorescence around pseudoislets several days after treatment. Fluorescent images demonstrated decreases in fluorescence intensity and significant increases in pseudoislet sizes accompanied with progression of incubation (Figure 4. 20). Decreases in fluorescence can occur due to internalization of particles, detachment of particles from cell surface or membrane restructuring due to proliferation.[300, 307] In our system, MIN6 beta cells that were used to make pseudoislets have proliferation capacity and hence remodeling of cell membrane occurs with time. Cellular proliferation was also confirmed with increases in the sizes of pseudoislets with incubation, on day 2 and day 3 (Figure 4. 20). To eliminate particle internalization, it is possible to link

CHPOA nanogels covalently on pseudoislet surface through the use of bi-functional or tetra-functional PEG-thiol. Cellular proliferation could also be avoided through nonproliferative human or non-human pancreatic islets.

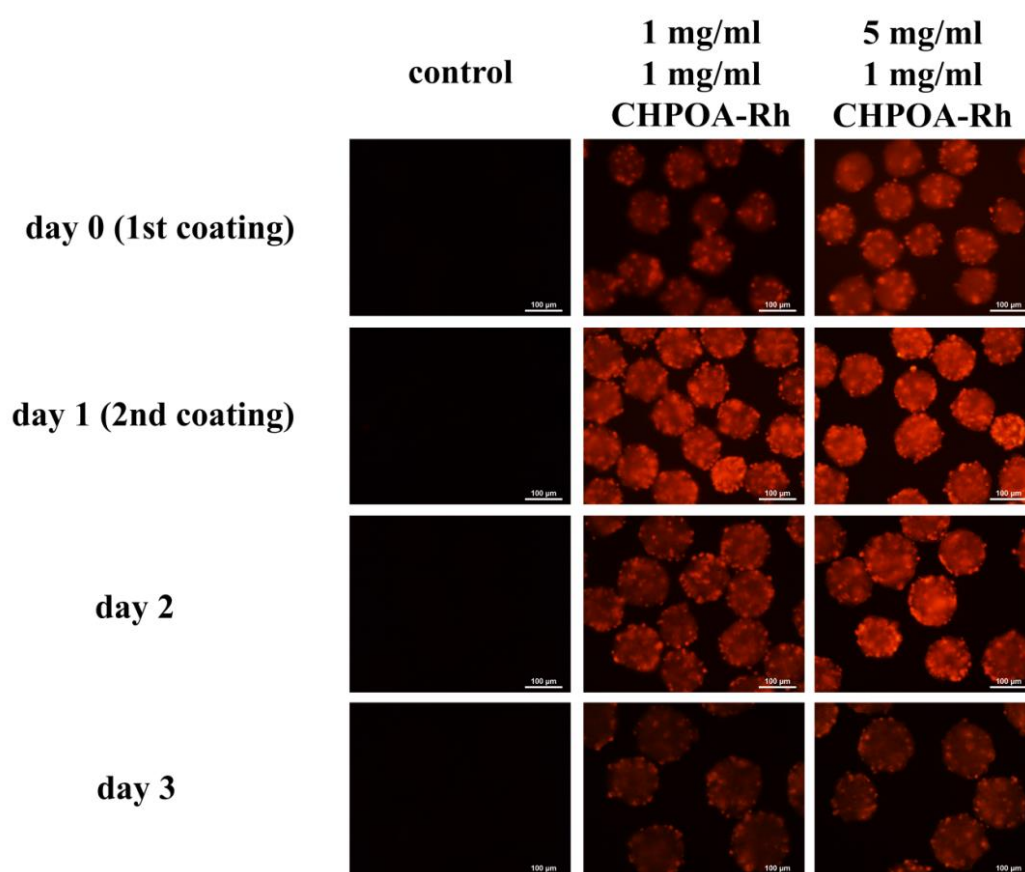


Figure 4. 20: Fluorescence images of CHPOA coated pseudoislets with bilayer nanogel treatment after coating and during in vitro incubation. Initial MIN6 beta cell number is 200 for each droplet and droplet volume is 30 μ l. First treatment was performed with 1mg/ml or 5 mg/ml CHPOA-Rh concentration. For the control group, PBS treatment was used. For the second treatment, 1 mg/ml nanogel treatment was after 1mg/ml and 5 mg/ml nanogel treatment. For the control group, second PBS incubation was applied. Scale bars: 100 μ m.

During this incubation period, MIN6 pseudoislet size continued to increase (Figure 4. 21A) and quantification of pseudoislet size showed that initial diameters of 105.98 ± 10.06 μ m, 110.07 ± 6.88 μ m and 106.44 ± 9.3 μ m significantly increased by 44%, 34% and 30% in control, 1 mg/ml-1 mg/ml and 5 mg/ml-1 mg/ml nanogel treated groups, respectively (Figure 4. 21B and C). This increase in pseudoislet sizes due to cell proliferation was the

main reason that contributed to decreases in the fluorescence intensity of CHPOA nanogels.

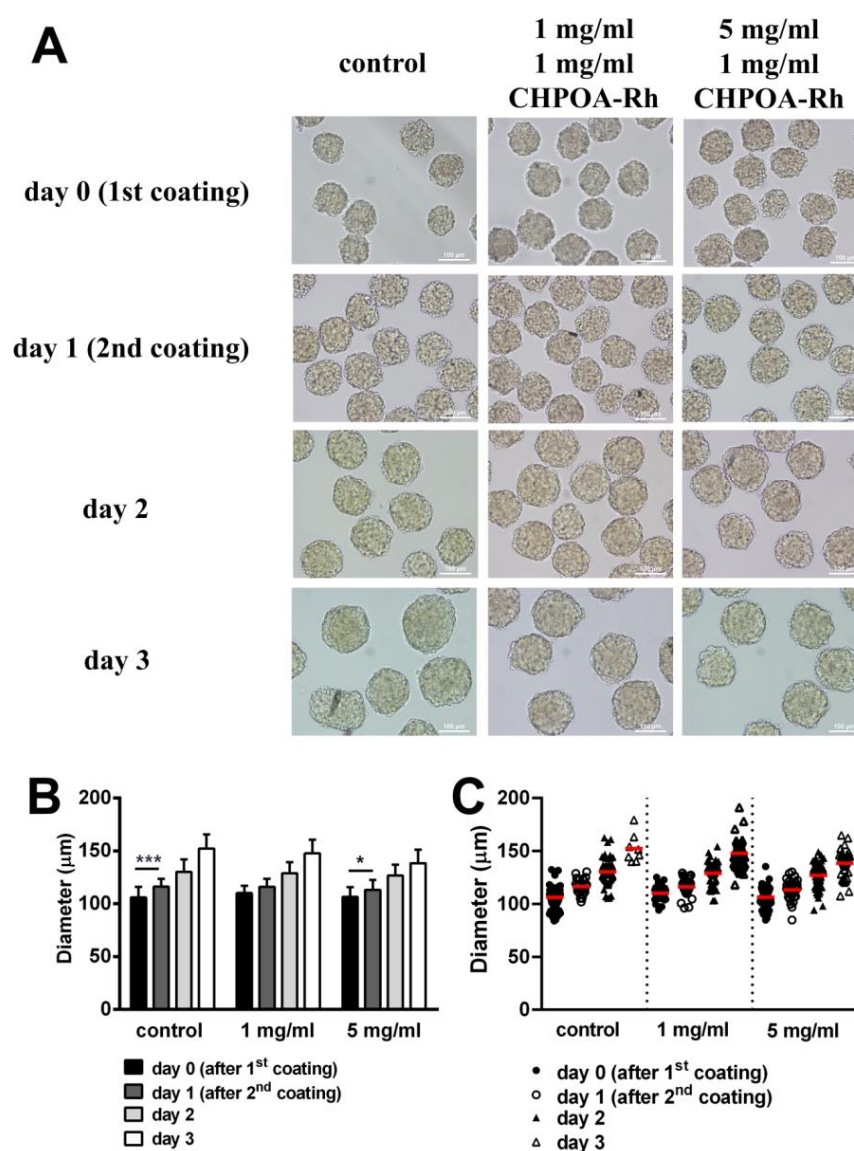


Figure 4. 21: *In vitro* culture of CHPOA nanogel coated MIN6 pseudoislets. A) Optical microscope images of CHPOA coated pseudoislets with double layer coating. Initial MIN6 beta cell number is 200 for each droplet and droplet volume is 30 μ l. Scale bar: 100 μ m. B) Diameters of pseudoislets after multi-layer coating. Mean $\pm$ SD.n=8-77. *** p <0.001. * p <0.05. Within each group, remaining comparisons except day 0 vs. day 1 in 1 mg/ml nanogel treated group, showed statistical significance (**** p <0.0001). C) Scatter plot of diameters (μ m). Red lines represent mean.

4.4.6. Analysis of Cytotoxicity after Bilayer Coating with CHPOA Nanogels

To analyze and investigate the influence of CHPOA nanogels coating on MIN6 pseudoislet survival, we have used live/dead staining assay (Figure 4. 22A). Quantitative

analysis demonstrated that, after first coating, viabilities were measured above 80% for all conditions (Figure 4. 22B, C). After second coating (day 1), viabilities were slightly compromised where decreases in survival to 82%, 77% and 76% for the three groups were measured on the same day of coating. However, prolonged culture resulted in cell death (Figure 4. 22B, C).

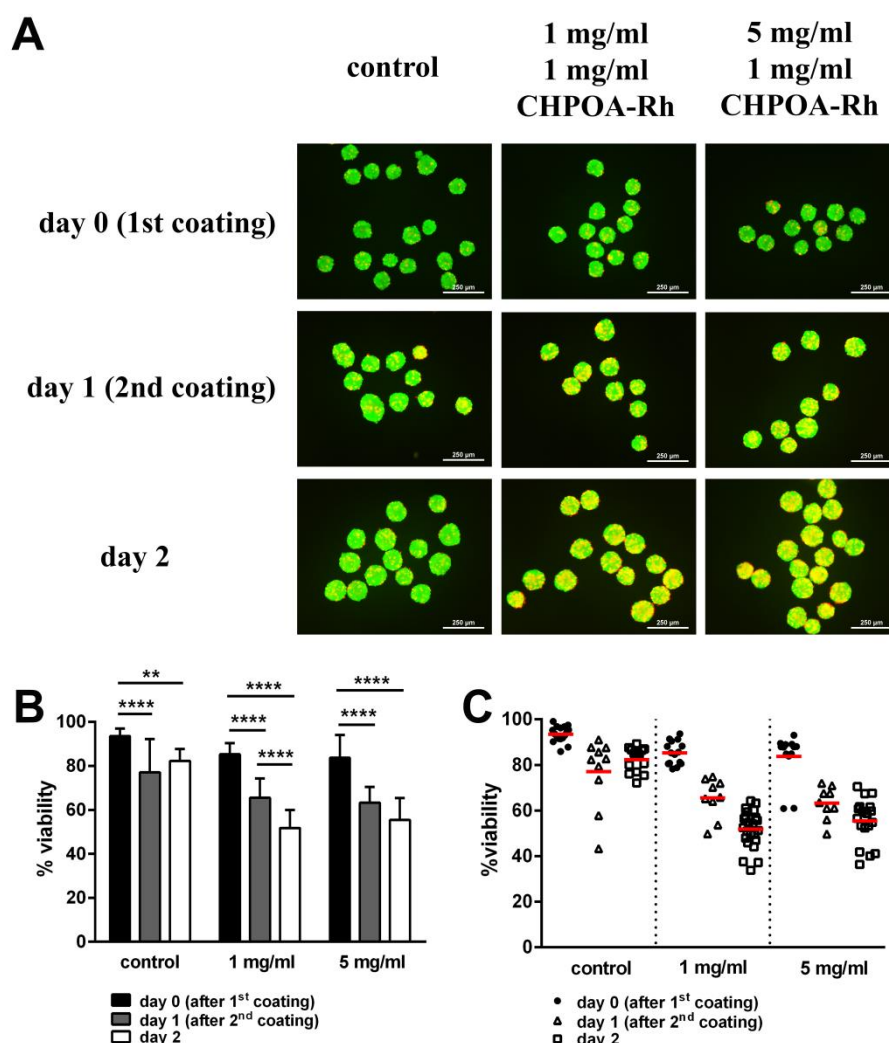


Figure 4. 22: Live/dead staining of MIN6 pseudoislets after nanogel coating. Initial MIN6 beta cell number is 200 and droplet volume is 30 μ l. Green: Viable (fluorescein diacetate). Red: Dead (propidium iodide). A) Fluorescence images of coated MIN6 pseudoislets after live/dead staining. Scale bars: 250 μ m. B) % viability of MIN6 pseudoislets calculated from fluorescence images. % viability represents ratio of green area to total area. Mean $\pm$ SD. **** p <0.0001. ** p <0.01. n =9-23. C) Scatter plot of %viability. n =9-23. Red lines represent mean.

We further examined apoptotic activity of coated MIN6 pseudoislets and observed

increases in apoptotic activity compared to control groups (Figure 4. 23A). During this experiment, we also assessed the insulin secretion potential of MIN6 pseudoislets after bilayer coating and as in Figure 4. 23B, stimulation index was above 1 for control group and observed to decrease around 0.85 for CHPOA nanogel treated groups. However, no significant differences were obtained among all three groups studied. These results are in agreement with the viabilities of MIN6 pseudoislets (Figure 4. 22), as decreases in pseudoislet viability was accompanied by elevated levels of caspase activity. Electron-withdrawing potential of carbonyl group located near vinyl group drives Michael reaction with amino and thiol groups of proteins. This reaction is suggested to play a pivotal role of toxicity of (meth)acrylates on MC3T3-E1 cells [308]. It was also reported that poly(ethylene glycol) methyl ether acrylate (mPEGA) can lead to cellular toxicity by decreasing the level of reduced glutathione in L929 cells. Acrylate group can react with -SH group in glutathione and this interaction destroys redox balance of cells in induces apoptosis [309]. These events can also be contributing factors in our case, as acrylate groups in this study is proposed to react with thiol present on MIN6 beta cells. Use of CHP particle without acrylate functionalization can be a solution for current limitation of coating.

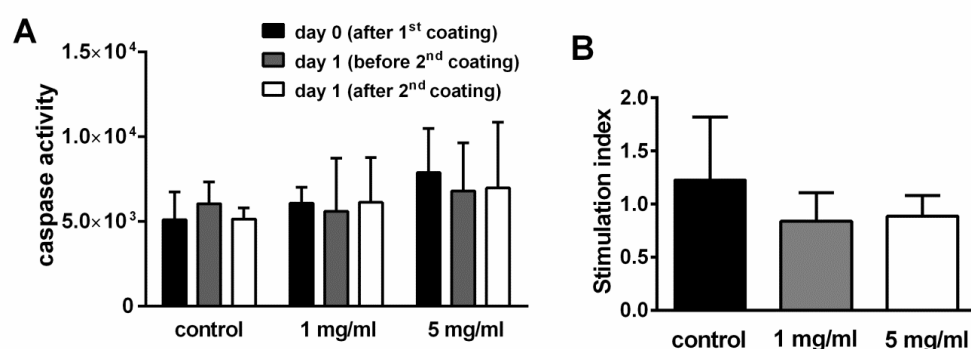


Figure 4. 23: Apoptotic activity and insulin secretion from double layer coated MIN6 pseudoislets. A) Caspase activity levels during nanogel coating after treatment or 24 h *in vitro* culture period. Mean $\pm$ SD. n=3. B) Stimulation indices of MIN6 pseudoislets after second layer coating. Mean $\pm$ SD. n=3.

4.5. Conclusion

We developed sequential coating of insulin secreting beta cell aggregates, so called pseudoislets, within nano-thin layers of CHPOA nanogels. First, we optimized

preparation of pseudoislets from MIN6 beta cells using hanging drop approach, where we studied the influence of initial cell number in the droplets and incubation time on pseudoislet size, morphology and function in detail. We observed negative correlation between initial cell number in the droplet and incubation time. Significant decreases in viabilities were noted with higher cell numbers in the droplet and incubation time. Droplet volume was also an important parameter to optimize during the preparation of pseudoislets, as larger droplet volumes contributed to pseudoislet viability. Next, we described a novel approach to coat beta cell clusters within thin nanogel layers in a single-step, where physiologically compatible medium was used for conjugation of nanogels on pseudoislet surface. This approach is superior compared to existing approaches in that fragile islets were not brought in contact with toxic prepolymer solutions.

This approach offers use of multiple thin-layer coating with negligible effects on viability and insulin secretion of beta cells after coating. However, for sustained viability and thus function, CHP nanogels without acrylate moiety could be applied. The results in the present study are encouraging for beta cell transplantation and in the future part of this research and this coating approach can be implemented to enhance immuno-isolation capacity as a physical barrier and a drug carrier. This study is promising and offers new opportunities through coating of insulin secreting islets with advanced functional materials under completely physiological conditions for clinical translation of cell transplantation technology and particularly for the treatment of T1D.

Chapter 5

Investigation of Poly(ethylene glycol) Hydrogel Encapsulated Pseudoislets in a Diabetic Mouse Model

5.1. Abstract

The aim of this study was to investigate beta cell behavior in PEG hydrogels *in vivo*. To date, there have been various reports on investigation of beta cell function *in vitro*. However, support of these results with animal models is required as a first step for clinical applications. For this purpose, MIN6 beta cells were used as models and were clustered into pseudoislets. Chemical induction of diabetes in CD1 mice was successfully performed to mimic hyperglycemia in diabetic patients. To these animals, IKVAV and GLP-1 functionalized PEG hydrogel encapsulated pseudoislets were transplanted subcutaneously. The results in this study point that although blood glucose levels were not normalized in diabetic recipients, obtained data is a clear indicator of requirement of careful consideration of transplant region and transplanted cell number.

5.2. Introduction

DM is a chronic disease of pancreas, the insulin secreting organ of the body. Typically, loss of insulin secretion or insulin function drives occurrence of DM [310]. According to International Diabetes Federation, in 2015, 415 million people suffered from DM worldwide. It is also reported that 5 million people lost their lives in 2015 due to DM and this number is estimated to be higher than people deceased as a result of AIDS, tuberculosis or malaria in 2013. With 39% increase in patient number, 19% increase in healthcare expenditure is also predicted for 2040. For the first time, T1D child patient number passed over 500,000 [4].

In T1D, multiple doses of insulin injection and in serious cases, pancreas/pancreatic

islet transplantation are applied [311]. In the case of insulin injection, although insulin analogs are developed, factors such as day-time based variabilities or requirement of multiple injections can be limiting. If a pump is preferred, infection or contact dermatitis may occur at the pump insertion region. For pancreas/pancreatic islet transplantation, mainly, donor (thus, cell number to be transplanted) and use of immunosuppressive drugs define which therapy will be applied [5].

Alternative to exogenous insulin supply and pancreas/islet transplantation, a biomaterial coated environment can be promising for the future treatment strategy. By this way, it is proposed that specifically designed barrier will allow oxygen, nutrient uptake and will not interfere with the removal of cellular wastes and with the transfer of hormones (e.g. insulin). This approach also opens the idea of use alternative insulin secreting cell sources by decreasing the risk of immune reaction [14]. Today, pancreatic islets encapsulated in alginate/poly(ethylene glycol) beads (Novocell), alginate/poly-L-lysine beads (Amcyte/ReNeuron) and alginate sheets (Islet Sheet, Cerco Medical) are currently in phase I/II, phase II and clinical trials, respectively [78]. PEG based hydrogels are also one of such materials that have extensive use in tissue engineering and drug delivery due to biocompatibility, ease of modification and bio-inertness [93-95]. These properties make PEG-based systems ideal candidates for immune-camouflaging and functional support of pancreatic islets [97].

To further characterize insulin secretion of beta cells in macro-sized PEG hydrogels, visible-light induced photopolymerization of PEGDA was used to coat MIN6 beta cell clusters. Covalently bonded IKVAV was used to mimic extracellular matrix and GLP-1 was natural insulintropic ligand for beta cells. Implantation was performed subcutaneously and fasting blood glucose levels were used to monitor performance of transplanted bioartificial pancreas.

5.3. Materials and Methods

Culture of MIN6 Beta Cells: MIN6 beta cells are donated by Dr. Donald F. Steiner from University of Chicago, Biochemistry and Molecular Biology with the permission of Professor Jun-ichi Miyazaki from Osaka University, Medical School, Division of Stem Cell Regulation Research. For maintenance, MIN6 beta cells were cultured in DMEM (HG, Gibco), 10% FBS (heat inactivated, Gibco), 100 IU/ml penicillin-0.1 mg/ml

streptomycin (Sigma), 2 mM L-glutamine (Sigma), 1 mM sodium pyruvate (Lonza) and 0.10 μ M β -mercaptoethanol (Invitrogen) at 37°C with 5% CO₂ environment.

Formation of MIN6 Pseudoislets through Hanging Drops: MIN6 beta cells (p25) grown in 60 mm dishes for a week were rinsed with sterile 1X PBS and were treated with 1 ml trypsin-EDTA (0.25%, Sigma) for 3 min. Following this incubation, growth medium was added and detached cells were centrifuged for 3 min at 1500 rpm in 15 ml tubes. In the next step, after removal of cell-free supernatant, cells (p26) were counted with a hemocytometer and were placed onto the lids of 100 mm cell culture dishes in 20 μ l droplets at a final concentration of 0.01 million/ml. Then, lids were inverted as in Figure 5. 1, 10 ml 1X PBS (sterile) supplemented with 1% penicillin-streptomycin was placed into the dishes and these cells were cultured in the form of droplets at 37°C, 5% CO₂ environment for 3 days.

