Development of an in vitro Screening Platform for Research of Type 1 Diabetes Therapies

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For Bob, Gloria,

Mary & Nigel…

Abstract

Diabetes is a chronic disease wherein the afflicted lose their ability to maintain stable blood glucose levels. In both type 1 and type 2 diabetes, the peptide signaling molecule insulin is implicated. The insulin hormone is secreted into the blood by endocrine cells within the pancreas organ at times of high blood glucose. Insulin signals organs and tissue tissues throughout the body to absorb glucose from the blood to be used in energy production by metabolism into ATP and cues the synthesis of glycogen by the liver for storage. The effect of insulin is to lower heightened levels of blood glucose back towards homeostatic levels. Too much insulin can cause a crash into dangerous and even lethal hypoglycemic levels. Type 2 diabetes is known to be a metabolic disorder in which the receptors for insulin throughout the body become overworked, exhausted and damaged, leading to a loss of insulin's effect of lowering blood sugar. This form of the disease comprises roughly 90% of all cases, though has a known mechanism of onset and can be treated and even reversed by sufficient dietary and lifestyle strategies. Type 1 diabetes presents a similar end result of the body losing the capability to regulate blood glucose levels, however in this case due to a lack of insulin circulating the bloodstream. While the insulin receptors remain undamaged, they are never cued towards their function since insulin is not being produced by the endocrine pancreas in the first place. The underlying pathologies of this condition are not entirely understood, however the mechanism is an autoimmune mediated attack, specifically targeted to the insulin producing β-cells of the pancreas. Once the body's entire population of β-cells has been depleted blood glucose levels become dangerously uncontrolled, and one must administer exogenous insulin to restore glucose balance. Insulin can be self-administered through subcutaneous injection, however this requires great care and constant monitoring to avoid dangerous low blood sugar. While this has been the standard of care for many diabetics, intermittent dosing fails to appropriately balance constantly fluctuating glucose levels. More dynamic control can be attained with continuous glucose monitoring devices coupled to wearable insulin infusing pumps. These systems make use of advanced

machine learning algorithms and smartphone communication to offer the user comprehensive insight and vastly improved control over their blood glucose levels. Even so, the quantity of carbohydrates and sugars must be accurately estimated prior to a meal and entered into the system as input for the predictive release of short and long-acting insulin analogues. Close monitoring of blood levels after the meal will inform the user if adjustments are needed or if it is safe to resume physical activity.

While advances in technology and understanding have significantly improved disease management, type 1 diabetes remains a lifelong condition, severely impacting quality of life with dangerous short-term and long-term complications due to low and high blood glucose, respectively. Future developments in knowledge may reveal underlying aspects of the disease onset allowing for prediction and prevention of the condition altogether. Present strategies with the highest impact involve early and informative diagnostics, along with sustainable measures to mediate the loss of insulin production. To date, one of the most promising techniques for maintaining blood glucose control is not by supplementing insulin itself but looking further upstream to the biological source of the missing hormone. Live cell transplantation can replace β-cells lost to immune destruction, providing a continuous supply of fresh insulin to the bloodstream, with a dynamic release profile thanks to the natural glucose sensing capabilities of these same cells. Not only does isolating the β-cell containing clusters of the pancreas obviate the need for highly invasive transplants of the entire organ, but these clusters, known as the ‘islets of Langerhans’, are capable of producing other endocrine hormones that balance the glucose lowering effects of insulin in the blood. In this way, live islet transplantations offer complete control of blood glucose and resolution of the diabetic condition as long as the graft remains intact and functional. Islet implants have already displaying promising success, giving hope of freeing those who suffer from type 1 diabetes from the constant threat of glucose mismanagement, however obtaining

enough cells from matching donors and ensuring long-term protection of the graft remain major hurdles to making this therapy widely available.

In this thesis project, limitations to islet transplantation are addressed on three main fronts: (i) Development of a biosensing system for detection of insulin antibodies in a flowing liquid to improve the speed and accessibility of islet functionality characterization in-vitro, to improve islet screening during transplant and facilitate basic research, (ii) investigation of peptides as insulin stimulating agents, to increase the output in response to glucose and maximize the impact of a given population of islets, and (iii) engineering a renewable cell model to represent native human cells in drug discovery and proof of concept studies in vitro, lifting the burden of using scarce and valuable human islets at the level of fundamental research. Each of these strategies represent entire fields of specialty, the marriage of which is required to tackle such a complex issue as the treatment of diabetes, and perhaps more importantly uncovering a deeper understanding to fuel future methods improving the lives of many.

Résumé

Le diabète est une maladie chronique dans laquelle les personnes atteintes perdent leur capacité à maintenir une glycémie stable. Dans les diabètes de type 1 et de type 2, la molécule de signalisation peptidique insuline est impliquée. L'hormone insuline est sécrétée dans le sang par les cellules endocrines de l'organe du pancréas en cas d'hyperglycémie. L'insuline signale aux organes et aux tissus du corps entier d'absorber le glucose du sang pour être utilisé dans la production d'énergie par métabolisation en ATP et déclenche la synthèse de glycogène par le foie pour être stocké. L'effet de l'insuline est d'abaisser les niveaux élevés de glucose sanguin vers des niveaux homéostatiques. Trop d'insuline peut provoquer une chute vers des niveaux hypoglycémiques dangereux et même mortels. Le diabète de type 2 est connu pour être un trouble métabolique dans lequel les récepteurs d'insuline dans le corps entier sont surmenés, épuisés et endommagés, ce qui entraîne une réduction de l'effet de l'insuline sur la baisse de glycémie. Cette forme de la maladie représente environ 90 % de tous les cas, bien qu'elle ait un mécanisme d'apparition connu et puisse être traitée et même inversée par des stratégies diététiques et de mode de vie suffisantes. Le diabète de type 1 présente un résultat final similaire sur le corps. De la même façon, il perd la capacité de réguler la glycémie, mais, dans ce cas, en raison d'un manque d'insuline circulant dans le sang. Bien que les récepteurs d'insuline restent intacts, ils ne sont jamais orientés vers leur fonction puisque l'insuline n'est pas produite par le pancréas endocrinien en premier lieu. Les pathologies sous-jacentes de cette affection ne sont pas entièrement comprises, mais le mécanisme est une attaque à médiation auto-immune, spécifiquement ciblée sur les cellules β productrices d'insuline du pancréas. Une fois que toute la population de cellules β du corps a été épuisée, la glycémie devient dangereusement incontrôlée et il faut administrer de l'insuline exogène pour rétablir l'équilibre glycémique. L'insuline peut être auto-administrée par injection sous-cutanée, mais cela nécessite une grande prudence et une surveillance constante pour éviter une hypoglycémie dangereuse. Bien que cela ait été la norme de soins pour de nombreux diabétiques, le dosage intermittent ne parvient

pas à équilibrer de manière appropriée les niveaux de glucose qui fluctuent constamment. Un contrôle plus dynamique peut être obtenu avec des dispositifs de surveillance continue du glucose couplés à des pompes à perfusion d'insuline portables. Ces systèmes utilisent des algorithmes avancés d'apprentissage automatique et la communication par smartphone pour offrir à l'utilisateur un aperçu complet et un contrôle considérablement amélioré de sa glycémie. Même ainsi, les quantités de glucides et de sucres doit être estimée avec précision avant un repas et entrée dans le système comme entrée de données pour la libération prédictive d'analogues de l'insuline à action de courte et longue durée. Une surveillance étroite des taux sanguins après le repas informera l'utilisateur si des ajustements sont nécessaires ou s'il est sécuritaire de reprendre une activité physique.

Alors que les progrès de la technologie et de la compréhension ont considérablement amélioré la gestion de la maladie, le diabète de type 1 reste une maladie qui dure toute la vie, affectant gravement la qualité de vie avec des complications dangereuses à court et à long terme dues à une glycémie basse et élevée, respectivement. Les développements futurs des connaissances pourraient révéler des aspects sous-jacents de l'apparition de la maladie permettant de prédire et de prévenir la maladie dans son ensemble. Les stratégies actuelles ayant le plus grand impact impliquent des diagnostics précoces et informatifs, ainsi que des mesures durables pour atténuer la perte de production d'insuline. À ce jour, l'une des techniques les plus prometteuses pour maintenir le contrôle de la glycémie ne consiste pas à compléter l'insuline elle-même, mais à rechercher plus en amont la source biologique de l'hormone manquante. La transplantation de cellules vivantes peut remplacer les cellules β perdues à cause de la destruction immunitaire, fournissant un apport continu d'insuline fraîche à la circulation sanguine, avec un profil de libération dynamique grâce aux capacités naturelles de détection du glucose de ces mêmes cellules. L'isolement des grappes contenant des cellules β du pancréas évite non seulement le besoin de greffes hautement invasives de l'ensemble de l'organe, mais ces grappes, connues sous le nom d '«îlots de

Langerhans», sont capables de produire d'autres hormones endocrines qui équilibrent l'abaissement du glucose effets de l'insuline dans le sang. De cette manière, les transplantations d'îlots vivants offrent un contrôle complet de la glycémie et une résolution de l'état diabétique tant que la greffe reste intacte et fonctionnelle. Les implants d'îlots ont déjà affiché un succès prometteur, donnant l'espoir de libérer ceux qui souffrent de diabète de type 1 de la menace constante d'une mauvaise gestion du glucose. Cependant, obtenir suffisamment de cellules de donneurs compatibles et assurer une protection à long terme du greffon restent des obstacles majeurs à la réalisation de render cette thérapie largement disponible.

Dans ce projet de thèse, les limites de la transplantation d'îlots sont abordées sur trois fronts principaux : (i) Développement d'un système de biodétection pour la détection d'anticorps anti-insuline dans un liquide en écoulement afin d'améliorer la vitesse et l'accessibilité de la caractérisation de la fonctionnalité des îlots in vitro, afin d'améliorer le dépistage pendant la greffe et faciliter la recherche fondamentale, (ii) l'étude des peptides en tant qu'agents stimulant l'insuline, pour augmenter la production en réponse au glucose et maximiser l'impact d'une population donnée d'îlots, et (iii) concevoir un modèle de cellule renouvelable pour représenter des cellules humaines naturelles dans la découverte de médicaments et les études de preuve de concept in vitro, allégeant le fardeau de l'utilisation d'îlots humains rares et précieux au niveau de la recherche fondamentale. Chacune de ces stratégies représente des domaines de spécialité entiers, dont le mariage est nécessaire pour s'attaquer à un problème aussi complexe que le traitement du diabète, et peut-être plus important encore, découvrir une compréhension plus approfondie pour alimenter les futures méthodes améliorant la vie de nombreuses personnes.

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This project was made possible by my supervisor, Dr. Maryam Tabrizian, whose constant support and belief in the project carried my dedication throughout the ups and downs of this degree. Her fundamental understanding of the vast field of principles in biomaterials and medical translational research was a backboard of reassurance while I found my footing as an expert in this field. Moreover, her experience with establishing collaborations, presenting to and sharing advances with top leading scientists and medical professionals provided a framework to give me the chance of unfolding the true potential of this work and my own skills. I am forever grateful she saw light enough in my motivations to add me to her team of aspiring professionals. I am filled now with the purpose and resolve to fulfill my contribution to discovering means of improving quality of life for the largest demographics possible.

Without the assistance of open discussions with my fellow members of the BioMat'X lab, I could never have results of the calibre presented in this thesis. Each individual scientist coming from a unique set of experiences all displayed a level kindness making us into friends striving together through this journey. My first mentor, Rafael Castiello, was like a safety net as I plunged into the technical aspects of biosensing, introducing me to the chemical and biological techniques to address challenges within the scope of diabetes. As a comparative veteran in the task I was now facing, Rafael guided me like an older brother as I began to understand how to apply my expertise to limitations we were facing. The figure, who I perceived then, much as I do now, as a juggernaut of solid advancement to evolve my know how was Jamal Daoud. His experience with islet biology, microfluidic systems and commercial applications inspired ideas for fabrication of my own devices. Reza Rasouli offered his friendship and a calming brilliance which gave me hope to achieve great heights. As a partner working on parallel aspects of my own project, Michael Yitayew was a constant source of brightness and a wealth of knowledge in areas which complimented the gaps missing in my own. I had the privilege to meet many scientists who I came to admire through the years, Celine, Ibukun, Oscar, Mika, Agnese, Feriel, Selya, Alex, John, Nic,

Adrien, Kaushar, Ling, Nick, Antoine, Chris, Karina, Anthony, John, Marina, Antonio and others collectively played a support role instrumental to my experience. Of course, I owe many thanks to Dr. Lorenzo Piemonti and the islet biology team at San Raffaele in Milan, as well as Dr. André Charette and Léa Guerassimoff whose collaborations from outside institutions fostered a deeper validity of this work, opening doors for new directions and greater possibilities than I ever expected. Raphael Scharfmann, Masaya Oshima and Marco Gasparrini provided a gateway to the living cells that would serve as models to the drug screening and biosensing methods developed herein. Finally, my two supervisory committee members, Dr. Milica Popovic and Sara Mahshid nurtured my progression from program entry to candidacy and graduation. The insight of their perspectives led to a strengthening of this project into a packaged whole that I could be proud of.

Undoubtedly, it goes without saying, though worth illuminating broadly is the unwavering and unconditional love and support from my parents. Though I did not always feel it was deserved, their spirit and exuberance were paramount to my surviving this endeavour.

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List of Abbreviations

11-MUA	11-Mercaptoundecanoic acid
ABIF	Advanced BioImaging Facility
ACET	Alternating current electrothermal
ATP	Adenosine triphosphate
DAPI	4’, 6-diamidino-2-phenylindole
ECL	Electrochemical luminescence
EDC	N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide
ELISA	Enzyme linked immunosorbent assay
GHSA	Glycated human serum albumin
hPSC	Human pluripotent stem cell
HUVEC	Human umbilical vein endothelial cell
IBMX	3-isobutyl-1-methylxanthine
IDE	Interdigitated electrode
iPSC	Induced Pluripotent Stem cell
kDa	Kilo Dalton
LSPR	Localised surface plasmon resonance
MSC	Mesenchymal stem cell
NHS	N-Hydroxysuccinimide
PBS	Phosphate buffered saline
PDMS	Poly (dimethyl siloxane)
SPR	Surface plasmon resonance
T1D	Type 1 diabetes
T2D	Type 2 diabetes
VEGF	Vascular endothelial growth factor
VSV	Vesicular stomatitis virus

Original Contributions

Throughout the preparation of this dissertation, experimental and academic work led to the publication of novel results in the following original publications:

[1] "Interfacial capacitance immunosensing using interdigitated electrodes: the effect of insulation/immobilization chemistry", F. R. Castiello, **J. M. Porter**, P. Modares, Maryam Tabrizian. *Phys. Chem. Chem. Phys.* (2019)

[2] "Capacitive detection of insulin antibody enhanced by AC electrothermal mixing", **J. M. Porter**, F. R. Castiello, P. Modares, Maryam Tabrizian. *IEEE Xplore* (2019)

[3] "Synthesis and Screening of Novel Peptides on Human Pancreatic Islets for Type 1 Diabetes Therapies", **J. M. Porter**, L. Guerassimoff, F. R. Castiello, Maryam Tabrizian. *IEEE Xplore* (2020)

[4] "INGAP-Peptide variants as a novel therapy for type 1 diabetes: effect on human islet insulin secretion and gene expression", **J. M. Porter**, L. Guerassimoff, F. R. Castiello, Andre Charette, Maryam Tabrizian. *Pharmaceutics* (2022)

[5] "Renewable Cell Model for Type 1 Diabetes Research: EndoC-βH5/HUVEC Coculture Spheroids", **J. M. Porter**, M. Yitayew, Maryam Tabrizian. *Journal of Diabetes Research* (accepted 2023)

[6] "3D Cell Spheroid Model Characterization using Surface Plamson Resonance Imaging: Focus on Type 1 Diabetes Research", **J. M. Porter**, Maryam Tabrizian (*ready for submission*)

Furthermore, the work conducted in this thesis project has contributed to knowledge in the field beyond the aforementioned publications, which serve to share the culmination of such efforts to the research community. Following review and testing of various microfluidic strategies and fabrication techniques, gold electronic hormone sensors on glass were selected for the purpose of type 1 diabetes biomarker detection in fluids. Mathematical theory guided the testing of varying electrode sizes and spacing for

effect, the result confirming smaller geometries performed best. Microfluidic channels are often patterned atop the sensing array for fluid sample interfacing using PDMS gels via photolithography. As the device was designed to hold living islets for future biosensing studies, cleanroom fabrication methods proved cumbersome in defining sharp channels with large heights in the range of 300 μm. In the face of this challenge, 3D printing proved a suitable alternative for custom designed fluid chambers to be coupled with the electronic sensors on glass substrates. In addition to the flexibility of chamber dimensions and material, this strategy allowed for the temporary and removable attachment of the fluid chamber to the sensing device. This streamlined research and allowed for reuse of valuable electrode arrays. With this tool in hand, electrode array sensing parameters were tested and optimized for biomarker quantification, allowing detection of the insulin antibody in < 10s. High-fidelity antibody detection is needed for measuring insulin in solution via competitive immunoassay. Employing an optical transduction system in plasmonic biosensing, coupled with dextran surface matrices for biorecognition element attachment made the direct detection of insulin molecules possible, with high accuracy and reproducibility.

Following the functionality testing of living human islets, spheroidal 'pseudoislets' made first of immortalized mouse, then human beta cells were also tested. To more accurately represent human islets, composite pseudoislets containing insulin producing beta cells as well as supporting endothelial accessory cells were also formed and tested. The results of these studies show the relative unreliability of human organ donor tissue for research, and the strength of lab-derived imitation islets for drug screening and development of in vitro functionality characterization methods. Overall, this project makes tangible contributions to multiple aspects of islet transplant research, spanning fields of biology, medicine, engineering, and physics.

Contribution of Authors

Chapter 5: Porter, J. M., Guerassimoff, L., Castiello, F. R., Charette, A. & Tabrizian, M. (2022). INGAP-Peptide as a Novel Therapy for Type 1 Diabetes: Effect on Human Islet Insulin Secretion and Gene Expression. *Pharmaceutics*, 14(9), 1833, DOI: 10.3390/pharmaceutics14091833.

- Porter, J. M. cultured human islets, performed stimulation and gene expression experiments, took confocal images, analyzed data, and wrote the manuscript text.
- Guerassimoff, L. research INGAP-P peptide chemistry and synthesized and purified custom INGAP-P sequence variants.
- Castiello, F. R. contributed to islet culture and stimulation.
- Charette, A. provided lab space and theoretical advice for peptide synthesis.
- Tabrizian, M. contributed to the experimental design, data interpretation, and manuscript editing, review, and approval.

Chapter 6: Porter, J. M., Yitayew, M. & Tabrizian, M. (2023). Renewable Human Cell Model for Type 1 Diabetes Research: EndoC-βH5/HUVEC Coculture Spheroids. Accepted for publication in the *Journal of Diabetes Research*.

- Porter J. M. researched cell biology, conceived of the experiment design, cultured cells, prepared molds and formed spheroids, carried out stimulation, gene expression and metabolic activity experiments, analyzed data and wrote the manuscript text.
- Yitayew, M. stained fixed spheroids, acquired confocal images, and contributed to spheroid formation with advice on microwells, spheroid stimulation and reviewed the manuscript text.
- Tabrizian, M. contributed to the experimental design, data interpretation, and manuscript editing, review, and approval.

Chapter 7: Porter, J. M., Castiello, F. R., Modares, P., & Tabrizian, M. (2019). Capacitive Detection of Insulin Antibody Enhanced by AC Electrothermal Mixing. *IEEE Xplore*, DOI: 10.1109/ENBENG.2019.8692504

- Porter, J. M. researched the theory and methods, designed and conducted the electrical experiments, designed the fluid chamber, analyzed data and wrote the manuscript text.
- Castiello, F. R. support the background chemistry knowledge used for sensor surface functionalization with insulin antibodies.
- Modares, P. helped with microfabrication of the interdigitated electrode arrays.
- Tabrizian, M. contributed to the experimental design, data interpretation, and manuscript editing, review, and approval.

Thesis Outline

Chapter 1 presents the general introduction of this thesis, describing islet transplantation for type 1 diabetes, it's promises and limitations. Next, the motivations of this project are described, and how this contributes to address shortcomings in the field. Chapter 2 presents the rationale of developing improved biosensing methods, the potential of peptide therapeutics and the need to establish a standardized research model. This is followed by Chapter 3, which states the overall hypothesis and objectives of the project.

Chapter 4 gives a review of the relevant subject matter and scientific literature to situate this project within the current state of the field, and build a background understanding of the medical, biological, and technical concepts involved. This chapter concludes with a focused review on the use of surface plasmon resonance biosensing and spheroid cell models for in vitro T1D research.

Chapter 5 covers a detailed exploration of molecular agents for stimulating the release of insulin from human pancreatic islets. First, a library of peptides is synthesized based on variations of the INGAP-peptide, which are then tested for their effects on human islet functionality.

Chapter 6 examines the use of incorporating endothelial cells within EndoC-βH5 insulin-producing spheroids for establishing a renewable cell model for in vitro T1D research. Cell culture and spheroid formation methods are discussed, followed by functionality testing by similar methods as used for human islets in Chapter 5.

Chapter 7 covers an investigation of the AC electrothermal effect for dynamic detection of the insulin antibody in a flowing liquid. This article represents a specialized application of capacitive biosensing for immunoassays.

Chapter 8 discusses the significance of this project's contributions to the field, followed by project limitations and future works to address those.

Chapter 1. General Introduction

Type 1 diabetes (T1D), also known as juvenile diabetes, is a life-threatening condition with no specifically known cause or cure. The disease often develops in children or adolescents as no result of their lifestyle, though can also appear in adults as so called ‘late-onset’ T1D [1]. Whether diagnosis comes early or later in life, T1D is a permanent condition that requires constant management to avoid immediately fatal episodes, as well as long term complications eventually causing death [2].

Both type 1 and type 2 diabetes result from a loss of the body’s functional use of insulin, leading to uncontrolled blood glucose levels. The peptide hormone insulin signals organs and tissues throughout the body to uptake glucose from the blood, and to either use for energy immediately (eg. muscle fibers) or store for later use (eg. as glycogen in the liver) [3]. In type 2 diabetes, insulin receptors become desensitized and thereby unable to recognize the signal to intake and utilize circulating glucose. Type 1 diabetics, on the other hand, lose the ability to produce insulin at all. While both conditions suffer a similar fate, the situation is generally more dire in type 1, due to the complete loss of any insulin, as opposed to partial desensitization in type 2. Insulin sensitivity can be regained through improved cardiovascular health and diet, whereas destroyed insulin producing cells cannot be recovered. Pancreatic beta cells, housed within the endocrine islets of Langerhans are non-proliferative and can only be restored through transplant from deceased donors, as no stem cell or otherwise gene edited alternatives have been established [4].

The prediabetic state begins to develop long before overt symptoms appear. Even though increased autoantigens cause beta cell death in presymptomatic T1D, only once irreversible damage is done and hyperglycemia requiring insulin develops do symptoms appear [5]. A diagnosis of T1D is made when fasting blood glucose higher than 126 mg/dL (7 mmol/L) is measured on 2 separate occasions, or over 200 nm/dL at a random time. Tracking a patient’s glycated hemoglobin (HbA1c) can

be used to reflect the average blood glucose over the previous months, with a treatment target of under 7%. Type 1 diabetics require lifetime administration of exogenous insulin to prevent fatal hypoglycemic episodes. Self-administered subcutaneous injections are performed multiple times a day and must be accurately timed and dosed according to food intake and metabolic activity. Take-home blood glucose monitors allow patients to check their current blood glucose several times a day by a finger-prick blood sample. Continuous glucose sensing can now be done with wearable subcutaneous monitors, which can be worn in conjunction with a subcutaneous injector, to infuse insulin by electronic control without need for a new puncture of the skin each time [6].

Implanting living islets from donors represents an avenue to directly monitoring blood glucose levels and dynamically release of insulin in response. Since Shapiro et al. presented the 'Edmonton Protocol' for islet allotransplantation to treat T1D, the procedure has demonstrated a 44% success rate 1 year post implant, and become the first FDA approved cellular therapy under the name Lantidra [7]. The main challenges involved with this procedure include the limited lifespan of human donor islets post isolation, and their susceptibility to hypoxia and immune destruction once implanted [8]. Furthermore, lifetime immune suppression via enzyme supplements is required to mediate the foreign body rejection response and maintain graft function over time. Nonetheless, islet allotransplantation offers diabetics hope for a cure, if these hurdles can be addressed. Many efforts have already been made, yet there is still work to develop more efficient and readily available implants to make islet transplantation truly useful for the roughly 150,000 children and adolescents diagnosed with T1D each year [9].

Efforts over recent years have pushed the field of T1D research to uncover deeper knowledge of the beta cell containing islets of Langerhans, how they function and which major weakness or limitations remain to be addressed. One of the most pressing, for instance, is the scarcity of human islets for transplant, or research alike, due to a shortage of donors. Stem cell and gene editing methods are under

development, yet no protocol has been widely established for differentiating progenitors or reprogrammed cells as a renewable source [10]. Increased computing power has led to miniaturized electronic sensors exploding into numerous aspects of disease research and understanding. While having the advantage of diverse functional properties and applications, microelectrode fabrication techniques and strategies for measurement present a likewise vast parameter space which is far from fully explored. As the machining of controlled fluid handling systems progresses, integration of biological assays with automated data collection provides a new generation of platforms, facilitating comprehensive understanding of islet function [11]. In combination with streamlined monitoring systems, advanced culture methods provide cell models to allow testing various conditions and strategies to improve allograft success rates [12, 13].

This thesis paper is relevant to clinicians, researchers and those afflicted by the disease of diabetes. The project aimed both to improve transplant outcomes and facilitate in vitro research of pancreatic islets. It was hypothesized that integrating existing techniques with modern advances would allow faster, cheaper, and more accessible research in the field of islet biology. Methods were developed in hormone sensing, fluid handling, drug discovery and sustainable cell models of coculture tissue constructs. 3D AutoCAD design of assay components allowed for cheap and simple printing of reusable fluid chambers. These chambers interface antibody-based biosensors with fluid solutions for protein analysis. Microelectrode arrays were shown to specifically detect insulin antibodies within a matter of seconds. For higher throughput and target multiplexing, however, optical signal transduction was deemed more suitable. In search of new and helpful therapies, a library of peptides was synthesized and screened for effect on human islets. Finally, a stable cell model was developed to facilitate in vitro cell studies, by creating 3D ‘spheroids’ comprised of insulin producing insulinoma cells, with or without

secondary accessory cells to improve spheroid structure and performance. The positive effect of a second cell type suggests a mutually beneficial signaling between the two cell types.

The hypotheses and objectives described herein address the need to improve in vitro islet sensing and searched for novel therapies using custom cell models. The thesis begins with an overall literature review to familiarize the reader with the concepts at hand and situate the project within the field. The next chapters detail the experimental findings of the project, covering the electronic detection of insulin antibodies, followed by the screening of peptide drugs for effect on human islets. The third experimental section covers the development of a sustainable synthetic alternative to human donor islets for research in vitro. Lastly, the enhancement of microcapacitors for insulin antibody detection, INGAP-P therapies for T1D and renewable coculture cell models for research are presented in a final discussion of the main achievements and findings. Future works will continue to profit from increasing technological ability to gain fundamental biological understanding, increasing quality of life for ever expanding demographics. There is an ongoing need for improved specificity and multiplexing capabilities of biosensors, and to reduce their cost for widespread use. Additionally, the advancing complexity of progenitor-derived multicellular synthetic tissues will springboard in vitro research and lead to vast improvements in clinical implants.

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Chapter 2. Motivation & Rationale

This project begins with screening the effect of synthetic peptides based on the INGAP protein on donor islets. Next, an alternate tissue model for research is developed by coculturing human EndoC-βH5 insulinoma cells with human endothelial cells in gel molds. Finally, rapid hormone sensors are developed using gold-on-glass substrates functionalized with bioactive surface layers, for the purpose of measuring human islet secretion in vitro.

2.1 Opportunity for peptide therapeutics

Research into short peptide therapies is on the rise in the 100 years since the advent of insulin replacement therapy, due to their promising advantages in customizable targeting, cheaper and faster than longer proteins to manufacture, easier to model their structure, low toxicity and immunogenicity and ability to pass through cell membranes [1]. The use of peptide drugs in clinics however is limited by their susceptibility to enzyme degradation, and therefore suffer from short circulation times and lower oral bioavailability. Insulin for example, is subcutaneously injected directly into the bloodstream to avoid rapid digestion.

Human islets for transplant suffer from a severe shortage of suitable donors, preventing the widespread applicability of the method. The need for donor-recipient matching and limited long term survival rates of the graft further exacerbate this methods usage [2, 3]. There certainly exists a desperate need to maximize both the longevity and the secretory impact of smaller or limited populations of donor islets in transplant. A naturally derived biological agent which could improve the viability and insulin secretion attributes of human islets could reduce the load of donor numbers needed, vastly opening the demographic of diabetes who would become eligible for this life changing treatment.