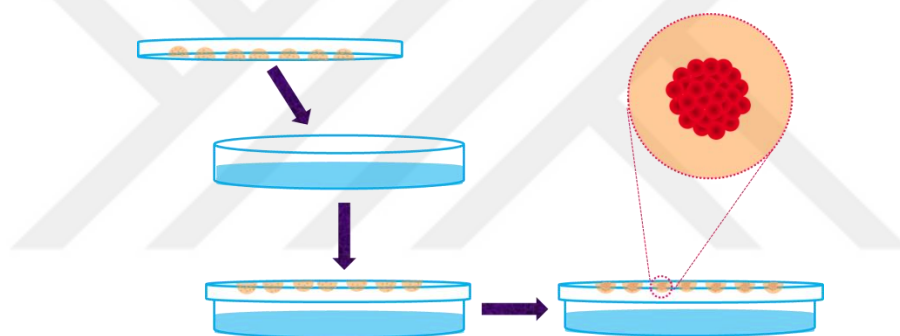


Figure 5. 1: Formation of MIN6 pseudoislets in hanging drops.

Encapsulation MIN6 pseudoislets in Functionalized PEG Hydrogels: For each mouse, 300 pseudoislets were coated with PEG hydrogels. For this purpose, prepolymer solution with final concentrations of 10% (w/v) PEGDA (MW=10 kDa, Laysan Bio), 225 mM TEA (Merck), 37 mM NVP (Aldrich) and 0.03 mM eosin Y (Sigma Aldrich), 2.5 mM acrylate-PEG-IKVAV and 20 μ M acrylate-PEG-GLP-1 was prepared. pH was adjusted to 8 and the solution was filter sterilized (0.45 μ m). Newly formed 300 pseudoislets were suspended in this solution and were exposed to visible light (514 nm) for 3 min (Figure 5. 2).

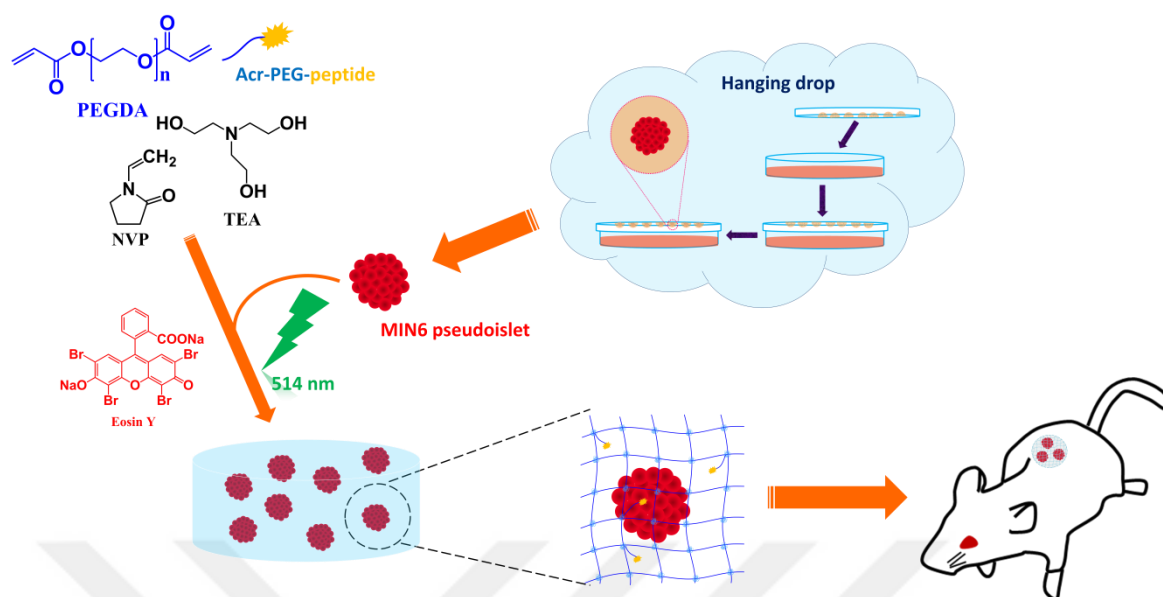


Figure 5. 2: Encapsulation and transplantation of MIN6 pseudoislets.

At the end of reaction, PEG hydrogel encapsulated MIN6 pseudoislets were cultured overnight at 37°C, 5% CO₂ environment before implantation. At the day of implantation, encapsulated cells were incubated in PBS.

Formation of Diabetic Mouse Model: All animal procedures were approved by Koc University Animal Research Local Ethics Committee. Animals were injected intraperitoneally with 50 mg/kg or 230 mg/kg body weight streptozotocin (STZ) in citric acid buffer (pH 4.5) consisting of 25.5 mM sodium citrate and 22 mM citric acid. Mice were injected with 50 mg/kg STZ (Sigma) for 5 consecutive days or with 230 mg/kg as a single bolus dose, and animals with blood glucose ≥ 300 mg/dl were accepted as diabetic.

Implantation of Encapsulated MIN6 Pseudoislets and Follow-Up: Encapsulated MIN6 pseudoislets were implanted subcutaneously under general anesthesia. For this purpose, a solution of ketamine (100 mg/ml) and rompun (23.32 mg/ml) was injected intraperitoneally. Anesthetized animals were placed onto warming table to prevent hypothermia and physiological saline was used to protect cornea. Implantation was performed into the back of animals. 0.5-1 cm incision was made on the back, right of the animals and implantation was performed on this region subcutaneously. Skin was closed with absorbable sutures. After this surgical procedure, animals were kept at 37°C for 15-20 min. In the following days, mice were weighted and fasting blood glucose levels were measured for a month. At post-implantation day 3, 8 and 29, cervical dislocation was

applied.

5.4. Results and Discussion

5.4.1. Implantation of Encapsulated MIN6 Pseudoislets into Diabetic Mice

To address donor limitation (repeated isolations), MIN6 beta cells were in the form of pseudoislets, as insulin secreting cells require adhesion and cell-cell interactions to manage their regular function and maintain their differentiated state. Also, in their natural environment, beta cells are a part of 3D cell cluster of pancreatic islets and dissociation of islets negatively affect insulin secretion [182]. As pseudoislets are formed, increases in E-cadherin (for cell-cell interaction), α and β -catenin (for actin and E-cadherin interaction, respectively) and connexin-36 (for molecule transfer between cells, electrical coupling, insulin secretion) expression were reported [312, 313]. For this purpose, hanging drop method was used to form pseudoislets. In addition to source, donor limitation is associated with the number of cells transplanted. For example, Shapiro et al. reported that repeated islet isolation might be required for proper regulation of blood glucose level [68, 314]. Further, if transplanted islet number to the portal vein was decreased from 500 to 300, % reversal of the diabetes would change from 30% to 10% [28]. As a result, finding a continuous proper choice of insulin source is a crucial step to cure T1D.

Beta cell environment supports cell survival and improves insulin secretion, so mimicking this environment is also important to determine required cell number to be transplanted. With this approach, number of beta cells can be decreased and this may help the address donor limitation. In this study, extracellular matrix derived peptide, IKVAV and insulinotropic peptide, GLP-1 were used.

To test MIN6 pseudoislet incorporated IKVAV and GLP-1 functionalized PEG hydrogels, a diabetic mouse model of CD1 male mouse was induced chemically. For this, a glucose analog known as streptozotocin [STZ, 2-deoxy-2-(3-(methyl-3-nitrosoureido)-Dglucopyranose] was used. This chemical is synthesized by *Streptomyces achromogenes* and is applied intraperitoneally or intravenously for diabetes induction. When it reaches pancreas, it uses GLUT2 as in the case of glucose, and causes DNA alkylation. In addition, it activates PARP which depletes NAD stocks. As ATP levels in the cell decreases, insulin secretion is inhibited. STZ can also form free-radicals and can

lead to DNA damage and cell death [315].

In this study, we used CD1 male mouse for diabetes induction with low dose STZ. For this, first, STZ dose was tested for this strain, because sensitivity of mouse strains to STZ can differ. Previously, Gurley et al. applied low dose STZ model (5 consecutive days, 40 mg/kg body wt) for 5 mouse strains and showed that sensitivity order was DBA/2 > C57BL/6 > MRL/MP > 129/SvEv > BALB/c [316]. In addition, lack of technical detail, changing death rate, different levels of diabetes and lack of standard protocol restrict a diabetic model development, as factors such as strain, circadian rhythm, feeding, STZ dosage, buffer used for STZ preparation and STZ injection site control diabetes induction [317]. For this purpose, for the diabetes model, CD1 male mice starved for 6.5 h and intraperitoneal injection of low dose STZ (50 mg/kg body wt) was performed. The reason for starvation was that in the case of feeding, glucose in the blood circulation will compete with STZ for GLUT2 and decrease the effectiveness of STZ [315]. In the case of starvation, blood glucose level will decrease and number of glucose molecules competing with STZ will decrease.

In the low dose STZ treated group, it was observed that 4 of 6 animals had blood glucose level around/more than 300 mg/dl indicating that these animals became diabetic (Figure 5. 3B). Among STZ treated animals, at most ~7% of the weight was lost. In contrast, healthy animals gained weight (Figure 5. 3A). Injection of a single dose of 230 mg/kg body weight STZ resulted in 50% death (3/6 mice). Among remaining 3 mice, 1 became diabetic with 28% weight loss (Figure 5. 3C) with blood glucose level of 346 mg/dl (Figure 5. 3D). The reason for this might arise from the fact that organs such as liver and kidneys have GLUT2 and during treatment of STZ, this chemicals also affects those organs [318]. As a result, in our study, we chose multiple low dose (50 mg/kg body wt, 5 days) STZ injection for induction of diabetes model for the following part.

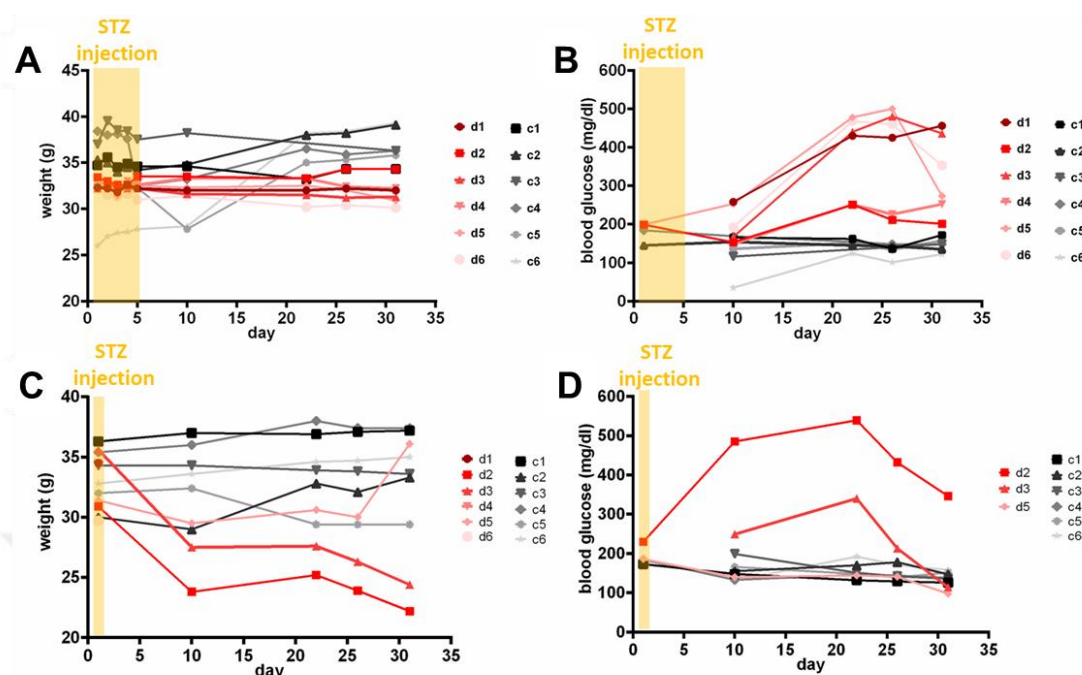


Figure 5. 3: Changes in weight (A) and blood glucose levels (mg/dl) (B) in 50 mg/kg wt streptozotocin treated CD1 mice during 30 days. Weight (C) and blood glucose levels (mg/dl) (D) in 230 mg/kg wt streptozotocin treated CD1 mice during 30 days. Orange region represents 5 consecutive days of STZ or buffer injection for low dose and date of STZ or buffer injection for high dose. n=6 for STZ treated and healthy (control) animals.

In the model used here, it is observed that fasting blood glucose levels increased above 300 mg/dl in 2-3 weeks (Figure 5. 4). Similarly, Iwakari et al. showed that with use of 30 mg/kg body wt STZ for 5 consecutive days, diabetes was observed in 21 days and increase in immune cell infiltration into pancreas was clear [319]. Low and high dose STZ treatments on CD1 mice were also reported by Ventura-Sobrevilla et al. and it was indicated that STZ amount in each dose and dose number were critical for diabetes induction. In that study, non-fasting blood glucose levels were used and mice having blood glucose level with ≥ 200 mg/dl were accepted diabetic. After use of 40 mg/kg body wt STZ for 5 consecutive days, 2/6 and 4/6 mice had hyperglycemia at the end of 21 and 28 days, respectively [320]. In this study, at the end of ~3 weeks, 22/23 mice had fasting blood glucose levels ≥ 200 mg/dl (Figure 5. 4). When 300 mg/dl was accepted as limit, this number decreases to 17/23. In the same study, it is also reported that 150 mg/kg body wt STZ can be used to induce diabetes. However, death rate was 50% [320].

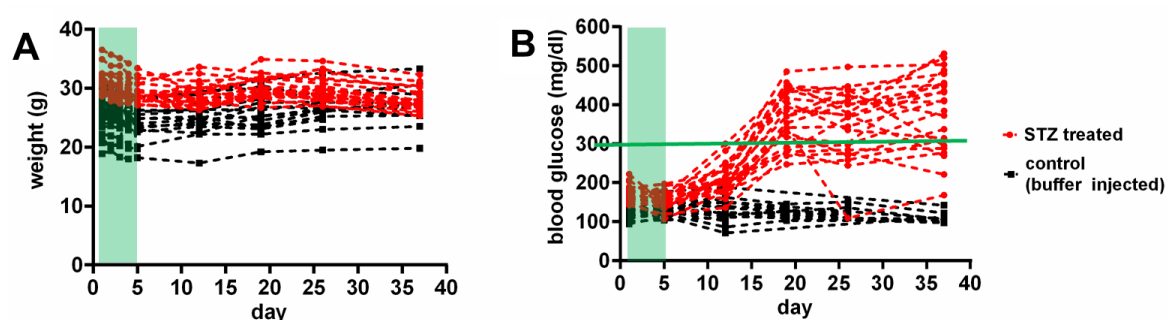


Figure 5. 4: A) Body weight, B) fasting blood glucose levels of STZ and buffer injected mice before implantation. Green region represents 5 consecutive days of STZ or buffer injection. n=23 for STZ treated group. n=18 for healthy (control) animal group.

After STZ treatment, mice were followed up to a month. Diabetic animal number was estimated with fasting blood glucose levels (Figure 5. 4). According to these results, 3-19% decrease in body weight was observed in STZ treated group. In healthy animals, this value changes in 9% decrease to 14% increase in body weight (Figure 5. 4A). When fasting blood glucose levels were investigated, in STZ treated group, 18/23 mice had blood glucose level ≥ 300 mg/dl (295-531 mg/dl) whereas healthy animals fasting blood glucose levels were below 200 mg/dl (97-142 mg/dl) (Figure 5. 4B).

For implantation, a subcutaneous pocket was chosen as an implant region. Today, there is no optimum site for beta cell transplantation. Subcutaneous region is preferred due to being safe and clinically accessible, but subcutaneous vascular network is not as rich as native environment of pancreas [5, 321].

Encapsulated MIN6 pseudoislets or only functionalized hydrogels were implanted subcutaneously to the back (right side) of mice. An example of this procedure is summarized in Figure 5. 5. For this, 0.5-1 cm incision was made (Figure 5. 5A) and implantation was performed into a subcutaneous pocket (Figure 5. 5B-D). At the end of implantation, this incision was sutured (Figure 5. 5E-F). In healthy animal-physiological saline group, saline injection was performed to the same region.

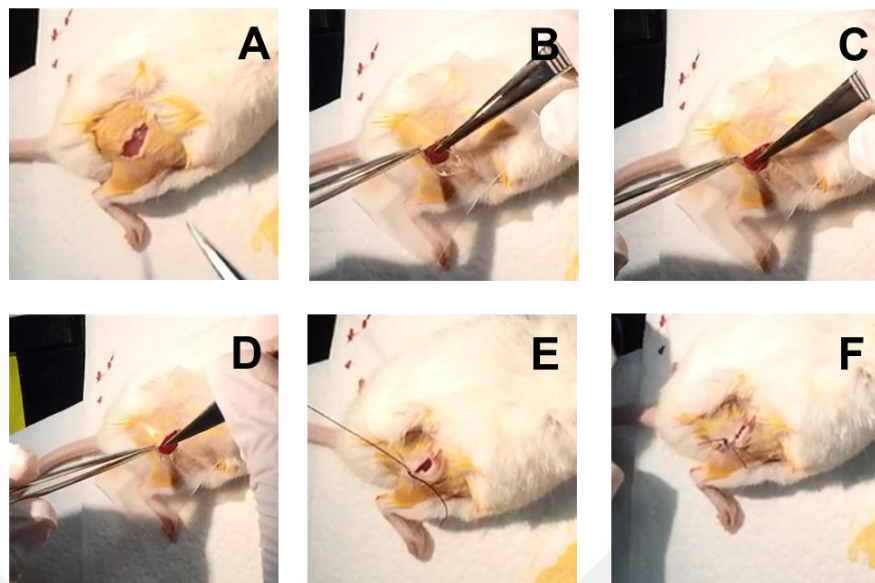


Figure 5. 5: Implantation procedure. 0.5-1 cm incision was made to the back of mice (A), implantation was made into the subcutaneous pocket (B-D). Finally, incision area was sutured with absorbable suture (E-F).

5.4.2. One Month Follow-Up after Implantation

In Figure 5. 6, at post-implantation day-29, body weight of diabetic-FN+cell group showed 0-10% increase in body weight (Figure 5. 6A). When diabetic-FN group was compared, 0-5% increase was obtained. In healthy animal groups, healthy-FN group showed decrease up to 4% and increase at most 6%. Healthy-SF group had 4-20% increase in body weight (Figure 5. 6A).

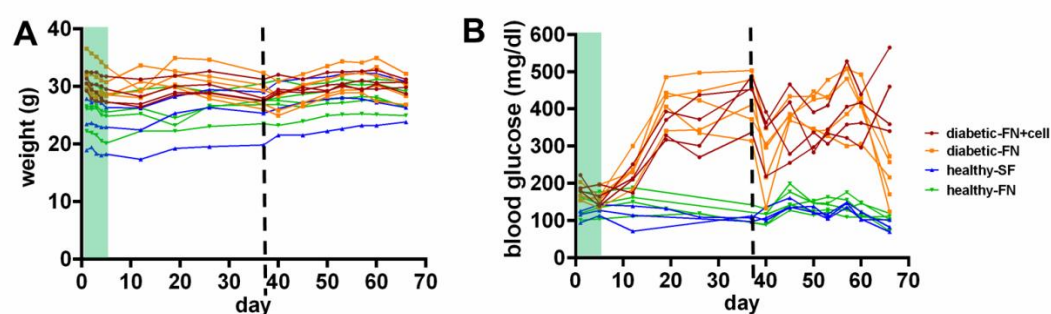


Figure 5. 6: Post-implantation day-29 A) body weight, B) fasting blood glucose levels of diabetic and healthy mice. Green region represents 5 consecutive days of STZ or buffer injection. Dashed line is the implantation day. **Diabetic-FN+cell:** Encapsulated MIN6 pseudoislet in IKVAV and GLP-1 functionalized PEG hydrogel implanted diabetic mouse group. n=4. **Diabetic-FN:** IKVAV and GLP-1 functionalized PEG hydrogel implanted diabetic mouse group. n=5. **Healthy-SF:** Physiological serum injected healthy mouse group. n=3. **Healthy-FN:** IKVAV and GLP-1 functionalized PEG hydrogel implanted healthy mouse group. n=5.

In the case of fasting blood glucose levels (Figure 5. 6B), diabetic-FN+cell group had blood glucose level around >300 mg/dl. However, diabetic-FN group showed ≤ 273 mg/dl. Finally, in all healthy animals, fasting glucose levels were <120 mg/dl. As represented in Figure 5. 6, fasting blood glucose levels were not decreased to normal levels. The reason for inability to decrease blood glucose is not due to use of MIN6 cells. Recently, for example, Yang et al. used different methods to form pseudoislets and showed that blood glucose levels were decreased to healthy animal levels [313]. The reason might be heavy immune system response, implanted cell/pseudoislet number, lack of sufficient vasculature and implantation region. As PEG hydrogels were classified as biocompatible, the reason might be related to other previously mentioned factors. For example, it was reported that C57BL/6 mice was subcutaneously transplanted with 1000 islets could not sufficiently regulate blood glucose levels (20% reversal). However, use of fibrin gels and growth factors with islets increased reversal to 60%. In addition, when 250 islets were transplanted into epididymal fat pad, reversal reached to 60% [322] suggesting that implantation region is important to regulate blood glucose levels and to determine implanted cell number. As in their natural environment, islets receive 5-10% of blood reaching pancreas and this vascular network is crucial for nutrient transfer, removal of wastes and transfer of hormones to the blood circulation [28]. When the number of pancreatic islets decreased from 600 to 500, reversal changed from 83% to 30%. In the same study, when vascularization was supported, for 500 islets transplanted, reversal is 100% [28]. These results support the idea of requirement of optimum cell number and vascularization around the implant.