2.2 Towards a cell model for research

There are several difficulties in using human islets for in vitro research and improving transplant outcomes. Some of the most pressing issues include limited islet lifetime in vitro, limited number and timing of islets available for transplant, and much less for research, high donor-to-donor variability which confounds drug screening, graft hypoxia, necrosis, and difficulty re-establishing vasculature, and inflammation mediated response leading to destruction of many cells implanted. Researching and understanding to biology and behaviour of human islets is also difficult due to their complex cytoarchitecture, with cell-to-cell communication through a dense capillary enervation of the multicell micro-organ. Not only have stem cell differentiation protocols so far failed to entirely reproduce the comprehensive blood chemistry sensing and control of native islets, but there is also still no widely accepted benchmark for their secretory fingerprint (SF) [4]. In conjunction, therefore, with a need for improved and standardized biosensing there is an unfilled void of subjects for researchers to test new therapies, fluidic handling and sensing technologies. Coculturing 3D spheroids of immortalized insulin producing cells with vascular accessory cells provides a consistent and renewable substitute for human islets in vitro. Challenges remain, however, in discovering the optimal cellular components and culture strategies for the most comprehensive cell model replication of islets possible.

2.3 Need for improved biosensing

Pancreatic islets do not expand in culture as many other native cell lines, meaning transplantation procedure require cadaveric donor islets for grafts [2]. The relative rates of prediabetic rates of beta-cell replication and neogenesis have not previously been well understand, though data now suggests both may play a role in balancing beta-cell apoptosis [5, 6]. Once they are isolated and purified from the enzymatically digested organ, islet lifetime is limited to 1-3 weeks in suspension culture [7]. Only a slim window then exists for researchers and clinicians to screen islet functionality for implant and adds a time constraint to studies of their biology. Despite recent advancements in multiplex hormone analysis, there

is yet to be widespread studies involving more than 2 islet hormones, let alone agreement [4]. Label free, time resolved measurements of insulin alone remain challenging for researchers today [8]. Improved streamlining of biosensing techniques is needed to gain understanding of islet behaviour and to improve transplant outcomes.

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Chapter 3. Hypothesis and Objectives

This doctoral thesis dissertation hypothesizes that further understanding of human islet function can be gained in vitro by:

1. Investigating INGAP-P synthetic variant peptides for their effect on human islet insulin secretion and gene expression, and
2. Establishing a standardized 3D cell spheroid model for renewable and consistent in vitro research, reducing the need for animal or human islets, and
3. Advancement of the electrical detection capabilities of microfluidic biosensors related to the screening of human islet hormones in vitro

which would make strides towards improving the outcomes of islet transplantation as a cure for T1D.

The field of diabetes research involves aspects from several different disciplines, from fluid physics and molecular biology to materials sciences and clinical applications. To familiarize the reader with the scope of topics, we begin with a review of the disease, followed by the latest techniques available at the leading edge of cell analysis and culture in vitro. Finally, this chapter ends with a draft article exploring the use of SPR for live cell secretion quantification, focusing on spheroid models for type 1 diabetes.

4.1 Background

Diabetes itself is a blanket term referring to a set of underlying pathologies, resulting in the presentation of lost glucose control in the blood. With the focus on type 1 autoimmune diabetes, the next sections outline the most pertinent biological and clinical aspects, including disease presentation, outcomes, difficulties and limitations of established methods.

4.1.1 Type 1 Diabetes

Glucose is a fundamental source of energy, from which the body derives ATP by glycolysis. Excess glucose that is not readily useable is stored as fat or connected into larger branched polysaccharide molecules and stored in the liver in the form of glycogen. Insulin is a critical endocrine hormone which has the ability to regulate glucose levels in the bloodstream, in part by stimulating the synthesis of glycogen. Diabetes is a chronic condition in which the ability to utilize glucose for energy is lost, resulting from a number of underlying pathologies [1]. While type 1 diabetes stems from a loss of insulin production, type 2 is a result of losing sensitivity to insulin, until it has no effect. As type 2 diabetes is a metabolic syndrome, it can be mediated and even reversed through lifestyle changes. The loss of insulin production seen in Type 1 diabetes, however, is caused by an autoimmune destruction of pancreatic beta-

cells which is not entirely understood. According to studies 150,000 people under 20 will be diagnosed each year, more than 70% of whom are under 15 [3]. Unfortunately, there are still major gaps in global data across different countries. General symptoms presented by juvenile type 1 diabetics include loss of weight, related to polyuria and polydipsia (excessive urination and excessive thirst, respectively), and one in three may experience bouts of ketoacidosis [4]. In the absence of insulin to allow glucose metabolism, the body begins to break down fat for energy, leading to increased ketone by-products and blood acidity, becoming lethal if left untreated. Measuring the glycated hemoglobin, Hb1Ac, gives longer term average glucose levels which complement the snapshots obtained from fingerprick blood readings. A by-product of the cleavage of insulin from the proinsulin molecule, C-peptide (cleavage peptide) levels indicate the production of insulin. While measuring blood glucose, insulin and C-peptide levels can reveal the presence of the diabetic state, there are not currently efficient ways to evaluate changes in beta cell mass, a critical factor and precursor to loss of beta cell endocrine function [6].

Once in the diabetic state, prognoses may vary. Complications of type 1 diabetes may include neuropathy, retinopathy, cardiovascular disease and severe hypoglycemia [7]. Following islet transplantation, immunosuppressive drugs are administered daily, which carry their own host of complications. Immunosuppressive medications following transplant have serious side effects including hypertension, renal failure, anaemia, increased risk of certain cancers and opportunistic infections [8]. It was found, however, that certain difficulties with memory and cognition were more due to repeated hypoglycemic episodes than immunosuppression [9].

In type 2 diabetes, insulin sensitivity can be regained through exercise and diet, however treating type 1 requires replacing insulin by injection or transplant. Subcutaneous injections can be done manually at each meal or infused by a wearable pump throughout the day. Whole organ transplants of the pancreas can restore the production of insulin, though the surgery is highly invasive with significant

risk of rejection. Roughly 20% of pancreas allografts end with rejection within the first year, half of which are antibody mediated [10]. Despite research and clinical efforts, chances of rejection have only improved ~2% over the last 10 years [11]. The Edmonton protocol of extracting solely insulin producing cells from the rest of the pancreas (largely made of fatty exocrine tissue), has presented the possibility of restoring insulin production through minimally invasive one-time infusion of beta-cells in suspension [12]. Islet allotransplantation still has limited success rates due to challenges re-establishing vascular integration coupled with the instant blood mediated inflammatory response (IBMIR), destroying roughly 60% the implanted cells within the first few hours [13]. There is an urgent need to improve *in vitro* drug screening and implant outcomes in order to benefit the millions worldwide who deal with this unpreventable, life-threatening illness.

While the exact underlying causes are not entirely known, it is clear that the loss of glucose regulation in type 1 diabetes is due to a direct attack specifically on the beta-cells of the pancreas. Antigen presenting cells trigger an autoimmune response wherein the body targets its own cells. Both the innate and specific immune responses take part in attacking and killing beta-cells during the onset of T1D [14]. The autoantibodies; islet cell cytoplasmic autoantibody (ICA), insulin autoantibody (IAA), glutamic acid decarboxylase autoantibody (GADA), insulinoma associated-2 autoantibody (IA-2A), zinc transporter-8 autoantibody (ZnT8A), and tetraspanin-7 autoantibody (Tspan7A) are detectable in 90% recently diagnosed T1D cases, making them prime clinical targets for diagnosis and tracking onset progression [15-17]. In a study of late-onset T1D, a connection between islet autoantibodies GADA, IA-2A and ZnT8A led to a recommendation for regular clinical testing of islet autoantibodies in adults [18]. It is thought that T cells cause beta-cell death by a cascade of ‘assisted suicide’ events when they become activated against autoantigens. Research into beta-cell antigen vaccination aims to induce immune tolerance by balancing populations of autoactivated and regulatory T cells [19]. Within the endocrine pancreas, the insulin producing beta-cells, along with other hormone releasing cells, are housed within

the membrane of larger (~150μm) clusters called the 'Islets of Langerhans' [20]. Pancreatic beta-cells within the islet control high blood glucose by releasing insulin, which has also been shown to have immunomodulatory properties [21]. Insulin appears to reduce inflammation in immune cells, which in turn help mediate insulin levels. Immune cells may therefore be making use of insulin when dealing with infection. Counterpart to the insulin-producing beta-cells, are the glucagon-releasing alpha cells, which have the effect of raising blood glucose [22]. Islets control blood chemistry by secreting signalling molecule directly into circulation, dynamically maintaining homeostasis. To perform this task, they are highly perfused with capillary networks, to sense and adjust blood chemistry, as well as to meet their metabolic demands. As such, islets receive ~10% of the total blood flow through the pancreas, despite making up only 2% of the organ's mass [23]. Activated T-cells can sweep through an islet, dismantling only beta-cells and leaving the other types unaffected. Why this misidentification of the native beta-cells as foreign pathogens occurs is less understood. An inflammatory microenvironment in the gut, or previous exposure to viruses are among the key suspects for initiating the diabetic state, keeping in mind multiple pathways might exist leading to the same outcome. While GAD65 has been identified as a pervasive autoantigen in T1D, insulin autoantibodies are among the first signs of developing the diabetic state [24]. Immune reactions are also implicated in stress of the endoplasmic reticulum accompanied by neutrophil recruitment, associated with loss of beta-cells in mice [25].

4.1.2 Management Strategies & Quality of life

Once autoimmune destruction has wiped out the pancreatic beta-cells, the body loses control of glucose levels in the blood. Without insulin to facilitate metabolism and storage, blood glucose accumulates to toxic levels while muscles are simultaneously starved of energy. The good news for type 1 diabetics, in contrast to type 2, is that supplementing insulin exogenously can restore glucose

absorption and balance blood levels to that of a non-diabetic. Precision dosing is difficult to attain through manual administration, however, as insulin must match the glycemic contents of each meal, based only on estimation. If a diabetic injects themselves with too much insulin, they could fall into a hypoglycemic coma which can be fatal. Modern glucose sensors address this challenge by continuous measurement of the blood, allowing for high accuracy in dose determination. Wearable insulin pumps can then transfuse the required insulin to the bloodstream in an automated fashion. So-called 'closed loop' infusion systems incorporate glucose sensors to predict insulin needs, though are still outperformed by 'open-loop' delivery systems which require carbohydrate counting meal inputs [26]. Effective closed-loop systems cannot rely on blood measurements alone, due to the slower absorption of insulin than glucose. The user is still required to alert the system that a meal is coming to allow a pre-emptive insulin dose preventing extended periods of hyperglycemia. While a simple notification is much less burdensome to the user than estimating meal content, careful attention is still required to avoid dangerous blood glucose fluctuations. Modern closed-loop insulin therapy system employing advanced models and machine learning to measure glucose and predict insulin adjustments yet still struggle to match the natural capability of endocrine cells to detect glucose and dynamically respond with insulin secretion, maintaining blood homeostasis [27]. A promising solution for exogenous insulin replacement now exists through the infusion of living Islets of Langerhans, which monitor glucose and actively release insulin and other endocrine hormones autonomously, when needed. While this approach has the potential to completely cure late-stage type 1 diabetes, long term graft survival faces major challenges yet to be overcome, forming the basis of many ongoing research efforts.

4.1.3 Islet Transplantation

As opposed to entire pancreas organ transplant, which is highly invasive and requires extensive recovery, there is now an option to replace insulin indefinitely by a single infusion of beta-cell containing islets in suspension. The Edmonton protocol has been established as a successful transplant method, though many works are being done to improve the overall long-term success rates, given no suitable renewable cell source has been identified beyond isolated cadaveric islets [12].

Islet transplantation is normally done by infusion to the hepatic portal vein, located by angiographic imaging. Islets in suspension are transfused in a single dose, with the aim establishing stable vasculature within the parenchyma of the liver. While the liver is the currently accepted site for islet transplant, other locations have been studied, such as behind the eye and beneath the kidney capsule [28]. Requiring a minimum dose of 5,000 IEQ per kilogram of body mass, islet transplantation became the first cellular therapy approved by the Food and Drug Administration (FDA) in June 2023, under the name donislecel, or Lantidra [29]. In addition to the severe shortage of islet donors, the robustness of graft longevity is not guaranteed, with most patients eventually reverting to an insulin deficient state requiring treatment. The majority of cells lost are in the first hours following transplant due to innate immune response, with subsequent autoimmune destruction by T-cell attack [25]. Roughly 60% of transplant recipients maintain freedom from exogenous insulin after the first year, with less than 50% of those making it to the end of year 2, and dropping to 8% after 20 years [30, 31]. Over a 20-year cohort, insulin independence was estimated at only 8% across 255 participants, dropping from the 61% after 1 year [32]. Immunosuppressive therapy preventing graft rejection is only suitable for few select recipients, with graft rejection disqualifying many. In addition to the slim applicability, islet transplants are subject to loss of nearly 40% of intrahepatically infused donor islets [33]. Concomitant pregnancy raises insulin needs and may exacerbate stress on islets grafted into the portal vein, increasing chances of the implant failing. One case study showed the pre-emptive administration of insulin to 2 pregnant women helped alleviate metabolic load, leading to maintained graft survival and insulin independence

[34]. As researchers gain better understanding of the biology at play, there is increasing success in differentiating progenitor cell-types into functional pancreatic tissue, as well as using endothelial coculture methods or preparing the implant site for increased vascularization [35]. With such demand on viable islets for transplant, as well as researching ways of reducing immune response and improving vasculature, there is a great need to fill the missing gap. Efforts to establish such in vitro models are discussed in the following section.

4.1.4 Renewable Cell Sources for Transplant

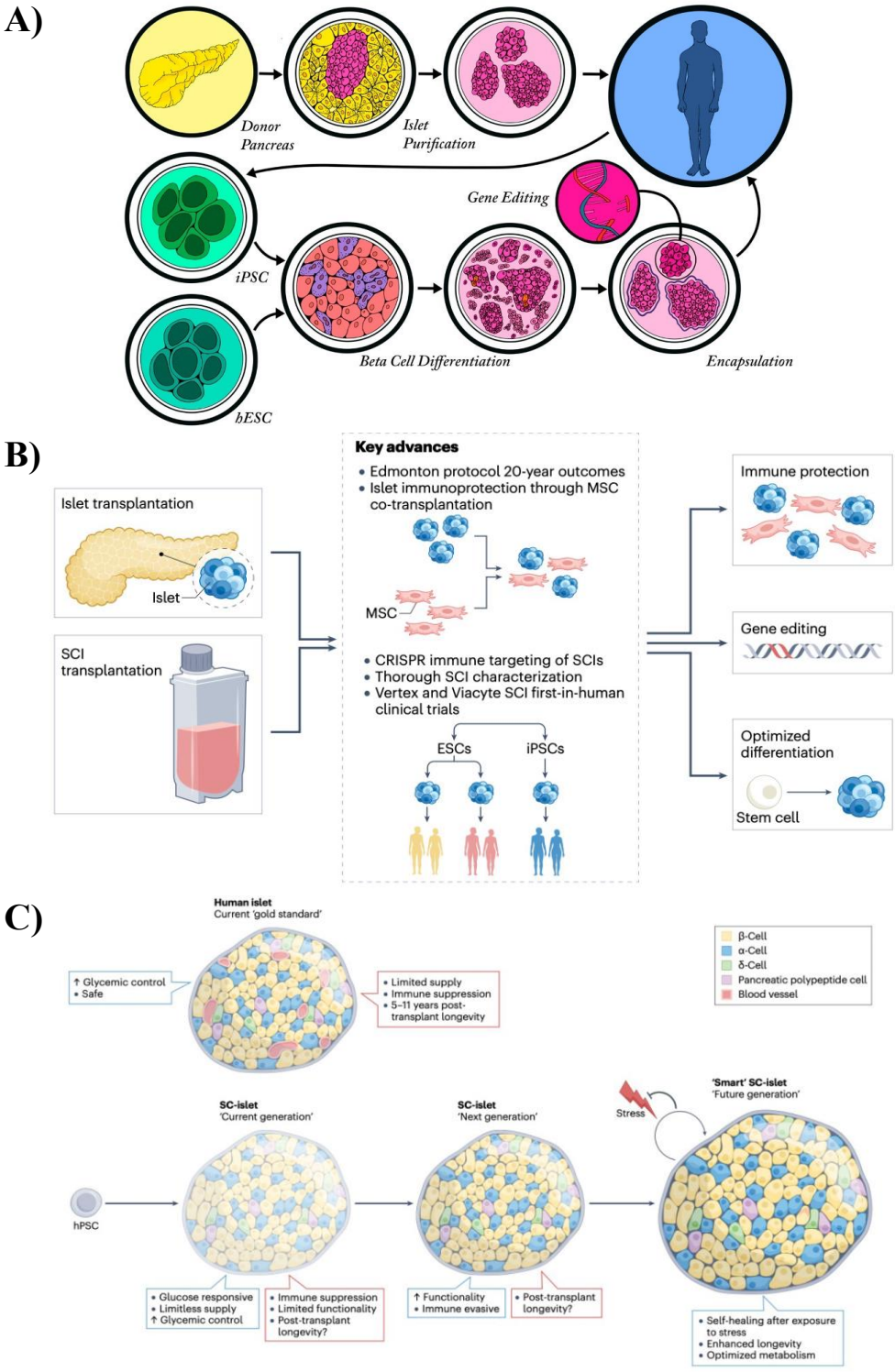

Figure 4.1: Exogenous islet sources for transplant A) Harvesting iPSCs for differentiation in beta-like cells and encapsulated for transplant (Docherty, 2021) B) Whether obtain from human donor isolation, or stem cell differentiation, coculture with MSCs can provide3 immunoprotection to islet implants (Shapiro, 2023) C) Fully functional, multicellular pseudoislets differentiated from human pluripotent stem cells (Cochrane, 2023)

As a means of reducing cost and removing time constraints, a renewable cell source for experimentation is highly sought after. Although a proliferative cell line would offer vastly improved stability in function batch-to-batch, a suitable model has yet to be established which sufficiently replicates the form and behaviour of native islets. Exogenous insulin sources could be islets retrieved from a cadaveric donor pancreas via the Edmonton protocol, differentiated from pancreatic progenitor cells and even modified for implantation (figure 4.1) [12, 31, 36, 37]. As medications have thus far only been able to mitigate the symptoms, but not address the actual loss of beta-cells, researchers are continually exploring renewable sources for cell replacements therapies. Stem cells differentiated to give insulin responses to glucose may be cultured with capillary forming endothelial cells, to achieve a vascularized implant resistant to hypoxia [38]. Native islets are perfused by a network of capillaries which control blood flow through islet and allow them to send and receive hormones and nutrients through the bloodstream, as well as communicate through paracrine interactions within the pancreas [39]. As the effective differentiation of progenitors into suitable beta-cell replacements is labour intensive and not yet fully understood, other options have been explored to enhance the impact of isolated human islets. For example, cadaveric islets have been encapsulated in alginate containing relevant ECM components, which improved their insulin response to glucose [40]. Proteins found in the intra-islet space known as the basement membrane can also be used to avoid severe rejection reactions. ECM proteins, known as integrins, such as collagen IV and VI were shown to have a protective effect on implanted islets, increasing their survival in the face of foreign body inflammatory insults [41]. While the limited lifetime of human pancreatic islets in vitro remains a challenge for research and transplantation, biomimetic, ECM simulating peptides have been used in culture to sustain islet functionality up to 4 weeks [42]. A microencapsulation platform was used in a study of murine islets, showing that porcine ECM significantly improved functionality, maintaining insulin responses over time, even reducing the toxic effects of inflammatory cytokines [43]. So-called ‘pseudoislets’ are clusters or spheroids comprised of

cell types chosen to mimic the behaviour of native islets. These can include differentiated pancreatic progenitor stem cells, immortalized insulinoma beta cells, along with endothelial, fibroblast, or non-beta other accessory cells. Some results, perhaps surprisingly, show that reaggregated islet clusters of previously dissociated human islets were superior to native islets as a subject for drug discovery research [44]. By breaking islets apart their constituents can be sorted by cell type, characterized for functionality, and reassembled accompanied by angiogenesis promoting accessory cells. Transdifferentiation of other cell types into beta cells presents one avenue of restoring beta cell populations for T1D treatment [45]. Even so, this does not help recreate beta cell hubs which act as communication centers, based on their position with respect to other cells within an islet. Differentiation protocols however can be difficult, time consuming, and are yet to be fully understood.

As stem cells present a practically unlimited supply, they can address one of the major challenges to the availability of this treatment. While perfused vasculature and low immunogenicity remain just out of reach, spheroidal cell aggregates could eventually outperform donor islets in many respects. Pluripotent mesenchymal stem cells, for instance, elicit only a small immune response, and replicate readily [46]. Mesenchymal stem cell homing, a cell migration process crucial to wound healing, still has limited efficacy in transplant survival. Research is ongoing, however, to explore enhancement through gene and receptor modifications [47]. Nonetheless, MSC still function as accessory cells beneficial to islets by promoting functionality, viability, improving angiogenesis and reducing immunoreactivity [14]. Induced pluripotent stem cells (iPSCs) present a promising alternative to generating beta-cell like insulin producing cells, as undifferentiated cell populations can be reprogrammed from somatic cells [48]. Gene editing via clustered regularly interspaced repeats and associated protein 9 (CRISPR-Cas9), however, gives researchers the power to develop fully functional beta-cell replacements in time. As knowledge of islet cells deepens, the immensity of the challenge of fully replicating native hormone production becomes more clear. Recently, four different subtypes of pancreatic beta-cells have been identified,

including bi-hormonal cells expressing insulin as well as proteins from the other islet cells in either somatostatin ($\beta/\delta-$like), ghrelin ($\beta/\gamma-$like), glucagon ($\beta/\alpha-$like) or pancreatic polypeptide ($\beta/\epsilon-$like) [49]. Several groups have obtained pancreatic progenitor cells from hPSCs that express PDX1 and NKX6.1, which are both critical to the hormone response to glucose as seen in beta-cells [50]. The use of hSPCs is severely limited, however, by inconsistent differentiation characteristics among different cell lines. Embryonic stem cells (ESCs), for instance, offer a limitless supply of source cells, but do not yet fully replicate the behaviour of native pancreatic cells, under current differentiation protocols. In addition to the costly and time-consuming differentiation protocols, implanting stem cell grafts carries an increased risk of certain cancers and tumour growth. A need for widespread standardization of culture techniques used in differentiation would further the applicability of this promising yet incompletely developed strategy of pancreatic cell renewal.

A promising candidate for replacing lost pancreatic beta cells and in vitro T1D research can be found in the EndoC-βH1 insulinoma line, created in 2011 by Human Cell Design [51]. Derived from human fetal pancreatic buds, EndoC-βH1 cells display the heat shock protein HSP70, much like native islets do, which acts to lower inducible nitric oxide synthase (iNOS) and interrupt cytokine mediated cell death [52]. Furthermore, proteomic studies of EndoC-βH1 secretions found a 90% similarity with mature human beta cells [53]. The latest generation EndoC-βH5 line has had immortality reversed, which improves the insulin response and helps to prevent dangerous proliferation, hypoglycemia, and malignancies. This identical clone population releases human insulin in response to glucose, expresses the beta-cell maturation markers INS, PDX1, MAFA and NKX6.1, with total insulin content similar to native beta cells (~5μg/millions cells) [54, 55]. As these cells are representative in many ways of human beta cells, originate from a renewable stock and present a consistent phenotype, they are prime candidates for first phase in vitro testing T1D therapies and biosensing techniques. Implanting cell grafts consisting of stem cell or otherwise insulinoma generated synthetic islets however is still out of reach,

with many challenging obstacles yet to overcome. Before entering the earliest clinical trials, differentiation protocols will need to become more standardized, full characterization of the immunogenic effects and potential safety risks associated with xenotransplanting lab designed imitation islets. Pluripotent and proliferative cells carry the potential to differentiate into unwanted cell types and lead to tumour growth. The current upside being an unlimited supply for research and flexible culture possibilities. Just as with other pseudoislet constructs, the EndoC-βH1 and 5 cells are amenable to spheroid formation and coculture with accessory cells. These concepts will be explored further in the review article draft at the end of this chapter, as well as in the research article presented in chapter 7.

4.2 Motivation for Development

The field of islet transplantation offers much promise for type 1 diabetics, though is in need of further advancement to realize the true potential. The disease can strike seemingly at random, often afflicting children, with no lifestyle causes for onset. The breakdown into a full diabetic state is a complex autoimmune dysfunction, which is not yet entirely understood, preventing full early diagnosis or intervention. It appears that antigen presenting cells are triggered by an inflammatory environment in the gut endothelium, leading to immune targeting of the pancreatic beta cells and a cascade into hypoinsulinemia and hyperglycemia [56]. Once triggered, immune cells will sweep through an islet, selectively destroying only the beta cells, leaving other islet cells untouched. There is currently no method of slowing or interrupting the assault on beta cells, with treatments only available to mediate the symptoms of the condition afterwards. Strategies of managing the disease are very burdensome, to the patient and the healthcare system collectively, require constant attention and have limited efficacy. Needless to say, there are several large gaps in the field from fundamental biological understanding to in vitro cell handling and sensing and discovery of improved medicinal therapies. The breadth of

application, and yet common limitations of in vitro cell study platforms is exemplified in table 4.1. Developments in microfabricated fluid devices promote research capabilities and facilitate experimental progress towards a cure. In these next sections the engineering and biomedical aspects involved are reviewed in greater detail.

Author	Target/Disease	Detection Method	Microfluidics	Year
G. Addario et. al	Kidney disease	Fluorescence	3D-printed channels in PDMS	2023
O. Candini et. al	Breast/pancreatic cancer	Fluorescence/histology	3D Matrix	2018
A. Magadum et. al	Heart disease	Fluorescence	none	2017

Table 4.1: In vitro screening platforms for live cell disease therapy research

4.3 Microfluidics in Biosensing and Cell Handling

As technology continues to advance, biomedical applications profit from the increased capabilities. While raw machining power and small-scale manipulations offer many new possibilities, researchers still must apply creative applications of their fundamental knowledge to best address the needs of the field. A structural understanding of key concepts lays a crucial ground for imaginative solutions. Next, a review of the latest tools at the disposal of academic and industrial efforts towards improving quality of life for diabetics, leading to a discussion of the limitations and most pressing developments needed.

4.3.1 Fluid Handling at the Microscale

The motivation for developing microfluidic devices is more than simply downscaling bulky lab equipment and saving cost by reducing reagent volumes. At these smaller length scales and lower flow

rates, the effects of viscosity and surface tension lead to a change of regime in the prevailing physics. As fluids carry less momentum, laminar flow dominates, and the Navier Stokes equations can be reduced to allow suitable approximation through numerical methods [57]. Microfluidic chips can closely mimic the flow rates of blood between cells within an islet (~1-5μm/s) [58]. By introducing multiple liquids of varying hydrophilicity, for example oil and water, droplets within a carrier liquid can readily be formed. By varying the nozzle width and flow rate, the size and even the content of microcapsules be finely controlled.

Micromixing is an important tool for optimizing in vitro assays. By increasing the frequency of analyte molecules encountering the sensing surface, mixing the sample fluid can benefit detection sensitivity and time to result. AC electrothermal flow can be efficiently generated in conductive fluids using the imposed electric field from sensing electrodes. Temperature gradients in the ionic sample solution can be induced by a Joule heating of the electrodes, controlled by input signal amplitude [59, 60]. A gradient of fluid temperature due to heating in the region of the electrodes leads to corresponding non-uniform fluid conductivity and permittivity, causing a mixing force in the presence of the ambient field applied to the actuating electrodes [61]. Greater fluid control allows faster target binding to a surface sensor, as well as novel 3D spheroid culture possibilities [62]. Asymmetric electrode designs and custom fluid chamber geometries based on dynamic modeling are among the leading efforts in this area. The size and shape of microfluidic channels can promote laminar or turbulent flow; however the choice of construction material also offers flexibility of design for researchers. Material considerations for microbiological assays are discussed in the next section.