5.5. Conclusion

In this part of research, MIN6 pseudoislets were encapsulated in PEG hydrogels and transplanted into diabetic mouse model. However, blood glucose levels were not decreased to levels that healthy animals have. In order to reach normoglycemia, cell in transplanted pseudoislet and site of transplantation should be reconsidered for the future studies. Overall, it can be concluded that implanted cell/pseudoislet number, lack of sufficient vasculature and implantation region affect the outcome of encapsulated beta cell transplantation studies and highlight the requirement of optimization for the future treatment trials of T1D.

Chapter 6

Development of Polysaccharide-Poly(ethylene Glycol) Composite Hydrogels for Biomedical Applications

6.1. Abstract

Composite hydrogels are promising candidates for engineering cell environments. Currently, development of these composite materials takes great interest and clinical use of such materials requires detailed characterization. In this study, we focused on composite hydrogels of PEGDA and CHPOA where physically crosslinked CHPOA nanogels were incorporated into PEG hydrogels through visible light photopolymerization. Subcutaneous implantation of composite hydrogels revealed insignificant immune response supporting the idea of application of this system for cell delivery. For the future studies, this new system is a promising candidate for cell/biomolecule delivery in PEG-CHPOA composite hydrogels.

6.2. Introduction

Advances in nano- and bio-technology lead to versatility in engineering of biomaterials [323]. Such improvements create various hydrogel-nanoparticle design possibilities with different chemical and physical properties [324]. As a result, these composite polymers are under extensive research from vascular grafts to bone cements [325] and are reported to be promising for both tissue engineering and growth factor delivery. For example, it was previously shown that nanoparticle-fibrin system provided more sustained release of VEGF compared to fibrin only system. In hindlimb ischemia model, higher blood pressure and capillary network formation in nanoparticle incorporated group was obtained [210]. Further, nanoparticle systems can convert a cell-

resistive surface to a cell-promoting surface. Hydroxyapatite nanoparticle modified PEG hydrogels were prepared for such purpose and it was reported that osteoblast attachment to composite hydrogel was superior compared to PEG hydrogel itself [326]. In addition, cell function can be improved in the presence of nano-sized molecules where cardiomyocytes seeded on carbon nanotube-gelatin methacrylate composite hydrogels produced enhanced electrophysiological activity and mechanical integrity [327].

Self-assembly of gel particles is a promising strategy to form both microscopic and macroscopic matrices (particle aggregates) [328, 329]. Cholesterol units in CHP nanogels provide hydrophobic crosslink points that promote self-assembly of polymeric particles [330]. Pullulan is a non-toxic, biodegradable, FDA-approved natural polymer with the use of pharmaceutical, food and cosmetics industries [331, 332]. Pullulan gels formed under mild conditions have been used in support of cells including ECs and osteoblasts and shown to be promising in tissue engineering and regenerative medicine [331, 333]. Pullulan is also proposed to be a good cytoadhesive polymer, as it was previously reported that MSC retention in healing cartilage can be improved with the reparative environment provided by pullulan [334]. CHP nanogels have also use in biomedical applications. These self-assembled particles were used as a part of cancer vaccine to deliver NY-ESO-1 to evoke and facilitate T-cell response in a clinical trial [239]. In a calvarial defect, CHPOA nanogels releasing fibroblast growth factor-18 and bone morphogenic protein-2 accelerated osteoprogenitor cell infiltration and thus, healing [240].

In addition, covalent crosslinking is a critical parameter for the integrity of hydrogel matrices used as immunoisolation barriers [333]. PEG is an FDA approved polymer use in tissue engineering and drug delivery due to its biocompatibility, ease of modification and bio-inertness. It has been extensively used in hydrogel formulations to form a covalent network structure [93-95]. Such applications include hydrogels in the form of particle or macroscopic hydrogel to coat cells for immuno-camouflaging and deliver bioactive molecules [97, 109, 335, 336]. PEG hydrogels were used as physical immunobarrriers for beta cell delivery. For example, it was previously shown that IKVAV, RGDS and GLP-1 immobilized hydrogel were promising to create a ECM-mimic environment for viability and insulin secretion of beta cells [336]. In the form of

PEG nanoparticles with covalent immobilization of CREKA, a tumor targeting peptide through fibrin affinity, it was shown that doxorubicin delivery could be achieved efficiently [335].

In this study, it is aimed to design a novel composite hydrogel with use of these two approaches. CHPOA nanogels were assembled through hydrophobic interaction of cholesterol units. Further, these nanoparticles were incorporated into PEG hydrogel networks under mild reaction conditions (Figure 6. 1). In this part, mainly acrylates of PEGDA and NVP were reacted with visible light induced photopolymerization to form chemical crosslinks.

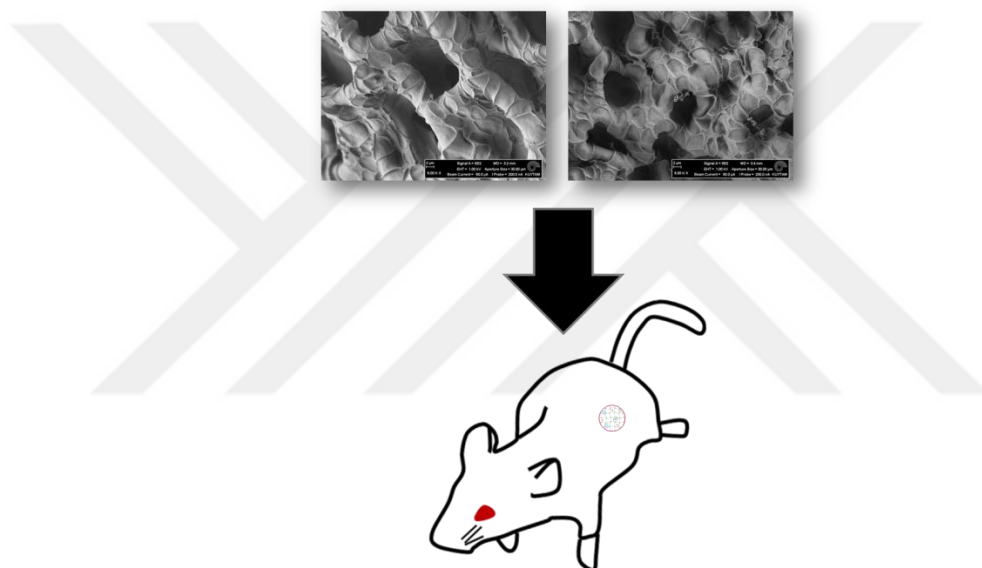


Figure 6. 1: Development of biocompatible PEG hydrogel-CHPOA nanogel composites.

This new system was then investigated *in vivo* and these composite hydrogels were shown to be promising in tissue engineering applications (Figure 6. 2). For the future studies, this new system is a promising candidate for dual delivery of cell and biomolecules in macro-sized PEG hydrogels with CHPOA nanogel incorporation.

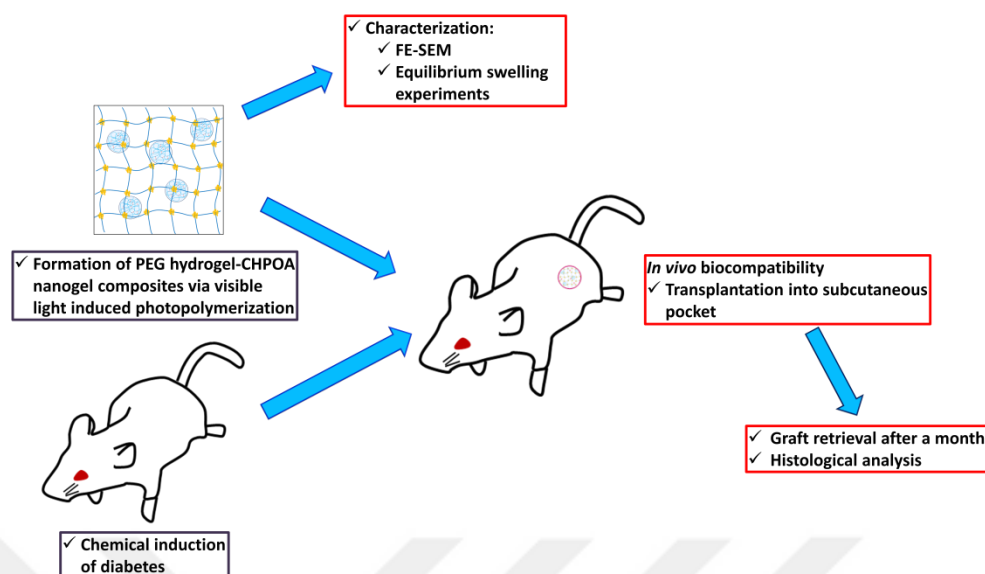


Figure 6. 2: Flow diagram of experiments in PEG hydrogel-CHPOA nanogel composite development.

6.3. Materials and Methods

Synthesis of CHPOA: CHPOA and CHPOA-Rhodamine B molecules were synthesized by Prof. Yoshihiro Sasaki and Prof. Kazunari Akiyoshi's Laboratory at Department of Polymer Chemistry, Graduate School of Engineering, Kyoto University, Katsura, Nishikyo-ku, Kyoto 615-8510, Japan.

Preparation of PEG-CHPOA Composite Hydrogels: CHPOA (10 mg/ml) nanogels were formed in PBS (pH 7.4) at room temperature under constant stirring and nanogels solution was filtered. PEG prepolymer solution with final concentrations of 10% (w/v) PEGDA (10 kDa, Laysan Bio, Inc), 225 mM TEA (Merck), 37 mM NVP (Aldrich) and 0.03 mM eosin Y (Sigma Aldrich) was prepared. Nanogels were incorporated into prepolymer solution at a concentration of 0.08, 0.4 or 0.8 mg/ml (Figure 6. 3).

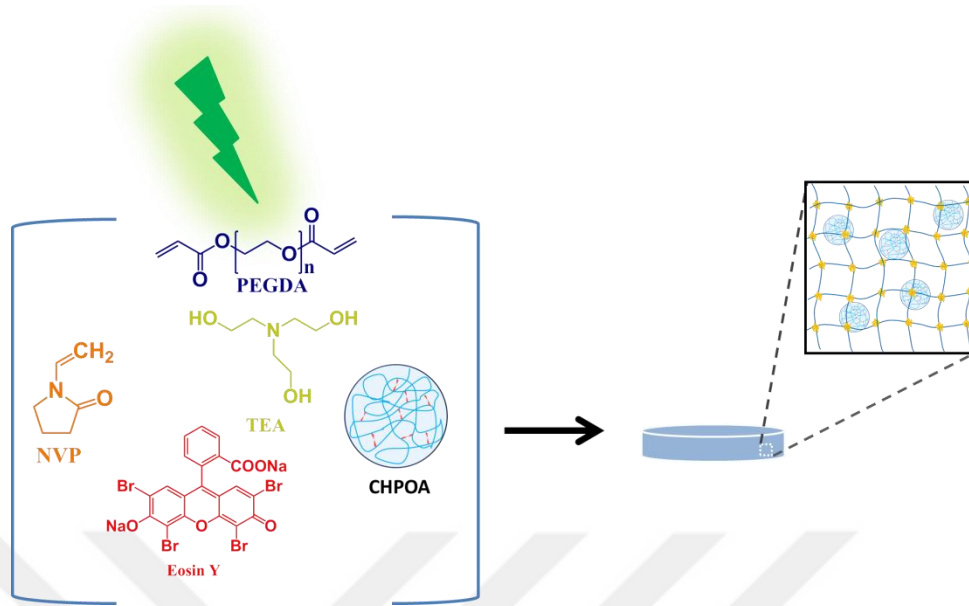


Figure 6. 3: Photopolymerization of PEG-CHPOA composite hydrogels.

Swelling of PEG-CHPOA Composite Hydrogels: PEG-CHPOA composite hydrogels were swollen to remove unreacted molecules. Freeze-dried composite hydrogels were weighted for dry weight and were incubated in PBS (7.4) for 48 h at 37°C to measure the swollen weight. These values were used to calculate swelling ratio (Equation 1) and mesh size (Equation 2-4) of the composite hydrogels [337].

$$q = \frac{W_s}{W_d} \quad (\text{Equation 1})$$

In Equation 1, W_s and W_d represent swollen and dry weights. q is the mass swelling ratio. To calculate mesh size, molecular weight between crosslinks (M_c) was calculated with Peppas-Merrill equation as follows [337, 338]:

$$\frac{1}{M_c} = \frac{2}{M_n} - \frac{\left(\frac{\bar{v}}{V_1}\right) \left(\ln(1 - v_{2,s}) + v_{2,s} + \chi v_{2,s}^2 \right)}{v_{2,r} \left[\left(\frac{v_{2,s}}{v_{2,r}} \right)^{1/3} - \left(\frac{v_{2,s}}{2v_{2,r}} \right) \right]} \quad (\text{Equation 2})$$

M_n is the molecular weight of PEGDA, $\bar{v}$ and V_1 are specific volume of the polymer (PEGDA) and the molar volume of water. χ is the Flory-Huggins polymer-solvent interaction parameter taken as 0.426 for this case. $v_{2,r}$ and $v_{2,s}$ represent polymer volume fraction of the hydrogel in the relaxed and swollen state, respectively [337-339].

Obtained M_c values were used to calculate $(r_o^{-2})^{1/2}$, the root mean squared end-to-end distance of the unperturbed (solvent-free) state. In Canal-Peppas equations (Equation 3 and 4), l is the bond length (1.46 Å); M_r is the molecular weight of PEGDA repeat unit (44 g mol⁻¹). C_n characteristic ratio for PEG (4) and ξ is the mesh size (Å) [339, 340].

$$(r_o^{-2})^{1/2} = 1 \left(2 \frac{M_c}{M_r} \right)^{1/2} C_n^{1/2} \quad (\text{Equation 3})$$

$$\xi = (r_o^{-2})^{1/2} v_{2,s}^{-1/3} \quad (\text{Equation 4})$$

M_c value was also used to calculate crosslinking density of the hydrogels as follows[341]:

$$\rho = \frac{1}{\bar{v} M_c} \quad (\text{Equation 5})$$

where ρ is crosslinking density (mole/cm³), $\bar{v}$ is the specific volume of the polymer (cm³/g) and M_c is molecular weight between crosslinks.

Field Emission Scanning Electron Microscopy (FE-SEM): Water-swollen composite hydrogels were frozen in liquid nitrogen, freeze-dried and spur-coated with Au (5 nm) (Cressington Sputter Coater, 108 auto). Dried samples were observed with FE-SEM (Zeiss Ultra Plus) with EHT voltage of 1.0 kV.

In vivo Biocompatibility Tests: All animal experiments were approved by Koc University Animal Research Local Ethics Committee. Eight week old CD1 male mice (~30 g) were obtained from Koc University Animal Research Facility (KUARF). Animals were injected intraperitoneally with 50 mg/kg streptozotocin (STZ) in citric acid buffer (pH 4.5) consisting of 25.5 mM sodium citrate and 22 mM citric acid. Animals with blood glucose ≥ 300 mg/dl were accepted as diabetic. After ~5 months of first injection, subcutaneous transplantation of composite hydrogels was performed into back of mice (Figure 6. 4).

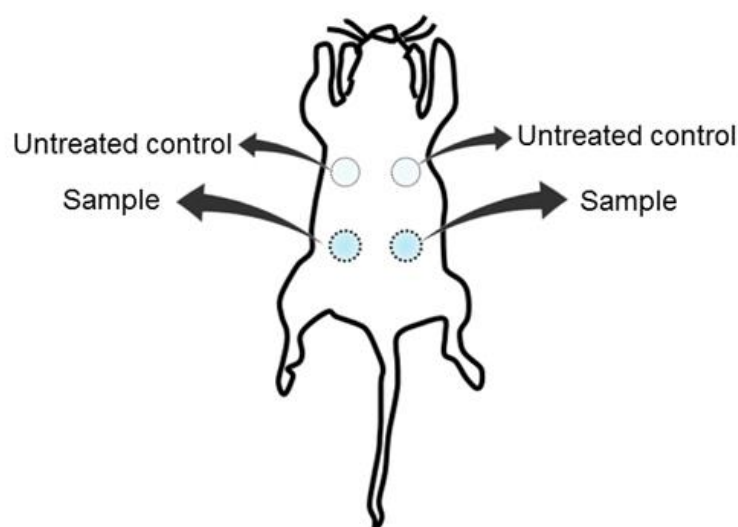


Figure 6. 4: Implantation regions of CD1 mouse.

At the end of 1 month, animals were sacrificed and muscle tissues in direct contact with implants were fixed with formaldehyde (10%). After dehydration, samples were embedded in paraffin and 5 μ m-thick sections were stained with hematoxylin-eosin. Muscle tissue sections were analyzed according to ISO 10993-6 standards according to scores in Table 6. 1 and Table 6. 2.

Table 6. 1: Scores for immune system cells.
yba is at high magnification(400.) per area.

Cell type/reaction	Score				
	0	1	2	3	4
Polymorphonuclear cells	0	Rare, 1-5/yba a	5-10/yba	Heavy infiltration	Cluster
Lymphocytes	0	Rare, 1-5/yba a	5-10/yba	Heavy infiltration	Cluster
Plasma cells	0	Rare, 1-5/yba a	5-10/yba	Heavy infiltration	Cluster
Macrophages	0	Rare, 1-5/yba a	5-10/yba	Heavy infiltration	Cluster
Giant cells	0	Rare, 1-5/yba a	5-10/yba	Heavy infiltration	Cluster
Necrosis	0	Rare, 1-5/yba a	5-10/yba	Heavy infiltration	Cluster

Table 6. 2: Scores for immune reactions.

Reaction	Score				
	0	1	2	3	4
New vessels	0	Little capillary proliferation, focal, 1-3 branches	4-7 capillaries and fibroblastic structures	Structures with extensive bands and capillaries	Structures with extensive bands with capillaries and fibroblastic structures
Fibrosis	0	Narrow bands	Partially thick bands	Thick bands	Extensive bands
Fat infiltration	0	Fat deposition with little fibrosis	A few layers of fat and fibrosis	Large and elongated structures with fat cell deposition around implantation area	Extensive fat tissue covering whole implant region

6.4. Results and Discussion

6.4.1. Characterization of Composite Hydrogels

In FE-SEM images (Figure 6. 5), PEG hydrogels and PEG-CHPOA composite hydrogels had porous network.

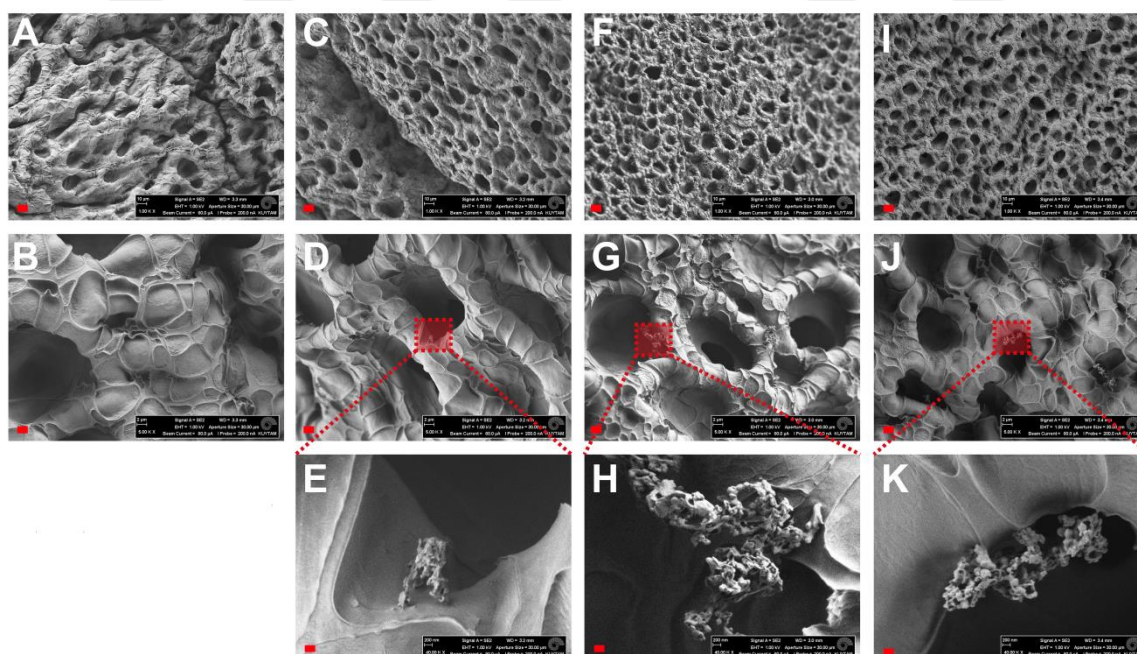


Figure 6. 5: Microscopic analysis of PEG-CHPOA composite hydrogels. A-K) FE-SEM images of PEG hydrogel and PEG-CHPOA composite hydrogels, A-B) PEG hydrogel. C-E) 0.08 mg/ml CHPOA incorporated composite hydrogel. F-H) 0.4 mg/ml CHPOA

incorporated composite hydrogel. I-K) 0.8 mg/ml CHPOA incorporated composite hydrogel. Scale bar (A, C, F, I) is 10 μm . Scale bar (B, D, G, J) is 2 μm . Scale bar (E, H, K) is 200 nm.

When swelling of these hydrogel networks were analyzed, mass swelling ratios were 12.35 ± 0.42 , 12.60 ± 0.23 , 12.49 ± 0.68 and 12.54 ± 0.69 for PEG hydrogels and PEG-CHPOA composite hydrogels (0.08, 0.4 and 0.8 mg/ml), respectively (Figure 6. 6A) with similar crosslinking densities. As represented in Figure 6. 6C, mesh sizes were in angstrom ($\text{\AA}$) range being $55.56 \pm 1.25 \text{ \AA}$ for PEG hydrogels. This value was around $56.94 \pm 1.34 \text{ \AA}$, $57.09 \pm 3.34 \text{ \AA}$ and $56.76 \pm 2.63 \text{ \AA}$ for 0.08, 0.4 and 0.8 mg/ml CHPOA containing composite hydrogels, respectively (Figure 6. 6C). Micrographs and swelling experiment showed that there is no significant change in porosity of hydrogels. These mesh sizes are larger than hydrodynamic radius of the glucose which is about 3.9 $\text{\AA}$ [342] and can allow glucose transfer when the constructs are used as scaffolds for beta cell transplantation.

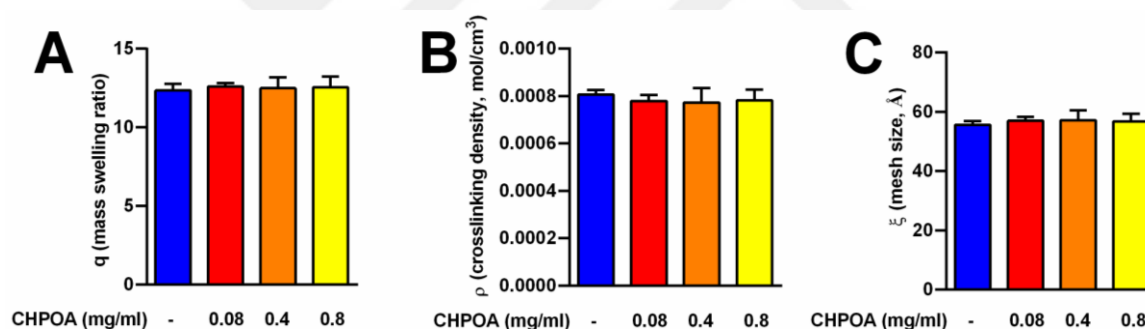


Figure 6. 6: Swelling capacity of PEG-CHPOA composite hydrogels (0.08-0.8 mg/ml CHPOA). A) mass swelling ratio, B) crosslinking density, C) mesh size. $n=4$. Mean $\pm$ SD.

During swelling, change in Gibbs free energy of mixture and contribution of elastic retractive forces control total free energy. At equilibrium swelling at constant temperature and pressure, chemical potential difference of swelling reagent between hydrogel and pure fluid will be zero. The elastic forces are calculated with rubber elasticity theory and are important to determine the M_c of the hydrogels [343]. Thus, crosslinking density might be an important factor for cells in hydrogels. By tuning the crosslinking density with changing PEGDMA monomer concentration in hydrogels, it was previously shown that in the absence of any strain chondrocytes adapted to a rounded morphology. During 6 day culture, chondrocytes in lower crosslinking density

(0.116 M) exhibited 13% increase in diameter whereas 4.5% increase was obtained with increasing crosslinking density (0.376 M). Upon introduction of strain, the changes in morphology of the cells were evident. Even at a single crosslinking density, the deformation response of the cells differed probably due to anisotropic internal strain and heterogeneous nature of the cell population and life cycle [344, 345].

As the sizes of pores are smaller than sizes of the cells and there is elastic retraction within hydrogels due to crosslinked polymer chains, cells will be trapped. There might be possibility to develop stress induced squeezing of the cells in hydrogels and cells do not freely move in hydrogels. However, if this factor was dominant over cells, insulin secretion and cell viability would be compromised and cells would not be stimulated in glucose challenge. The results through this study showed that beta cells were viable and functional in PEG hydrogels during incubation time studied here.

As can be observed from Figure 6. 5, electron micrographs showed larger sized pores, mainly in micrometer range. However, as can be observed from calculations of mesh size, a few nanometer sized (average 5.56-5.71 nm) pores should be present in the network structure. This contrast in micrograph and calculations was also reported by Lin and colleagues where a similar mesh size of 7.78 nm (10%, 10kDa) for PEG hydrogels had micrometer sized pores [346]. Further, it was also reported that calculated mesh size can differ from SEM images in which carbon nanotubes (CNTs) were incorporated into PEG hydrogels. It was suggested that this can occur during flash freezing in liquid nitrogen, as growing ice crystals might have caused mechanical disruption. Moreover, hydrophobicity of CNTs and ability of CNTs to expel hydrophilic units were suggested for this difference [347]. In this case, as it was observed that unmodified PEG hydrogels also showed the same behavior, possible reason for this might be due to mechanical disruption or during the removal of coverslips around the hydrogels.

6.4.2. Chemical Induction of Diabetes

It was observed that 4 of 6 animals had blood glucose level around/more than 300 mg/dl indicating that these animals became diabetic (Figure 6. 7B). Among STZ treated animals, at most ~7% of the weight was lost. In contrast, healthy animals gained weight (Figure 6. 7A).

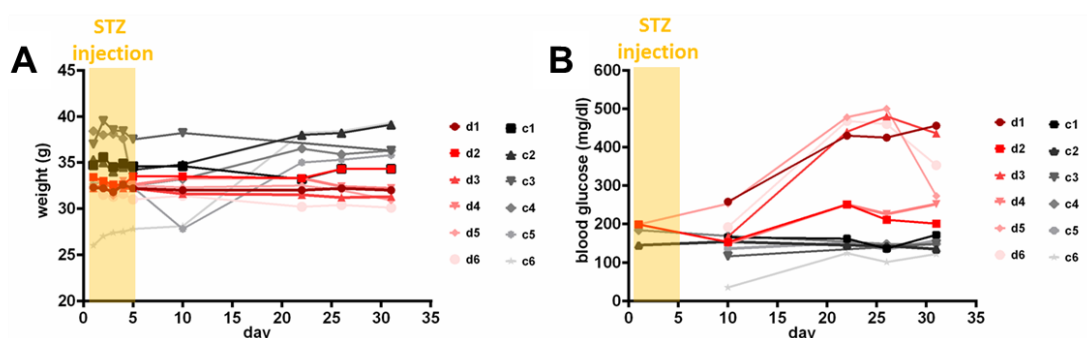


Figure 6. 7: Changes in weight (A) and blood glucose levels (mg/dl) (B) in 50 mg/kg wt streptozotocin treated CD1 mice during 30 days. Orange region represents 5 consecutive days of STZ or buffer injection for low dose. n=6 for STZ treated and healthy (control) animals.

6.4.3. Histological Analysis of Implant Regions for Immune Response

Implants retrieved from diabetic animals are represented in Figure 6. 8 and scores for immune reaction against implants are summarized in Table 6. 3. In Figure 6. 9 and Table 6. 4, results of implantation into healthy animals are presented.

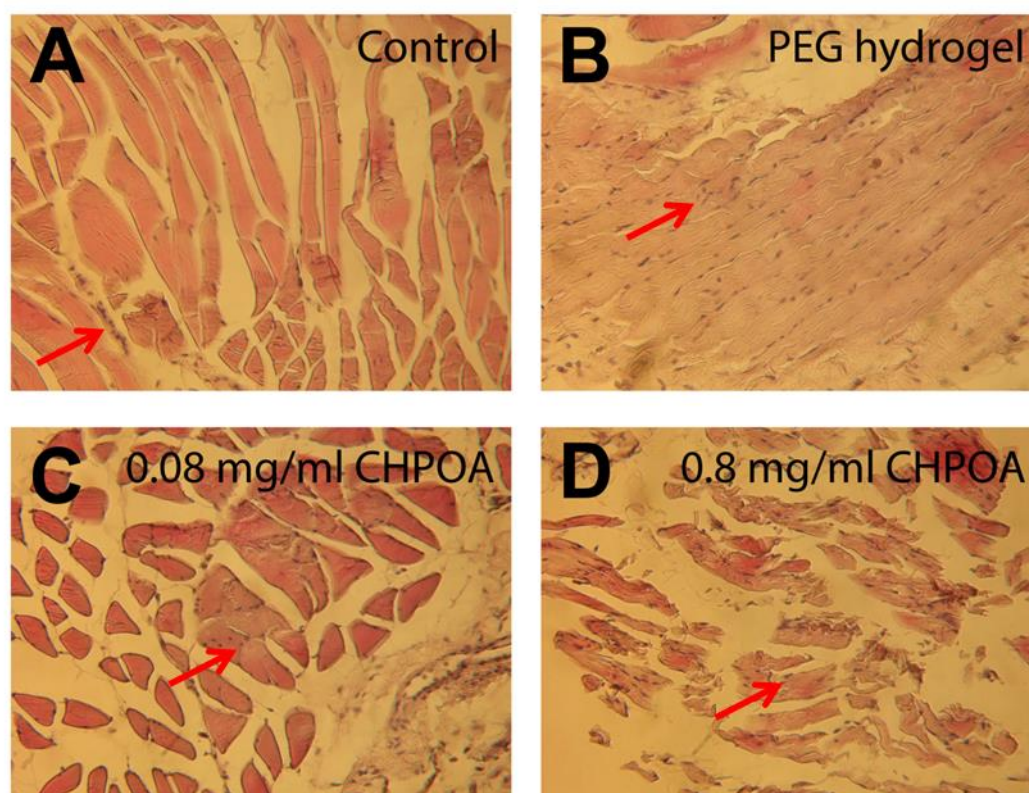
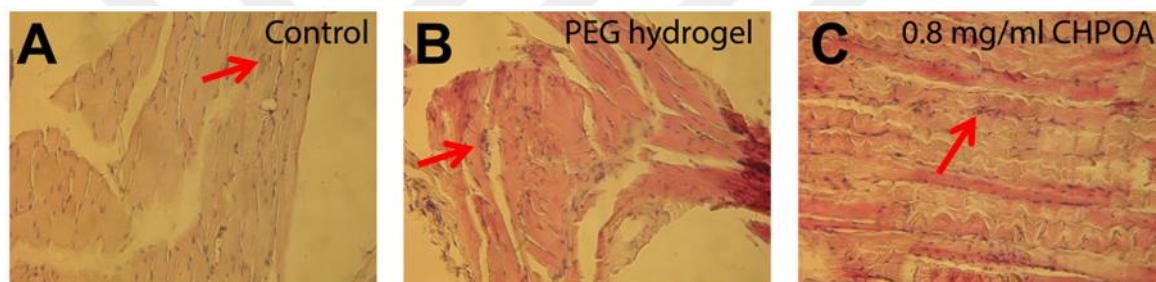


Figure 6. 8: Histological analysis of A) Control (untreated tissue), implanted PEG-CHPOA composite hydrogels with CHPOA B) 0 mg/ml, C) 0.08 mg/ml and D) 0.8 mg/ml in diabetic mice. Red arrows point examples of immune system cells.

Table 6. 3: Scores for immune reaction in diabetic mice.

	PEG hydrogel	0.08 mg/ml CHPOA	0.8 mg/ml CHPOA	Controls
Blood vessel	1	1	0	0
Fibrosis	1	1	1	1
Fat	0	0	0	0
Polymorphonuclear cells	0	0	0	0
Lymphocyte	2	1	1	1
Macrophage	1	1	1	0
Plasma cell	0	0	0	0
Giant cell	0	0	1	0
Necrosis	0	0	0	0

**Figure 6. 9:** Histological analysis of A) Control (untreated tissue), implanted PEG-CHPOA composite hydrogels with CHPOA B) 0 mg/ml, C) 0.08 mg/ml and D) 0.8 mg/ml in healthy mice. Red arrows point examples of immune system cells.**Table 6. 4:** Scores for immune reaction in healthy mice.

	PEG hydrogel	0.8 mg/ml CHPOA	Controls
Blood vessel	0	1	0
Fibrosis	1	2	0
Fat	0	0	0
Polymorphonuclear cells	0	0	0
Lymphocyte	1	2	1
Macrophage	1	1	0
Plasma cell	0	0	0
Giant cell	0	0	0
Necrosis	0	0	0

When the results were analyzed, healthy and diabetic animals had normal muscle

tissue morphology (Figure 6. 8 and and Figure 6. 9) and inflammation scores (Table 6. 3 and Table 6. 4). In 0.08 mg/ml containing diabetic mouse group, muscle tissue was similar to controls. However, presence of capillaries and small amount of macrophages in the implant region were also observed in this group (Table 6. 3). 0.8 mg/ml CHPOA containing hydrogel transplanted group was similar to controls and 0.08 mg/ml CHPOA containing diabetic mouse group. Compared to 0.08 mg/ml CHPOA containing diabetic group, this group did not have new capillary formation and small amount of giant cells were present (Table 6. 3). In healthy mice, 0.8 mg/ml CHPOA containing nanogels attracted more lymphocytes compared to diabetic mice (Table 6. 3 and Table 6. 4).

In diabetic mice, PEG hydrogel implanted group had the highest lymphocyte count (Table 6. 3). In contrast, in healthy mice, immune response was similar to control (Table 6. 4).

Overall, it is possible to say that PEG hydrogels and composite hydrogels can be accepted as biocompatible and they evoke a mild immune response.

5.5. Conclusion

Results in this study suggest that newly developed PEG hydrogel CHPOA nanogel composite system is biocompatible and can be used in islet cell research. PEG hydrogels were formed through visible-light induced polymerization by radical-based covalent bonding and CHPOA nanogels were obtained by physical crosslinking of cholesterol units on pullulan. By combining such two unique approaches, we observed that this system is applicable to not only use in islet cell research, but also possibility to use in cellular applications. In the future, these newly established platforms can be used to deliver drugs or proteins to regulate immune system during transplantation.

Chapter 7

Heterospheroid-Laden Engineered Poly(ethylene glycol) Hydrogels for Beta Cell Niche

7.1. Abstract

Poor engraftment of beta cells after transplantation define the outcome in clinical setting. Heavy immune reaction plays a central role on this limitation, as transplanted beta cells are under severe immune attack that causes failure of transplantation therapy. To advance engraftment, deleterious effects of immune system should be managed for beta cell function and survival, ultimately, for control of beta cell mass. In this study, we investigated the effect of mesenchymal stem cells (MSCs) in heterospheroids for immunoprotection and functional improvement of MIN6 beta cells. PEG hydrogels were also applied to form a physical barrier to support MSC activity. Encapsulated MIN6-MSC heterospheroids sustained beta cell survival after proinflammatory cytokine treatment in both naked and PEG hydrogel coated groups, and insulin secretion was promoted with MSC incorporation. Insulin secretion was further controlled with GLP-1 immobilization into PEG hydrogels. Such combination of MIN6 beta cells and MSCs as a heterospheroid in GLP-1 functionalized PEG hydrogels offer a platform to enhance therapeutic efficacy in beta cell transplantation.

7.2. Introduction

T1D is a pancreas-specific autoimmune disease characterized by immune cell infiltration. T1D can be triggered environmentally and/or genetically. Each one of these reasons have negative effect on beta cell survival as they destroy pancreatic beta cells which eventually initiates hyperglycemia episodes effecting whole body [46]. To restore normoglycemia, pancreatic islet transplantation has emerged as alternative to regulate

glucose imbalance in the body. In this system, immunosuppressive drugs that often include sirolimus and tacrolimus have been utilized as an essential therapy to sustain islet graft survival [2]. However, these immunosuppressive drugs have adverse effects on transplanted beta cells and patients' whole body. For example, tacrolimus (FK 506), an inhibitor of calcineurin, seriously affects insulin secretion and islet viability, as drastic changes in insulin content and integrity of pancreatic islets have been reported previously [6-9, 65]. Sirolimus was designed to bind 12-kDa FK506 binding protein (FKBP-12) to inhibit mechanistic target of rapamycin (mTOR) for cell cycle arrest. However, it was shown that sirolimus activated autophagy of beta cells and down-regulation of insulin expression [10-12]. Further, side effects such as mouth ulcer, nephrotoxicity, anemia and hypercholesterolemia were also induced with sirolimus uptake [13].

Instead of weakening immune system and increasing the vulnerability to other diseases with immunosuppressive drugs, use of immuno-modulatory cells is offered as an alternative during transplantation. In this study, we considered heterospheroids rather than use of monolayer of dispersed cell suspensions. The 3D morphology that heterospheroids provide has been suggested to be promising as it improves the potency of the cells used in transplantation. For instance, co-localization of MSCs and beta cells can be facilitated with use of heterospheroids. MSC-secreted ECM and anti-inflammatory proteins can improve function and survival of beta cells [132]. Further, spheroids are superior to promote cell-to-cell interactions of same or different type. In addition to cellular communication, deposition of extracellular matrix (ECM) can be provided by one of the cell types used to control behaviour of cells in spheroids. For example, heterospheroids of MIN6-MAEC and MIN6-NIH3T3 with hanging drops were formed and improved MIN6 insulin secretion was only observed in heterospheroids where no significant effect was detected with monolayers. These results suggest that 3D cell-cell interaction and possible deposition of ECM from MAECs and NIH3T3 cells are critical factors for beta cell function [183]. In portal vein transplantations, possibility of washing of co-transplanted MSCs lessens the potency of designed system. However, in heterospheroids, this effect can be minimized and co-localization of cells can be achieved [154]. Also, spheroid configuration is proven to control transcriptome of human MSCs, as anti-inflammatory and anti-apoptotic protein expression was reported to be enhanced

in this configuration. It was previously shown that human MSC spheroids were more efficient than monolayers to silence proinflammatory responses of lipopolysaccharide activated cells and peritonitis model [348].

For immunoprotection, heterospheroids of beta cell-Treg, beta cell-sertoli cell and beta cell-MSC have been investigated. Tregs play a role in self-tolerance, as they are influential to control graft tolerance by regulating activation and effector function of T and B cells. For instance, Iwata and his group have shown that in portal vein transplantation of C57BL/6 mouse, 400 islets managed glucose levels transiently for approximately 10 days. In contrast, Treg-islet cell heterospheroids had prolonged graft survival over 100 days where 6 of 9 recipients were normoglycemic [155]. Sertoli cells are providers of immunoprivileged environment in testis and this property makes them promising to use in beta cell transplants. The same group also reported that 800 sertoli cell-islet cell heterospheroid transplantation yielded in re-establishment of normoglycemia in portal vein transplantation of C57BL/6 mouse. In heterospheroid transplanted group, 6 of 7 diabetic recipients had sustained normoglycemia for more than 100 days compared to 10 days in islet cell spheroid group [19].

MSC are also versatile tools to facilitate engraftment. These cells are reported to have outstanding ability on immuno-regulation, anti-apoptosis and vascularization. For example, they can control effector T cell function with secretion of a variety of soluble factors such as hepatocyte growth factor (HGF), transforming growth factor β (TGF- β) and indoleamine 2,3-dioxygenase (IDO). Some of these factors such as TGF- β secreted by MSCs have potential to activate Tregs for self-tolerance. They have capacity to inhibit T cells by utilization of cell-cell contact. [146]. Ability of MSCs in heterospheroids to protect beta cells against proinflammatory cytokine induced apoptosis was established recently where MIN6 beta cell-MSC heterospheroids in phosphorylcholine-modified poly-L-lysine and heparin layered coats or without coating had decreased level of apoptosis [132]. In addition, transplantation of MSCs for beta cell therapy showed promise in a cynomolgus monkey trial [17]. In their native environment, beta cells are surrounded by a dense network of blood vessels and during transplantation; this network is required to be re-established to prevent hypoxia induced beta cell death. Proteins such as vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs)

secreted by MSCs are favorable in vascularization of beta cells [247]. Reported by Ito and colleagues, transplantation of islets and MSCs into portal vein of LEW rat recipients decreased the number of islets to reach normoglycemia. After transplantation into renal capsule of NOD SCID mice, higher capillary density was observed around islets infused with MSCs [28]. Another distinct feature of MSCs is their ability to promote insulin secretion [281, 336, 349]. MSCs are the only cell type reported so far to have both anti-apoptotic and insulin secretion improving property. Previously, HUVEC, NIH3T3 and MAEC were reported to increase insulin secretion of beta cells in heterospheroids or co-culture, probably due to deposition ECM proteins [183-185]. However, none of these cells has been reported to have immunoprotective function and aforementioned immunoprotective cells do not regulate insulin secretion from beta cells.

In addition to using a cell type as an immunological support, it is common to benefit from a physical barrier to prevent direct contact with immune system. This barrier can act as a filter to prevent diffusion of large sized proteins such as immunoglobulins. Further, it can mimic the native environment of beta cells and be a physical support for integrity of beta cell clusters. To date, various coats with different functionalities have been developed and shown to be promising [5, 109, 132]. These devices offer the advantage of implantation through minor surgery and ease of graft retrieval [117]. One of such coats are PEG hydrogels which have been previously shown to be biocompatible with beta cells [176, 336].