4.3.2 Materials

Microfluidic or point of care devices can be constructed with various materials, depending on the applications. Clear materials such as glass, PDMS or thermoplastics are often used, allowing for optical analysis of islets during screening [58]. PDMS is often used to define microchannels due to its low cost, porous, oxygen perfusable nature. The photolithographic protocols used to define fluid channels are well established due to their extensive use in fabricating microelectronics. Resolution, in terms of the size of any feature in the microfluidic channel pattern, is however limited by the wavelength of UV lamp light source. Electronic beam lithographically can thus drastically improve size resolution, at which point the channel material becomes the limiting factor. Softer materials like PDMS would not be as well suited for sub-micron feature definition as polycarbonate or glass. Which however generally require laser micromachine etching. Glass, polycarbonate, and to some extent PDMS all maintain optical transparency for in situ imaging, staining and light stimulation. 3D printed plastics or biogels at best retain partial transparency, though low cost and quick manufacturing times allow rapid turnaround of successive design prototypes. Cross linkable gels can be 3D printed into more macrostructures, though carry the power of incorporating cells directly into the structure, embedded in culture factors. Whether the target of study is a biomolecule or coordinated cell populations will determine the methods of fabrication required. Modern techniques now allow for repurposing surgically extracted whole organs as perfused flow devices for islet culture. This and other concepts are discussed in the following section.

4.4 Cell Scaffolding and Encapsulation

Many efforts today focus on recreating *in vivo* tissue models for study *in vitro*. A movement from adherent monolayer cell culture to more relevant 3D architecture has deepened the understanding of tissue formation, drug/cell interactions and provides an arena to uncover disease causes and outcomes [63].

During the isolation from human donors, a collagenase enzyme solution is used to break apart the organ structure which houses the islets. Those purified islets which are recovered following this process are often lost to anoikis, a subset type of apoptosis, referring to the programmed cell death of connective (endothelial) cells due to disruption of the extracellular matrix [64]. The subsequent reduction of islet populations in culture, coupled with significant numbers of cells dying due to failure re-establishing vasculature following transplant represent a major challenge in successful islet transplantation. It is possible, however, to improve transplant outcomes by including ECM components. Decreased oxygen perfusion through isolated islet also reduces their function and survival, though can be partially recovered by culturing within detergent-free ECM scaffolds [65]. These scaffolds may take the form of hydrogels, supplemented with ECM extracted from pancreas and other organs tissues, improved islet culture stability and integration with endothelial cells when embedded within the hydrogel scaffold [66]. As with any foreign implant, grafted scaffolds face immune rejection immediately following transplant, destroying many, if not all of the vital cells contained therein. In addition, cell-scaffold constructs must quickly and effectively establish perfused vasculature throughout, with higher risk of internal hypoxia compared to suspension cells. Endothelial cells can be cultured in decellularized rat liver scaffolds to increase their ability to carry and hold blood *in vitro* [67]. Applied to *in vivo* mouse models, grafts of islets cultured within rat lung scaffolds, prevascularized with endothelial cells achieved normoglycemia faster and maintained lower fasting glucose over 30 days when implanted subcutaneously, compared to an infusion of the same amount of islets in suspension [68]. By first seeding the endothelial cells into the acellular lung scaffold a vascular bed is prepared for receive islets. Culturing the islets together with endothelial cells within the scaffold prior to implant allows the coculture to form a robust and well-integrated organ for transplant.

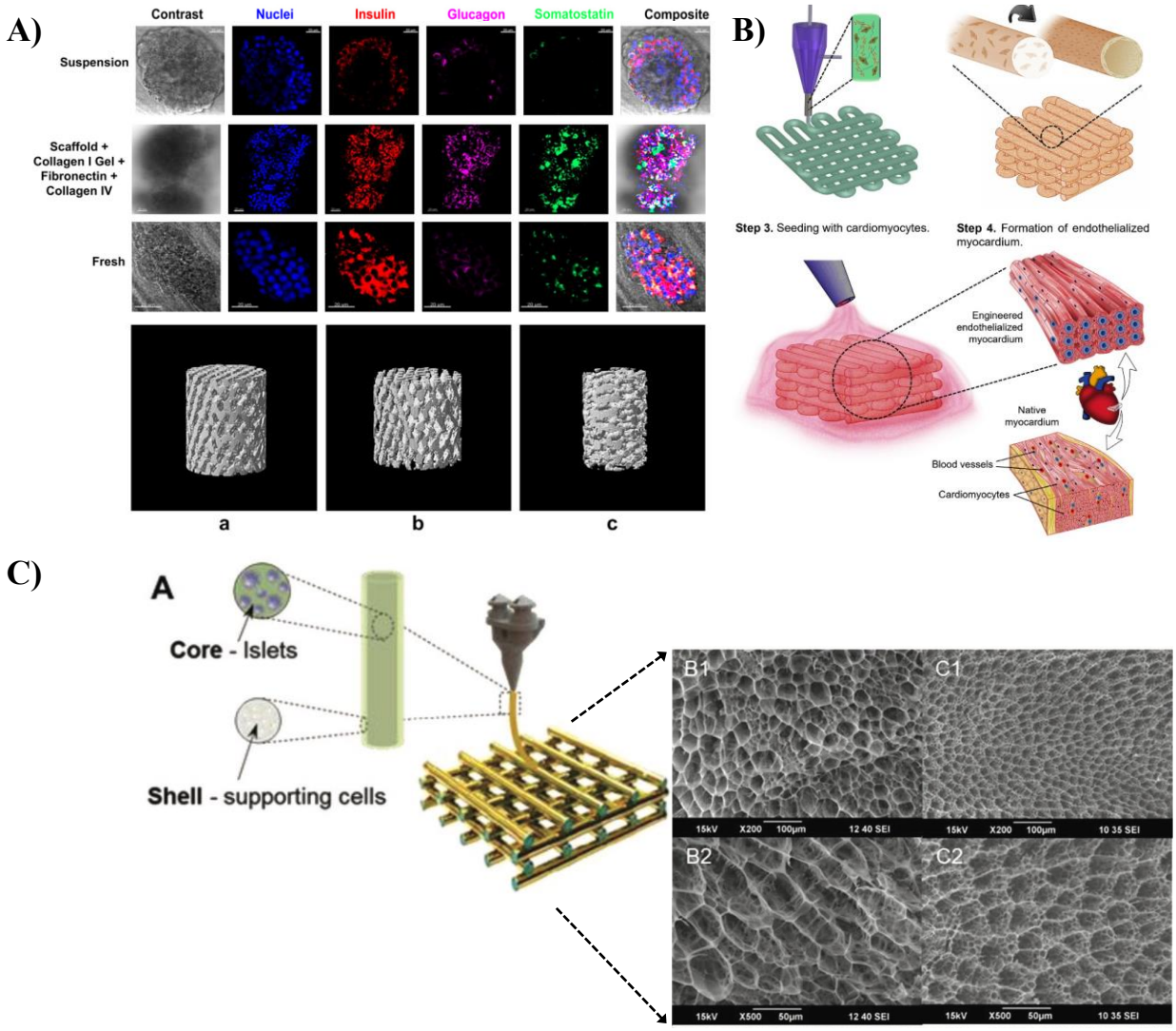

Figure 4.2: Bioscaffolds for 3D. cell culture A.) Immunofluorescent imaging reveals highly interconnected populations of islets cultured on 3D printed scaffolds with ECM proteins (Daoud, 2012) B) Use of coaxial 3D printing to obtain 3D cocultures (Zhang, 2016) C) Coaxial printing of islet embedded microporous scaffolds (Liu, 2019)

As using decellularized organs for transplant scaffolds presents a limited resource supply, is costly and time consuming to prepare, synthetic fabrication is sought for the development of renewable alternatives. Advancements in machining capabilities has brought 3D printing into the realm of biology, providing a unique control over spatial distribution of cells and ECM components. Both the macro- and micro-structure of the scaffold can be controlled to suit the individual characteristics of cell types to be involved [69]. Figure 4.2 displays a few implementations of 3D printing in biocompatible scaffolding for islet

culture in vitro [69-71]. Macroencapsulation devices are a popular strategy for immunoprotection, such as the Encaptra device which utilizes a semipermeable barrier to prevent rejection in subcutaneous implantation [72]. Furthermore, human embryonic stem cells were differentiated to produce insulin as a renewable source of cells with which to load the device. While larger populations of islets may retain viability when cultured together, large scaffolds also face fibrotic over growth due to a foreign body response. Obtaining a successful implant strategy will require further understanding of islet biology, and therefore necessitate in vitro scanning and characterization methods.

4.5 Biosensing and Detection Methods

Given the wide range of molecule type, size, charge and mass that maybe be of interest, different methods are required for measuring analytes in different circumstances. Attributes such as cost, detection limit, and user interface are each important considerations. Certain instances may require monitoring numerous analytes at once, with fast sampling rate and high temporal resolution. Various as the biological applications, biosensing devices are similarly diverse. General grouping classifications may be instructive, however, for determining the most suitable tool in each case. Below are descriptions of some of the most pervasive and powerful techniques in use today.

4.5.1 Optical Methods

Having been implemented for many years, optical characterization has been well explored. For that reason, an in-depth review of optical biosensing techniques is not discussed here. Instead, the reader is directed to such excellent articles such as the review by C. Chen and J. Wang [73].

Biosensing strategies can be broadly categorized into those which require physical contact and those which do not. Antibody based immunosensors such surface plasmon resonance, which can detect

disease markers in the form of proteins or RNA, applying to many pathologies, require direct physical interfacing with the fluid sample [74]. Optical or magnetic based sensing mechanisms, however, are contactless, giving them an entirely different application base. Fluorescent lifetime imaging, for instance, can be used to monitor glucose metabolism in vascularized islets within the optically accessible chamber of the eye, or *in vitro* using such an open with glass or translucent covering [75]. A drawback of direct optical observation, however, is difficulty detecting analytes in more opaque serums such as blood, or within tissues without the use of histological sectioning. Incredibly fast and readily miniaturizable, optical biosensors make up a valuable component in the toolbox of researchers and should be considered for integration with other sensors in versatile platforms.

4.5.2 Electronic biosensing

The development of transistor technology in recent years has made electronic devices more cost effective, vastly more compact with increasingly diverse functional capabilities. In the scope of biomedical devices for T1D, microchip electronics have direct applications in miniaturized point of care diagnostics and streamlined in vitro screening assays. Electrode surfaces can be biologically functionalized towards a variety of targets such as enzymatic products, antibody-antigen binding interactions, and nucleic acids allowing analysis of otherwise optically inaccessible fluids. Enzymatic biosensors employ colorimetric or electrochemical transduction methods to detect glucose, hormones and other biomolecules, yet their widespread point-of-care application is limited by high cost [76]. Electrochemical impedance sensing (EIS) provides means for evaluating numerous physiologically

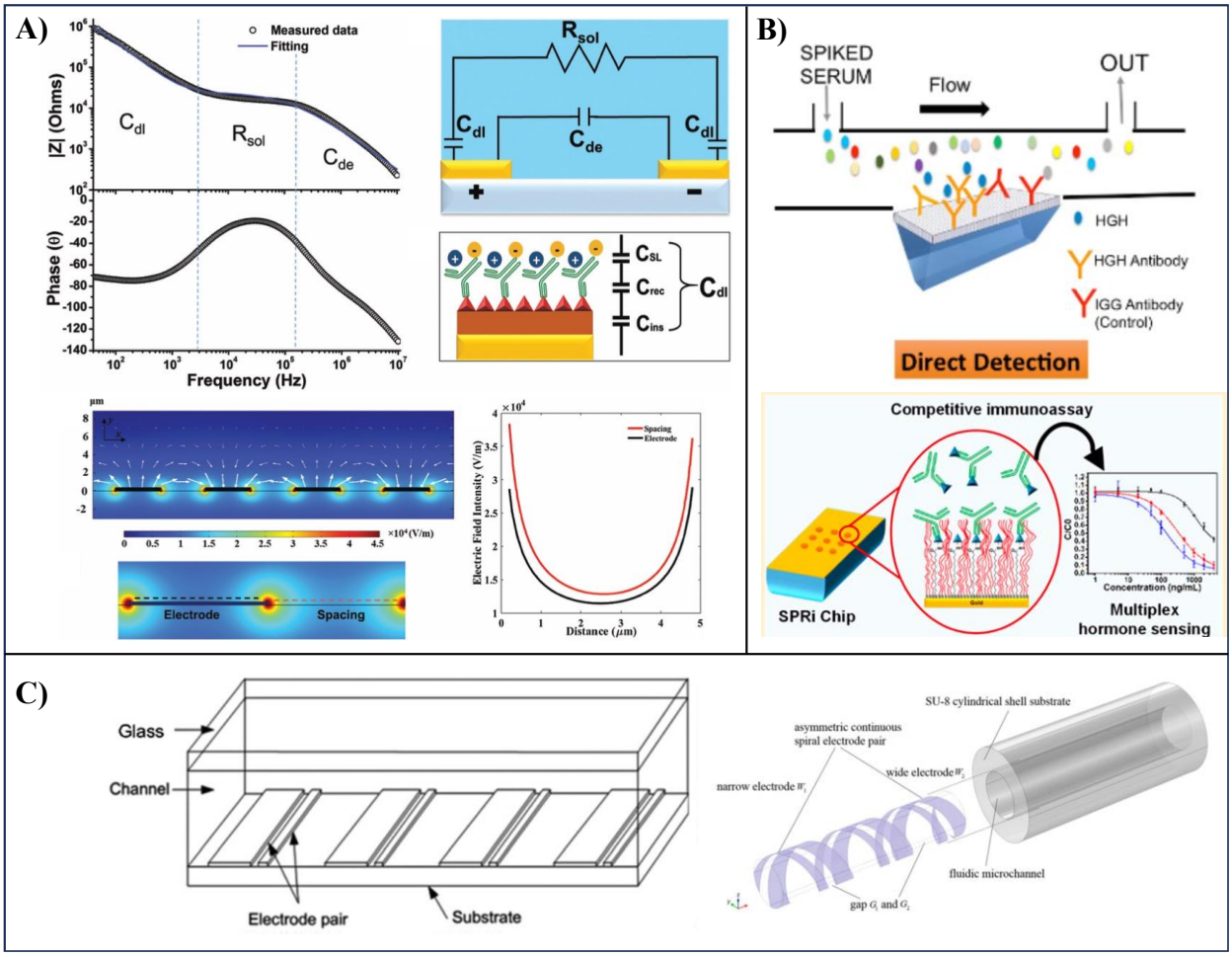

Figure 4.3: A) Frequency spectrum, circuit diagrams and field simulations for coplanar microcapacitors (Castiello, 2019) B) Premise of flow through SPR detection (Vance, 2016), Multiplexed detection using distinct target sensing regions (Castiello, 2018) C) Asymmetric electrode geometries suitable for inducing AC electrothermal mixing (Salari, 2019)

relevant parameters, such as the solution resistance, capacitance at the sensor surface, among others [77]. Generally requiring only small sensing voltages, on the order of 0.01V, microelectrodes can also be used to manipulate samples at higher potential differences. Controlling the frequency and voltage of the AC signal applied to the electrodes one may tune through remiges of AC electroosmosis, dielectrophoresis, and AC electrothermal effects. Thus, there are numerous phenomena which offer the possibility to not only measure, but to manipulate the system under study. Figure 4.3 displays capacitive, plasmonic, and asymmetric electrode-based biosensor configurations [59, 78-80]. At voltages of 100mV up to 5V, and frequencies from 100kHz to nearly 200MHz, the AC electrothermal effect can be used to impose

microstreams and vortexes in ionic fluids [59]. Strongest effects are seen in higher electrical conductivity fluids, making ACET ideal for biological fluids such as blood, urine and saliva. Independent of particle size, ACET mixing can generate directed flows that bring analytes into contact with the sensor surface, improve detection efficiency [81]. Carboxylated graphene aptasensors employ electrochemical insulin sensing down to 1.5pM detection limits [82].

Ion selective electrodes (ISE's) interface with polymer membranes to measure potentiometric changes, related to biological processes. ISE's can be equipped with bioreceptors for sensitive detection of targets of different sizes, from ions and small analytes to whole cells, in a time-dependent manner [83]. The use of machine learning or artificial intelligence could be especially useful in the case of rich chemical data. Soon artificial intelligence, or digital neural networks (DNN) will aid major advancement in all aspects of microfluidic biology, from dynamic fluid modelling, to drug discovery and disease diagnoses [57].

Electronic biosensing devices are cost effective and easy to use, making them a dependable choice for complex, biologically relevant serum samples. There is, however, an immense parameter space left to explore. Between electrode design, target molecule(s), electrode frequency, voltage and fluid temperature. Though promising, much legwork is still needed to realize the full benefits of electrical-based biosensing.

4.6 Chemical Therapies for T1D

Improvements in biosensor sensitivity, selectivity and throughput have opened a floodgate of data for drug screening trials. Actual experimentation will determine which leads should be followed. Metformin, for instance, can improve glucose management, reducing insulin reliance and even slowing atherosclerosis in type 1 diabetics [84]. Research of oral alternatives to subcutaneous insulin delivery has been slowed due to difficulty overcoming enzyme degradation, and short half-lives of proteins in

circulation [85]. For this reason, efforts have turned towards encapsulating insulin with protective polymeric or micelle nanocarriers. In clinical human trials INGAP was administered subcutaneously at doses up to 600mg/day for 90 days [86]. While a significant increase in Argenine-stimulated C-peptide was observed ($p = 0.058$), local reactions to the high-dosage injections pose a considerable obstacle.

While many synthetic agents can achieve specific effects, they are often not without side effect and cost. In search of a naturally derived beta cell promoting agent, experiments are now looking into the exocrine-derived islet neogenesis-associated protein (INGAP). The INGAP peptide is released from acinar cells in the exocrine portion of the pancreas, which secretes the signaling molecule to promote the function and viability of neighboring endocrine cells [87]. INGAP's proposed beta cell protection mechanism is through reducing oxidative stress, though how the specific expression in exocrine tissue helps to normalize blood glucose is not entirely understood [88]. Beneficial effects in T1D and T2D patients have spurred clinical studies, reaching phase 2 human trials. The INGAP protein is 115 amino acids long, however the sequence from bases 104 to 118, the pentadecapeptide fragment of INGAP (INGAP-PP), exhibits pleiotropic behaviours which retain many of the benefits of the full chain while facilitating faster and cheaper synthesis. INGAP-PP has been shown to improve beta cell mass, insulin secretion and promote angiogenesis [89]. The bioactive region of INGAP-PP, thought to be a 15 amino acid fragment, has shown inhibition of apoptosis and iNOS expression in rat insulinoma cells treated with proinflammatory cytokines IL-1β and IFN-γ, similar to the complete INGAP sequence [90]. Smaller proteins fragments can be more difficult to measure due to lower molecular weight, however screening for effects on cellular performance can be used as first indicators.

Further discussions of the INGAP molecule and it's bioactive pentadecapeptide region can be found in Chapter 6.

4.6.1 In-vitro Drug Discovery

Standardized in vitro screening methods for human islets employed in the drug discovery experiments of chapter 6 are adopted from clinical protocols used in screening isolated islets for transplant. The quickest and most direct characterization of islet functionality is done by finding the 'stimulation index'. Calculated as the fold increase of insulin released in response to high glucose, compared to basal levels, the stimulation ratio should be above 2 to be sent for transplant. As the cells used for insulin quantification cannot be easily recovered, a sample population is taken to represent the batch of purified islets.

Stimulating human islets can be done using cell culture inserts with pore size on the order of 10μm, which allow the immersion and recovery of suspension islets into successive buffers of increasing glucose concentration. Islets may be taken for lysis following glucose stimulation and subsequently subjected to nuclear mRNA gene expression analysis. To screen new potential therapeutics, molecular agents may be added at varying concentrations to the stimulation buffer, which is monitored for fluctuations in insulin released by islets, compared to controls. Each test condition should be repeated in triplicate, for statistical fidelity. As the standardized protocol uses ~150 IEQ per test replicate, testing different compounds at multiple doses, including positive and negative control groups quickly amounts to significant time and work for lab technicians and consumes valuable human islets. This strain puts a bottleneck on testing new therapies in vitro. High-throughput biosensors with multiplexed target capabilities and better sensitivity are powerful for extracting detailed information within the confines presented. In addition, engineering advancements in device fluid and cell handling provide a backbone of support on which developing assays may be conducted. Many efforts to combine cutting-edge biosensors with current cell models incorporate novel 3-dimensional cell culture methods, to be reviewed with SPR sensing in the following section.

3D Cell Spheroid Model Characterization using Surface Plasmon Resonance Imaging: Focus on Type 1 Diabetes

The final section of chapter 4 is prepared in the form of a literature review article draft to be submitted for standalone publication. This section covers the use of real-time SPR biosensing for monitoring the secretions of cell models in vitro. This review helps relate the concepts cell culture and sensor physics as they pertain to dynamic screening assays for diabetes. Native and renewable cell sources are discussed, along with means of enhancing sensing capability through surface modification. A comparison of strategies that have been used to monitor cellular activity points the way to what is available and what is missing for the purpose of a live islet testing system.

3D Cell Spheroid Model Characterization using Surface Plasmon Resonance Imaging: Focus on Type 1 Diabetes

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Abstract

Type 1 diabetes (T1D) is an endocrine dysfunction leading to an imbalance in several signalling proteins. While much progress has been made, little is still known about the mechanism of disease, proper diagnosis methods, and avenues for treatment. Surface plasmon resonance (SPR) is a versatile and powerful biosensing method which could shed light on puzzling aspects of diabetes. In order to apply SPR to T1D study, sample interfacing and cell modelling must be used to bridge the gap. Standardized screening is needed to establish a benchmark across human islet samples, and to measure efficacy of designer cell models a replicating or surpassing donor cells. This article presents a review of in vitro sensing and cell models for T1D research, avenues of technological development and aspects of the field that face hinderance or could be improved.

4.7 Introduction

There are many ongoing research efforts today to obtain a user friendly, real-time, sensitive and multiplexed biosensor. While no singular technique can wield the best of all worlds, surface plasmon resonance (SPR) is poised to become a leading in vitro method for biological assays alongside traditional methods such as ELISA, mass spectrometry and PCR. For monitoring binding affinity interactions in solution, SPR sensing falls in line with affinity chromatography, western blot, radioligand binding and digital autoradiography [91]. There are many recent comprehensive reviews compiling the use of SPR for biomolecule and cell analyses. To name a few: Ma et al. focused of the detection and analysis of cells using SPR including direct cell analysis such as internal organelle transport, cell secretions, surface binding effects, and detection/identification of bacterial species [92]. The review by Su et al. explores the use of SPR for directly monitoring cell morphology, proliferation, interactions with ECM and

subcellular activities [93]. Ngyuyen et al. detail SPR sensing mechanisms across various surface immobilization detection methods, while Masson et al. describe the use of SPR to detect antibodies and disease biomarkers in serum or plasma [94, 95]. Table 4.7.1 gives examples of surface plasmon resonance being applied to biomarker detection for T1D diagnosis.

One advantage of SPR biosensing is the ability for multiplexed detection of numerous target analytes, increasing throughput by flowing buffer across multiple reaction chambers in parallel. This is particularly attractive for simultaneous detection of a panel of biomarkers, in the context of many diseases such as cardiovascular diseases, cancers and diabetes [96-98]. The strength of SPR biosensing is to detect small concentrations of a variety of molecules in a multiplexed and high throughput fashion. In order to increase the dynamic range of detection, as well as limit of detection, various strategies have been proposed. For instance, the review by Puiu et al. discusses details of SPR enhancement with nanoparticles and nanostructured sensor surfaces or combination of SPR with other technologies [99]. The integration of other technology with the SPR is also used to overcome the limitations of the high throughput screening with SPR. Researchers attempted to combine microfluidic technology with SPR-based sensors. The most recent reviews including the ones published by Bogdanowicz et al., Ahmadsaidulu et al., and Liu et al., to name a few, describe microfluidics based SPR biosensing for rapid, multiplexed and high throughput detection of various drug and biomarkers [100-102].

4.7.1 Type 1 Diabetes

Type 1 diabetes is a chronic and life-threatening condition resulting from a loss of endocrine cells controlling blood glucose homeostasis. Clusters of microorgans housed in grapevine-like structures within the pancreas, called the Islets of Langerhans, are crucial to maintaining control of blood chemistry, and their dysfunction is pivotal in development of diabetes. Roughly 46 million people around the world are affected by T1D [38]. When it comes to a target HbA1c of 7% or less, less than 17% of adolescents and 21% of adults meet that goal, respectively [103]. In order to understand the underlying

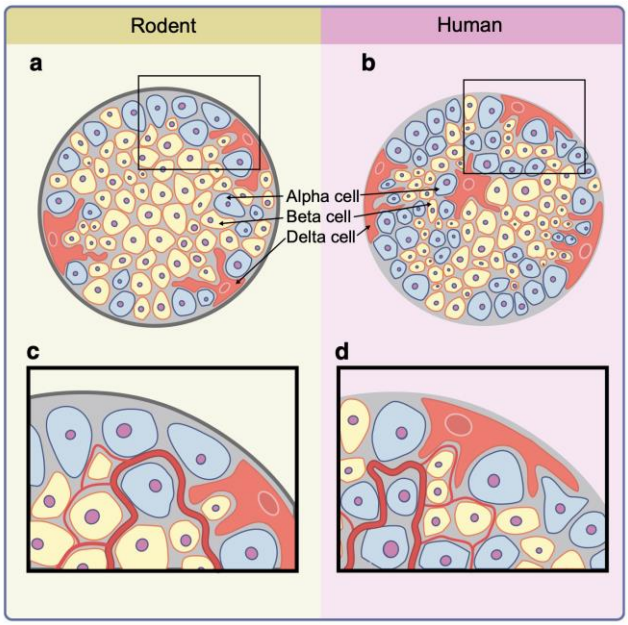

Figure 4.7.1: Schematic of rodent (A) and human (B) pancreatic islets. Islet ECM depicted as grey. (C) and (D) exemplify the strong peri-islet basement membrane in rodents *(Moede, 2020)*.

mechanisms of Diabetes, it is crucial to interrogate the pancreatic islet. Differences between human and rodent islets motivate advanced in vitro systems for human islet study (figure 4.7.1) [104].

The pancreatic islets of Langerhans make up 1-2% of the pancreas, and can be implanted to avoid subcutaneous insulin injections, however requiring lifelong immunosuppression [45]. Responsible for secreting numerous endocrine proteins, the islets of Langerhans maintain balance of blood chemistry. A dysfunction of these hormonal regulators leads to a cascade of devastating effects, including hyperglycemia, hypertension, blindness, atherosclerosis and kidney disease [7]. There is currently no agreed upon standard established for the secretion profile of a healthy islet, and therefore no foundation for early diagnosis or understanding pathologies. Collective responses by multiple islet cells are mediated through paracrine interactions. β-cell secreted insulin (among other factors) inhibits α-cell release of glucagon, in contrast, α-cell released glucagon stimulates insulin, though not in the absence of glucose [104]. Monitoring the dynamic hormone response of islets to various stimuli will require an *in vitro* screening platform coupled with a multiplexed flow-through sensor capable of detecting biomarkers relevant to diabetes (table 4.7.1). Target biomarkers for T1D diagnosis include the insulin antibody (IA), insulin autoantibody (IAA), and GAD65 [105-107]. Furthermore sorbitol and GHSA are also useful in diagnosis [97].

Author/Year	Target	Significance	Sensitivity	Surface Type	Assay Type	Method of Attachment	Notes
Kaur (2023)	GHSA, sorbitol	biomarker to diagnose diabetes	50 μg/mL, 1 μmol/L	multiple	Fluorescence/enzyme	DNA aptamer to GHSA	Enzymatic biosensor avoided due to unstable immobilization
Trabucchi (2013)	IA/IAA	T1D diagnosis	100nM	carboxymethylated-dextran CM5	antigen-antibody	standard proinsulin	semi-quantitative, limited to bound % titre
Zhang (2014)	IAA, GAD65, insulin, IgG AA	T1D diagnosis	sensitivity of 100% and specificity of 85%	gold islands on glass	sandwich	multiplexed islet antigen microarrays	better sensitivity than RIA
Nogues (2010)	GAD65	T1D diagnosis	4.8 nM	ethylene glycol	antigen-antibody	Cysteine residue	Amenable to multiplexing

Table 6.7.1: Applications of SPR for disease biomarker detection

It is thought that impaired autophagy triggers activation of the inflammasome in diabetic pathology [108]. One interesting finding is that neurodegenerative diseases are, in large part, endocrine related [109].

4.7.2 SPR in T1D Research

Surface plasmon resonance imaging (SPRi) makes use of antibody-antigen affinity interactions to detect the presence of target analytes in solution. With the use of a collimated light source, the system measures changes in the optical refractive index of a conductive film as molecules adsorb onto the functionalized surface. While fluid flow introduces the biological sample to the sensing surface, this technique has the advantage over other optical methods as the light does not need to pass through the actual liquid, interacting only with the backside of the chip. Having direct interaction with sample analytes through antibody binding without needing to pass through the fluid sample allows for target

specific measurement even of opaque or higher optical density fluids, such as blood or plasma. The need for non-invasive diagnostic sensing continues to drive the innovation of biosensing technology.