In the present study, effectiveness of MSC incorporation into MIN6 pseudoislets was investigated (Figure 6. 1) and improved activity of MIN6 beta cells are reported. PEG hydrogels were also used to provide a local microenvironment of beta cells to be transplanted and with further functionalization with GLP-1, insulin secretion was increased in PEG hydrogels. The results presented here suggest that heterospheroids of MSCs and MIN6 beta cells are practical tools to analyse cell behaviour in GLP-1 functionalized PEG hydrogels for therapeutic applications and to treat autoimmune pancreatic disease.

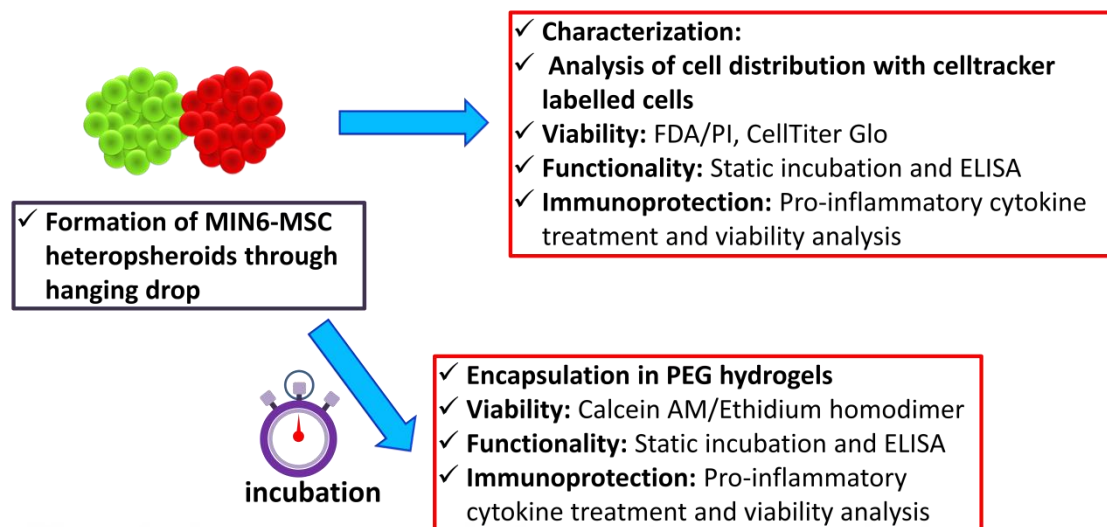


Figure 7. 1: Flow diagram for investigation of free and PEG hydrogel encapsulated MIN6-MSC heterospheroid activity.

7.3. Materials and Methods

MIN6 Beta Cell Culture: MIN6 beta cells were a kind gift of Dr. Donald F. Steiner from University of Chicago, Biochemistry and Molecular Biology. MIN6 cells (p24-44) were propagated in DMEM (Gibco) supplemented with 10% FBS (Gibco), 100 IU/ml penicillin-0.1 mg/ml streptomycin (Sigma), 2 mM L-glutamine (Sigma), 1 mM sodium pyruvate (Gibco) and 0.10 μ M β -mercaptoethanol (Gibco) under standard culture conditions.

MSC Culture: Rat bone marrow MSCs were isolated as described previously [152]. These cells (p9-14) were cultured in DMEM (Gibco) supplemented with 10% FBS (Gibco) and 100 IU/ml penicillin-0.1 mg/ml streptomycin (Sigma) at 37°C, 5% CO₂ environment.

Labelling of MIN6 Beta Cells and MSCs: MIN6 beta cells and MSCs were labeled with CellTracker Red and Green (Life Technologies), respectively to observe distribution of cells within heterospheroids. Five μ m dye solution was added and cells were incubated for 1 h. Following this treatment, cells were rinsed with 1X sterile PBS twice. Labelling was confirmed with fluorescence microscopy (Nikon Eclipse).

Heterospheroid Assembly with MIN6 Beta Cells and MSCs: After labelling, MIN6 beta cells and MSCs were suspended in DMEM with supplements. Following suspension, MIN6:MSC ratios of 1:1, 2:1, 5:1 and 10:1 were analyzed and ratios of 1:0 and 0:1 were

used as control groups. Ratio of 1:0 (MIN6 only spheroids) was referred as pseudoislet. For each ratio, total cell number in each 30 μ l droplets was kept constant as 200 or 500. Following hanging drop formation on petri dish lids (Corning), cells were culture for 3 days under standard incubation conditions (Figure 7. 2).

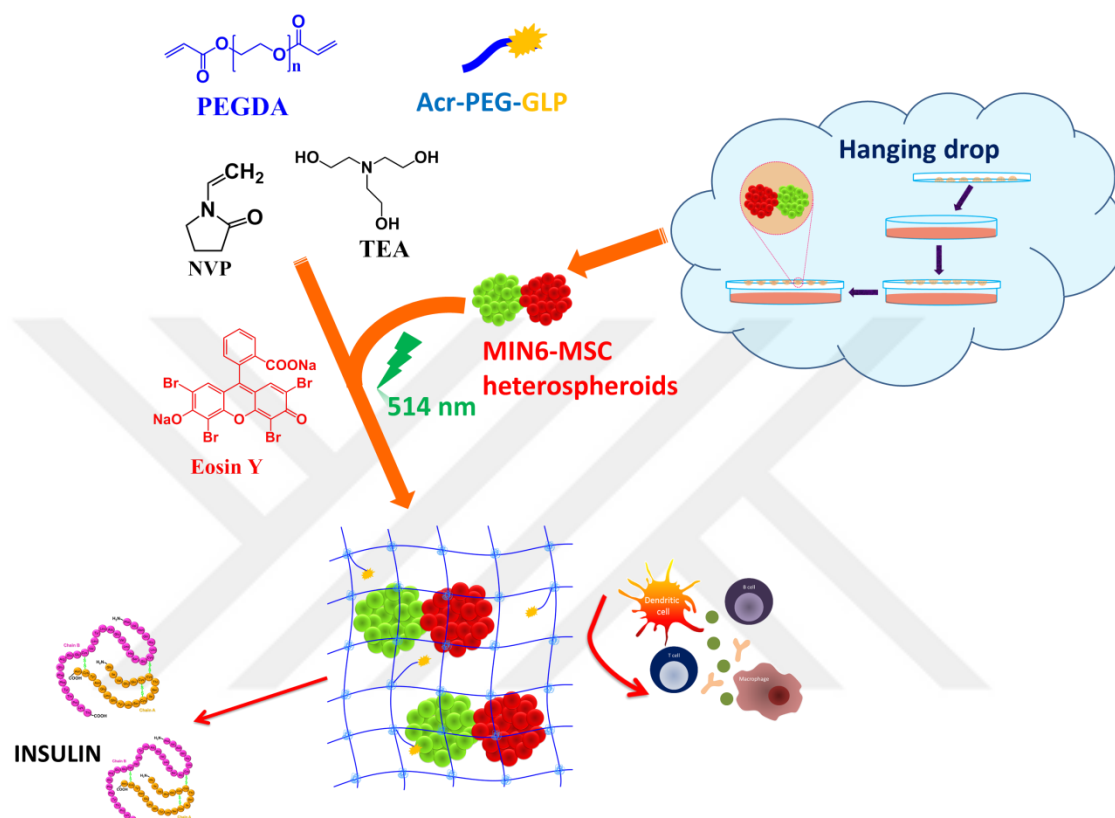


Figure 7. 2: Use of MIN6-MSC heterospheroids in PEG hydrogels.

Analysis of Heterospheroid Viability: To investigate heterospheroid viability, unlabelled MIN6 beta cells and MSC have been used in hanging drops. After 3 days of incubation, viability of heterospheroids and control spheroids was examined with fluorescein diacetate (FDA)/propidium iodide (PI) staining and intracellular ATP levels. Newly formed heterospheroids or control groups were incubated in dye solution of 42.4 ng/ml FDA and 8.5 μ g/ml PI for 5 min. Images of heterospheroids were taken with fluorescence microscopy (Nikon Eclipse). Green areas and red areas were calculated with ImageJ and green area/total area was used to calculate % viability.

Intracellular ATP amount of heterospheroids and control spheroids was measured with CellTiter Glo Reagent (Promega). Briefly, 10 spheroids were treated in 50% reagent solution in medium for 15 min with shaking. Luminescence was measured with a plate

reader (Synergy-H1 Multi-Mode Plate Reader, Biotek). ATP standards were used to calculate ATP amounts.

Proinflammatory Cytokine Treatment of Heterospheroids: A cytokine cocktail of 50 U/ml interleukin 1- β (IL1- β , ID Labs), 1000 U/ml tumor necrosis factor- α (TNF- α , ID Labs) and 1000 U/ml interferon- γ (IFN- γ , Peprotech) in culture medium was added to newly formed 40 spheroids. These heterospheroids were incubated at 37°C, 5% CO₂ environment for 24 h. Control groups were incubated in culture medium without cytokine supplement. After treatment, FDA/PI staining was performed.

Insulin Secretion from MIN6 Beta Cells in Heterospheroids: Heterospheroids and control pseudoislets were incubated in Krebs Ringer Bicarbonate buffer containing 2.8 mM glucose (low) and 28 mM glucose (high) sequentially and insulin secreted in each buffer was analyzed with mouse insulin ELISA (Mercodia). Stimulation index was used to represent insulin secretion of MIN6 beta cells under glucose challenge and it was calculated by dividing insulin secreted at high to insulin secreted at low glucose buffer.

Encapsulation of Heterospheroids in PEG Hydrogels: Prepolymer solution was prepared with following: 10% (w/v) PEGDA (10 kDa, Laysan Bio, Inc), 225 mM TEA (Merck), 37 mM NVP (Aldrich) and 0.03 mM eosin Y (Sigma Aldrich) (Figure 7. 2). Twenty μ M acr-PEG-GLP-1 was incorporated into this solution where necessary. Thirty pseudoislets or heterospheroids were suspended in this solution and were exposed to 514 nm for 3 min (Coherent Inc., Innova 70C).

Investigation of Antiapoptotic and Insulin Secretion Promotion Effects of MSCs on Heterospheroids in PEG Hydrogels: After 30 hours of incubation under standard culture conditions, encapsulated spheroids were treated with 50 U/ml IL1- β (ID Labs), 1000 U/ml TNF- α (ID Labs) and 1000 U/ml IFN- γ (Peprotech) for 24 h. Following treatment, 16-h long alamar blue (10%) (Invitrogen) incubation was used to assess mitochondrial activity. After this incubation, caspase activity of each respective sample was measured with Caspase Glo (Promega). 50% Caspase Glo reagent was added on spheroid incorporated hydrogels and incubated for 1.5 h. Following incubation, luminescence of each sample was measured with a plate reader (Synergy-H1 Multi-Mode Plate Reader, Biotek). To calculate apoptotic activity, caspase activities were normalized with mitochondrial activity obtained from alamar blue.

Viability of encapsulated spheroids was investigated with calcein-AM and ethidium homodimer (Invitrogen) according manufacturer's instructions. Briefly, dye solution with 2 μ M calcein-AM and 4 μ M ethidium homodimer was used to stain spheroids. Hydrogels were incubated in dye solution for 45 min with shaking. After PBS wash, viability was assessed with fluorescence microscopy (Nikon Eclipse).

To observe the effect of MSCs on MIN6 beta cell insulin secretion, encapsulated spheroids were incubated in glucose solutions of 28 mM and 2.8 mM and, secreted insulin was used to calculate stimulation index for each condition.

Statistical Analysis: Statistical analysis was performed either with Mann-Whitney or student's t-test. $p < 0.05$ was considered statistically significant.

7.4. Results and Discussion

7.4.1. Aggregation and Distribution of MIN6 Beta Cells and MSCs in Heterospheroids

MIN6 beta cell co-culture with MSC was investigated with hanging drop by labeling with celltracker dyes to visualize distribution of both cell types. In fluorescence images of heterospheroids with 200 initial cell number (Figure 7. 3), MIN6 beta cells or MSCs alone formed clusters with same type of cells. In the case of co-culture within hanging droplets, cell clusters of different types attached to each other to form a single cluster rather than forming a homogeneous spheroid. To note that, as MSC number decreased in droplets, smaller MSC cluster size, thus larger MIN6 cluster in each heterogenous spheroid was observed. This behavior was also evident in 500 cell number as in Figure 7. 4.

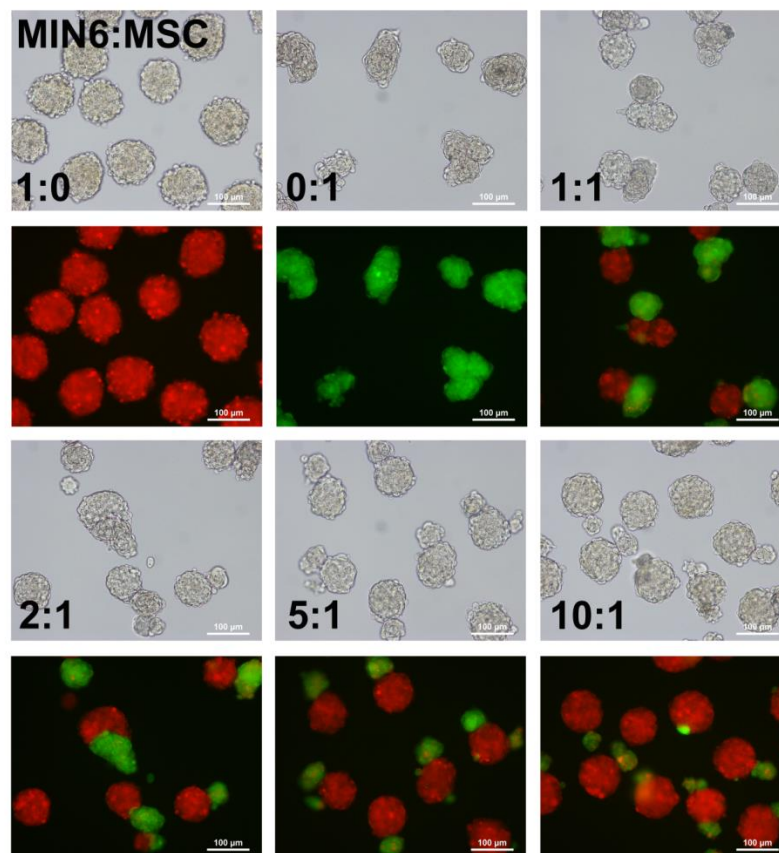


Figure 7. 3: Fluorescence images of MIN6-MSC heterospheroids. MIN6 beta cells and MSCs were stained with CellTracker dyes. Heterospheroids with different MIN6:MSC ratios were formed with hanging drops and images were taken at day 3. Initial cell number in each droplet was 200 and droplet volume was 30 μ l. Red: MIN6 beta cells. Green: MSCs. Scale bars: 100 μ m.

Similar behavior between beta cells and MSCs was also reported where dispersed islet cells and MSCs were mixed in hanging drops. Cells showed preferential aggregation with cells having similar characteristics and then, heterogeneous spheroids were assembled by interaction of these two spheroids [154]. The theory behind this adhesion tendency can be explained with differential adhesion hypothesis (DAH) and differential interfacial tension hypothesis (DITH). DAH describes cell sorting and tissue spreading where intercellular bonding is a crucial factor. Cell aggregates tend to maximize strong adhesive bonding by also replacing the weaker bonds. This behavior exists in irregular pieces of tissues to obtain spherical morphology and to explain coverage of cell aggregates having high surface tension with cell aggregates having lower surface tension [350, 351]. For example, L cells having higher N-cadherin expression were shown to

have higher surface tension and were covered by L cells expression lower level of N-cadherin [350]. It was also reported that expression levels of different cadherins regulate cell-to-cell interactions. For example, surface tension of α TC cells is larger than MIN6 beta cells where no significant expression of E-cadherin was present in α TC cells and high expression level in MIN6 beta cells shown in western blot analysis. Heterospheroids formed with these cells resulted in complete envelopment of MIN6 beta cell by α TC cells. P-cadherin transfected α TC cells expressed higher levels of protein compared to E-cadherin expression level of MIN6 beta cells and this change resulted in partial engulfment of MIN6 beta cells. The reason for this behavioral change was suggested to be cross-adhesion between E- and P-cadherins and induction of cohesion of α TC cell to each other with the remaining P-cadherin molecules that were not cross-adhered [296].

In addition, DITH is used to study distribution of cells in co-aggregates. It shows that the contractile force of microtubules, force of cell membrane with its proteins, force of cell-cell adhesion and net contractile force due to other structures form net interfacial tension between cells ($\gamma^{\text{cell1-cell2}}$). These forces are also sources for surface tension between a cell and medium ($\gamma^{\text{cell-medium}}$). If $\gamma^{\text{cell1-cell2}} > \gamma^{\text{cell1-medium}} + \gamma^{\text{cell2-medium}}$, cells separate. If $\gamma^{\text{cell1-cell2}} < \gamma^{\text{cell1-medium}}$ and $\gamma^{\text{cell1-cell2}} < \gamma^{\text{cell1-medium}} - \gamma^{\text{cell2-medium}}$, total engulfment of cell1 by cell2 will occur. If there is no total engulfment or separation, partial engulfment may occur [352, 353]. These forces can be the reason why MIN6 beta cells and MSCs formed segregated clusters within a heterospheroid. Probably, cells of the same type preferred to aggregate and because of interfacial force between different types of cells, cells did not completely separate, but segregated and weakly attached to each other compared to intercellular interaction within each cluster. From these results, it can be concluded that homotypic interactions were preferred for these heterospheroids.

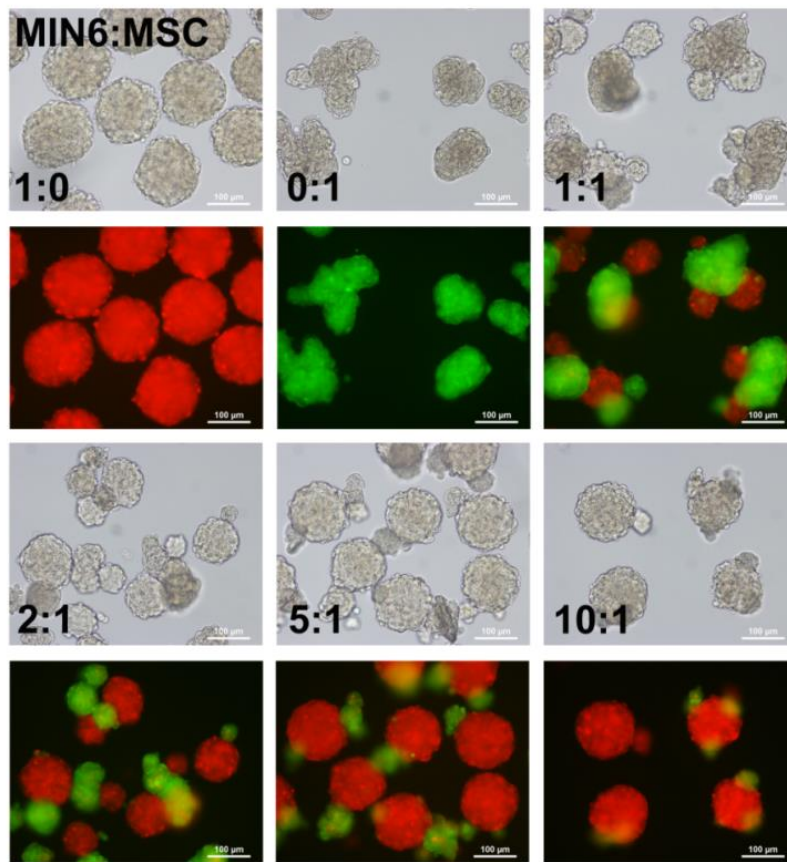


Figure 7. 4: Fluorescent images of MIN6-MSC heterospheroids. MIN6 beta cells and MSCs were stained with CellTracker dyes. Heterospheroids with different MIN6:MSC ratios were formed with hanging drops and images were taken at day 3. Initial cell number in each droplet was 500 and droplet volume was 30 μ l. Red: MIN6 beta cells. Green: MSCs. Scale bars: 100 μ m.

7.4.2. Survival of MIN6-MSC Heterospheroids

Newly formed heterospheroids were stained with FDA/PI and analyzed with Image J to calculate viability of heterospheroids. As shown in Figure 7. 5 and Figure 7. 6, MIN6 pseudoislet viability was around 84% and 73% for 200 and 500 initial cell numbers, respectively (Figure 7. 7A). With the same initial cell numbers, MSC spheroid (0:1) viability was 98% and 99% being higher than MIN6 pseudoislets. In a recent study, 3-D culture of 10 000 and 25 000 human MSCs in hanging drops, 90% of the cells in spheroids were viable [348]. In the present study, more than 90% of the cells in MSC spheroids were viable. The reason for this slight difference is that we have applied a low number of initial cells in hanging drops (200, 500 vs. 10 000 and 25 000) to halt cell death due to medium depletion. At 1:1 and 2:1 ratios, average viability was calculated to

be 95-96%. However, in heterospheroids of ratio to 5:1, 95% and 89% were noted being higher in 200 initial cell number. Further decrease of MSC ratio to 10:1 contributed higher number of cell death in heterospheroids. Even at this 10:1 ratio, total viability of heterospheroids was greater than MIN6 pseudoislets (Figure 7. 7A).