As islets release numerous peptide hormones and communicate via paracrine interactions, they present an ideal subject of study for SPR. Although there have been tremendous efforts in developing various SPR-based detection methods, alone or in combination of with other spectroscopic techniques. Additionally, lab-on-a-chip approaches making use of the evanescence wave for diabetes research are still in infancy, particularly in type 1 diabetes, where donor islet transplantation is required to establish glucose homeostasis. Xing et al. give an excellent review for researchers or clinicians, interested in islet screening for transplant [110]. The authors focus on islets from human and rodent sources, interrogated by PDMS or glass microfluidic devices for T1D-relevant targets such as insulin, glucagon and C-peptide. A valuable related review can be found by F. R. Castiello et al., which gives comprehensive coverage over microfluidic structures for characterization of islets for diabetes, though nearly all were applied to mouse or rodent islets [111]. While there is great potential for insulin detection by SPR, there were only limited publication reports on this application of the biosensing technique. This is due partly to the lack of an established in vitro model to be combined with the SPR to better understand the fundamental concepts of T1D. One of the in vitro models of interest is the 3D construct known as cell spheroids. The cell spheroids can be constructed with any cell type of interest.

In the context of diabetes, with the recent developments in iPSC biology and understanding of differentiation protocols, there is a surge of interest in using these as renewable islet replacement cells and investigate their fate in 3D spheroid configuration. Coculturing stem cell derived α and β cells, as discussed by the comprehensive review by J. Siehler et al. [38], the iPSC derived MIN6 β-cell spheroids cocultured with dermal fibrobalsts and HUVECs for prevascularized implant by S. Takaichi et al., or from immortalizing human β-cells by retrovirus transfection, such as seen with the EndoC-βH1 insulinoma line established by R. Scharfmann, P. Ravassard et al. are examples the cells for constructing

spheroids to help bridge of the modern in vitro platforms with the SPR biosensors for islet secretome analysis [51, 53, 112, 113]. While suitable for single molecules and low molecular weight peptides, SPR may also be applied to measurement of larger structures, whole cells and ECM proteins (table 4.7.2) [96, 114-117].

Author/Year	Target	Size	Sensitivity	Surface Type	Assay Type	Method of Attachment	Notes
Y. Yanase (2012)	Basophils (reacting)	< 500nm	~20 RU	anti-basophil on DSP	individual basophil RI response to anti-IgE	amine bound anti-basophil	stimulate immobilized cells - watch RI change
P. Zhang (2022)	CD-63, HeLa EV binding exosome	30 nm	1 x 10^9 /mL	NHS/EDC on PEG6-COOH	plasmonic Scattering (SPR)	anti-CD63	unique scattering setup
H. Xiong (2022)	pancreatic cancer exosomes	~100nm	4 /mL	AuNP	sandwich	antibody / Au-N bond	LSPR based ECL
W. Chen (2021)	$HER2^+$	30-150nm	$1x10^4$ to $1x10^7$ particles/mL (linear range)	NHS/EDC with tyramine	enzyme assisted amplification	aptamer region of MAB immobilized	amplified by molecular aptamer beacon
A. W. Peterson (2009)	serum proteins	0.01-1μm	signal-noise ratio 3 ng/cm	fibronectin protein on hexadecane thiol coating	passive deposition	PDMS arrays stamped onto gold surface	units of μg/mL to quantify immobilized protein

Table 4.7.2: Cellular and structural applications of SPR biosensing

4.7.3 Cell Models for T1D Research

Due to the fact that endocrine hormone producing β-cells do not proliferate, and native isolated cells have limited culture lifetimes *in-vitro*, there is a large field of research dedicated to the development of lab grown alternatives to endogenous islets. The advancement of pluripotent stem cells and insulinoma-line spheroids has brought researchers to the edge of creating suitable alternatives to human islets for research. As designer cell types and aggregated spheroids approach a viable mimicry of human cell behavior, it gives motivation to developing comprehensive quantification of the human pancreatic

islet hormone secretion profile as a benchmark. If a well-defined standard can be widely accepted, it could provide a measure with which to compare synthetic hormone producing cell alternatives.

4.7.4 Spheroid Models in Diabetic Mice

K. Kusamori et al. demonstrated the use of PDMS microwell arrays to form spheroids with mouse insulinoma NIT-1 cells for transplant into streptozotocin-induced diabetic mice [118]. When implanted under the kidney capsule, it was shown that the NIT-1 spheroids could restore blood glucose homeostasis in less time compared to an implant of the same cells in suspension alone. Regardless of the cell formulation used for implant, a major hurdle to overcome, in both mice and humans alike is graft rejection due to the immune response. Nearly half of islets in a graft population do not survive the first few hours after transplant due to this instant blood mediated inflammatory reaction (IBMIR). M. Kim et al. demonstrate a layer-by-layer nanofilm coating to protect implanted spheroids from the immune response, and show that MIN 6 β-cell spheroids encapsulated by a 6-layer hydrogel could protect from NK cells while maintaining glucose stimulated insulin secretion [119].

4.7.5 Coculture Cell Models

The complex cytoarchitecture of the pancreatic islet by now is well known, with numerous endocrine cell types each releasing their signaling hormones into the blood. Not only is the islet interior a multicellular environment, but survival and function importantly relies on integration with connective tissue in the basement membrane and vascular network. The inclusion of supporting cells into spheroids can provide a co-beneficial signaling, improving the performance and longevity of 3-dimensional cell culture models. A. C. Bowles et al. developed coculture and triculture spheroid models using human organ donor islets with HUVECs and MSCs to promote robust vasculature, and used a bed of endothelial

cells seeded onto basement membrane extract in monolayer culture as a simulated implantation site [120].

Within the context of interrogating the secretory behavior of endocrine cells involved with the development of diabetes, it is essential to consider the effects of perfusion. Islets communicate with one another as well as tissues throughout the body by secreting signaling hormones directly into the bloodstream. Highly enervated and vascularized, the flow of blood over these microorgans is a key factor to their normal function. In order to develop an *in vitro* platform which simulates the islets' natural environment, and perform dynamic stimulation testing, we should hold the islets under a continuous perfusion flow. A hormone sensor capable of continuous flow-through measurements would be a powerful tool to quantify and monitor the secretory release profile.

In addition to being able to read the hormone contents from a continuous fluid stream, the desired sensor would optimally have the added capacity for detection of multiple analytes. While the stimulation of insulin release has been widely studied, pancreatic islets are highly complex regulators of the body's blood chemistry – making use of numerous autocrine and paracrine factors to maintain blood homeostasis. By considering other disease biomarkers such as glucagon and somatostatin, we may garner a more comprehensive understanding of the behaviour of pancreatic islets in the context of diabetes. If clinicians had access to quick and easy tests such as the blood glucose monitor, which could reveal more information on each patient's unique situation, it could lead to more accurate prediagnostic classifications, and therefore individually tailored treatment plans. In the work by F.R. Castiello et al., the human pancreatic hormones insulin, glucagon and somatostatin were simultaneously quantified in a cocktail solution by flowing the sample to a multiplexed SPRi chip [80]. In this instance, islet antigens were immobilized at discreet locations on the gold sensor surface, and solution concentrations of the 3 hormones were detected by parallel competitive immunoassays. This represents a method of flow-through sensing that could be developed for integration with microfluidic or point-of-care assays.

SPR systems are readily adapted to high throughput screening applications, as shown in the proteomic analysis of serum by C. Lausted et al. [121]. Here, researchers used an array of 83 antibodies to evaluate the proteomic profile of serum from the liver, and to compare the healthy vs. diseased state.

4.7.6 SPR Enhancement

An SPR system uses a biological recognition system composed of an organic functionalized layer coated onto a sensing surface. Mass detection or target analytes is performed by monitoring the refractive index of a conductive gold film. Selectivity of surface binding is achieved through immobilizing

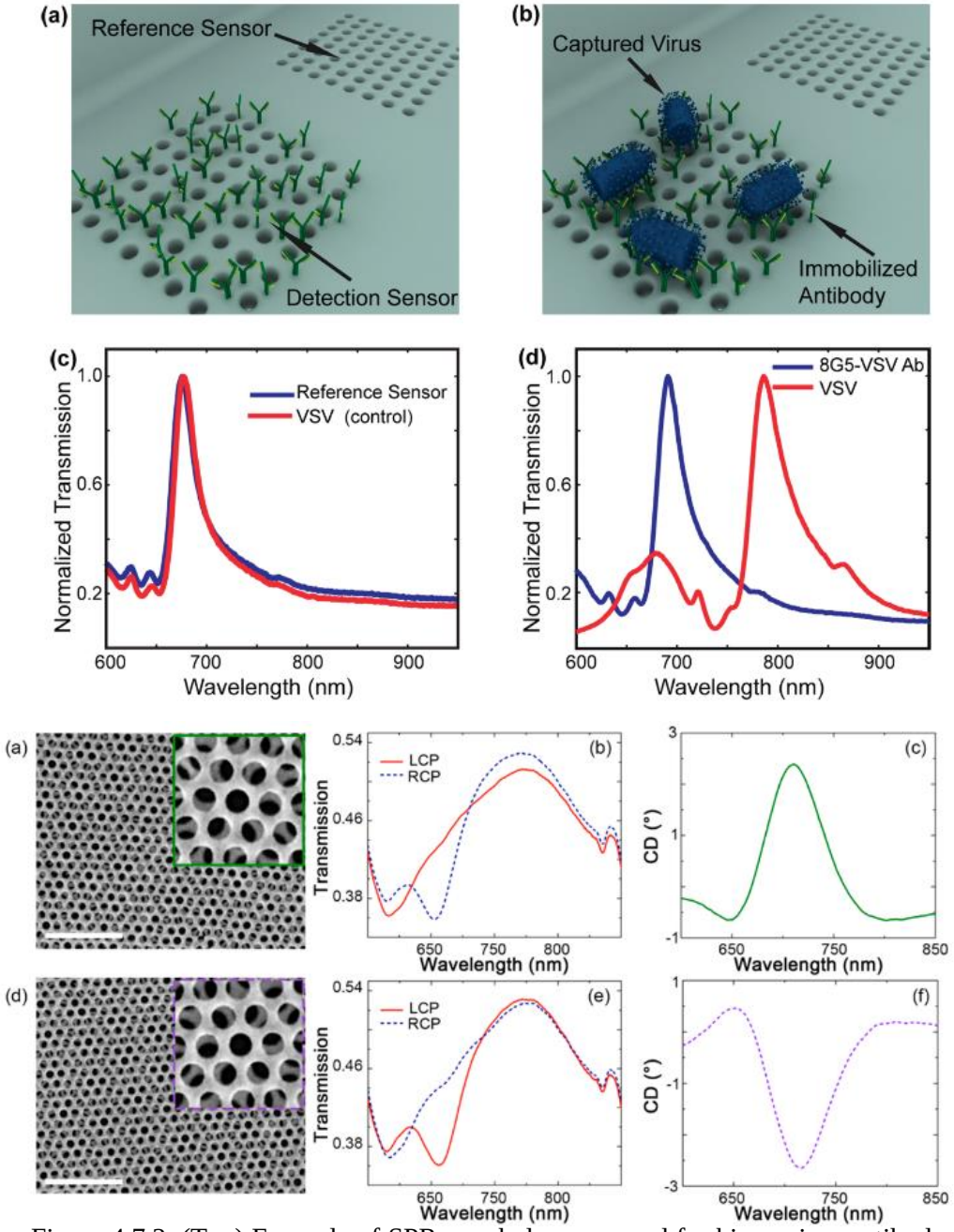

Figure 4.7.2: (Top) Example of SPR nanohole array used for biosensing: antibody capture of VV (Yanik, 2010) [2] (Bottom) Overlapping periodic nanohole arrays produce subwavelength periodic Moire patterns (Wu, 2018) [5].

complementary partner molecules. Making use of lock and key recognition mechanisms, an antibody/antigen binding can provide highly specific target molecule capture. The orientation, type and density of antibody sites fixed to the sensing surface will subsequently affect binding kinetics, detection time and sensitivity. One method of increasing available binding sites is by making use of a dextran matrix. With an amino N-terminal available as a surface linker, carboxylated C-terminals are distributed evenly along the length of the polymer which can attach to chosen targets particles. As such, dextran molecules of chosen length (/molecular weight) form a self-assembled monolayer which provides a 'forest' of binding sites for the functionalized target analyte. With a multitude of open docking ports for incoming particles, the saturation capacity of the sensor is increased, and low concentration limits are improved enough to allow for direct detection over competitive immunoassays. This eliminates the need for procedural steps and fluid handling in prior mixing of antibody/target molecules en route to detection. For an on-chip consumable microfluidic point-of-care detection system, minimizing the number of steps is crucial to opening the technique to more users.

Increased SPR sensitivity can be gained by introducing topographical features to the sensing surface. For instance, nanohole arrays are a popular means of achieving better signal, as demonstrated by the SPR detection of vesicular stomatitis virus (VSV) by antibodies (figure 4.7.2) [2]. By introducing topography to the otherwise 2-dimensional gold sensing surface, fine tuning of the evanescent wavestates becomes possible. Localized surface plasmon resonance occurs when particles or structures at the nanometer scale interact with light of a similar sized wavelength [122]. An excellent review covering the mechanisms of LPSR and SPR enhancement by nanostructures is given by Q. Duan et al. [123]. The in-depth discussion of the underlying physics conveys the power of SPR sensing at the current cutting edge and shows how this technology will see much development in the years to come. Patterned nanostructures on the order of the optical wavelength are more often seen as a means of enhancing detection through LPSR. In order to access finer detail of nanostructures without putting the brunt of

work on the microfabrication process, researchers are making using of Moiré patterns, which can be established by an overlapping set of periodic structures [5]. This technique can provide the sensitivity enhancement of subwavelength features without need for the same lithographic resolution. Using 2 overlapping nanohole arrays, a Moiré pattern of subwavelength features can be attained, the plasmonic properties of which vary with the angle of overlap (figure 4.7.2). Similarly, the recent review by Y. Liu et al. describes SPR biosensing strategies employing nanostructured arrays [124].

Conducting SPRi in an angle-resolved setup gives higher resolution and dynamic range than reflectivity-based, using phase interrogation mode offers ultrahigh sensitivity [125]. Using an SPR nanohole array, E.H. Lin et al. found a 4.5x enhancement of sensitivity when interrogating the surface from high incidence angles (>20°), compared to regular prism based sensors [126]. N. Polley et al. employed the novel approach of fixing a nanohole array onto the cross sectional end of a fiber optic cable, instead of a glass prism [127]. SPR biosensing can be modified to increase sensitivity, number of targets, or cellular secretion assays. Figure 4.7.3 demonstrates SPR modalities of amplification by magnetic nanobeads or plasmonic scattering, as well as direct cell secretion monitoring [96, 114, 128].

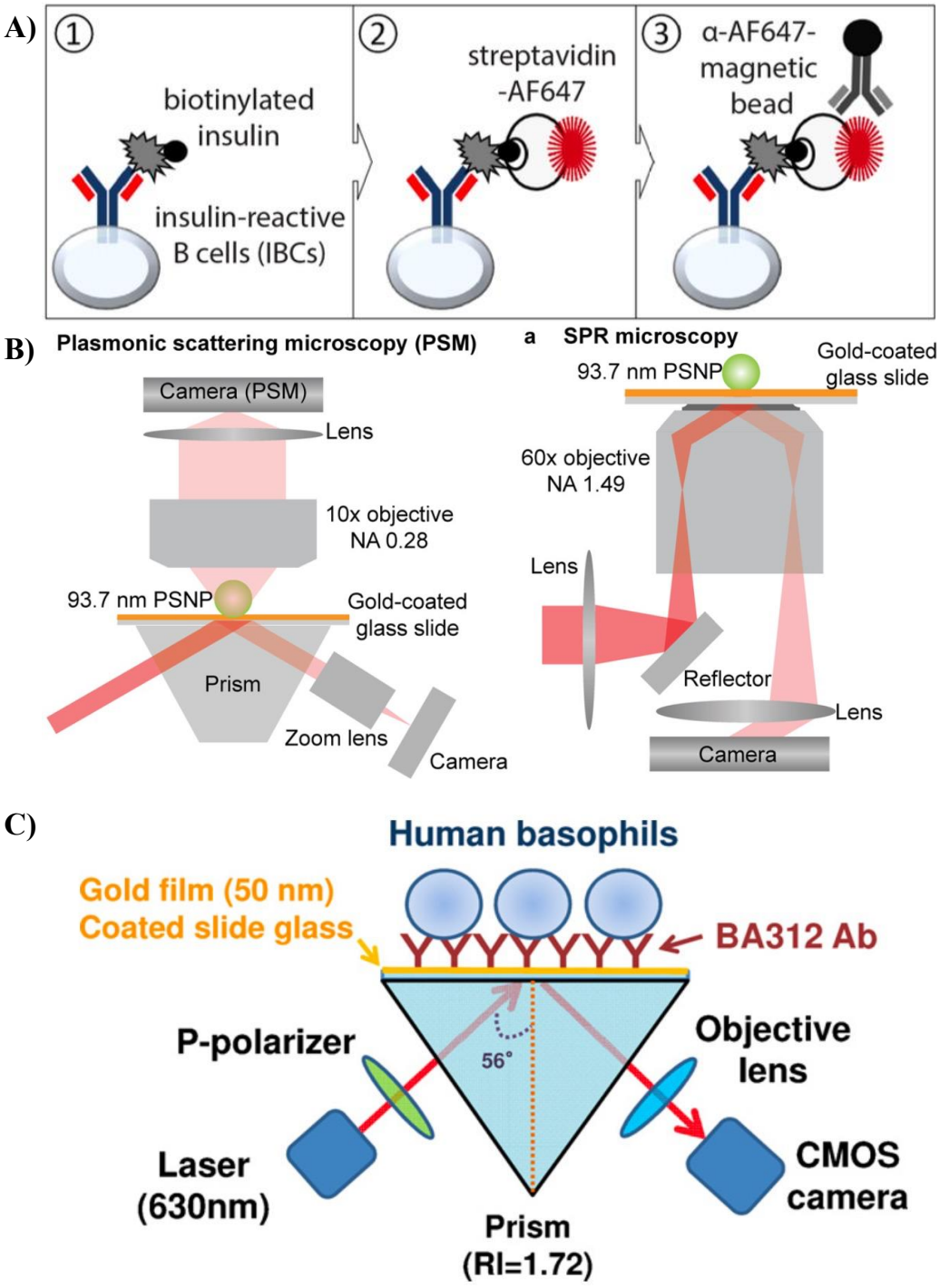

Figure 4.7.3: SPR modalities A) Sandwich assays using biotin-streptavidin complexes with magnetic beads comparative analysis of the repertoire (Banach 2022) B) Plasmonic Scattering Microscopy (Zhang 2022) C) evaluation of peripheral blood basophil (Yanase 2012)

4.7.7 Membrane Interactions

Membrane proteins are the target for roughly 60% of current therapeutic drugs [129]. A powerful but somewhat less seen application of SPR is to identify cell surface binding receptors and quantify their affinity to certain targets. U. Anders et al. demonstrated this capability by immobilizing RNA binding targets to the sensing surface and flowing whole cell bacterial lysate into the chamber [130]. In this way, they were able to identify binding regions on the cell surface, by performing mass spectrometry on the captured fractions. This article gives a useful description of the on-chip proteolysis method necessary to detach bound complexes from the sensor surface for identification. With development, the capture of lysed membrane proteins could be applied to uncover the mechanisms of interaction between therapeutic compounds under investigation and their target cells, to allow engineering of more specific and effective

compounds. Figure 4.7.4 exhibits ways SPR has been applied for measuring proteins, aptamers, and antibody production in clinical trials [131-134].

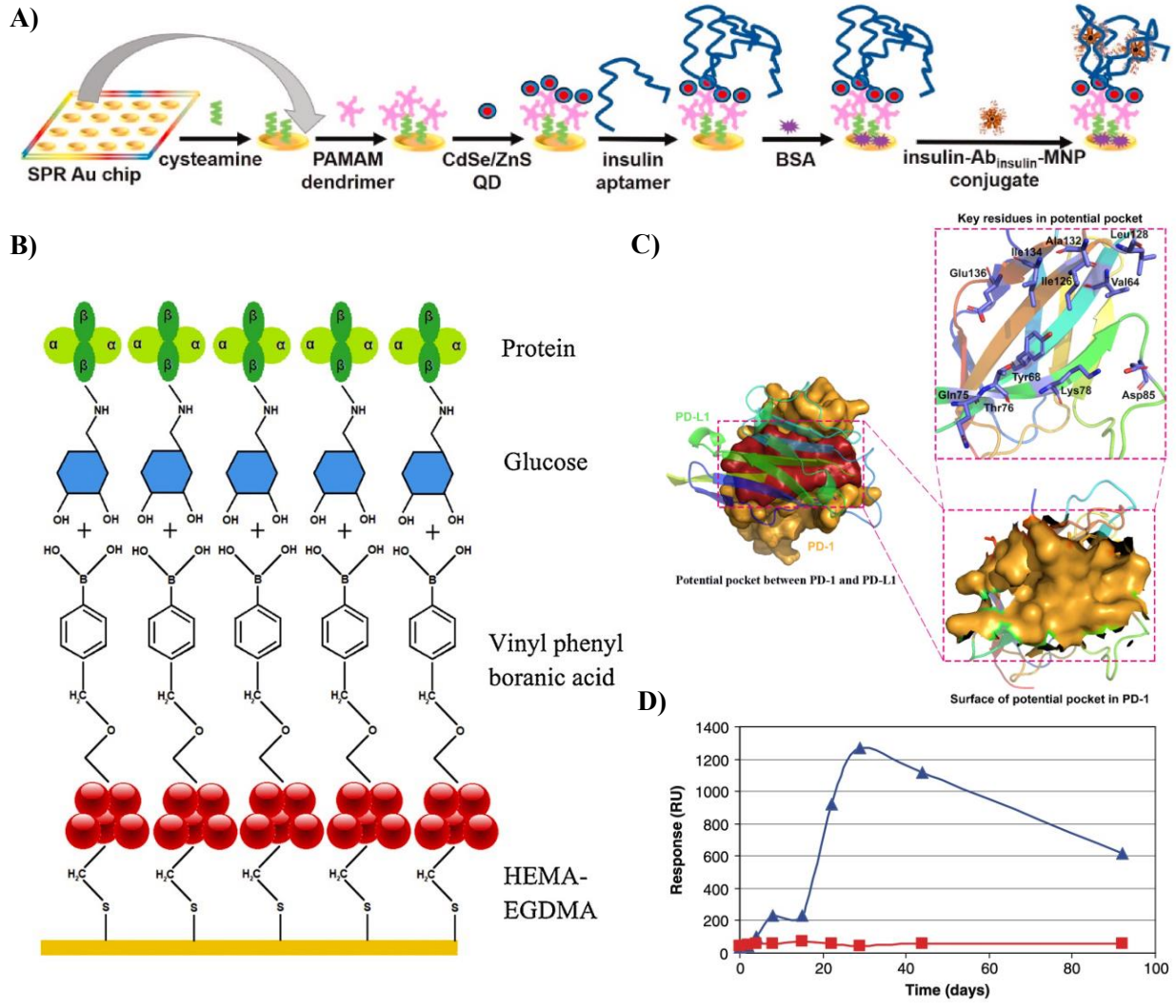

Figure 4.7.4: A) A multiplex, quantum dot coupled ultrasensitive SPR strategy (Singh 2020) B) SPR surface chemistry for detection of T1D diagnosis biomarker HbA1c (Calisir, 2020) C) In-depth modeling combined with SPR to study binding orientation and epitope affinities in diabetes biomarker PD-1 (Wang, 2019) D) SPR measurement of patient antibodies, developed over 30 days to T1D drug otelixizumab (Hale, 2010)

4.7.8 Bioreactors for SPR

As much effort and attention has been directed towards improving sensing capabilities, so too has science advanced for *in vitro* cell culture. In order to obtain a realistic representation of an islet's hormone expression, their environment during testing should mimic the native *in vivo* conditions as much as possible. Adherent monolayer culture in flasks is no longer the top standard for modelling disease. Without the extracellular matrix that provides support structure and vascularization, pancreatic islets suffer from anoikis-induced apoptosis, a process mediated by caspase enzymes [64, 135]. One strategy to enhance the survival of islets in culture is to chemically block these death signaling cascades, another is to physically provide an alternative structure to house the cells. A 3-dimensional scaffold providing attachment also facilitates cell-to-cell interaction, crucial to facilitate paracrine signaling across a network of pancreatic islets. A fascinating example of scaffolding used in a coculture heart tissue is given by Y.S. Zhang et al., who used 3D bioprinting to create a prevascularized endothelial scaffold, upon which they seeded cardiomyocytes [71]. HUVEC cells were able to populate the printed lattice, and self-organize into tube like structures, providing an excellent support platform to culture cardiac cells, strongly resembling native myocardial tissue (figure 4). The ability to form a perfused interconnected network is clearly beneficial to building a heart tissue model, as the cells need to act in cooperation to perform their physical function of beating – just as in the pancreas endocrine cells act to release their chemical signals in concerted fashion.

4.7.9 Perifusion Cell Chambers

While protocols have been widely tested and optimized for straightforward lab work, static media cell culture has its limitations. Endothelial cells, for instance, proliferate outwards from an initial seeding point, until they reach confluence or collide with another colony. If they are grown under a constant flow of media however, the cells will replicate and align themselves with the direction of fluid movement

[71]. This directional growth can be instrumental for emulating *in vivo* conditions, for instance in engineering a heart tissue construct with myocardium cells seeded onto an aligned endothelial scaffold. Having a continuous perfusion culture system not only ensures a constant supply of fresh oxygen and nutrients, but also allows for stimulating cells with potential therapeutic agents and reading the secretion response from the perfusate.

In the case of pancreatic islets, where we are interested in studying the effects of paracrine and autocrine signaling, a colony of islets can communicate by releasing endocrine factors into the fluid stream. Glucose can be added to the fluid input to stimulate the release of insulin, which is measured at the output of the perfusion culture system, by in line real-time sensing or offline with standard assays such as ELISA. The insulin response to glucose can be used to verify the functionality of pancreatic islets *in vitro*. One may then add other known secretagogues, such as IBMX, exendin-4, or screen potentially therapeutic compounds. There exist numerous approaches to applying a fluid flow across the cells' surface, for instance the microgravity flow reactor developed by NASA for instance [136-138]. Cells are cultured in a rotating cylinder, kept in a state of perpetual freefall in the moving media. This, however, represents a closed system and does not have an outlet for media analysis. For interrogating pancreatic islets, a perifusion assay can flow a stimulant through a cell trap, and into aliquots for subsequent analysis by ELISA. Each of the 96 wells in the ELISA plate then represents a different time point in the stimulation, allowing the user to reconstruct a dynamic response curve. With the development of SPRi, live output of the secretion measurement has become a possibility. This allows for a real-time readout of the hormonal components of media leaving the chamber. Towards developing inexpensive and portable systems, pumpless perfusion can be achieved using the wicking mechanism of a porous membrane attached to the chamber outlet. This allows for the removal of bulky equipment and miniaturization needed for point-of-care testing.

4.7.10 Summary

There are many adjacent fields of study which must collaborate to understand type 1 diabetes. A flow-through sensor based on surface plasmon resonance detection could offer many insights for research and in practice. The ability of an SPR sensor to detect molecular binding in real time, as the interactions occur, for an array of highly specific single molecule targets in unprecedented. Combined acquisition of data across an array of detection chambers can further be automated, with many of the surface preparation, activation and regeneration cycles able to be programmed into methods of operation. Once the steps of an assay are encoded, modern systems can retrieve, mix, and deliver fluids from a 96 or even 384 well plate into 30 or more individually addressable reaction chambers. The bottleneck to developing more advanced SPR systems is partially due to its low user base. Obtaining less expensive, more compactable SPR systems which are widely standardizable will be critical to advancing protocols and methods to their full potential.

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Chapter 5. INGAP-Peptide Variants as a Novel Therapy for Type 1 Diabetes: Effect on Human Islet Insulin Secretion and Gene Expression

Following the exploration of biosensor development in the previous chapters, focus was turned to identifying molecular agents for boosting islet function. This chapter presents the results of this drug screening study which is published in the journal of *Pharmaceutics* by MDPI. Herein a library of peptide modifications were tested for their effect on increasing the insulin secretion of living islets obtained from human donors. This work explores the use of chemical engineering for custom designing biocompatible peptides to improve islet transplant outcomes.