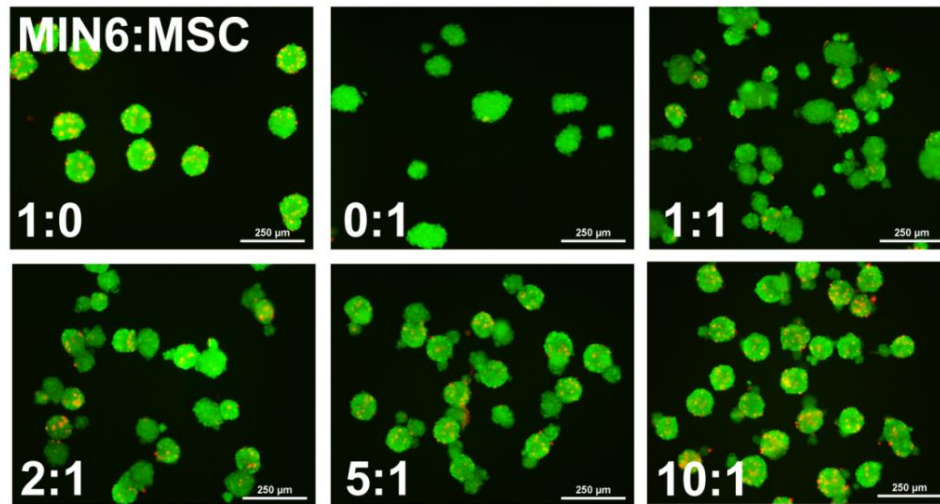


Figure 7. 5: Live/dead staining of MIN6-MSC heterospheroids of 200 initial cells. After 3 days, droplets were destroyed and newly formed heterospheroids were stained with FDA/PI. Scale bars: 250 μ m.

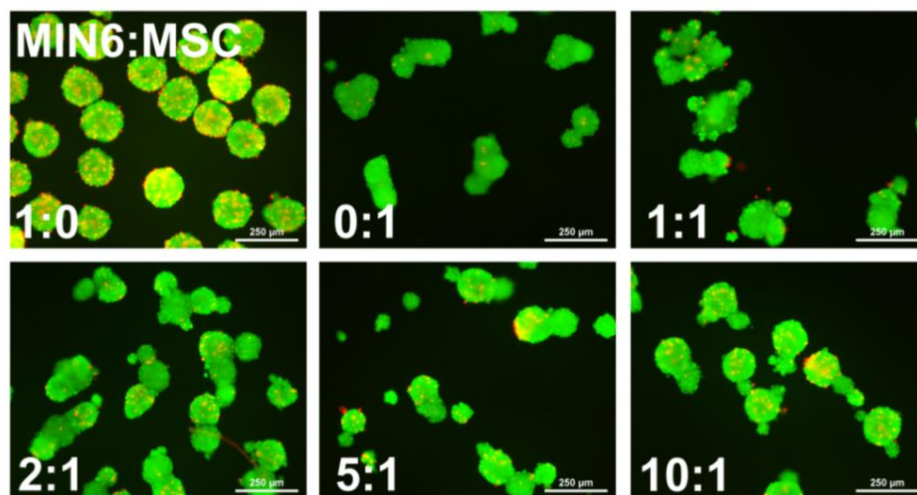


Figure 7. 6: Live/dead staining of MIN6-MSC heterospheroids of 500 initial cells. After 3 days, droplets were destroyed and newly formed heterospheroids were stained with FDA/PI. Scale bars: 250 μ m.

Investigation of intracellular ATP levels support the idea of higher viabilities in MSC incorporated groups (Figure 7. 7B). Comparison of MIN6 pseudoislets of 200 and 500 initial cell number showed that intracellular ATP levels were 1.66 and 3.13 pmole being

higher in 500 initial cell number. This was probably due to incorporation of more cells into spheroids in larger cell number. MSC clusters had slightly higher ATP amounts (1.83 and 3.31 pmole) than MIN6 pseudoislets. In heterospheroid groups, ATP levels were slightly higher than MIN6 pseudoislet and MSC spheroid groups. Similar to MIN6 pseudoislets case with different initial cell numbers, 500 initial cell numbers always had increased levels of ATP.

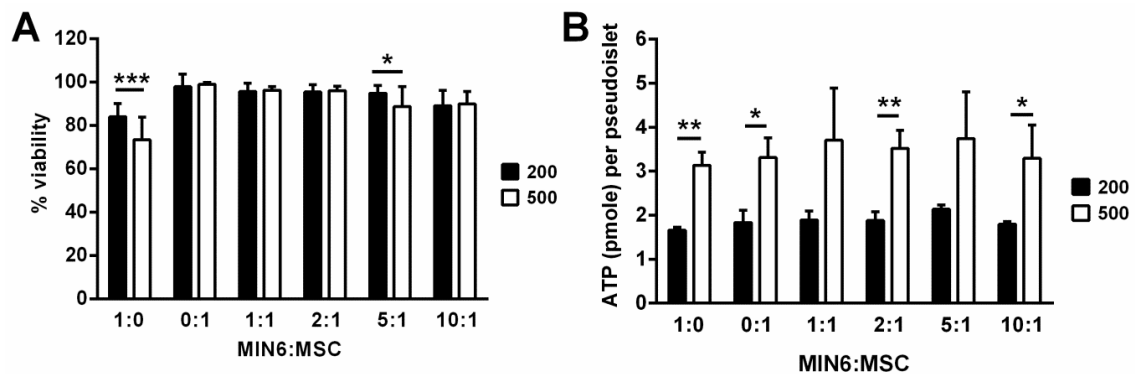


Figure 7. 7: Viability of heterospheroids and control groups of different initial cell numbers. A) Percent viability of heterospheroids of 200 and 500 initial cells calculated from FDA/PI staining. Live/Total area was used to calculate % viability with Image J. Mean $\pm$ SD. n=10-25. B) Intracellular ATP amount for each MIN6:MSC ratio. Mean $\pm$ SD. n=2-3. ***p<0.001. **p<0.01. *p<0.05.

7.4.3. Insulin Secretion from MIN6-MSC Heterospheroids

Insulin secretion of MIN6 beta cells in heterospheroids was explored to analyze whether influence of MSCs on insulin secretion in these 3D spheroids developed or not. Stimulation indices were examined for heterospheroids after assembly in hanging drops. These indices were used to represent the ability of beta cells to respond changing environmental glucose levels. Stimulation indices of MIN6 pseudoislets were 1.41 and 1.36 indicating healthy beta cell response against environmental glucose alterations (Figure 7. 8). For a specific initial cell number, incorporation of MSC into spheroids improved function as well. For ratio of 1:1, insulin secretion was increased 24% and 65% compared to MIN6 pseudoislets. Further improvement was identified, as 31% and 83% increase to glucose responsiveness for 200 and 500 initial cell number in 2:1 ratios compared to MIN6 pseudoislets was summarized in Figure 7. 8. Increases in stimulation indices might be due to partially by increased total viability upon incorporation of MSC (Figure 7. 7). If a comparison is made at constant initial cell ratio in heterospheroids, it

can clearly be observed MSC enhanced insulin secretion. As an example, at 1:1 ratio, viabilities were 96% for both initial cell number with stimulation indices of 1.76 and 2.25 (Figure 7. 8) showing improvement of insulin secretion in heterospheroids with the effect of MSCs. In a previous study of Wittig and her colleagues, bone marrow cell incorporated spheroids with 1:1 ratio showed 93% increase in stimulation index compared to islet cell pseudoislets (2.7 vs. 1.4) [349] confirming increase in stimulation index observed here.

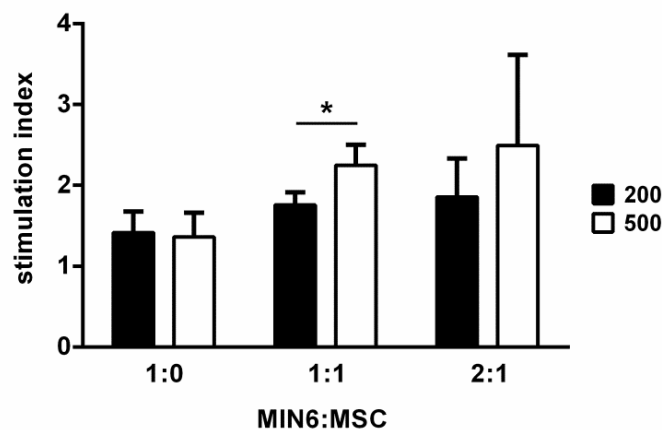


Figure 7. 8: Insulin secretion from heterospheroids. Insulin secretion potential of MIN6 beta cells during glucose challenge is represented as stimulation index. Mean $\pm$ SD. n=3. *p<0.05.

MSC have also been reported to activate glucose responsiveness of beta cells due to secretion of trophic factors. For example, in a previous report of Park et. al, human islets co-cultured with human bone marrow MSCs had stimulation indices of 2.69 ± 0.45 for islet alone and 3.37 ± 0.13 for co-culture group. Trophic factors of IL-6, HGF, TGF- β , and VEGF-A were suggested for advancing in insulin secretion [354]. This process can also be other effector for enhanced glucose responsiveness in our case. For example, factors secreted from MSC such as HGF, TGF- β and IL-6 have positive responses on beta cell function [152, 281, 354]. HGF (through c-Met receptor) and TGF- β activate PI-3K-PKB/Akt pathway [281-284], but the negative effect of TGF- β on beta cells was also observed previously [285]. Although the effect of IL-6 on beta cells have controversial reports [286-288], IL-6 signals through PLC-IP3 were reported to take part in insulin secretion [289].

7.4.4. Analysis of Immunoprotection Potential of MSCs in Heterospheroids

We also investigated ability of MIN6 beta cells to tolerate cytokine environment during *in vitro* culture. For this, control spheroids and heterospheroids were incubated in a cocktail of IL1- β , TNF- α and IFN- γ and viabilities were analyzed with FDA/PI staining. These proinflammatory cytokines are secreted by macrophages and effector T cells to induce apoptosis of beta cells [132]. Heterospheroids were stained after cytokine exposure and results are represented in Figure 7. 9 and Figure 7. 10 for two different initial cell numbers. In Figure 7. 9A and Figure 7. 10A, red areas corresponding to dead cells in spheroids show significant changes and a proof of elevated level of cell death due to cytokine attack. In MIN6 pseudoislets (1:0), cytokine induced apoptosis contributed further cell death within pseudoislets, as viabilities decreased up to 35% and 26% for 200 and 500 initial cell numbers. These corresponded to 47% and 34% of the control groups (untreated MIN6 pseudoislets) (Figure 7. 9B and Figure 7. 10B). In MSC spheroids (0:1), more than 90% viability was evident after cytokine treatment indicating that MSCs can tolerate cytokine induced apoptosis. In heterospheroids, although red areas were observed to be increased compared to control counterparts, viability was higher than 60% being highest in 1:1 ratio in 200 cell group. These results prove that cytokine induced cell death can be prevented in the presence of MSC to a certain level.

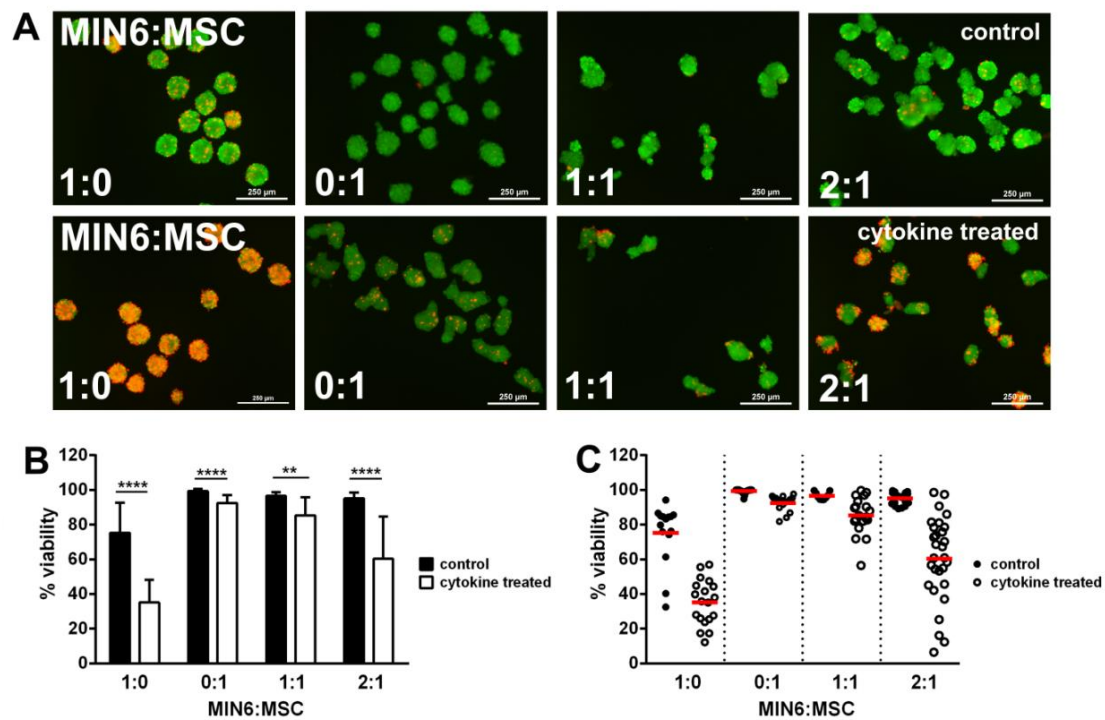


Figure 7. 9: Viability after cytokine cocktail treatment. Initial cell number is 200. A) Fluorescence images of control and cytokine treated heterospheroids. B) Percent viability of each group (control and cytokine treated) for different MIN6:MSC ratios in droplets. Mean $\pm$ SD. n=9-29. ****p<0.0001. **p<0.01. C) Scatter plot of %viability. Red lines represent mean.

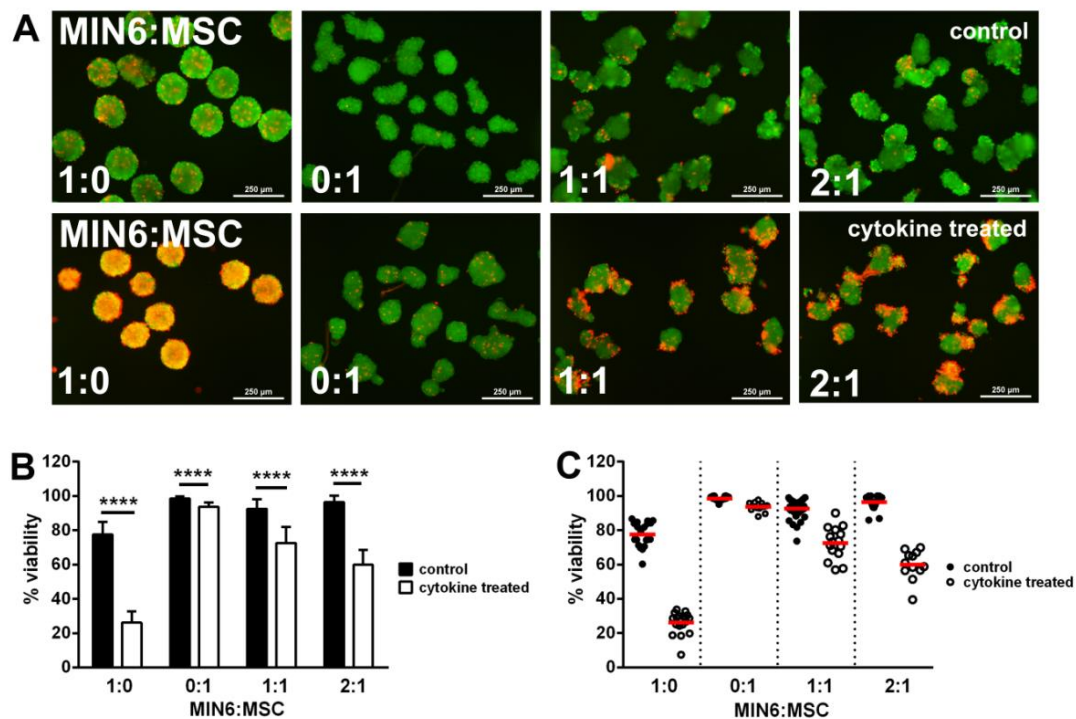


Figure 7. 10: Viability after cytokine cocktail treatment. Initial cell number is 500. A) Fluorescence images of control and cytokine treated heterospheroids. B) Percent viability of each group (control and cytokine treated) for different MIN6:MSC ratios in droplets. Mean $\pm$ SD. n=12-35. ****p<0.0001. C) Scatter plot of %viability. Red lines represent mean.

Bhajii et al. also showed that after cytokine treatment, viability of MIN6 pseudoislets formed on uncoated dishes was less than 60% of the control showing large amount of cell death. In contrast, viability of 3:1 MIN6:MSC heterospheroids was around 100% of MIN6 only group [132]. In our study, MIN6:MSC (1:1) heterospheroid viability was around 128% and 119% compared to untreated MIN6 pseudoislet control and these values change to 113% and 93% in cytokine treated counterparts compared to untreated MIN6 pseudoislet group in 200 and 500 initial cell numbers. In heterospheroids of 2:1 ratios, normalized viability was around 126% and 124% and decreased to 80% and 77% after cytokine challenge compared to untreated MIN6 control group. Similarly, in culture of human islets, high level of apoptotic cells shown by TUNEL staining in islets was established after cytokine treatment. However, minimal cell death was evident in co-culture group confirming that MSCs actively take part in protection of beta cells from cytokine-induced apoptosis [151].

Anti-apoptotic properties of MSCs are reported to arise from trophic factors

including IL-6, TGF- β , HGF and SPP-1 (OPN) [17, 152]. For example, MIN6 beta cells and islet cells treated with cytokine mixture of IL1- β (50 U/ml), TNF- α (1000 U/ml) and IFN- γ (1000 U/ml) showed reduced viability. However, pre-treatment with IL-6 was shown to be powerful to preserve metabolic activity and western blot analysis proved that increased level of anti-apoptotic protein, Bcl-xL. This approach was also shown to be effective in syngeneic transplantation into diabetic C3/HeJ mouse kidney capsule [288]. In another study, OPN was shown to decrease the NO and iNOS expression of RINm5F beta cells treated with IL1- β through inhibition of the activity of NF- κ B [355]. In ECs, HGF increases expression of Bcl-2 (anti-apoptotic protein) and prevents Bax translocation to mitochondrial membrane [356]. All these events reveal as possible mechanisms for the anti-apoptotic pathways against cytokine induced cell death.

Overall, these results suggest that 1:1 MIN6:MSC heterospheroids are more beneficial over 2:1 heterospheroids against proinflammatory cytokines. For this purpose, for the following part of our study, 1:1 MIN6-MSC heterospheroids of 500 initial cell numbers were chosen for encapsulation in PEG hydrogels.

7.4.5. Encapsulation of MIN6-MSC Heterospheroids in PEG Hydrogels

Despite the fact that immunoprotection was achieved through trophic factor secretion by MSCs, a physical barrier around transplanted beta cells aid effectiveness of engraftment for clinical setting by further filtering cells and proteins communicating with beta cells. For this purpose, MIN6-MSC heterospheroids were encapsulated in PEG hydrogels and viability was investigated within these hydrogels. In some conditions, PEGylated GLP-1 was incorporated due to reported positive effect of GLP-1 on beta cells, as we and others have previously showed that GLP-1 can improve islet function and intracellular ATP levels in PEG hydrogels [98, 274, 336, 357]. Superiority of GLP-1 over IKVAV and RGDS was also previously shown [336], so we have used GLP-1 for functionalization of PEG hydrogels. Fluorescence images of calcein AM/ethidium homodimer stained heterospheroids and control pseudoislets are represented in Figure 7. 11. As observed from optical images, spheroids were intact for all conditions tested.

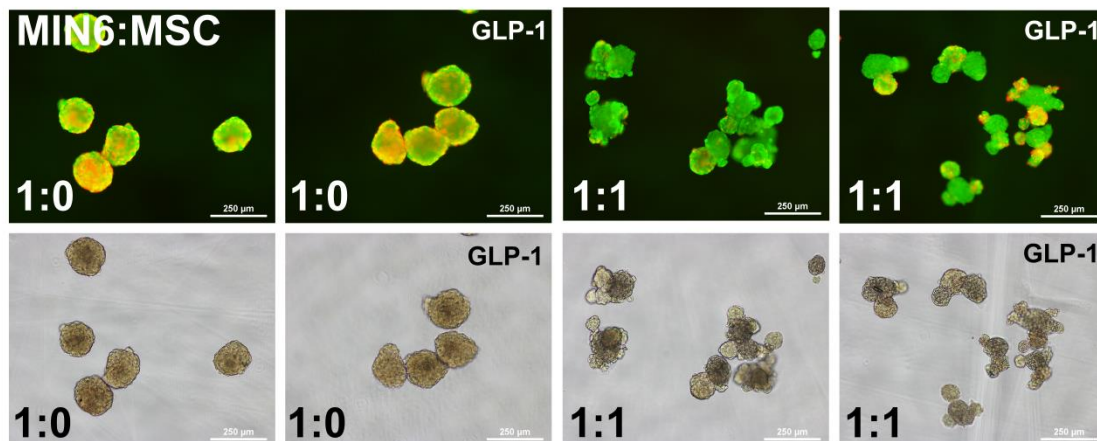


Figure 7. 11: Live/dead staining images of encapsulated spheroids. Top images are fluorescence images taken after staining. Bottom images are optical images of fluorescent spheroids. Scale bars: 250 μm .

From these images, calculated viabilities were 50% and 53% for encapsulated MIN6 in unmodified and GLP-1 modified PEG hydrogels (Figure 7. 12A) with a slight increase in viability in GLP-1 treated MIN6 pseudoislet group. However, % viability of MIN6 pseudoislets was lower compared to uncoated groups (50% vs. 73% and 78%). In uncoated groups more than 20% of the cells were dead and encapsulation in PEG hydrogels may have limited transfer of nutrients contributing in increased cell death. In heterospheroids (1:1), 94% and 85% viability were obtained for the same conditions. In our previous study, it was also reported that ISLET+MSC group had similar viability in PEG hydrogels compared to ISLET+MSC+GLP. Insignificant increase in ISLET+GLP group was obtained compared to ISLET group [336]. In uncoated heterospheroids, viabilities are above 90% (Figure 7. 7A and Figure 7. 10). GLP-1 incorporated heterospheroids had higher viability compared to MIN6 pseudoislets. These results suggest that heterospheroids are viable in PEG hydrogels independent from peptide functionalization.

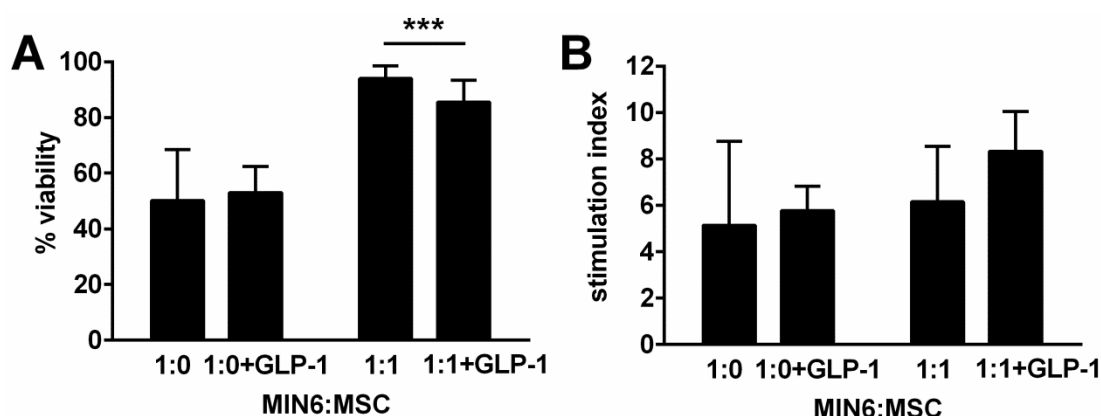


Figure 7. 12: Cellular activity in PEG hydrogels. A) Percent viabilities of encapsulated spheroids. Mean±SD. n=9-21. ***p<0.001. B) Stimulation index of MIN6 pseudoislets and MIN6-MSC heterospheroids in PEG hydrogels. Mean±SD. n=2-3.

Encapsulated MIN6 pseudoislets were stimulated under glucose challenge (Figure 7. 12B) and glucose responsiveness was sustained in PEG hydrogels. When GLP-1 functionalized hydrogels have been used for encapsulation of MIN6 pseudoislets, stimulation index was improved as expected (5.1 vs. 5.8) probably through cAMP-mediated PKA and EPAC1/2 pathway [259]. In heterospheroids, stimulation index was 6.1 being higher than MIN6 pseudoislets, showing that in encapsulated heterospheroids, MSCs can trigger increase insulin secretion. Ability of MSCs to improve insulin secretion was also previously reported in silk hydrogels where stimulation index increased from 1.4 to 2.3 in the presence of MSCs [149]. Kerby et al. improved islet function in alginate hydrogels with MSCs up to one fold increase and showed MSC concentration dependent effect on islet function [150]. We also have shown that co-encapsulation of pancreatic islets and MSCs increased stimulation index 2-fold [336]. Further improvement up to 8.3 in insulin secretion of heterospheroids was evident with incorporation of GLP-1. In our previous study, we have observed that viability of ISLET+MSC were similar with ISLET+MSC+GLP in PEG hydrogels as in our present study and 75% increase in stimulation index was observed upon GLP-1 incorporation [336]. In this study, we have observed 35% increase which still shows the contribution of GLP-1 to insulin secretion in the presence of MSCs.

7.4.6. Investigation of Immunoprotection by MSCs in PEG Hydrogels

During development of T1D, proinflammatory cytokines of IL1- β , TNF- α and IFN- γ have a central role to mediate immune system based destruction of beta cells. These

factors are secreted by lymphocytes and monocytes starting local inflammation. Deleterious effects of these cytokines on beta cell function and survival were established in literature [358-360] and these factors are secreted as a part of immune response against implanted biomaterials [74]. The apoptotic effect of cytokines is initiated by interaction with membrane-associated receptors and in intracellular pathway, these cytokines downregulate antiapoptotic Bcl-2 and Bcl-xL expression while promoting proapoptotic protein Bax. NF- κ B and STAT1 activation lead to increased expression of apoptotic proteins. p53 pathway is activated and resultant ROS production damages cells. Further, well-known effector protein, caspase-3 is activated [358, 359, 361, 362].

To mimic this stress environment, we treated encapsulated spheroids with cytokines for 24 h and checked the caspase-3 activity of spheroids. To test the anti-apoptotic properties of MSCs in PEG hydrogels, cytokine treatment was performed similar to uncoated heterospheroids and caspase activity was analyzed. As in Figure 7. 13, apoptotic index was increased in MIN6 pseudoislets treated with cytokines compared to untreated controls and 70% increase in apoptotic activity was noted showing although there is a physical barrier around the cell, cytokine freely pass through this barrier and can induce apoptosis. In MIN6 pseudoislets encapsulated in GLP-1 functionalized PEG hydrogels, cytokine attack resulted in 34% increase in apoptotic activity compared to untreated counterpart. This result indicates that GLP-1 can be useful to prevent apoptosis in MIN6 pseudoislets after cytokine treatment even though all the destructive effect was not inhibited. It has been shown GLP-1 prevents caspase activation through PKC and activation of ERK1/2 leads to phosphorylation of a pro-apoptotic protein, Bad and inactivates it [273]. Increase in activation of Bcl-2 and Bcl-xL, anti-apoptotic proteins, further contributes to survival of the cells [274, 275, 363].

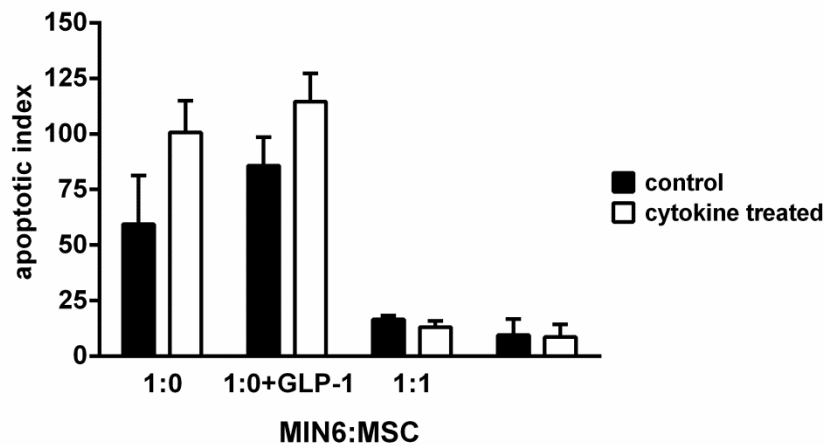


Figure 7. 13: Apoptotic activity of MIN6 pseudoislets and MIN6-MSC heterospheroids in PEG hydrogels after treatment with a cocktail of IL1- β (50 U/ml), TNF- α (1000 U/ml) and IFN- γ (1000 U/ml). Mean $\pm$ SD. n=2-3.

In MIN6-MSC heterospheroids, either in unmodified or GLP modified hydrogels, apoptotic activity did not significantly altered before or after proinflammatory cytokine treatment and caspase activity was recorded as lower than MIN6 pseudoislets. These results indicate that MSCs are far more effective than GLP-1 to prevent cytokine induced apoptosis and PEG hydrogels can be used to prevent physical contact between encapsulated cells and immune system cells. This approach offers a way to prevent apoptosis with combinatorial use of a physical barrier, an insulintropic peptide and cells having capacity to secrete anti-apoptotic proteins and insulin secretion stimulating proteins.

7.5. Conclusion

In summary, incorporation of MSCs into MIN6 pseudoislets has been shown to be a powerful tool for future therapy of diabetic patients. Although cells have been segregated in heterospheroids, viability of cells in spheroids was improved and glucose responsiveness of beta cells was enhanced in both initial cell numbers of 200 and 500. Protection against cytokine induced stress in the presence of MSCs was presented. Analysis of MIN6-MSCs heterospheroids potency was also confirmed in PEG hydrogels barriers. Decreased amount of caspase activity before and after cytokine treatment was clearly obtained and improved insulin secretion was provided. Such improvements were also supported by covalent incorporation of GLP-1. Incorporation of MSCs, a supportive cell with both immune-regulatory and insulin secretion enhancement function, and GLP-

1 benefit to expand a controlled microenvironment for transplanted beta cell mass in treatment of T1D. Ability of MSCs to support vascularization may be used to profit vascularization of transplanted beta cell for future diabetes treatment.



Chapter 8

Development of MIN6-HUVEC Heterospheroids for Beta Cell Vascularization

8.1. Abstract

Organs are vascularized in body and this network carries essential molecules for proper functioning of whole body. In addition, deposited ECM by vascular network cells supports the native environment of surrounding cells. In pancreatic islet transplantations, vascular network within and around islets are destroyed and is needed to be re-developed. One of the promising approaches is formation of co-aggregates which can be used as models for clinical vascularization. Here, MIN6-HUVEC heterospheroids were developed with hanging drop and characterized *in vitro*. Distribution of MIN6 beta cells and HUVECs were analyzed and tendency of HUVECs to coat MIN6 beta cells was observed. Further, total spheroid viability and stimulation index for insulin secretion was improved in heterospheroids.

8.2. Introduction

In their natural environment, beta cells reside in a dense vascular network which allows transfer of insulin to target tissues and nutrients, wastes, O₂. Although islets are 1-2% of total pancreatic mass, they have capillary density 10 times higher than surrounding exocrine tissue. However, after isolation of islets, this network is destroyed and is required to be re-established. Until it is re-formed, hypoxic environment contributes to failure of >50% of islet engraftment [154, 364].

In literature, there have been reports on either *in vivo* or *in vitro* vascularization of islets using cells, biomaterials, growth factors or combination. In cell based strategies, co-transplantation or coating of islets with MSCs and ECs is proposed. For example, co-

transplantation of MSCs and islets to the renal capsule of NOD-SCID Lewis rats yielded high number of capillaries and showed vWF⁺ and VEGF⁺ MSC (differentiation into endothelial cell lineage) compared to poorly vascularized islets. This strategy not only might be considered for vascularization, but also to decrease the number of islets required to reach normoglycemia [28]. Male Lewis rats having islet infusion with endothelial progenitors cells through portal vein showed improved blood glucose and similar endothelial layer thickness [365]. Johansson and her colleagues also coated islets with EC-MSC in fibrin gels and, sprout formation and interaction of ECs with intra-islet ECs were observed [15].

Intraportal transplantation of islets may trigger instant blood mediated inflammatory reaction and negatively affects islet engraftment. To prevent this, EC coating of islets were suggested, as ECs face flowing blood and are promising to form a blood-compatible surface around islets. EC coverage of human islets was shown to induce decreased coagulation and complement cascade activation [366].

Alternatively, expression of angiogenic factors might be useful. For example, when VEGF cDNA transduced mouse islets were transplanted to athymic NIH nude mice, increase in the level of CD31⁺ islets with improved glycemic control was achieved [227]. In prevascularized tissues, for instance, stainless steel cylindrical mesh with a plunger can be implanted subcutaneously. Pileggi and his colleagues proved that after 40 days, implants were vascularized. At the time of islet transfer, plunger was removed and islets were transplanted into these regions. During next ~3 months, blood glucose was successfully controlled, animals gained weight and vascularization was enhanced [228]. In biomaterials, VEGF and RGDS functionalized PEG degradable hydrogels were also effective for re-establishment of vascular network of pancreatic islets with transplantation to small bowel mesentery. This strategy had significant effect on islet functionality, weight and engraftment potential compared to islet delivery to hepatic vein [208]. Also, these hydrogels were shown to be superior over alginate gels [367] and epididymal fat pad was suggested to be a better transplant site compared to subcutaneous area [231]. Further, pancreatic islets coated with HBPA-GF (VEGF and FGF) in peptide amphiphile based gel show effective vascularization and number of sprouts emanating from islets was significantly higher than GF untreated groups [229]. Combination approaches of

biomaterials and cells are also reported. For example, pancreatic islets encapsulated in collagen gels were seeded with EC cells and in omental implantation model, host vessels were anastomosed with vessels around islets. This system was more successful than naked islet transplantation, but 2/5 animals remained normoglycemic for 2 months [232].

Although not strictly required for *in vivo*, ECs are needed for *in vitro* analysis of islet vascularization. Coating of islets with ECs is further can be promising as described above. For this purpose, HUVECs and MIN6 beta cells were co-cultured in hanging drops (Figure 8. 1). It was observed that HUVEC try to form a coat around beta cells spontaneously and such coating improved viability of heterospheroids and stimulation index of beta cells. This model for the future use is promising for *in vitro* evaluation of beta cell vascularization where sprouting can be investigated in detail.

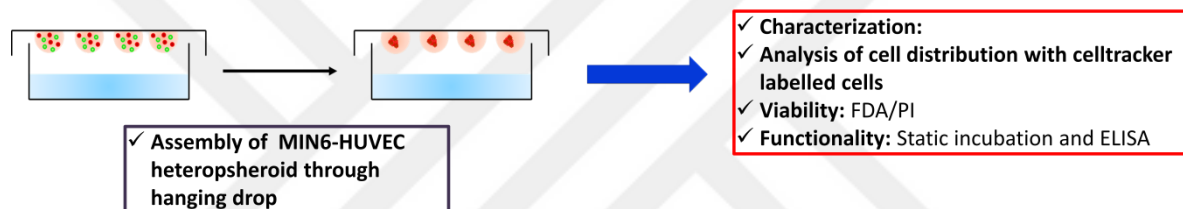


Figure 8. 1: Characterization of MIN6-HUVEC heterospheroids.

4.3. Materials and Methods

MIN6 Beta Cell Culture: MIN6 beta cells were a kind gift of Dr. Donald F. Steiner from University of Chicago, Biochemistry and Molecular Biology. MIN6 beta cells (p28-34) were cultured in DMEM (Gibco) supplemented with 10% FBS (Gibco), 100 IU/ml penicillin-0.1 mg/ml streptomycin (Sigma), 2 mM L-glutamine (Sigma), 1 mM sodium pyruvate (Gibco) and 0.10 μ M β -mercaptoethanol (Gibco) under standard culture conditions.

HUVEC Culture: HUVECs were a kind gift of Prof. Menemşe Gümüşderelioğlu from Hacettepe University. These cells (p10-14) were cultured in RPMI-1640 (Gibco) supplemented with 10% FBS (Gibco) and 100 IU/ml penicillin-0.1 mg/ml streptomycin (Sigma) at 37°C, 5% CO₂ environment.

Labelling of MIN6 Beta Cells and HUVECs: MIN6 beta cells and HUVECs were labeled with CellTracker Green and Red (Life Technologies), respectively to observe distribution of cells within heterospheroids. Five μ m dye solution was added and cells were incubated for 1 h. Following this treatment, cells were rinsed with 1X sterile PBS twice. Labelling

was confirmed with fluorescence microscope (Nikon Eclipse).

Heterospheroid Assembly with MIN6 Beta Cells and HUVECs: MIN6 beta cells and HUVECs were suspended in DMEM with supplements. Following suspension, MIN6:HUVEC ratios of 1:1, 2:1, 5:1 and 10:1 were analyzed and ratios of 1:0 and 0:1 were used as control groups. Ratio of 1:0 (MIN6 only spheroids) is referred as pseudoislet. For each ratio, total cell number in each 30 μ l droplets was kept constant as 200-5000. Following hanging drop formation on petri dish lids (Corning), cells were culture for 3 days under standard incubation conditions (Figure 8. 2).

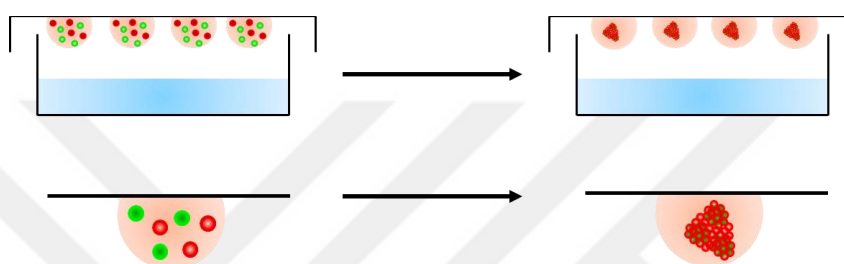


Figure 8. 2: Heterospheroid assembly in hanging drops. MIN6 beta cells and HUVECs are suspended medium drops and culture for 3 days.

Viability Analysis of Heterospheroids: Viability of heterospheroids and control spheroids was investigated with FDA/PI staining. Newly formed heterospheroids or control groups were incubated in dye solution of 42.4 ng/ml FDA and 8.5 μ g/ml PI for 5 min. Images of heterospheroids were taken with fluorescence microscope (Nikon Eclipse). Green areas and red areas were calculated with Image J and green area/total area was used to calculate % viability.

Insulin Secretion from MIN6 Beta Cells in Heterospheroids: Heterospheroids and control pseudoislets were incubated in KRB buffer containing 2.8 mM glucose (low) and 28 mM glucose (high) sequentially and insulin secreted in each buffer was analyzed with mouse insulin ELISA (Mercodia). Stimulation index was used to represent insulin secretion of MIN6 beta cells under glucose challenge and it is calculated by dividing insulin secreted at high to insulin secreted at low glucose buffer.

Statistical Analysis: Multiple comparisons were performed with Kruskal-Wallis test with Dunn's test and One-way ANOVA with Tukey's post hoc test. p values less than 0.05 were considered as statistically significant.

8.4. Results and Discussion

8.4.1. Assembly of MIN6-HUVEC Heterospheroids

Distribution of cells within each spheroid was analyzed visually by using CellTracker dyes as labels. An example is shown in Figure 8. 3 where MIN6 beta cells were labeled with CellTracker Green (Figure 8. 3A) and HUVECs were labelled with CellTracker Red (Figure 8. 3B).

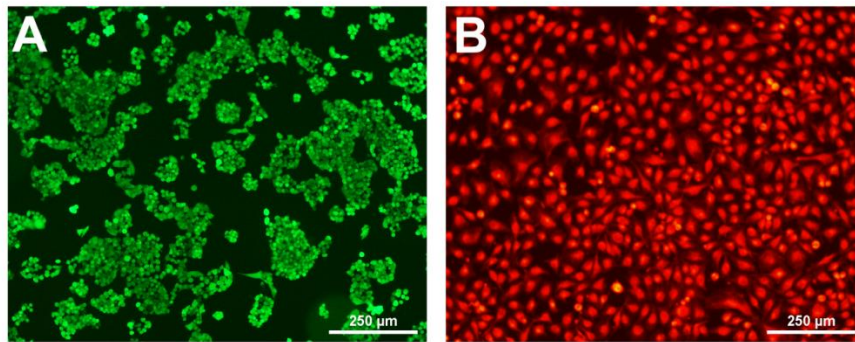


Figure 8. 3: Labelling of cells with CellTracker dyes. A) MIN6 beta cells, B) HUVECs. Scale bars: 250 μm .

CellTracker labeled MIN6 beta cells and HUVECs were suspended in 30 μl droplets with different cell ratios and initial cell number. Initial cell numbers were 200-5000 and ratios of 1:0, 0:1, 1:1, 2:1, 5:1 and 10:1 were investigated (Figure 8. 4-Figure 8. 8). For each initial cell number, MIN6 beta cells and HUVECs only formed spheroids in 1:0 and 0:1 ratios. In different MIN6:HUVEC ratios, HUVEC tendency to attach MIN6 pseudoislets was clearly observed. Increase of HUVEC ratio showed higher number of HUVECs attaching to MIN6 pseudoislets. This type of cell behavior can be explained with Differential Adhesion Hypothesis (DAH) and Differential Interfacial Tension Hypothesis (DITH). DAH suggests that cells of higher surface tension are surrounded by the ones having lower surface tension [350, 351]. This explains why MIN6 beta cells occupy the core and HUVECs tend to coat MIN6 as a shell. According to DITH, if $\gamma_{\text{cell1-cell2}} < \gamma_{\text{cell1-medium}}$ and $\gamma_{\text{cell1-cell2}} < \gamma_{\text{cell1-medium}} - \gamma_{\text{cell2-medium}}$, total engulfment of cell1 by cell2 will occur. If there is no total engulfment or separation, partial engulfment may occur [352, 353]. In our case, cell1 and cell2 correspond to MIN6 and HUVECs. Engulfment of MIN6 beta cells by HUVECs was more evident in high cell number due to incorporation of higher number of HUVEC into droplets.