INGAP-Peptide Variants as a Novel Therapy for Type 1 Diabetes: Effect on Human Islet Insulin Secretion and Gene Expression

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Abstract

Islet transplantation offers a long-term cure for Type 1 Diabetes (T1D), freeing patients from daily insulin injections. Therapeutic peptides have shown potential to increase the insulin output of pancreatic islets, maximizing the impact of grafted cells. The islet neogenesis-associated protein (INGAP), and its bioactive core (INGAP-P), stimulate beta-cell function and viability, offering the possibility for islet treatment prior to implant. However, dosing efficacy is limited by low circulation time and enzyme degradation. This proof-of-concept study presents the investigation of novel molecular variants of INGAP-P to find a more bioactive form. Custom-designed peptide variants of INGAP-P were synthesized and tested for their effect on the insulin secretion and gene expression of live human islets. We exposed the live islets of five donors to varying glucose concentrations with INGAP-P variants in solution. We identified four peptide variants (I9, I15Tyr, I19 and I15Cys) which displayed statistically significant enhancements over negative controls (representing a 1.6–2.8-fold increase in stimulation index). This is the first study that has assessed these INGAP-P variants in human islets. It highlights the potential for customized peptides for type 1 diabetes therapy and provides a foundation for future peptide-screening experiments.

Keywords: islet transplantation; peptide therapeutics; insulin secretion; gene expression

5.1 Introduction

Three major umbrellas of diabetes include autoimmune mediated beta-cell-specific destruction—type 1 diabetes mellitus (T1D) mellitus, metabolic exhaustion of pancreatic endocrine cells due to acquired insulin resistance—type 2 diabetes (T2D) mellitus, and the breakdown of insulin-

producing cells by inflammatory effects of chronic exocrine pancreatic disease in type 3c diabetes (T3cD) mellitus [1–3]. Together, these forms of diabetes affect nearly half a billion people [4]. These various pathologies of the diabetic state reflect the major needs to be addressed in terms of cell replacement therapies. Islet grafts must be protected from host-immune rejection, not only to ensure insulin independence but to protect from the adverse effects of long-term immunosuppression [5]. Islets must have stable vasculature, usually provided by integration with the basement membrane via endothelial cells and pericytes [6,7]. Adequate perfusion of blood and oxygen is essential for islets' metabolism, efficient hormone delivery and paracrine communication [7]. Finally, inflammatory effects are responsible for the death of 30–50% of implanted islets within the first hours to days of the procedure [8]. Addressing these main aspects of diabetes pathologies is essential to a robust islet transplantation.

For T1D, as with other diseases, both exogenous and endogenous cell replacement therapies are available. Endocrine function can be restored by infusing islets from an outside donor (allograft), by replacing a patient's own islets following a pancreatomy due to cancer or other injury/inflammatory disease (autograft), or by regenerating the native pancreas. Regeneration can occur through replicating remaining beta cells, generation of new endocrine cells through differentiation—pluripotent pancreatic progenitor cells can be attained through dedifferentiation of acinar ductal cells, and subsequently differentiated into insulin-producing beta cells—or through α to β-cell transdifferentiation [9–12]. Indeed, the islet neogenesis-associated protein (INGAP), is a member of the RegIII family of hormones produced by the exocrine pancreas that communicate with endocrine cells via acinar/islet paracrine interactions to improve islet survival and function [2].

Insulin is one of ~80 peptide drugs currently on the market, which are known for their selectivity and low toxicity, but are limited by degradation effects and poor stability [13]. Insulin analogues have been engineered for fast- or long-acting release, which together sold $25 billion US in 2019. Injecting the incretin hormone Glucagon-like peptide-1 (GLP1) stimulates insulin production in type 2 diabetics; however, it is rapidly cleared by the kidneys in under 2 min. Exendin-4 (EX4) peptide, extracted from Gila monster venom, agonizes the GLP1 receptor while maintaining circulation over 5 h, though it leads to inflammation and pyknosis of pancreatic acinar cells in rats [14]. Further examples of synthetic peptide drugs can be found in liraglutide and dulaglutide (also GLP1 receptor agonists).

Endocrine targets comprise ~17% of the current peptide therapeutic market, and INGAP is involved with the protection and neogenesis of the native endocrine pancreas by increasing beta-cell mass and insulin secretion [13,15,16]. INGAP could lead to a peptide therapy by emulating the exocrine pancreas to protect and promote islet function, whether a native population or newly implanted cells. Along with prevascularization and immune protection of grafts, peptide therapies are among the current strategies for in vitro enhancement of islets prior to implant. The bioactive subsequence of INGAP was initially identified through modelling to be residues 104–118 [17]. The so-called INGAP-pentadecapeptide (INGAP-P), and particularly its cyclized analogues have been shown to increase β-cell proliferation in RINm5F cells [18]. The mechanism of INGAP-P's interaction with the beta cell is not completely understood. It is suspected that the beta cell surface receptor of the KiR6.2/SUR1 channels may be a ligand candidate [19]. INGAP-P has been proven safe for humans, already having reached Phase 2 clinical trials, increasing the post-transplant viability of islet allografts, but the short half-life motivates improvement of the drug [15,20,21]. INGAP-P appears to activate the Ras/Raf/ERK pathway, suggesting alteration of the mRNA

translation [21]. Since INGAP-P has been shown to increase islet proliferation after 24 h, we focused on the insulin stimulatory and gene regulation properties of the peptide, which has shown to be internalized within 30 min by rat islet RIN-m5F cells, increasing insulin secretion and affecting gene expression in neonatal rat islets [22].

This study investigates the relation between molecular conformation and insulin-promoting capability of INGAP-P on the insulin secretion of human islets. Our aim was to discover which peptide characteristics are most important for maximizing the outcomes of transplanted islets in a clinical setting. We synthesized 7 variations of the 15 amino acid INGAP-P sequence [23] and tested their ability to stimulate insulin secretion in human islets [24]. Live islets from human organ donors were subjected to basal and stimulatory concentrations of glucose, with controls or peptide variants added to the high glucose solutions. Negative control was high-glucose media with no other additives, with known insulin promoter exendin-4 (EX4) or KCl, commonly known for its ability to simulate glucose-induced insulin release, serving as a positive control [25,26]. Supernatants were quantified for insulin content by Enzyme Linked Immunosorbent Assay (ELISA). Islets were subsequently lysed to assess gene regulation of nuclear mRNA expression. A panel of islet genes implicated in the insulin secretion pathway (triggering or amplifying) were evaluated using quantitative reverse-transcription polymerase chain reaction (RT-qPCR), relative to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene [15]. Gene expression is compared to insulin secretion for each therapeutic peptide candidate across human donors. Although the preliminary sample size was limited by the availability of human donors, this pilot study provides valuable insights on the variation between human donors and sheds light on the challenges of developing synthetic peptides to improve the outcomes of islet transplant for

the treatment of T1D that can face the high variability of insulin secretion patten among individuals.

5.2 Materials and Methods

5.2.1 INGAP-P Variant Peptide Synthesis

Synthesis of custom-designed peptides is detailed in our previous report [23]. Briefly, individual amino acid residues are sequentially added to the peptide chain using an Fmoc solid-phase method first described by Garcia et al. [18]. This Fmoc solid-phase protein synthesis was done using the Focus XC peptide synthesizer from AAPPTec (Louisville, KY, USA), with Rink-Amide 4-methylbenzhydrylamine (MBHA) resin and (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate/1-Hydroxybenzotriazole (Py-BOP/HOBt) coupling reagents. Piperidine in dimethylformamide (DMF) solvent is used for deprotecting the resin of the Fmoc group, then an acetyl group is used to cap the N-terminus. Dichloromethane (CH2Cl2) is used to evaporate the solvents before cleaving the peptide from the resin. Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) was employed to purify peptide products, using a C18 stationary phase column. The polar peptide spends more time in the hydrophobic stationary phase compared to faster-moving impurities, allowing separation by fractionation. A gradient of two polar solvents made up of water with 0.4% formic acid (solvent A), and acetonitrile (solvent B) was used to ensure adequate separation. Solvent B was progressively increased from 10–50%. Final purity was confirmed by comparing the expected exact mass to the measured value via liquid chromatography-mass spectrometry (LC-MS) direct injection analysis. Peptides were kept frozen

at −80 °C before overnight lyophilization, allowing for stable storage as powders until dilution for biological testing.

5.2.2 Study Subjects

Human pancreatic islets were obtained from listed organ donors through the United Network for Organ Sharing (UNOS), with informed consent from donors prior to their death. Chosen donors were non-diabetic and confirmed COVID-19 negative. These five consenting donors, between ages of 19 and 59, had known glycated hemoglobin, BMI and cause of death. Medical history and donor condition at the time of death emphasize the highly individual nature of each test subject. Experimental protocols were approved by the McGill Institutional Review Board, with project approval until February 2022. All methods were carried out in accordance with relevant guidelines and regulations. Human pancreatic islets were obtained from organ donors through Prodolabs (Aliso Viejo, CA, USA), within 1–2 days post-isolation. Non-diabetic, COVID-19 negative human subjects were selected between the ages of 19–59 years (informed consent was obtained from all subjects). Individual donor information including BMI, Hb1Ac and cause of death are shown in Table 5.1. The supplemental information details islet culture and handling methods, conducted following the McGill Human Islet Transplant Lab.

Donor	Islet Donor Information									
	Age	Islet Size Index	Purity (%)	Islet Viability (%)	HbA1c (%)	BMI	Cause of Death	Height (in)	Weight (lbs)	Gender
1	59	0.93	90–95	95	5.0	24.5	Stroke	68	161	Female
2	55	0.79	90–95	95	5.6	28.4	Stroke	68	187	Male
3	37	1.25	90	95	5.8	23.9	Head Trauma	76	198	Male
4	26	1.18	95	95	5.9	30.3	Head Trauma	70	209	Male
5	19	1.17	90	95	5.8	23.1	Head Trauma	71	157	Male

Table 5.1 Human islet donor profiles. All subjects were non-diabetic and COVID-19 negative.

5.2.3 Human Islet Handling and Stimulation

Islets were isolated by Prodolabs (Aliso Viejo, CA, USA) from digested human pancreases at the time of death and purified from acinar tissue. The islets were shipped in Prodo Islet transport media (PIM(T)) at 4 °C and transferred to culture media at 37 °C upon arrival. Full islet recovery and handling protocols were referenced in the supplemental information. Islets were cultured for a total of 2–3 days (at 37 °C and 5% CO2), including prior to shipping, before testing. Islet stimulation was carried out within this period to avoid any loss of functionality over time.

GSIS was tested using an in vitro static-well incubation, following the protocol of the McGill Islet Transplant Laboratory (full details in supporting information). Groups were tested in triplicate, with ~150 islet equivalents (IEQ) in each. Cell counts were verified using Quant-iT PicoGreen DNA Quantification kit, normalizing to 10.4 ng of DNA/islet equivalent on average.

Briefly, islets were taken from culture media and resuspended in 2.8 mM glucose Krebs buffer (preparation described in supporting information) to ensure basal insulin secretion rate after removal from 5.8 mM glucose culture medium. Islets remained in the low-glucose solution for 30 mins to stabilize prior to testing. Using 8 μm pore cell culture inserts (VWR), islets were transferred to a fresh well also containing 2.8 mM glucose solution and incubated for 1 h at 37 °C. Next, islets were moved into the stimulation well, with 28 mM glucose Krebs, plus peptide variants or controls. As a negative control we used 28 mM glucose Krebs with no other additives. Positive control was 28 mM glucose with 1 μg/mL EX4, or KCl. Exendin-4 is known to be a slow-acting GLP-1 agonist and insulinotropic factor [25,27,28]. Test groups consisted of 7 different variations upon the INGAP-P 15 amino acid sequence. Islets are then recovered from the inserts and lysed

or frozen at −80 °C for later qPCR analysis. Insulin was quantified using Mercodia Human ELISA (Cedarlane), within 24 h of sampling. After initial testing, 5-fold sample dilutions were used, plated in duplicate. Optical absorbance was measured at 450 nm with an i3 SpectraMax plate reader (Molecular Devices, San Jose, CA, USA). Aliquots of the supernatant were sampled at the beginning (t = 0) and end (t = 1 h) of the incubations. These two readings were subtracted to find the insulin released during a 1-h incubation. To calculate the stimulation index, the insulin concentration in the stimulation well was divided by that in the low-glucose buffer.

5.2.4 Live Cell Viability Imaging

Live/dead staining for fluorescence imaging was done using the Biotium Viability/Cytoxicity Assay Kit (for Animal, obtained from VWR). The green live dye consisted of an elasterase substrate, which was cleaved into fluorescent calcein, and only remained if the cell membrane was intact. The red dead dye was Ethidium Homodimer—III (EthD-III), a membrane-impermeable DNA dye, only penetrating cells when the cell wall was compromised. Islets were prepared for imaging first by resuspension in serum-free media (here: PBS). Staining media was 2.5 µL calcein AM (hydrolyzed, pH 8) and 10 µL EthD-III in PBS (5 mL total volume). Islets immersed in staining media were shielded from light and incubated at 37 °C for 30 m before imaging. Images were taken using the LSM 710 Confocal Scanning Microscope (excitation wavelengths: 488 nm for live and 543 nm for dead). Image reconstruction and analysis was performed using the Zen Microscope Software by Zeiss.

5.2.5 RT-qPCR Quantification of Islet Gene Expression

Following glucose-stimulated insulin secretion (GSIS), cells were lysed for gene analysis. Nuclear mRNA was extracted and purified using the PureLink RNA Mini Kit (Invitrogen by Thermo Fisher

Scientific, Mississauga, Ontario, CA) protocol, verified by NanoDrop quantification. Gel electrophoresis confirmed extracted mRNA quality. Purified RNA samples were kept at −80 °C until RT-qPCR with the Luna OneStep reaction kit. mRNA was reverse transcribed into cDNA, then amplified by thermal cycling. Although glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is commonly used as a reference gene, some variation may occur among different human donors [29]. Therefore, target gene expression was quantified relative to the housekeeping gene GAPDH within each group, and further normalized against the negative control (high-glucose stimulation with no peptides added). Table 5.2 shows selected islet genes related to the insulin detection, metabolism and secretion pathway of β-cells.

Gene	Function	Forward Primer Sequence	Reverse Primer Sequence
Insulin	Encodes preproinsulin	GAA-CGA-GGC-TTC-TTC-TAC-AC	ACA-ATG-CCA-CGC-TTC-TG
Glucagon	Encodes preproglucagon	ACC-AGA-AGA-CAG-CAG-AAA-TG	GAA-TGT-GCC-CTG-TGA-ATG
SUR1	Membrane protein; target of antidiabetic drugs	CGA-TGC-CAT-CAT CAC-AGA-AG	CTG-AGC-AGC-TTC-TCT-GGC-TT
GLUT2	Transmembrane carrier protein	CTC-TCC-TTG-CTC-CTC-CTC-CT	TTG-GGA-GTC-CTG-TCA-ATT-CC
PDX1	Insulin promoter factor 1	ATG-GAT-GAA-GTC-TAC-CAA-AGC	CGT-GAG-ATG-TAC-TTG-TTG-AAT-AG
GAPDH	Catalyzes glycolysis, can activate transcription	CAC-CCA-CTC-CTC-CAC-CTT-TG	CCA-CCA-CCC-TGT-TGC-TGT-AG

Table 5.2 mRNA primer sequences used for qPCR investigation of gene regulation related to the insulin secretion pathway of human pancreatic islets.

5.2.6 Data Analysis

All islet groups were tested in triplicate (n = 3). Insulin secretion data were presented as mean ± SD, and One-Way ANOVA was applied for significance.

Relative gene expression for the 5 chosen genes was normalized relative to the house-keeping gene of that group, then each group was normalized against the negative control. The relative expression factor, R, was calculated in this way, by the Livak-Schmittgen method: $R = 2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_{T(test)} - \Delta C_{T(calibrator)}$, with ΔC_T for each gene calculated in reference to the GAPDH housekeeping gene. The calibrator test group was our negative control, a high glucose (28 mM) stimulation well, with no other peptides added.

5.3 Results and Discussion

5.3.1 INGAP-P Variant Synthesis and Characterization

Table 5.3 shows modified peptides tested for enhancing insulin secretion, based on variations of the 15 amino acid sequence of INGAP-P. Following synthesis (experimental methods Section 5.2.1), peptides were purified by HPLC to ≥95%. Peptide identification was done by comparing exact expected mass to charge ratios (ex.: tyrosine-modified INGAP-P in figure 6.1A) to those measured by LC-MS in figure 5.1B (also supporting information for mass spectroscopy and for LC-MS analyses).

Peptide ID	Variant	Sequence	Interest
I15	INGAP-P	N'-Ile-Gly-Leu-His-Asp-Pro-Ser-His-Gly-Thr-Leu-Pro-Asn-Gly-Ser-C'	INGAP's bioactive region (already proven)
I6	INGAP-P conserved motif	N'-Ile-Gly-Leu-His-Asp-Pro-C	Synergistic effect with I9 conserved motif
I9	INGAP-P specific motif	N'-Ser-His-Gly-Thr-Leu-Pro-Asn-Gly-Ser-C'	Synergistic effect with I6 specific motif
I15Cys	Cyclic INGAP-P	N'-Ile-Gly-Leu-His-Asp-Pro-Ser-His-Gly-Thr-Leu-Pro-Asn-Gly-Ser-Cys-C'	Efficiency of the cyclization method (with cysteines)

Peptide ID	Variant	Sequence	Interest
I15Gly	Modified INGAP-P (C-terminal)	N'-Ile-Gly-Leu-His-Asp-Pro-Ser-His-Gly-Thr-Leu-Pro-Asn-Gly-Ser-Gly-C'	Effect of glycine at the C-terminal amino acid on the ligand/receptor mechanism
I15Tyr	Modified INGAP-P (C-terminal)	N'-Ile-Gly-Leu-His-Asp-Pro-Ser-His-Gly-Thr-Leu-Pro-Asn-Gly-Ser-Tyr-C'	Effect of tyrosine at the C-terminal amino acid on the ligand/receptor mechanism
I19	Modified INGAP-P	N'-Cys-Cys-Ile_Gly-Leu-His-Asp-Pro-Ser-His-Gly-Thr-Leu-Pro-Asn-Gly-Ser—Cys-Cys-C'	Effect of making a longer peptide with hydrophobic amino acids
I19Cys	Cyclic modified INGAP-P, longer peptide	N'-Cys-Cys-Ile-Gly-Leu-His-Asp-Pro-Ser-His-Gly-Thr-Leu-Pro-Asn-Gly-Ser—Cys-Cys-C'	Efficiency of the cyclization method, combination of longer peptide (19) and cyclization

Table 5.3 Custom designed and synthesized INGAP-P variants for testing on islet insulin secretion with shorthand ID.

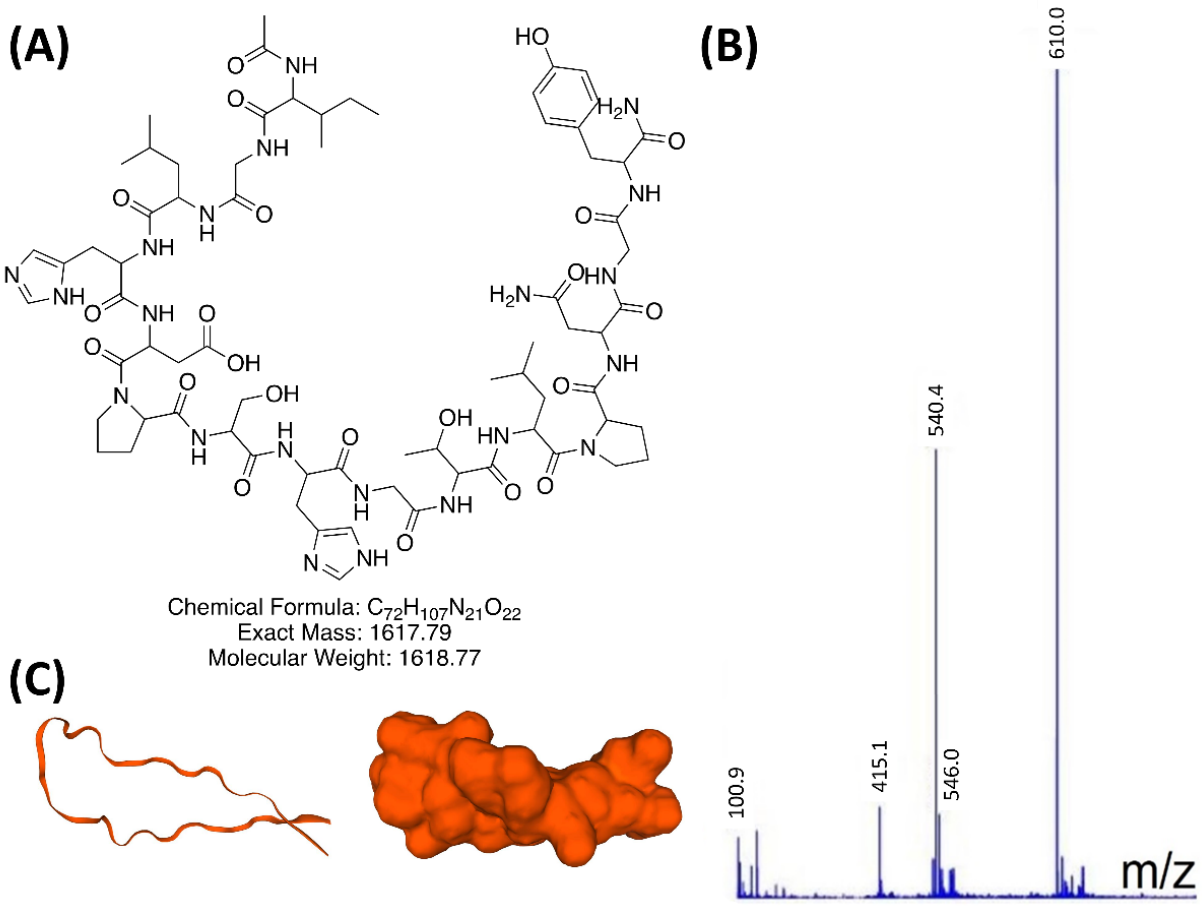

Figure 5.1 (A) I15Tyr, the INGAP-P sequence modified with tyrosine replacing the serine residue at the C-terminal position. (B) LC-MS measured mass to charge ratio of 810.0 kg/C, matching the expected value within 0.09%. Table 5.1 displays the variations made to INGAP-P ("I15") for testing. (C) 3D simulation of conformational folding using the INGAP-P sequence as input. (From SwissModel online tools https://swissmodel.expasy.org/interactive/H396J7/models/, accessed on 1 July, 2021).

Results showed m/z ratios accurate to within 0.09%. Cyclization by connecting the peptide's N- and C-terminals was shown to greatly improve stability and extend the circulating half-life [13]. Two cyclic modifications to the INGAP-P sequences were prepared in I15Cys and I19Cys. Using SwissModel's 3D protein folding simulator, images in figure 5.1C were created from the I15Tyr sequence. Simulations revealed the protein sequence as an F1 capsule/anchoring protein. Conserved hydrophobic clusters on the surface of the CAF1A usher C-terminal domain were important for antigen assembly. From the visual representation in figure 5.1C, we can see a twisting ribbon-like conformation, similar to the helical structures of GLP1 and exendin-4, combined with a self-intersecting loop shape which may contribute to higher stability.

5.3.2. Islet Culture and Peptide-Stimulated Insulin Secretion

Figure 5.2 shows confocal fluorescence imaging of intact islets from the youngest donor prior to glucose challenges. Live cell imaging revealed good islet morphology and viability following transport, with very few dead cells throughout each layer of the 20 image Z-stack. Staining and imaging procedures are detailed in experimental methods Section 5.2.4.

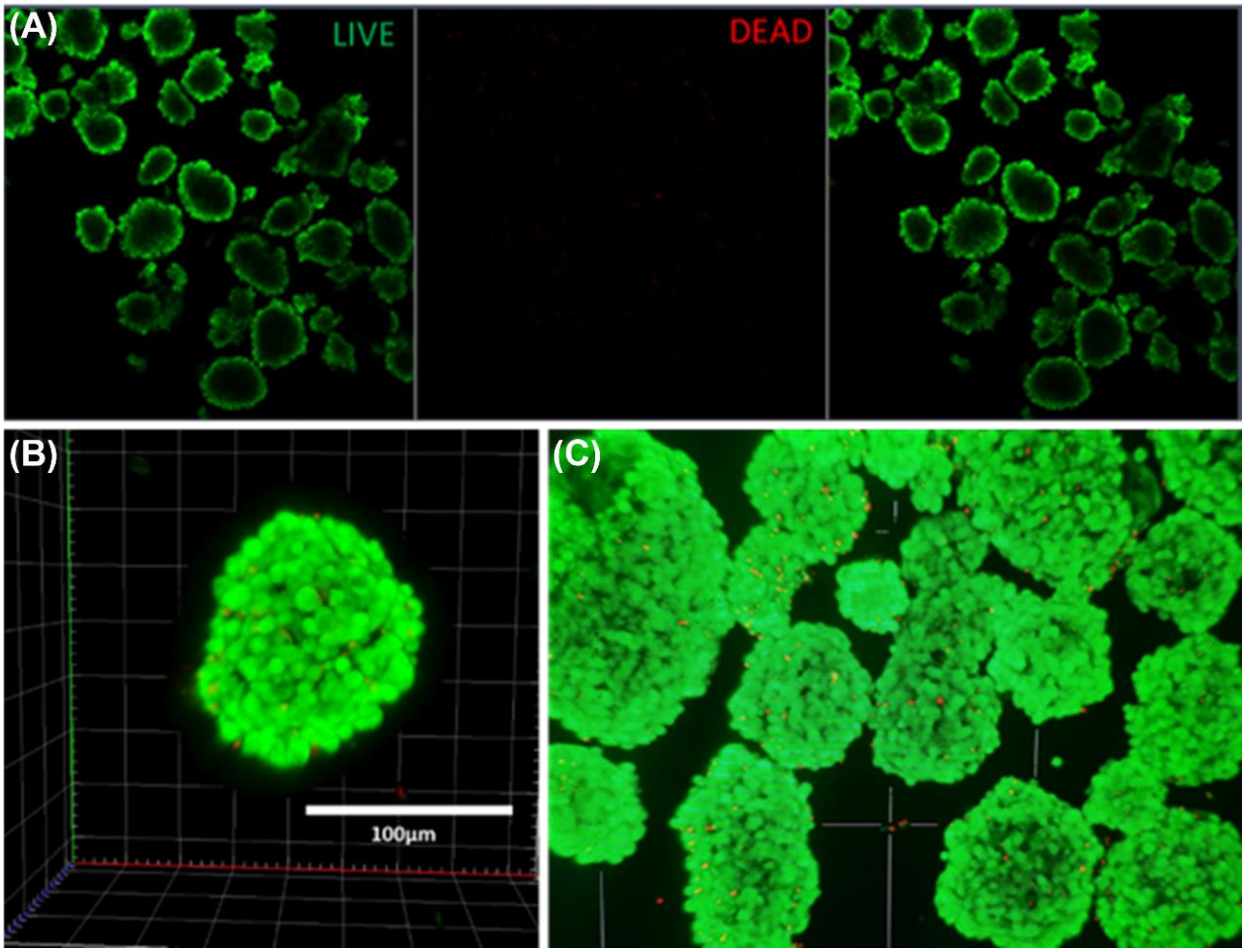

Figure 5.2. Confocal Live (Green: Calcein) and Dead (Red: EthD-III) fluorescence image of human pancreatic islets. (A) Single focal plane Live, Dead and Combined images, (B) 3D reconstruction of 20-image Z-stack shows single cell viability throughout a single islet, and (C) Consistent viability across a population of islets of various sizes, with small single dead cells on the surface of and detached from living islets.

Figure 5.3 shows the insulin concentration of supernatant sampled from basal and stimulation wells, shown as mean ± SD, quantified by ELISA (n = 3). Donors 1–3 were subjected to the complete panel of peptide variants, whereas 4 and 5 focused on smaller subsets. Donors 2 and 4

(age 55 and 26) are presented at scale for comparison to other donors, with inset graphs showing zoomed in data plots. Donor 3, age 37, produced an impressive insulin response across all peptide groups, compared to the other donors. The average concentration of insulin in stimulation wells for this donor was 36.4 ng/mL. On the other hand, islets from donor 4, age 26, were not secreting at an appreciable rate. This donor was exposed to a subset of the groups to focus on candidate peptide variants.

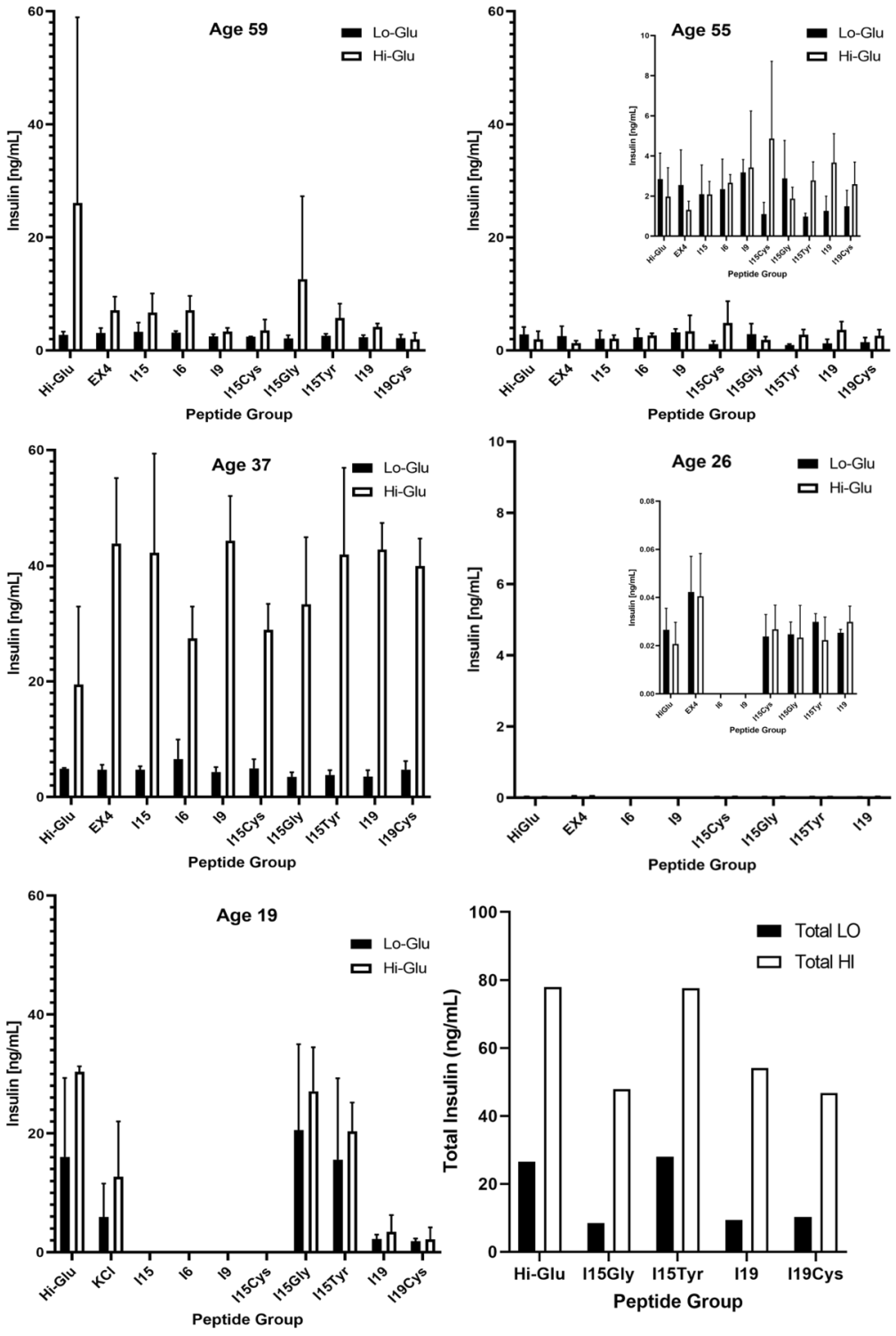

Figure 5.3. ELISA quantification of islet insulin release in low- and high-glucose wells, for each peptide variant. Each group was tested in triplicate. Final panel (bottom right) displays total peptide response for all donors.

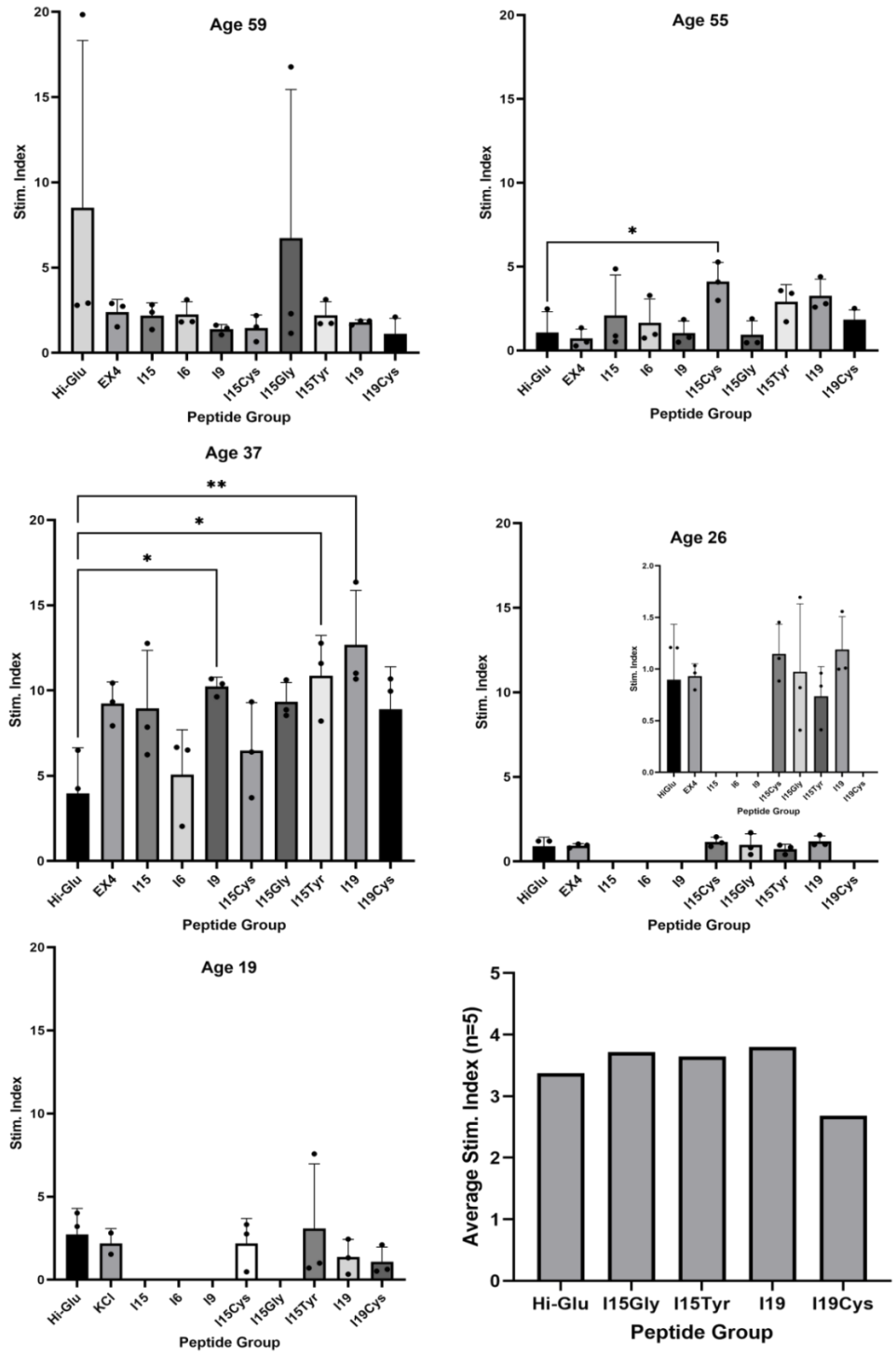

Figure 5.4. Stimulation index shows fold-increase in insulin from the low-glucose to high-glucose wells (individual samples appearing as black dots over the columns, "•"). One-way ANOVA was used to compare the stimulation index of each group to the negative control, "Hi-Glu" (significant differences between means are shown by "*", two standard deviations from the mean as "**", $p < 0.05$). Final panel (bottom right) displays total stimulation index for all donors.

Figure 5.4 displays the stimulation index, calculated as the ratio of insulin in the high-glucose to that in the low-glucose buffer wells, facilitating a comparison of functionality between groups. One-way ANOVA was applied between each peptide group and the negative control. In addition to the higher concentrations of insulin released, donor 3 also displayed the highest fold-change of insulin in response to stimuli. Here, the INGAP-P (I15) peptide displayed an insulin-boosting effect similar to EX4, compared to negative control. Significant improvements were seen for I15Cys (donor 2), and for I9, I15Tyr and I19 (donor 3). The last panel shows the combined stimulation index for all donors across peptide groups.

High glucose islet stimulation has been shown to result in AMPK inhibition in rats, mice and humans [30]. With recent advances in research, we are still only now learning what human rodent islets have specifically in common, and when animal findings can be translated to human applications [31]. Exposing human islets to 16.7 mM glucose-elevated thrombospondin-1 gene expression while decreasing vascular endothelial growth factor (VEGF) mRNA by 20% [32]. Human insulin ELISA quantification for the 5 donors is shown in figure 5.3. Donor 2 showed a statistically significant increase in output to I15Cys, and donor 3 had significant enhancement of insulin for I9, I15Tyr, and I19, compared to controls. Other donors had too much variation to identify beneficial peptides. Noteworthy is that two of the younger donors (3 and 5), at ages 37 and 19, respectively, displayed a heightened insulin production in response to all groups, compared to other donors. It is possible the observed increased insulin production of the third and fifth donors was at least a partial consequence of their age at the time of death. Donor 4 (age 26), however, showed little stimulation response, secreting much less insulin than other donors. With a BMI of 30.3, this donor would be considered obese.

From donor information in table 5.1, the cause of death for donors 3 and 5 being head trauma suggests that these islets may have been in better physiological condition at the time of isolation, reflected in lower BMI and higher islet size index. In contrast, the older donors 1 and 2 died from stroke. Although interruption of blood flow leads to a hypoxic environment within the brain, this does not drastically affect islet isolation and purification from acinar tissue as much as the cold ischemia time. Islet purity—one of the most critical factors in transplant—was consistent across all donors, showing oxygen conditions in the pancreas were not directly influenced, as reported by the isolation team.

Preliminary perifusion tests of human islets using known secretagogues displayed very much similar release profiles, despite varying degrees of insulin released (figure S6.1). Based on these initial observations, it was decided first to focus on quantifying the amount of insulin released during the primary response as a gauge for evaluation. Future works may utilize surface plasmon resonance biosensing or other such kinetic assays to elucidate the dynamics of peptide stimulated insulin release.

5.3.3 RT-qPCR Analysis of Islet Nuclear mRNA

Following islet lysis and nuclear RNA extraction and purification, PCR plates were prepared for amplification by mixing RNA with islet-specific primer sequences to quantify the relative gene expression of each group, in response to stimuli. Purity and quality of RNA was verified by Nanodrop quantification, yielding a 260/280 ratio of 2.09. Gel electrophoresis showed the presence of 28S and 18S RNA, shown in figure S6.2. Forward and reverse primers for qPCR were combined and tested for amplification, shown in table 5.2. Primer and target RNA amplifications were

validated using 1, 5 and 10-fold dilutions (figure S6.3). A linear slope of C_q vs log of the starting amount verifies the PCR reaction.

Due to scarcity of human islets for research coupled with a lack of islet cell lines, there is relatively little known of islet gene regulation in response to glucose stimulation [28]. Figure 5.5 shows expression levels for the panel of insulin secretion pathway genes for each peptide group, for the 5 donors tested. Quantified gene expression levels were found using the Livak-Schmittgen method relative first to the GAPDH housekeeping gene and then to high-glucose negative control (no peptides added). The colour bar legend on the right of figure 5.5 provides the scale of values, with red representing a downregulation, white being unaffected and blue the most upregulated. Donors 1 and 2 showed substantial downregulation of most genes in response to the conserved INGAP-P sequence (I6), and the specific motif (I9). We can see the peptide group for donor 2 having the highest stimulation index (I15Cys), generally upregulated mRNA expression among all islet genes on the target panel, except glucose transporter 2 (Glut2, "G2", also known as SLC2A2). The donor who displayed the most upregulation across all groups was 3, also releasing the most insulin in GSIS experiments than the other subjects. Donor 3 showed higher relative pancreatic-duodenal homeobox factor-1 (PDX1) expression across all peptide groups as compared to other donors. Generally unaffected, PDX1 was upregulated in response to the I15Gly peptide variant. In contrast, donors 1 and 2 showed downregulation of PDX1 in response to multiple groups. Looking at the gene expression of 5 donors, it appears that INS and PDX1 were the most stable across peptide groups, whereas Glucagon, SUR1 and Glut2 were more affected. I15Gly for donor 3 appears to be most upregulated among all groups, though it did not produce a noticeable difference in secretion of insulin. Donor 3 showed the most upregulation of islet genes related to the insulin

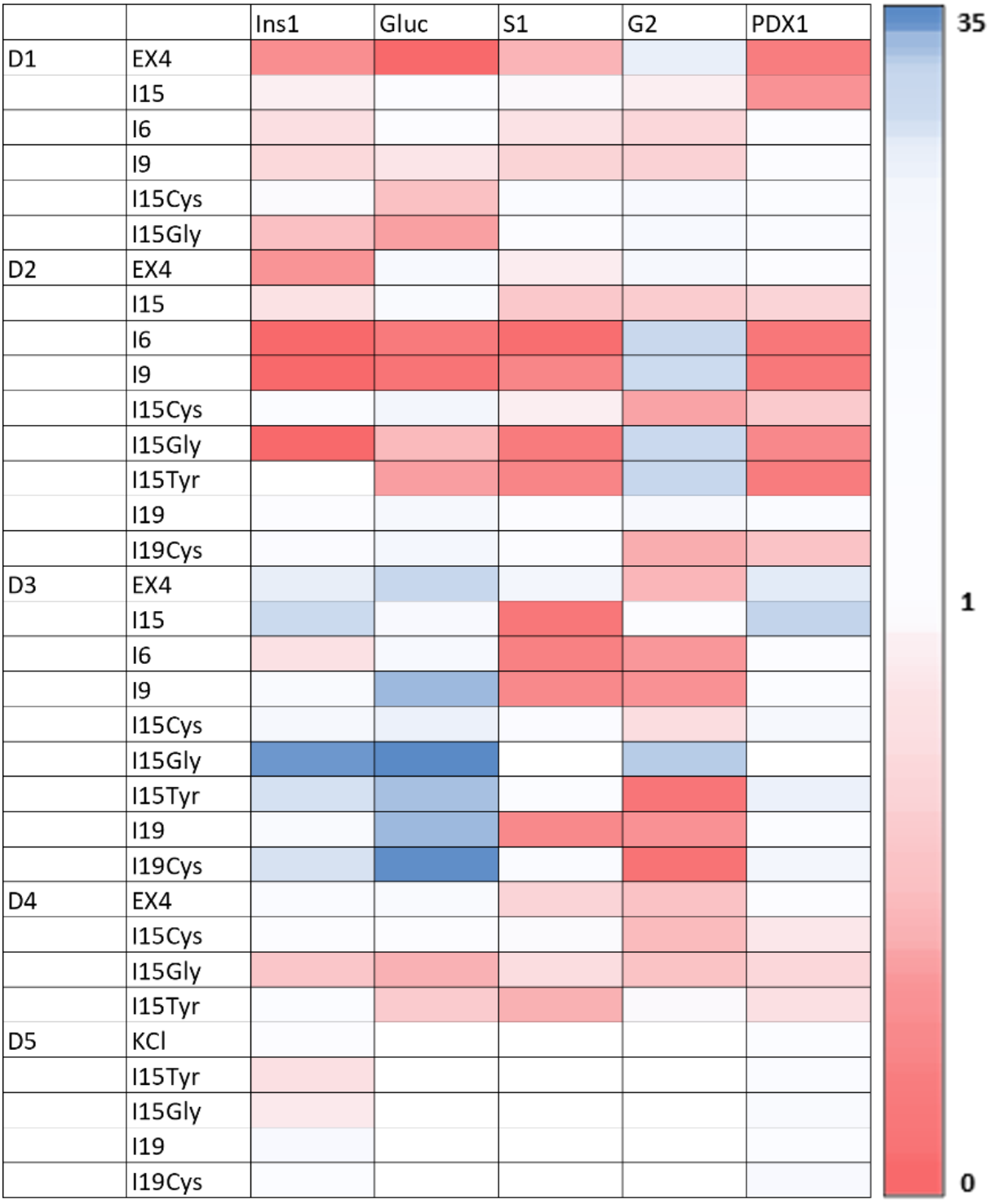

Figure 5.5. Heatmaps of islet gene expression. Bars are computed using the ddC_T method, with the y-axis representing $R=2^{(-\Delta\Delta C_T)}$. Side legend shows the colour scale, with blue indicating upregulation, and red downregulation.

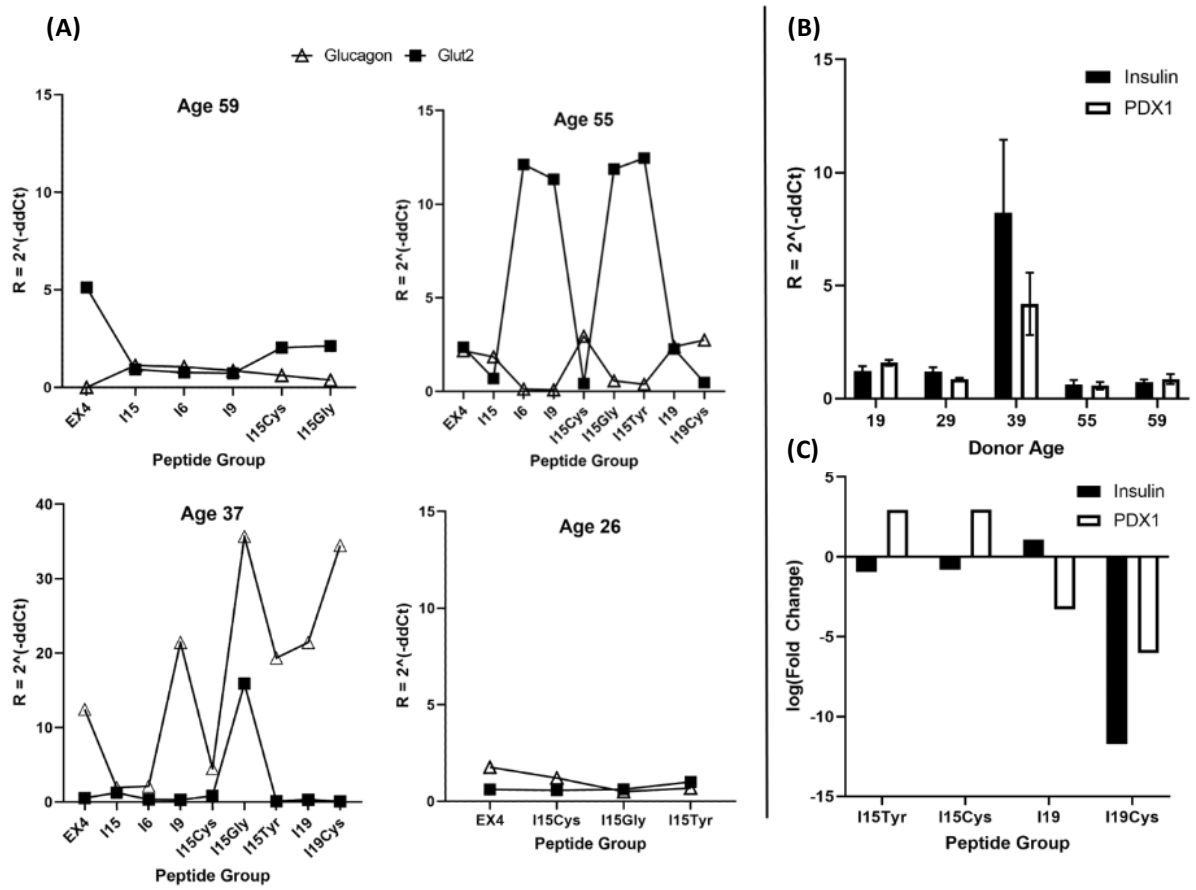

Figure 5.6. Panel (A) Expression of Glucagon (Δ) and Glut2 (■) genes, quantified relative to GAPDH, across peptide groups. These two genes appear to activate in opposition, though donor 3 (age 37) showed simultaneous activation of Glucagon and Glut2, along with producing the most insulin overall in ELISA tests. (B) Gene expression quantified relative to GAPDH. (C) Fold change for insulin and PDX1 expression at 1 μg/mL vs 100 μg/mL peptide concentration. Dose-response gene expression tested on donor 5 (age 19).

secretion pathway and showed remarkably higher insulin in response to all peptides, compared to other donors.

Figure 5.6 panel A shows the expression of Glut2 and Glucagon genes quantified relative to GAPDH. Donor 5 (age 19) is excluded from this figure since those islets were used for a dose-response study of INS and PDX1 genes (figure 5.6C). Glucagon acts to raise blood glucose by

stimulating glycogenolysis and gluconeogenesis, whereas Glut2 acts as a glucose sensor and to uptake glucose into the β-cell, initiating the insulin pathway levels [33,34]. By observing the scatter plots of gene expression in figure 5.6A, we see that 3 out of 4 donors did indeed exhibit a reciprocal action of these two genes. Donor 3 (age 37), however, remarkably displayed heightened glucagon expression simultaneous to Glut2. ELISA data shown in figures 5.3 and 5.4 show this same donor secreted the most insulin overall and produced the highest fold change in response to stimuli compared to other donors.

Figure 5.6B shows the expression of INS and PDX1 genes, averaged across all peptide groups for the 5 donors investigated. An increase in age relates to a corresponding increase in DNA methylation at multiple sites, affecting INS and PDX1 gene regulation [35]. In fact, aging has been connected with increased methylation of human pancreatic islets, resulting in decreased expression of INS and PDX1 genes and greater loss of β-cells with earlier diabetes onset [35,36]. The younger donors exhibited a slightly elevated expression of both INS and PDX1 genes, drastically so in the case of donor 3. Insulin-like growth factor-1 (IGF1) serum concentration reaches islets maximum during puberty and falls off roughly threefold by age 60 [37]. With age differences between donors up to 35 years, this represents a significant factor in the condition and functionality of the donors' pancreatic islets. As age has been negatively correlated with islet GSIS, lower insulin secretion may result from decreased ability to regenerate β-cell mass [38]. Changes in glucose concentration alone have been shown not to affect the human islet PDX1 gene, therefore observed regulatory changes are expected to result from exposure to INGAP-derivatives, or differences between individual donors. Although donor 4 was also younger in comparison to 1 and 2, the overall insulin response was much lower across all groups. Heterogeneity among islet donors highlights the need

for large sample sizes to accurately predict how therapeutic compounds may affect the diabetic population.

Figure 5.6C shows a mini study of dose-response for 4 peptide variants (I15Tyr, I15Cys, I19 and I19Cys) on INS and PDX1 gene expression. This test was comprised of exposing islet groups to the same peptide at either 1 or 100 µg/mL, as well as negative control. Data shown in the bar graph are the logarithm of fold change in gene expression from 1–100 µg/mL peptide exposure. I15Tyr and I15Cys show similar responses in that increasing peptide concentrations raised PDX1 and lowered insulin expression levels. Both I19 variants showed a steep decrease in PDX1, though they differed in their INS gene responses.

5.3.4 Challenges and Limitations

The primary limitation with identifying therapeutic compounds for T1D therapy, as in this study, is the need for extensive in vitro analysis and in vivo transplantation models. Due to the steps involved with synthesis and initial screening evaluation, along with the number of compounds investigated, the authors found it necessary and reasonable to first identify agents of interest to qualify for in-depth analysis.

Without a clear consensus on the characteristics of rodent cells that can be directly translated to human, we focused on studying primary human islets from organ donors. Difficulties involved with this methodology include the sporadic availability of islets, time of shipping and necessity to place islets into transport media, slowing their metabolism. Once the cells recover, stimulation must be carried out within 1 week to ensure maximal viability and functionality. Most importantly, each individual donor has their own medical history and unique physiology at the time of death,

introducing variance from one donor to another. In addition, analysing nuclear lysate for gene expression precludes normalization by cell counting with DNA quantification. Other non-β islet cells would contribute mRNA following lysis, leading to uncertainty of the regulation of insulin secretion related genes. This could be addressed by using immortalized cell lines or separating islet cells to specifically interrogate β-cells. Nonetheless, the overall islet response is a meaningful subject of study in terms of blood glucose regulation.

Consequently, the results presented here are significantly relevant, though initial sample size (n = 5) was limited by availability of suitable donors. The standard deviation in this experiment can be used to find how many subjects should be studied to discern the best of 10 groups tested. To lower the required sample group size, we recommend focusing on a subgroup (3 or 4) of the peptides of interest. Based on power calculations using experimental ELISA data, we offer the sample size estimation of n = 33. This was found with an online calculator (http://powerandsamplesize.com/Calculators/Compare-k-Means/1-Way-ANOVA-Pairwise-1-Sided, accessed on 1 July 2022), using the equation:

$$n_A = \left(\sigma_A^2 + \sigma_{\frac{\beta}{K}}^2\right)\left(\frac{Z_{1-\beta\backslash\tau}+Z_{1-\beta}}{\mu_A-\mu_\beta}\right)^2 \quad (1)$$

where: $\kappa = n_A/n_B$ is the matching ratio, σ is the standard deviation, σ_A is the standard deviation of Group "A", σ_B is the standard deviation of Group "B", α is Type I error, τ is the number of comparisons to be made, and β is Type II error (1-β is the power: 80%). This pilot study can therefore be used to guide future experiment design in choosing peptide candidates and human donor sample sizes.

5.4 Conclusions

Personalized medicine can be made possible through comprehensive diagnostic evaluation accompanied by a bank of knowledge regarding various therapeutic agents and their target effects. Although the preliminary sample size was limited by the availability of human donors, this work demonstrates the ability of INGAP-P to boost insulin secretion, and identifies several variants for further investigation, based on their ability to impact islet signaling genes. In addition, this pilot study provides valuable insights on the variation between human donors and sheds light on the challenges of developing synthetic peptides to improve the outcomes of islet transplant for the treatment of T1D that can face the high variability of insulin secretion patterns among individuals. However, there remains much to learn about the potential benefits for therapeutic peptides and their synthetic variants. In particular, the individual differences between human islet donors coupled with the shortage of native cells necessitate platforms for efficient mapping of regulatory effects of libraries of compounds. Future screening of therapeutic compounds could be conducted on immortalized human insulin producing cell lines (such as EndoC-βH1) in order to reduce variability. The varying reactions each donor displayed to the same media incubations also motivate individually tailored treatment plans. Further testing, aided by high-throughput kinetic-based assays such as surface plasmon resonance imaging would help to characterize peptides such as INGAP-P derivatives for medicinal benefits to reduce the need for insulin injections and improve islet transplant efficacy.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S6.1: Glucose stimulation, Figure S6.2: Gel Electrophoresis; Figure S6.3: qPCR Validation.

Author Contributions: J.M.P. and F.R.C. conducted the islet stimulation experiment; L.G. performed peptide synthesis and characterization under the supervision of A.C.; J.M.P. performed RT-qPCR gene expression experiments, statistical analysis and wrote the manuscript with the help of L.G.; M.T. supervised the project and actively participated in scientific discussion, analysing data, writing, and editing the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study was conducted in accordance with the Institutional Review Board of McGill University's Faculty of Medicine and Health Sciences (IRB#: A02-E28-12B).

Informed Consent Statement: Informed consent was obtained from all subjects in the study. Written consent has been obtained to publish this paper

Data Availability Statement: The data presented in this study are available upon request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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Chapter 6. Renewable Human Cell Model for Type 1 Diabetes Research: EndoC-βH5/HUVEC Coculture Spheroids

This article addresses the difficulties and limitations which became apparent in the previous work, specifically donor-to-donor variation and scarcity of human islets function for in vitro testing. This article has been submitted and is currently under review for publication in the journal *Scientific Reports* by Nature. The work described hereafter covers the development of a pseudoislet constructed by coculturing immortal insulin producing cells with structural and perfusion supporting endothelial cells. Establishing a stable model for cellular research is yet one of the critical missing aspects of islet transplant which this article aims to address.

Renewable Human Cell Model for Type 1 Diabetes Research: EndoC-βH5/HUVEC Coculture Spheroids

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Abstract

In vitro drug screening for type 1 diabetes therapies has largely been conducted on human organ donor islets for proof of efficacy. While native islets are the ultimate target of these drugs (either *in situ* or for transplantation), significant benefit can be difficult to ascertain due to the highly heterogeneous nature of individual donors and the overall scarcity of human islets for research. We present an *in vitro* coculture model based on immortalized insulin producing beta-cell lines with human endothelial cells in 3D spheroids that aims to recapitulate the islet morphology in an effort towards developing a standardized cell model for *in vitro* diabetes research. Human insulin producing immortalized Endo-βH5 cells are cocultured with human endothelial cells in varying ratios to evaluate 3D cell culture models for type 1 diabetes research. Insulin secretion, metabolic activity, live cell fluorescence staining and gene expression assays were used to compare the viability and functionality of spheroids composed of 100% beta-cells, 1:1 beta-cell/endothelial, and 1:3 beta-cell/endothelial. Monoculture and βH5/HUVEC cocultures formed compact spheroids within 7 days, with average diameter ~140 μm. This pilot study indicated that stimulated insulin release from 0-20 mM glucose increased from ~8-fold for monoculture and 1:1 coculture spheroids to over 20-fold for 1:3 EndoC-βH5:HUVEC spheroids. Metabolic activity was also ~12% higher in the 1:3 EndoC-βH5:HUVEC group compared to other groups. Stimulating monoculture beta-cell spheroids with 20 mM glucose + 1μg/mL glycine-modified INGAP-P increased the insulin stimulation index ~2-fold compared to glucose alone. Considering their availability and consistent phenotype, EndoC-βH5 based spheroids present a useful 3D cell model for in vitro testing and drug screening applications.