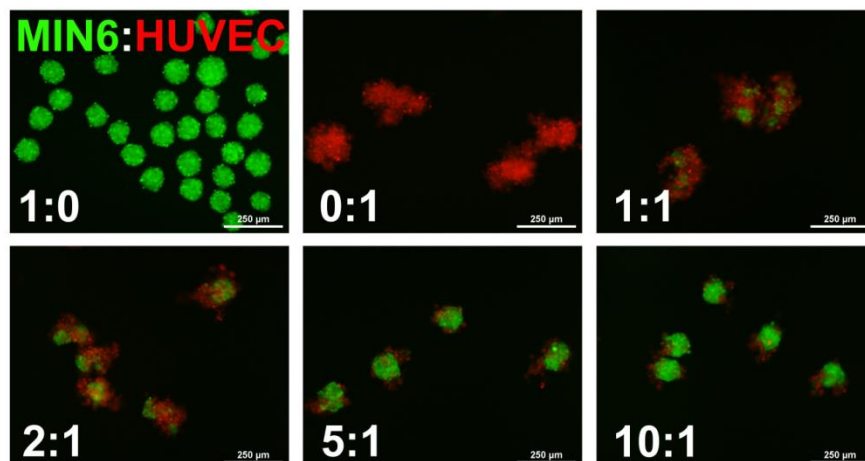


Figure 8. 4: Fluorescence images of MIN6-HUVEC heterospheroids. MIN6 beta cells and HUVECs were stained with CellTracker dyes. Heterospheroids with different MIN6:HUVEC ratios were formed with hanging drops and images were taken at day 3. Initial cell number in each droplet is 200 and droplet volume is 30 µl. Red: MIN6 beta cells. Green: HUVECs. Scale bars: 250 µm.

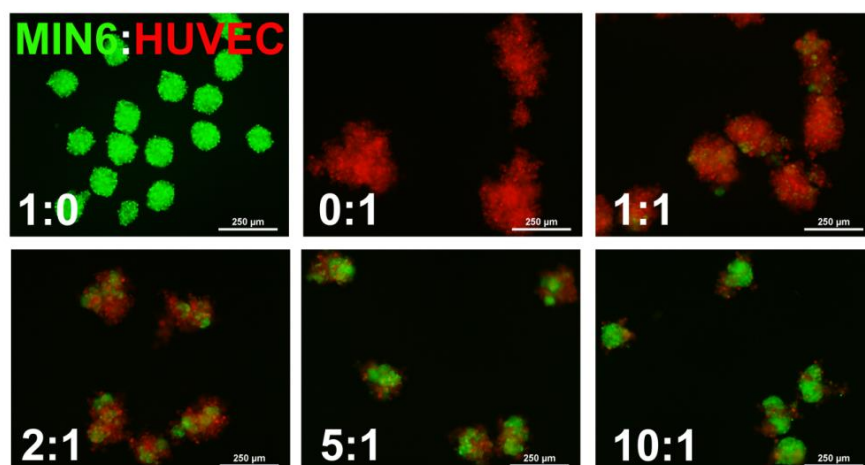


Figure 8. 5: Fluorescence images of MIN6-HUVEC heterospheroids. MIN6 beta cells and HUVECs were stained with CellTracker dyes. Heterospheroids with different MIN6:HUVEC ratios were formed with hanging drops and images were taken at day 3. Initial cell number in each droplet is 500 and droplet volume is 30 µl. Red: MIN6 beta cells. Green: HUVECs. Scale bars: 250 µm.

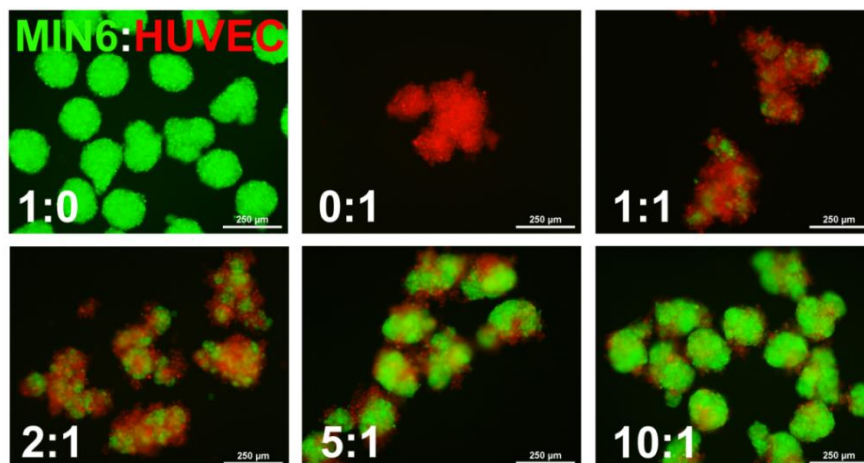


Figure 8. 6: Fluorescence images of MIN6-HUVEC heterospheroids. MIN6 beta cells and HUVECs were stained with CellTracker dyes. Heterospheroids with different MIN6:HUVEC ratios were formed with hanging drops and images were taken at day 3. Initial cell number in each droplet is 2500 and droplet volume is 30 μ l. Red: MIN6 beta cells. Green: HUVECs. Scale bars: 250 μ m.

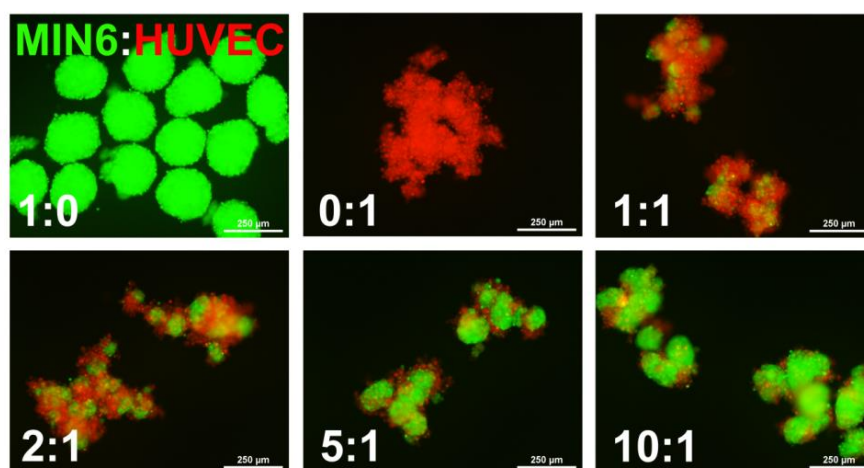


Figure 8. 7: Fluorescence images of MIN6-HUVEC heterospheroids. MIN6 beta cells and HUVECs were stained with CellTracker dyes. Heterospheroids with different MIN6:HUVEC ratios were formed with hanging drops and images were taken at day 3. Initial cell number in each droplet is 2000 and droplet volume is 30 μ l. Red: MIN6 beta cells. Green: HUVECs. Scale bars: 250 μ m.

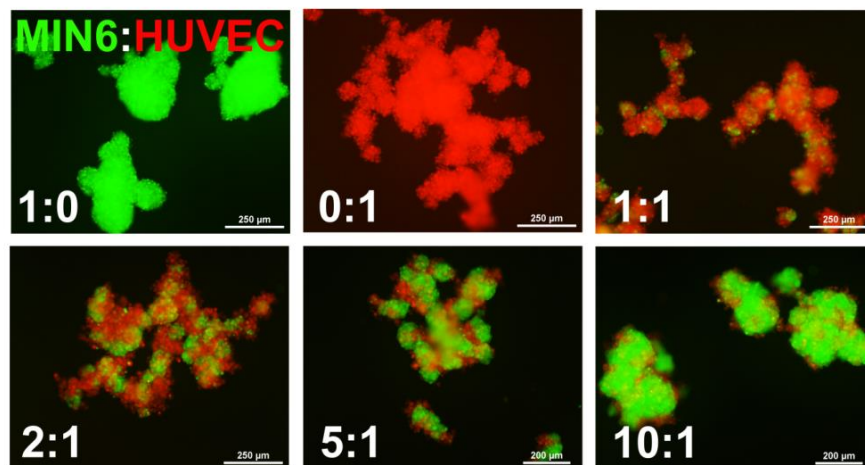


Figure 8. 8: Fluorescence images of MIN6-HUVEC heterospheroids. MIN6 beta cells and HUVECs were stained with CellTracker dyes. Heterospheroids with different MIN6:HUVEC ratios were formed with hanging drops and images were taken at day 3. Initial cell number in each droplet is 5000 and droplet volume is 30 μ l. Red: MIN6 beta cells. Green: HUVECs. Scale bars: 250 μ m.

Spheroids in Figure 8. 6 are summarized with higher magnification in Figure 8. 9. MIN6 beta cells (1:0) and HUVECs (0:1) formed clusters by themselves. In heterospheroids, clear tendency of HUVECs to coat MIN6 beta cells was pronounced.

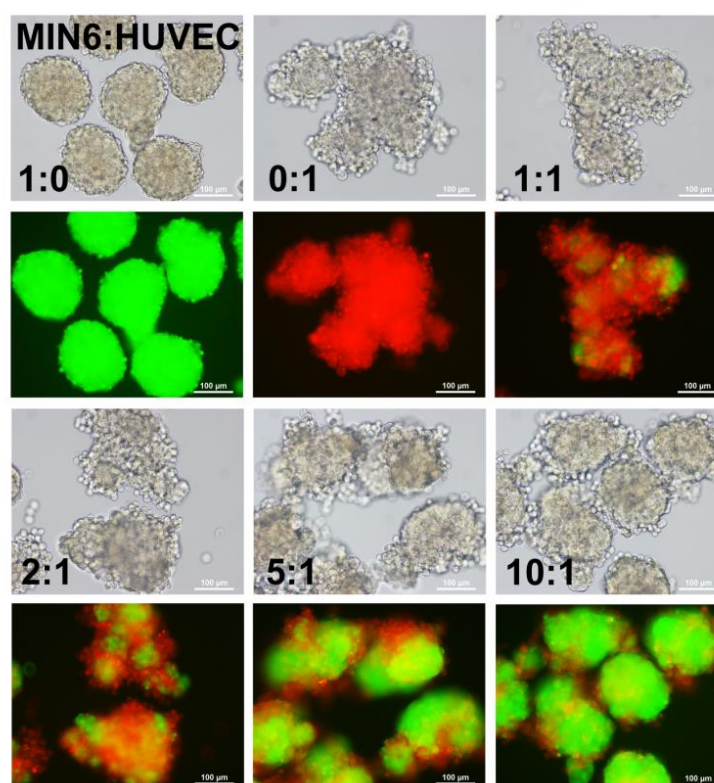


Figure 8. 9: Fluorescence images of MIN6-HUVEC heterospheroids. MIN6 beta cells and HUVECs were stained with CellTracker dyes. Heterospheroids with different MIN6:HUVEC ratios were formed with hanging drops and images were taken at day 3. Initial cell number in each droplet is 1000 and droplet volume is 30 μ l. Red: MIN6 beta cells. Green: HUVECs. Scale bars: 100 μ m.

Fluorescence images of viability of spheroids with initial number of 1000 are represented in Figure 8. 10A, calculation results of % viabilities with Image J are presented in Figure 8. 10B and viability distribution for each group are summarized in scatter plot as in Figure 8. 10C. Investigation of viability of these clusters showed that viabilities of MIN6 pseudoislets and HUVEC spheroids were 78% and 96%. In heterospheroids of 1:1 and 2:1 ratios, 95% and 94% viabilities were observed. Further increase in MIN6 ratios in heterospheroids (5:1 and 10:1) led to decrease in viability, as 92% and 90% of the cells in heterospheroids were viable.

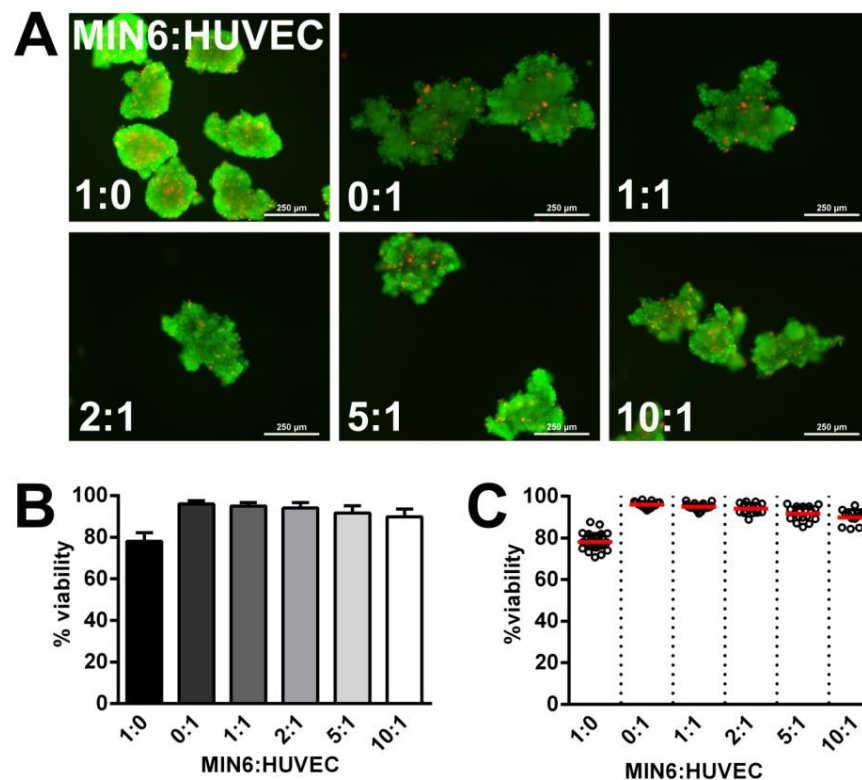


Figure 8. 10: Live/dead staining of MIN6-MSC heterospheroids of 1000 initial cells. A) Fluorescence images of heterospheroids. Scale bars: 250 μ m. B) Percent viability of heterospheroids of 200 and 500 initial cells calculated from FDA/PI staining. C) Scatter plot of %viability. Mean $\pm$ SD. n=14-26. Red lines represent mean. For 1:0 vs. 0:1, 1:0 vs. 1:1, 1:0 vs. 2:1, ****p<0.0001. For 1:0 vs. 5:1, ***p<0.001. For 0:1 vs. 10:1, **p<0.01. For 1:0 vs. 10:1, *p<0.05.

8.4.2. Beta Cell Function in Heterospheroids

Beta cell activity in these clusters were also analyzed (Figure 8. 11). MIN6 pseudoislets had stimulation index of 1.13 and insulin secretion index was increased to 2 in 1:1 ratio heterospheroids. It was around 2.2 and 2.12 for 2:1 and 5:1 ratios. Slight decrease was obtained in 10:1, as stimulation index was 1.88, but still higher than MIN6 pseudoislets.

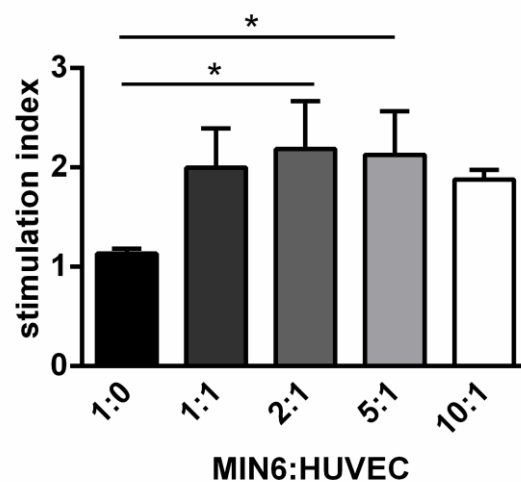


Figure 8. 11: Beta cell function in MIN6 pseudoislets and MIN6-HUVEC heterospheroids. Mean±SD. n=2-3.*p<0.05.

In heterospheroids, not only functional but also viability improvement was achieved. Enhancement in insulin secretion index was result of partly improved viability and partly HUVEC deposited ECM. Previously, MIN6 beta cell-MAEC and D11 beta cell-HUVEC spheroids were reported to show improved insulin secretion. Deposition of ECM proteins such as collagen IV was suggested to be underlying reason [183, 184]. INS-1 beta cells separated by either 100 or 200 μ m fibrin gel from HUVECs on RGD coated surface had enhanced insulin secretion after decreasing distance between cells or use of HUVECs. It was proposed that ability of ECs to secrete basement membrane proteins probably the contributor for increase in function [185]. These results suggest that, heterospheroids developed here can be tested as models for vascularization research and are useful to improve stimulation of beta cells in response to glucose.

8.5. Conclusion

MIN6-HUVEC heterospheroids were formed with hanging drop technique and were shown to be promising for future use in vascularization. Tendency of HUVEC coverage of MIN6 beta cells was reported. The results presented here showed improved insulin secretion and viability. Heterospheroids developed here constitute a part of an *in vitro* vascularization model for beta cells.

Chapter 9

Conclusions and Future Work

End-point solution for diabetic patients is pancreas transplantation with immunosuppressive drug therapy. Although classified as experimental, islet transplantation is an alternative for beta cell delivery. However, drawbacks of donor shortage, immunosuppressive drugs, lack of vascularization and repeated islet isolation convert this promise into a challenge for the patients. For this purpose, various coating materials from macro to nano-coating have been suggested for clinics. These materials would not only narrow the effects of immune mediated destruction but also maintain beta cell integrity. Use of 3D matrix of these materials is upgraded to accommodate ECM mimics, insulintropic peptides and support cells with alternative beta cells to take a step towards clinics.

The functionality of islets can be significantly improved within macroscopic 3D PEG hydrogels in the presence of MSCs, ECM derived cell adhesion ligands and GLP-1. Immobilized peptides acted as ECM mimic ligands to stimulate cell survival and insulin secretion pathways. GLP-1 increased insulin secretion in response to high glucose. Further addition of MSCs into the system resulted in significant improvements in functionality; most probably due to soluble factor release from MSCs. Synergistic effects of cell adhesion peptides on islet functionality were also observed. This study is promising in demonstrating the combination of fundamental parts of a tissue (cell and ECM) and insulintropic ligands for the future design of a bioartificial pancreas and for the treatment of T1D where immunosuppressive drugs are no longer necessary.

As a model beta cells line, MIN6 beta cells hold great promise. For this purpose, we described the aggregation behavior of MIN6 beta cells in hanging drops. We observed

negative correlation between initial cell number in the droplets and incubation day, as drastic decrease in viability was noticeable. Droplet volume was also a concern, as larger volume provided more support of viability. We further described a robust way to coat beta cell clusters in a nanogel thin-layer with a single-step strategy where CHPOA nanogels were applied to MIN6 pseudoislets. This approach offers use of multiple thin-layer coating with negligible effects on viability and insulin secretion of beta cells after coating. However, for sustained viability and thus function, CHP nanogels without acrylate moiety can be applied. The results in the present study are encouraging for beta cell transplantation and in the future part of this research, this coating approach can be implemented to enhance immunoisolation capacity as a physical barrier and a drug carrier.

Newly developed PEG hydrogel-CHPOA nanogel composite system is also biocompatible and can be used in islet cell research. PEG hydrogels were formed through visible-light induced polymerization by radical-based covalent bonding and CHPOA nanogels were obtained by physical crosslinking of cholesterol units on pullulan. By combining such two unique approaches, we observed that this system is applicable to not only use in islet cell research, but also has possibility to use in cellular applications. Biocompatibility of this system was observed *in vivo*. In the future, these newly established platforms can be used to deliver drugs or proteins to regulate immune system during transplantation.

Also, MIN6 pseudoislets can be delivered to test glucose regulation capacity. For this, MIN6 pseudoislets were encapsulated in PEG hydrogels and transplanted into diabetic mouse model. However, blood glucose levels were not decreased to levels that healthy animals have. In order to reach normoglycemia, cell in transplanted pseudoislet and site of transplantation should be reconsidered for the future studies. Overall, it can be concluded that implanted cell/pseudoislet number, lack of sufficient vasculature and implantation region affect the outcome of our encapsulated beta cell transplantation studies and highlight the requirement of optimization for the future treatment trials of T1D.

Incorporation of MSCs into MIN6 pseudoislets has been shown to be a powerful tool for future therapy of diabetic patients. Although cells have been segregated in

heterospheroids, viability of cells in spheroids was improved and glucose responsiveness of beta cells was enhanced in both initial cell numbers of 200 and 500. Protection against cytokine induced stress in the presence of MSCs was presented. Analysis of MIN6-MSCs heterospheroids potency was also confirmed in PEG hydrogels barriers. Decreased amount of caspase activity before and after cytokine treatment was clearly obtained and improved insulin secretion was provided. Such improvements were also supported by covalent incorporation of GLP-1. Incorporation of MSCs, a supportive cell with both immunoregulatory and insulin secretion enhancement function, and GLP-1, insulinotropic peptide, benefits to expand a controlled microenvironment for transplanted beta cell mass for treatment of T1D. Ability of MSC to support vascularization may be used to profit vascularization of transplanted beta cell for future part of diabetes research.

Finally, MIN6-HUVEC heterospheroids were formed with hanging drop technique and were shown to be promising for future use in vascularization. Tendency of HUVEC coverage of MIN6 beta cells was reported. The results presented here showed improved insulin secretion and viability. Heterospheroids developed here constitute a part of an *in vitro* vascularization model for beta cells.

In summary, this dissertation contributes the field of diabetes research by design and tailoring the properties of immunoisolation membranes. Here, combination of cell and materials based strategies offer a diverse area of applications for beta cell transplants. As demand for organ transplantation increases, alternative strategies presented here can be applicable for future trials. Function of immunoisolation barriers developed in this study can be practiced to transplantation of other organs. Besides, we believe that these unique approaches will be valuable tools to promote new clinical treatment methods to close the gap between demand for transplantation and transplantation practice as well as to find a cure for diabetes.

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VITA

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