Keywords: 3D cell culture, in vitro diabetes research, renewable cell transplants

Nomenclature

BMI	body mass index
BSA	bovine serum albumin
EDTA	ethylenediaminetetraacetic acid
GSIS	glucose stimulated insulin secretion
HUVEC	human umbilical vein endothelial cell
IBMIR	instant blood mediated inflammation response
IBMX	3-isobutyl-1-methylxanthine
IEQ	islet equivalent
INGAP-P	islet neogenesis associated protein – pentadecapeptide
T1D	type 1 diabetes
TBS	tris-buffered saline

6.1 Introduction

Human islet transplantation is a minimally invasive procedure which offers the hope for a one-time curative treatment for type 1 diabetes. Harvesting functional insulin-producing cells from

recently deceased donors provides a replacement strategy for beta-cells lost to autoimmune destruction. While a promising treatment option, long-term success of islet transplants could be improved, with recipients who maintain stable blood glycemia dropping from 87.5% to 71% after one year, with significant decline thereafter [1, 2]. The most direct threats to successful islet transplantation are the instant blood mediated inflammatory response (IBMIR) and hypoxia due to difficulty establishing sufficient vascular connectivity [3]. The importance of mitigating immune rejection by donor/recipient blood type matching further complicates the existing shortage of donor cells. Lifelong immunosuppressive medication is administered after transplant, which increases the risk of infection and burdens the medical system.

Accessory cells, such as endothelial, mesenchymal and other non-beta islet cells can benefit insulin secretion and vascular connectivity [4]. Culturing cells as 3D-dimensional 'spheroids' provides an avenue for *in vitro* functionality testing and drug screening. Due to limited donors, priority for successful isolations is given to transplantation, creating a severe shortage of primary islets for research. Islet-like cell clusters recapitulate native morphology and insulin production, providing freedom from timing of availability and number of biological replicates. Forming spheroids with fewer cell types reduces background noise in cellular crosstalk investigations. Incorporating accessory cells in models supports beta-cells grafts, by secreting performance-enhancing proteins [5, 6]. Recently, human umbilical vein endothelial cells (HUVECs) have been shown to improve vascularization and nutrient distribution when co-transplanted with islets [7-9]. Endothelial cells improve beta-cell survival by expressing basement membrane proteins and send paracrine signals through the bloodstream, playing a critical role in supporting insulin gene expression and glucose stimulated insulin secretion (GSIS) [10, 11]. Protein-secreting accessory cells may therefore be superior to adding molecular factors themselves [12]. In the future,

autologous endothelial cells could help reduce the foreign body immune response [13]. Endothelial cells regulate immune mediators, acting to protect the glucose sensing mechanism and control permeability on the outer boundaries of islets [14]. Factors such as INGAP-P, exendin-4 or IBMX promote insulin secretion, maximizing the effect of smaller beta-cell populations [15-17]. Identifying and therapeutic compounds relies on stimulating human islets, posing challenges due to their short *in vitro* lifetimes, lack of proliferation, and significant donor-to-donor variation [18].

The scarcity of human islets for research coupled with functional heterogeneity between isolations make drug screening costly and inefficient. Here, we present a 3D cell model to facilitate *in vitro* screening for type 1 diabetes (T1D) research. We first validate the spheroid formation protocol using the proliferative mouse insulinoma MIN6 line, then focus on coculturing HUVECs with the non-proliferative EndoC-βH5 human beta-cell line. The MIN6 line has been shown to increase the insulin response to glucose 2-fold in 3D spheroids compared to monolayer culture, providing a renewable *in vitro* model for animal islet testing [19]. EndoC-βH5 is the latest development of Human Cell Design, who first pioneered the immortalized human EndoC-βH1 cell line. While the insulin release of EndoC-βH1 cells can be suppressed using mannoheptulose, continued proliferation would present issues in terms of graft stability and hypoglycemia [20, 21]. To stop replication while promoting maturation, the EndoC-βH5 line has had immortality reversed, which can be accomplished by excision of hTERT and SV40LT, or even temporary inactivation of SV40LT mRNA [22]. EndoC-βH1 cells have shown improved function when combined with supporting cells in spheroids [17, 20, 23]. The non-replicating, newer generation EndoC-βH5 line, showing a higher response to glucose and insulin secretagogues has also recently been validated for drug screening applications [24]. Cocultured spheroids with interspersed endothelial cells have increased cell-cell communication compared to layered shell structures [25]. In this study, we

explore the impact of varying ratios of low-passage HUVECs to non-proliferative EndoC-βH5 cells in 3D spheroids, aiming to establish a robust model for T1D research. This comparative study evaluates 'pseudo-islet' spheroid groups by GSIS, gene expression, metabolic activity, and immunofluorescence.

6.2 Methods and Materials

6.2.1 Cell Culture

Human umbilical vein endothelial cells (HUVECs) purchased from Lonza (Cedarlane Labs, ON, Canada) were thawed and seeded in T75 tissue culture flasks at a density of 300,000 cells per flask. Supplier prescreening verified HUVECs showed TrypanBlue viability of 85-90% and 98% CD31/CD105 double positive. HUVECs were cultured in EGM-2 Endothelial Cell Growth media and supplemental BulletKit (Cedarlane Labs, ON, Canada). MIN6 mouse beta-cells (kindly gifted by Prof. Hoesli from McGill University) were seeded at 2-3 million cells per flask. The human beta cell line EndoC-βH5 (Human Cell Design, France) was seeded at 80,000 cells/cm^2 and maintained without proliferation, changing media weekly. Low passage HUVECs (p4-p8) and immortal MIN6 cells (p30-p40) were used for spheroid formation [26]. Cells were trypsinized using 0.05% trypsin with EDTA and resuspended at 2 million cells/mL for seeding onto agarose molds. To prepare the molds, 0.9% (w/v) saline solution was mixed by dissolving NaCl powder in D.I. H_2O to 9 mg/mL, then filtered by 0.2 μm syringe filter. 500 mg of agarose powder was then dissolved into 20 mL of the filtered saline solution in a 50 mL falcon tube, resulting in a 2.5% agarose (w/v) mixture. The mixture was heated in a microwave at 10 s increments until fully dissolved. 300 µL of warm agarose was pipetted into MicroTissue 3D Petri-Dish micro-mold casts

(Sigma-Aldrich, ON, Canada). After cooling for 10 min the agarose forms a gel and can be removed from the cast and stored at 8 ℃ in a saline solution.

6.2.2 Spheroid Formation

As cells expanded in monolayer culture, agarose microwell molds were prepared for spheroid formation. Molds were precondition by immersion in growth media in a 24-well plate for 30 min. The media was then removed, and cells were pipetted in a 130μL suspension onto the premade molds. To attain 1000 cells in each of the 256 wells of the gel microarray, a density of 256k cells/130μL (~2 million cells/mL) was used.

Based off previous experiments coculturing human islets with HUVECs and MIN6 insulinomas with HUVECs at beta:endothelial cell ratios of 1:2 and 1:6, respectively, we opted for 1:1 and 1:3 in our coculture groups [36,48]. For 1:1 beta-cell:HUVEC coculture spheroids, 62.5 μL of beta-cells and 62.5 μL of HUVEC containing cell suspensions were added, respectively, at the appropriate density. For 1:3 beta-cell:HUVEC constructs, 32.5 μL of beta-cell suspension is added to the mold, along with 97.5 μL HUVEC cell suspension (table S1).

The initial seeding was left undisturbed for 4 hours to allow the cells to sediment into the microwells and begin aggregating, at which point 1.3 mL of media was added to each well of the 12-well plate to submerge the molds and incubated at 37 °C. Growth media was changed every 3 days, as per normal culture protocols. Coculture spheroids were immersed in a 50:50 blend of Ulti-β EndoC-βH5 cell and EGM-2 HUVEC medias. Over 5-7 days cells aggregated into compact spheroids and could be flushed from the molds and resuspended (visual depiction given by X. Guo et. al [27]).

6.2.3 Immunofluorescent Confocal Imaging

Live/dead staining for fluorescence imaging was performed using the Biotium Viability/Cytotoxicity Assay Kit (for Animal, VWR, CA, USA). The green (live) dye consisted of an elasterase substrate, which was cleaved into fluorescent calcein, and only remained if the cell membrane was intact. The red (dead) dye was Ethidium Homodimer—III (EthD-III), a membrane-impermeable DNA dye, only penetrating cells when the cell wall was compromised. Spheroids were prepared for imaging by washing twice with PBS. Staining media was 2.5 µL calcein AM (hydrolyzed, pH 8) and 10 µL EthD-III in PBS (5 mL total volume). Spheroids immersed in staining media were shielded from light and incubated at 37°C for 30 min before imaging. Images were taken using the LSM 710 Confocal Scanning Microscope (excitation wavelengths: 488 nm for live and 543 nm for dead, Zeiss AxioObserver).

For cell-specific staining, spheroid samples were washed twice with PBS and fixed in 3.5% PFA for 10 min at 37°C, followed by two more PBS rinsing steps to remove the fixative and stored in the dark at 4°C until staining. Samples were washed twice in tris buffered saline (TBS; 50 mM Tris, 150 mM NaCl) + 0.05% Tween-20 and blocked for 30 min using TBS + 3% BSA. Samples were washed twice in TBS + 0.05% Tween-20 and once in TBS before staining overnight with the CD31-AlexaFluor 488 nm conjugate (Biotium, CA, USA) and insulin primary antibody, both diluted to 1 µg/mL, targeting HUVECs and beta-cells, respectively. Samples were washed 3x in TBS + 0.05% Tween-20 and AlexaFluor 647 nm secondary antibody was used to stain for 1 hour and washed a final 3x in TBS + 0.05% Tween-20. As a negative control, the 488 nm secondary antibody was incubated with samples without the insulin primary antibody (no fluorescence was

observed). Image reconstruction and analysis were performed using the Zen Microscope Software by Zeiss.

For cell tracker staining, adherent monolayer cells were dyed prior to spheroid formation, in contrast to target-specific antibody staining of fully formed spheroids. T75 flasks containing either EndoC-βH5 or HUVEC cells were incubated with the CellTracker red or blue dye, respectively (Invitrogen, ThermoFisher Scientific, ON, Canada), for 30 min then placed back in growth media until imaging.

6.2.4 Metabolic Activity Assay

Cell viability was measured using the AlamarBlue reagent from Invitrogen (ThermoFisher Scientific, ON, Canada), based off the reduction of resazurin to resorufin by healthy cells. The assay reagent was diluted 1:9 in media and incubated with living cells for 4 hours. Following incubation with living cells, the test reagent was collected, and replaced with fresh growth media. The percent reduction of the test reagent was calculated by measuring the absorbance or optical density at both 570 nm and 600 nm:

$$\%\, Reduction = \frac{((\epsilon_{OX}(600nm) * A_1(570nm) - (\epsilon_{OX}(570) * A_1(600nm))}{((\epsilon_{RED}(570nm) * A_2(600nm) - (\epsilon_{RED}(600nm) * A_2(570nm))} * 100\%$$

Where A_1 denotes the sampled supernatant, and A_2 the negative control comprised of the serum-free media diluted AlamarBlue reagent at a ratio of 1:9. The molar extinction coefficients for the

2 wavelengths are ϵ_{OX}(600nm) = 117,216, ϵ_{OX}(570nm) = 80,586, ϵ_{RED}(570nm) = 155,677 and ϵ_{RED}(600nm) = 14,652, respectively. Both samples and controls were measured for optical density at 570 nm and 600 nm incident light for determination of the percent reduction of AlamarBlue reagent.

6.2.5 Glucose Stimulated Insulin Secretion (GSIS)

After allowing cells to compact for 7 days, spheroids are flushed from the molds by gentle pipetting. Prior to glucose stimulation, cells were placed in a low-glucose starvation media for 24 hours. The test stimulation was carried out in Krebs buffered salt solution, prepared at 0 mM and 20 mM glucose for 'low' and 'high' solutions, respectively. For EndoC-βH5, high and low glucose solutions were made using β-Krebs purchased from Human Cell Design (Toulouse, France). Upon recovery from starvation media, cells are washed for 1 hour in the low glucose Krebs, then incubated for one hour in low glucose, followed by 1 hour in high glucose, at 37°C. Supernatant from each spheroid group, having 0, 50 or 75% HUVECs, was sampled in triplicate for insulin measurement. Once the stimulation was complete, the spheroids were lysed and stored at -80°C for mRNA quantification and gene expression analysis. Insulin secretion was quantified using the Mercodia human insulin ELISA kit (Cedarlane Labs, ON, Canada).

6.2.6 Beta Cell Gene Expression Assay (RT-qPCR)

Following GSIS testing, cells were lysed for gene analysis. Nuclear mRNA was extracted and purified using the Invitrogen PureLink RNA Mini Kit (ThermoFisher Scientific, ON, Canada)

protocol and evaluated by NanoDrop quantification. Purified RNA samples were stored at −80°C until gene expression analysis using the CFX Opus Real-Time PCR system (BioRad, ON, Canada). Primers specific to beta-cell genes were chosen to evaluate the spheroids in terms of insulin production and beta-cell maturation. Forward and reverse sequences for reference and target genes were obtained from Integrated DNA Technologies (IA, USA) and prepared for testing using the Luna OneStep reaction kit (New England Biolabs, MA, USA). mRNA was reverse transcribed into cDNA, amplified by thermal cycling, and detected by SYBR green fluorescence. Target gene expression was quantified relative to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) within each group and normalized to the monoculture spheroid control group for fold change analysis ($R = 2^{-ddCt}$). Glut2 is a transmembrane glucose transporter, PDX1 is involved with endoplasmic reticulum health and function, while MAFA is a beta-cell specific transcriptional activator (primer sequences and gene descriptions shown in table S2, visual diagram in figure S4) [28].

6.3 Results

6.3.1 Spheroid Formation and Viability Assay

Figure 6.1A shows the ImageJ quantified diameter of spheroids after 7 days of sedimentation. Monoculture aggregates compacted faster than cocultures during the first 5 days. However, monoculture and 1:1 coculture spheroids did not have significant differences in diameter or cross-sectional area by day 7, respectively. While 1:1 coculture spheroids exhibited a slightly narrower distribution, all groups fell under the target of 150 μm for ‘average’ human islets of 1 IEQ. Cross sectional spheroid areas are shown in figure S1.

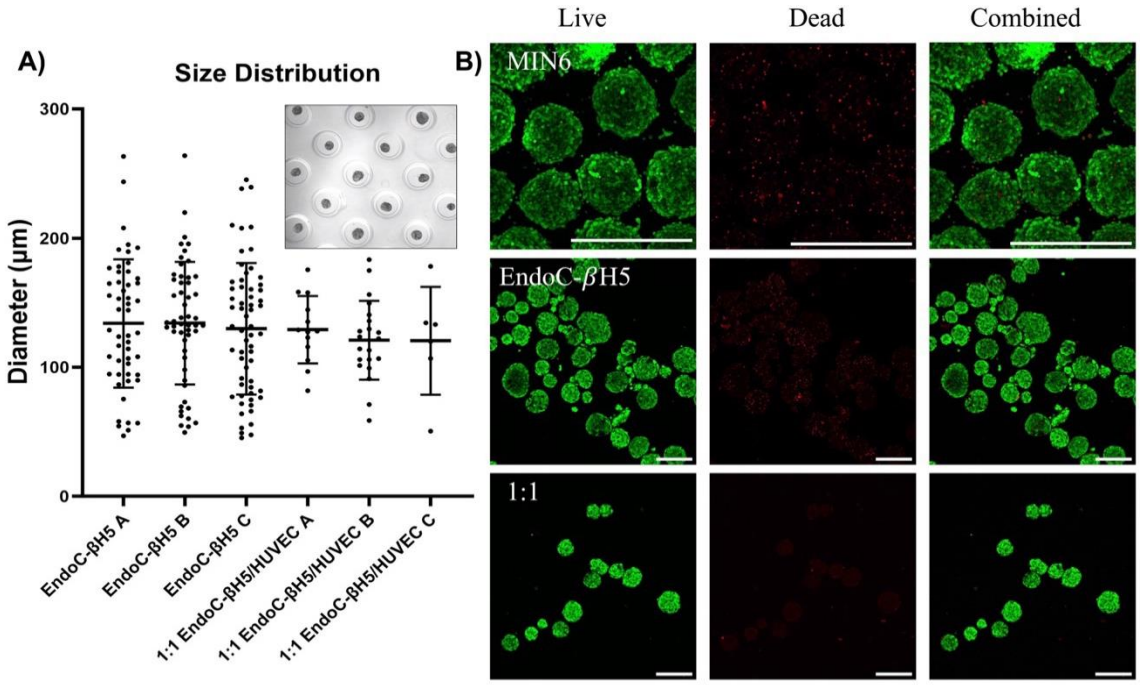

Figure 6.1: A) Diameters of individual spheroids in each of 3 batches for monoculture and 1:1 EndoC-βH5:HUVEC cocultures (Inset: Cells in agarose microwells, center-to-center spacing is 580μm), B) Live/dead confocal imaging for MIN6 monoculture, EndoC-βH5 monoculture and 1:1 EndoC-βH5:HUVEC coculture spheroids. Scale bar is 250 µm.

The live (green, 488 nm), dead (red, 647 nm) staining and combined images for different spheroid groups acquired by confocal fluorescence imaging on day 12 after spheroid seeding are shown in figure 6.1B. Recombined Z-stack images show both monoculture EndoC-βH5 and 1:1 cocultures display a compact structure with spheroidal morphology and few dead cells. Monoculture MIN6 spheroids appeared less tightly bound.

ImageJ quantification of live/dead cell images was used to quantify viability (figure S2A). Percent viability is calculated by the number of live cells divided by the total cell number. MIN6, EndoC-βH5 and 1:1 (βH5:HUVEC) spheroid groups showed statistically similar viabilities of ~90%. Figure S2B shows the metabolic activity of MIN6 monoculture and MIN6:HUVEC coculture spheroids at days 3, 5 and 8 after formation.

Using cell-specific stains for insulin and CD31, the beta and endothelial cells could each be resolved. Confocal imaging using separate captures for the 2 dyes (647 nm for insulin and 488 nm for CD31) is shown for 100% EndoC-βH5 monoculture and 1:1 EndoC-βH5:HUVEC spheroids in figure 6.2A. CellTracker dye stain of EndoC-βH5 (red) and HUVEC (green) monolayer cells prior to spheroid formation shows the incorporation of both cell types within the construct (figure 6.2B). Total fluorescent intensity quantification revealed HUVEC/beta-cell ratios of 0.9 and 1.4 for the 1:1 and 1:3 groups, respectively. Cell-specific staining including nuclear staining for 1:1 coculture spheroids is shown in figure S3.

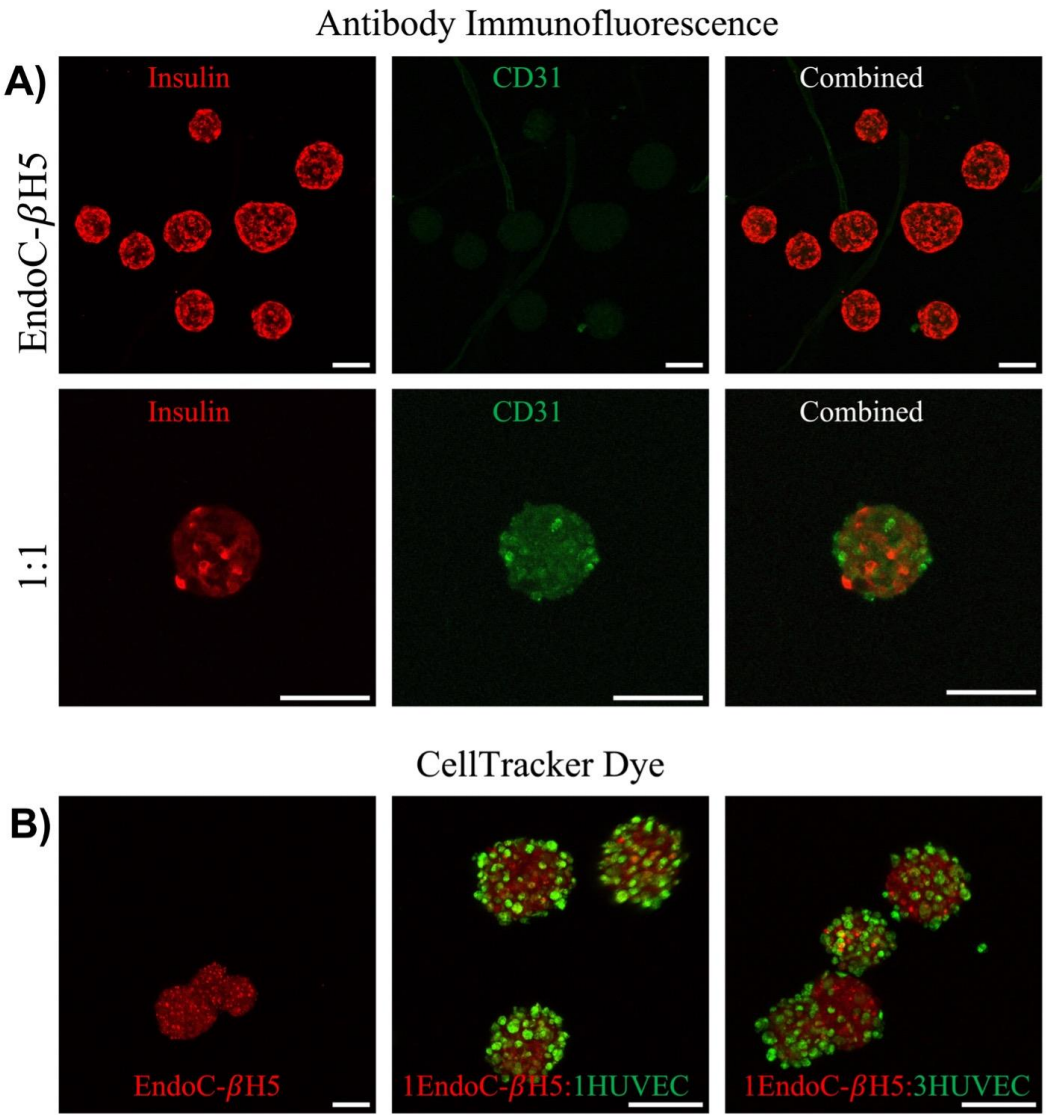

Figure 6.2: Spheroids stained with fluorescent antibodies specific to insulin in EndoC-βH5 (red) and CD31 in HUVECs (green), B) CellTracker cell dye on EndoC-βH5 (red) and HUVECs (green), stained prior to spheroid formation. Scale bar is 100 μm.

6.3.2 Glucose Stimulated Insulin Secretion

After 7 days of culture in molds, spheroids were recovered and starved for 24 hours in low glucose media before stimulation. Figure 6.3 shows the ELISA-quantified insulin concentration of the Krebs glucose buffer following incubation with different spheroid groups. Figure 6.3A presents the insulin concentrations measured from supernatant following incubation of the spheroids in low and high glucose stimulation buffer. The inset shows corresponding stimulation indices, calculated as the ratio of high to low insulin concentrations. Cocultured EndoC-βH5:HUVEC (1:1) spheroids displayed the highest consistency among triplicates and the most statistical significance in insulin production, with p = 0.0085 between high and low values (denoted as '**'). The 1:3 group exhibited the highest insulin release and stimulation index, though were not significantly different due to variation among triplicates. Figure 6.3B shows the response of 100% EndoC-βH5 spheroids to 0 mM and 20 mM glucose containing 1 μg/mL INGAP-P modified with cysteine (I15Cys) or glycine (I15Gly) residues, with inset showing the corresponding stimulation indices. Details of synthetic INGAP-P peptide screening are covered in our previous work [15]. Peptides were custom synthesized as analogues of the 15 amino acid sequence, INGAP-P, to find stable conformations with increased stimulatory effect. All 3 groups

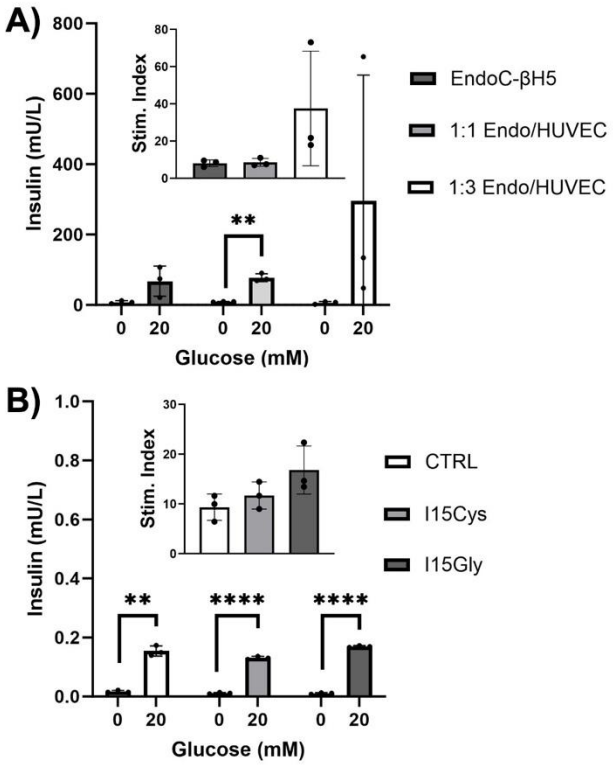

Figure 6.3: ELISA quantification of insulin secretion in response to low (0 mM) and high (20 mM) glucose (Inset: stimulation) (B) Insulin response to low (0 mM) and high (20 mM) glucose alone, and with 1 µg/mL INGAP-P variants I15Cys or I15Gly, (Inset: stimulation index)

showed a marked insulin response to elevated glucose (~10-15 fold). While exposing EndoC-βH5 spheroids to I15Gly nearly doubled the stimulation index, this alone is not enough to conclude the peptide's beneficial properties. The reproducible stimulation response to glucose, however, demonstrates the usefulness of this cell model for future studies. Investigating a range of dose concentration and exposure time would help identify peptides or other agents for stimulatory

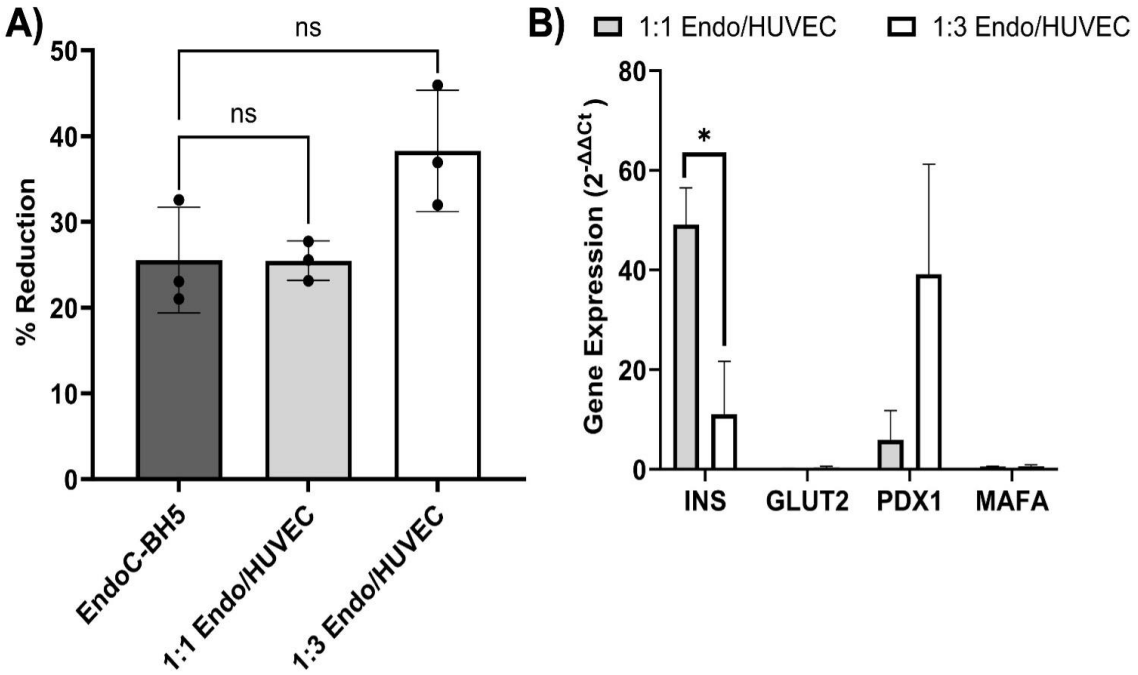

Figure 6.4: A) Metabolic activity, shown as percent reduction of the AlamarBlue reagent. 1EndoC-βH5:3HUVEC spheroids showed increased activity (p = 0.077), and B) RT-qPCR quantification of beta-cell specific mRNA targets, shown as $R = 2^{-ddCt}$, relative to the GAPDH housekeeping gene, with monoculture spheroids used as the control group. A paired t test found significant difference between the insulin expression of 1:1 and 1:3 coculture groups (p = 0.02).

effects.

6.3.3 Metabolic Activity and Gene Expression

Figure 4A displays the metabolic activity of the 3 spheroid groups as measured by the AlamarBlue assay, and 6.4B the gene expression values of target genes quantified relative to the internal GAPDH reference. The monoculture and 1:1 coculture groups had a similar percentage reduction, whereas the 1:3 EndoC-βH5:HUVEC group had a higher metabolic activity, compared

to other groups (p = 0.077). The Livak-Schmittgen value, $R = 2^{-ddCt}$, gives the fold change for mRNA levels in 1:1 and 1:3 coculture groups, normalized against the monoculture control [29]. Both coculture spheroid groups displayed insulin and PDX1 upregulation compared to monoculture spheroids, with significant increases of insulin expression in the 1:1 group compared to 1:3.

6.4 Discussion

Maximizing the insulin production and survival of implanted cells are critical to islet graft efficacy and longevity. Our previous work highlighted the variability in drug screening studies on human islets due to individual differences between donors [15]. Age, BMI, comorbidities and drug history all act to confound the accuracy of studies with limited donor access. Alternatively, immortalized cell lines provide a stable phenotype for greater consistency and an unlimited cell supply. 3D culture enables studying morphological effects on paracrine interactions. Endothelial cells cultured in support of insulin-producing beta-cells can provide molecular stimulation, regulation of immune mediators, and induce vascularization [5, 30, 31]. Mutually beneficial signaling occurs as beta-cells release angiogenic VEGF-A, and islet ECs in turn produce endothelins-1 and -3, which enhance GSIS [14]. To investigate the effect of endothelial cells on beta-cells, we studied three groups of spheroids in varying proportions of the two cell types. The first group, a 100% EndoC-βH5 monoculture, served as negative control for the cocultured spheroid groups at 1:1 and 1:3 beta-cell/EC ratios. The total cell number was held constant at 1000 cells/well.

3D structures increase cell-cell contact and improve cell function. The mouse insulinoma MIN6 cell line has consistent proliferation into high passages (<80), and releases mouse insulin in response to glucose [26]. In a GSIS assay, beta-cells are immersed in a low-glucose (or basal)

media, which is then replaced with a high glucose stimulation media. Supernatant sampling after 1 hour incubation in each condition provides the fold change and total insulin output. MIN6 spheroids formed in microwells demonstrated improved insulin release compared to monolayer cultures [32]. Increased cell-cell communication may play a role in the concerted insulin response. Previous spheroid studies combining MIN6 cells with mouse aortic ECs or with NIH3T3 fibroblasts showed increased insulin secretion and gene expression, compared to MIN6 alone [33, 34]. Following promising indications in the literature, we used MIN6 cells to test our spheroid formation and HUVEC coculture process as their proliferative nature facilitates multiple rounds of optimization. While pure MIN6 groups formed spheroids with acceptable morphology, mixed groups containing HUVECs were more dispersed, and disaggregated upon handling. As GSIS responses were variable across replicates, we focused on EndoC-βH5 for stimulation studies. This human beta-cell line has shown improved insulin response to glucose (>6-fold change) and is a species-match for coculture with human endothelial cells. Here, we constructed EndoC-βH5/HUVEC spheroids ("human pseudoislets") for evaluation as an *in vitro* model for T1D screening studies. Spheroid formation has been shown to increase the insulin response to glucose of EndoC-βH3 cells, and the presence of islet endothelial cells improved insulin release in EndoC-βH1 spheroids [23, 35]. Heterocellular spheroids with varying proportions of EndoC-βH5/HUVEC were evaluated for the effect of directly incorporating endothelial cells into the model. Interspersed beta-cell/HUVEC morphology was chosen over core/shell designs to reflect the capillary networks throughout human islets.

The use of preformed agarose molds allowed for precise formation of 3D spheroids without the need for microfabrication or photolithography used for PDMS based microfluidic channels. We formed spheroids of controlled size and shape by seeding 1000 cells into each microwell of the

array for aggregation. Acceleration of spheroid formation can be achieved by centrifugation or actuation of magnetically functionalized beads, though suspension cells can still form compact and robust spheroids by gravity over 5-7 days [36]. MIN6 cells have shown a synergistic effect and increased insulin secretion when cultured with HUVECs, compared to monoculture MIN6 spheroids [34]. 250 MIN6 cells were cultured for 1 day, then 1500 HUVECs were added in suspension before centrifugation at 150 g for 30 s. Here, we added beta-cells and HUVECs in suspension at the same initial time point, allowing sedimentation to occur by gravity. Due to this softer aggregation, we started with lower beta-cell:HUVEC ratios of 1:1 and 1:3, as opposed to the 1:5 used with centrifugation. Higher endothelial content was seen to result in increased cellular debris and more loosely bound aggregates.

Immunofluorescence staining revealed consistent spherical morphology, good viability (~90%) and metabolic activity among EndoC-βH5 coculture and monoculture spheroid groups. Metabolic activity as determined by the AlamarBlue cell viability assay showed an increased % reduction for the 1:3 group, with the highest proportion of endothelial to beta cells, compared to monoculture spheroids. No change was seen in the 1:1 spheroid group. A proliferation assay could help to distinguish metabolism between groups if there is continued expansion of HUVECs. Spheroids with higher endothelial cell content also showed increased insulin release per beta-cell, yet further statistics are required to determine significance. Stimulation in smaller volumes with lower spheroid numbers could produce a comparable response with less consumption of non-proliferating EndoC-βH5 cells.

Our previous work has shown the potential of INGAP-P based peptides to improve insulin secretion and expression of certain beta-cell gene mRNAs, but dealt with high variability among human donors [15]. Here, we selected I15Cys and I15Gly for their potential to improve insulin

secretion, possibly in conjunction with upregulation of PDX1 expression. While the increased stimulation indices seen with EndoC-βH5 spheroids exposed to I15Cys, and particularly I15Gly are not sufficient to conclude their use as insulin secretagogues, the EndoC-βH5 3D cell models demonstrate improved performance for in vitro drug discovery testing, compared to human donor islets. Further studies should determine the effects of varying peptide dosage concentration and exposure time.

Beta-cell specific gene markers related to the insulin secretion pathway were analyzed for each spheroid group following glucose stimulation. Insulin, GLUT2, PDX1 and MAFA were quantified relative to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene in each group. While GAPDH expression generally remains constant in response to different treatments, some variation may occur among different human donors [37, 38]. GAPDH has been found to have more stable expression compared to 18s RNA, beta-actin, and other reference genes in EndoC-βH5 cells when quantified by RT-qPCR [39]. Here, by using these 'de-immortalized' clone cells we expect little to no variation across housekeeping genes in different groups. In our study, the 1:1 coculture models displayed a significant upregulation of the insulin gene compared to the 1:3 group, and both coculture groups show increased insulin and PDX1 compared to monoculture controls. In the context of a T1D research model, future studies should evaluate any changes in gene and protein expression in response to proinflammatory cytokines. Coculture spheroids of varying composition could be exposed to interleukin-1β, interferon-γ and tumor necrosis factor-α, for instance, as has been done with adherent EndoC-βH5 cells in monoculture [39].

Researchers have shown endothelial signaling occurs via secretion of laminins and basement membrane proteins which enhance proliferation and upregulate insulin gene expression in beta-cells [40]. Islet endothelial cells produced the ECM protein β1 integrin when cocultured with

native beta-cells, the blockade of which reduced insulin gene expression and increased insulin secretion at basal glucose levels [6]. PDX1 expression is known to be reduced in apoptotic beta-cells due to loss of insulin and insulin-like growth factor (IGF) signaling associated with endoplasmic reticulum stress [41, 42]. Reduced expression of PDX1 and MafA can occur in response to oxidative stress, as seen in the early pathogenesis of T1D [43]. Loss of insulin and MafA expression is also associated with loss of beta cell identity through dedifferentiation [44]. Since GLUT2 functions as a glucose transporter, limited mRNA expression indicates lower activation of the pathway required for pancreatic beta-cells to initiate the insulin response [45]. As these genes are involved with differentiation and maturation, the upregulation of insulin and PDX1 indicates a strong expression of the beta-cell phenotype [21, 46]. Stimulation of the transmembrane glucose transport Glut2 would likely contribute to improved performance in triggering insulin release. Future studies could expand the gene panel to include expression markers of HUVEC integration and function, such as primers for CD31, CD105, VE-cadherin, as well as testing for VEGF-α and endothelin protein production [47].

A limitation to these experiments, and the field in general, is the lack of a renewable human alpha-cell line for incorporation within the spheroids. A promising step forward, these 3D cocultures cell are vastly simplified models of native human pancreatic islets. Current efforts employ fluorescence activated cell sorting (FACS) to reaggregate cells from previously dissociated islets, however stable alpha lines for in vitro culture are not widely established or available at this time [49, 50]. Furthermore, although live/dead confocal imaging confirmed viability throughout the 3D constructs, it would be instructive to employ perfused culture chambers to investigate the dynamic perfusion of oxygen and nutrients through the spheroids, over time and in varying levels

of hypoxia. Fluid shear stress matching physiological levels would also allow for accurate determination of the real-time secretion response to stimuli.

6.5 Conclusion

The human insulin secreting EndoC-βH5 line outperformed mouse-derived MIN6 in spheroid stimulation index. Cocultured spheroids containing beta-cells and HUVECs displayed increased insulin secretion and metabolic activity, compared to monoculture. Reduced variability in stimulation of EndoC-βH5 spheroids can help overcome limitations of drug screening on human islets. Insulin stimulation and metabolic assays show there is promise for endothelial/beta-cell coculture spheroids as *in vitro* models for T1D research. The two cell types integrate readily, forming robust aggregates that respond to glucose with insulin. Future studies should focus on establishing vascular connectivity with a support scaffold, and the immunogenic responses of the heterocellular constructs.

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Author Contributions

Prof. Tabrizian assisted with experiment design, text editing and provided access to laboratory supplies and equipment. Michael Yitayew conducted antibody staining and confocal fluorescence imaging, provided advice on cell stimulations and helped with seeding and culture of monolayer EndoC-βH5 cells. All other experimental data acquisition/analysis, cell and spheroid culture and writing was conducted by James Porter.

Data Availability Statement

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Conflict of Interest Statement

The authors declare that they have no conflicts of interest regarding this manuscript

Supplementary Materials

Table S1: Cell culture groups used for spheroid formation and testing. Table S2: RT-qPCR target genes and mRNA primer sequences. Figure S1: Cross sectional areas of EndoC-βH5 and Endo-βH5/HUVEC coculture spheroids, quantified by fluorescent confocal imaging. Figure S2: A) Percent viability of MIN6, EndoC-βH5 and EndoC-βH5/HUVEC spheroids determined by live/dead staining B) Percent reduction of the AlamarBlue metabolic test reagent incubated with MIN6 or MIN6/HUVEC spheroids on days 3, 5 and 8 after formation. Figure S3: Cell-specific staining for insulin and CD31 in 1:1 (EndoC-βH5) spheroid including nuclear staining with DAPI.

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Chapter 7. Capacitive Detection of Insulin Antibody Enhanced by AC Electrothermal Mixing

The following chapter provides a working proof of concept for using capacitive sensors to detect biomarkers in real time. These results were published as a first author proceeding in the *IEEE Xplore* digital research database. This work was a branch-off side study of my 2nd author contribution to the article "Interfacial capacitance immunosensing using interdigitated electrodes: the effect of insulation/immobilization chemistry", published in *Physical Chemistry Chemical Physics* of the *Royal Society of Chemistry (RSC),* as a part of the PhD thesis of Dr. Rafael Castiello (not presented in this thesis). This original contribution proceedings article covers the physical phenomenon of electrokinetic fluid mixing applied to reduce the time needed for immunoassays. This study places an important foothold for researchers to build off in using interactions between charged electrodes and fluid samples for microfluidic biomedical detection devices.

Capacitive Detection of Insulin Antibody Enhanced by AC Electrothermal Mixing

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Abstract

Capacitive immunosensors present a useful tool for non-invasive and label-free biosensing. Readily integrable with microfluidic platforms, interdigitated electrode (IDE) arrays can be specifically functionalized to detect antibody/antigen surface binding events. Here, AC electrokinetics are used to enhance target binding to overcome long incubation times and diffusion limited miniaturization. This represents the first application of ACET enhancement for human hormone detection to be used in modeling diabetes.

Keywords: electrokinetics, capacitive immunosensing, interdigitated electrodes, microfluidics

7.1 Introduction

Hormone sensors are widely used in the biomedical field for evaluation of cell functionality during transplant, disease modelling and drug screening. With increasing need for multiplex detection, high throughput and low cost, available biosensors are investigated for integration with in-vitro microfluidic platforms. As sensors comprise 40% of the Micro-Electromechanical Systems (MEMS) market, there is a great need for a highly specific label free assay [1, 2]. Capacitive immunosensors detect a change of surface dielectric resulting from direct antigen/antibody binding, leading to highly sensitive target specific readouts. Analyzing the biological fluid sample, incubation times on the order of hours are often required for detection, when relying on particles encountering the sensor surface based off diffusion alone. As the diffusion time scales with the

square of distance, it can take several hours for a particle to traverse a chamber with width on the scale of 100 μm based on diffusion alone [3]. In this study, in order to enhance particle interaction with the sensor, AC electrothermal (ACET) mixing is employed, increasing binding efficiency and drastically decreasing assay time. With this technique, IDE arrays use a single applied voltage to both stimulate fluid vortices and simultaneously sense changes in the surface capacitance.

7.2 Mechanism

7.2.1 Capacitive Interface Immunosensing

In general, a biosensor is comprised of a transducer element, and a biorecognition element. Here, antibody/antigen lock and key binding mechanisms are utilized for biorecognition, and a microelectrode capacitor is used for signal transduction. The coplanar capacitor is interdigitated to increase the sensing area, and functionalized with an insulating polymer with immobilized antigen probes. Capacitive electrode array sensors are readily miniaturized, and make use of a non-faradic (no charge transfer) detection scheme, in contrast to electrochemical methods, which require reduction or oxidation of a target probe [2].

When a low-frequency (<105 Hz) voltage is applied to the electrodes, ions from the conductive fluid accumulate at the cathode and anode, forming the so called ionic double layer, typically having a capacitance on the order of μF [4]. As surface binding occurs, fluid ions will be displaced by steric effects, leading to a detectable change in capacitance.

At frequencies lower than 100 kHz, the equivalent electric network can be simplified as a series connection of an electrolyte resistance (R_s) and two (one for each plate) interfacial capacitances

(C_{int}) [6]. These circuit elements are measured as the real and imaginary parts of the total impedance, respectively:

$$|Z| = [(R^2 + X^2)]^{0.5} \quad (1)$$

Where Z [Ω], is the circuit impedance, R [Ω] the resistance, and X [Ω] the reactance. The complex reactance, X, can be used to extract the interfacial capacitance (C_{int} [μF]) by:

$$C_{int} = -1/\pi f X \quad (2)$$

Where f [Hz] is the AC frequency of the signal applied. In a standard capacitive immunoassay, the impedance spectrum is captured before and after the antibody incubation period, including a wash step to remove non-specific binding. Here, the change in capacitance is monitored continuously, at a single frequency, by the ACET/sensing voltage as binding occurs. In order to compare capacitance changes across different sensors, the normalized capacitance (C_{norm}) data are presented as the interfacial capacitance (C_{int}) normalized to the starting value ($C_{int,0}$):

$$C_{norm} = C_{int,n}/C_{int,0} \quad (3)$$

for each time value from t = 0 → n seconds, to obtain the percentage change.

The dynamic range for an affinity sensor, however, will depend on the antibody/antigen interaction as well as the total functionalized surface area. Once all available binding sites become filled, the sensor surface will become saturated and need to be regenerated.

5.2.2 AC Electrothermal Enhancement

The alternating current electrokinetics (ACEK) family includes the various phenomena of dielectrophoresis (DEP), AC electroosmosis (ACEO), and AC electrothermal (ACET) effects. The ACET force scales with conductivity of fluid, and in contrast to other ACEK effects has no dependence on particle size [5]. In using micro-electrode structures, the effects of thermal convection are negligible compared with those of the electrical forces [6].

The ACET effect originates from a localized Joule heating of the ionic fluid, which generates a thermal gradient. This leads to gradients in conductivity and permittivity:

$\nabla\sigma=(\partial\sigma/\partial T)\nabla T$ and $\nabla\varepsilon=(\partial\varepsilon/\partial T)\nabla T$ [6], [7]. Fluid vortex flow above each planar electrode results from interaction of these induced charges with the applied electric field. The ACET fluid force can be expressed:

$$\vec{F}_{ACET} = -0.011 * \nabla T * \epsilon E_0^2 \quad (4)$$

where T [K] is the fluid temperature, ϵ the permittivity [F/m], and E_0 the ambient electric field [N/C]. This causes microflows in the sensing chamber for long range enhancement of target molecule detection [7].

The electrothermal fluid motion serves as stringency wash for removing non-specific binding, as the measurement is taken in a continuous and real-time manner [8]. Reaching fluid velocities on the order of 50 μm/s, the ACET force has been shown to be independent of frequency, given it is below a threshold frequency, defined by the charge relaxation time of the fluid [6].

7.3 Devices and Materials

Absolute ethanol and phosphate-buffered saline (PBS) 10X were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Phosphate-buffered saline 1X (PBS) tablets and Tween 20 were purchased from BioShop Canada Inc. (Burlington, Ontario, Canada). N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-Hydroxysuccinimide (NHS), (3-Aminopropyl)triethoxysilane 99% (APTES), bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tris-buffered saline (TBS) with 1% Casein from BIO-RAD. Anti-insulin antibody (6.2 mg/mL) and human insulin were purchased from PROSPECT (Ness, Ziona, Israel). Anti-glucagon antibodies (200 μg/mL) were purchased from Santa Cruz Biotechnologies, Inc. (Mississauga, ON, Canada). Poly(methyl methacrylate) (PMMA 950 A2) was purchased from MicroChem Corp.

7.3.1 Microfabrication of Interdigitated Electrode Arrays

IDEs were fabricated in a cleanroom environment on glass substrates using photolithography. The electrode 'finger' length was set to 1 mm, with total sensing area 0.5 mm^2. Glass substrates 1 mm thick were cleaned by sonication in acetone then rinsed with isopropyl alcohol (IPA) and distilled water then dried with N_2. Substrates were then primed in a YES oven at 100°C for 5 min then cooled with N_2. Next the substrates were spin coated with LOR5B (Microchem, Newton, MA) at 1000 rpm for 45 s, then baked at 180°C for 5min. After cooling, the glass wafer was spin coated with Microposit™ S1813™ (Shipley, Marlborough, MA) at 4000 rpm for 30s then baked at 115°C for 60 s. Electrodes were defined using chrome photomask with UV exposure dose of 136 mJ/cm^2. After 70 s development in Microposit MF®–319 (Shipley) developer, a 10 nm layer of titanium

and 50 nm layer of gold were deposited using a NexDep E-beam evaporator (Angstrom Engineering Inc). Liftoff was done at 70°C under sonication using Microposit Remover 1165 (Shipley).

7.3.2 Microfluidic Sensing Chamber

To provide a fluid interface for the biological sample to the capacitive sensor, a sensing chamber with O-ring seal was designed using AutoCAD. Circumventing the need for the use of PDMS fluid channels, the removeable nature of the chamber allows for washing and reuse of microfabricated electrode arrays. Protein functionalization can be repeatedly removed using solvents and reapplied, as long as the pristine metal electrode array is not damaged during handling. Once the conducting fingers of the sensor have been scratched or damaged in any way they must not be used.

The sensing chamber was 3D printed using FLGPCLO2 postcured resin (a mixture of (meth)acrylated monomers, (meth)acrylated oligomers and photoinitiators), with post-cured tensile strength of 65 MPa. The microfluidic device consists of inlet and outlet tubes with 1 mm diameter, and an elliptical chamber with height of 9.03 mm, small radius 2.06 mm, large radius 4.05 mm and total fluid volume approximately 25 μL (Fig. 7.1A).

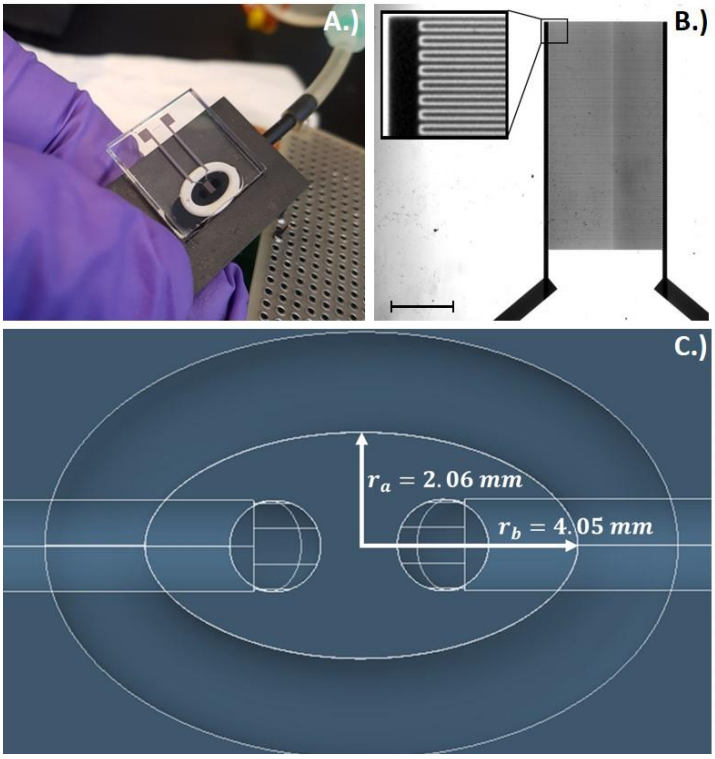

Figure 7.1: A.) Microfluidic chamber and O-ring seal to the electrode substrate (biointerface) B.) Interdigitated electrode array – the coplanar capacitor which acts as the sensor's transducer element. (Scale bar is 50 μm), and C.) AutoCAD design of the 3D printed chamber. Bottom view of the sensing chamber (interior ellipse), inlet and outlet channels and trough which seats the O-ring

7.3.3 Target Specific Sensor Functionalization

Prior to treatment, all IDEs were cleaned using 5 min sonication in acetone, then rinsed with IPA, distilled water and dried with N2. Kapton stickers were used to protect gold connection pads from coatings which could affect conductivity. The analyzer connection to sensor pads is depicted in Fig. 7.1B.

PMMA based functionalization was performed following a protocol reported by K. Kim et al. [9], with few modifications. Spin coating PMMA onto IDEs at 5000 rpm for 45 s followed by baking at 180°C for 90s resulted in a thickness of 66 nm, as measured by ellipsometry. Sensors were then treated with reactive O_2 plasma at 100 W for 2.5 min to create hydroxyl groups on the surface and submersed in 10% (v/v) APTES in water at 70°C. After 5 min, the sensors were removed from the solution, quenched in ethanol and baked at 110°C for 10 min.

After PMMA functionalization, sensor chips were loaded into the microfluidic chamber for the remaining steps of insulin immobilization. NHS (5 mg/mL) and EDC (2 mg/mL) were mixed in a 1:1 ratio in MES buffer (pH 6.0). 125 µL of the NHS/EDC was then added to 100 µL of an aqueous solution of insulin with concentration 1 mg/mL. After reacting 15 min, 825 µL of PBS 10x was added to raise the pH to 7.4, and the mixture was injected to the sensor. Incubating for 1 hr allowed covalent bonding between the amine groups on APTES and carboxyl groups of insulin.

After insulin incubation, the sensing surface was rinsed by flowing 1 mL of PBS (1x) with 0.05% Tween-20 (“PBS-T”) for 10 min. Finally, antibiofouling agent BSA with 1% Casein was used to occupy any remaining available binding sites and block the surface from non-specific interactions.

7.3.4 Real-Time Antibody Detection

Impedance measurements were carried out using a 4294A Agilent precision impedance analyzer in 0-span (single-frequency) mode. Insulin antibodies in PBS (1x) were injected to the system and carried to the sensing chamber at a 200 μL/min flow rate. Upon reaching the chamber, fluid flow is stopped and the AC sensing/ACET voltage is applied. The total impedance of the sensor is monitored for change over time a period of 1-2 min. Interfacial capacitance is calculated for each point using the complex component of impedance and normalized to the first value. The rate of change for antibody detection can be adjusted by subtracting the constant slope of control tests.

Sensors were tested with excitation voltages of 100 and 350 mV, at a frequency of 100 kHz, according to recent reports [5]. With insulin antigen probes immobilized on the sensor surface, anti-insulin concentrations of 1 and 10 μg/mL were tested. Positive controls consisted of sending 10 μg/mL of a non-specific, Glucagon antibody. Sensors used for negative controls were functionalized without the use of insulin, and had the entire surface blocked by BSA.

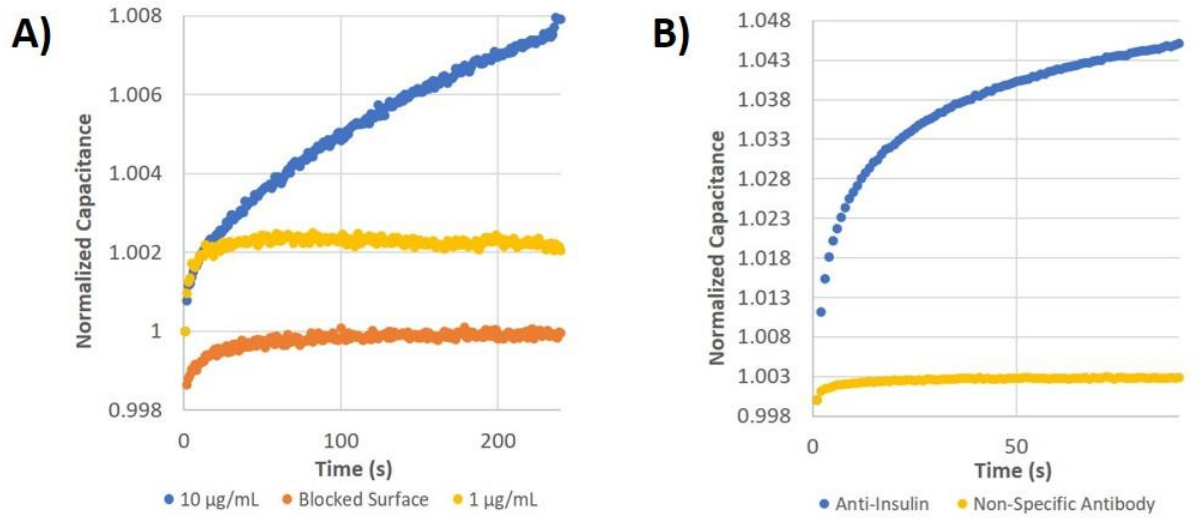

Figure 7.2: Normalized capacitance change with applied ACET/sensing voltage of 350 mV: A) Specific anti-insulin binding for concentrations of 10 $\mu g/mL$ (blue), and 1 $\mu g/mL$ (yellow), and 10 $\mu g/mL$ with a non-functionalized surface (orange) for control. B) Specific antibody, 10 $\mu g/mL$ (blue), and non-specific Glucagon antibody, 10 $\mu g/mL$ (yellow).

7.4 Results and Discussion

The ACET enhanced capacitive detection performed here targets the insulin antibody for future use within a competitive immunoassay for human hormones. Secondary motivation for immobilizing insulin on the sensor surface and detecting its antibody in solution stems from the relative sizes of the two molecules. The insulin peptide has a molecular weight (MW) of only ~5 kDa, as compared to the 150 kDa of its antibody. The larger particle size should have a larger physical footprint in displacing ions of the electric double layer, leading to a higher change in capacitance.

7.4.1 Quantitative results

Table 7.1 shows the representative data for tests done at both 100 mV and 350 mV. As can be seen, the magnitude of the signal, as compared to controls, was much lower when using 100 mV. For this reason, attention was focused on the higher voltage of 350 mV, which produced significant measurable changes in capacitance. Not shown in the 1st 350 mV row of Table 7.1 is the change for a diluted antibody sample.

Sensing/ ACEK Voltage	**Normalized Capacitance Change (%)**			**Time (s)**
	Specific Antibody [10μg/mL]	***AC***	***CA***	
100 mV	0.72	0.280	0.130	60
350 mV	3.91	0.024	0.027	60
350 mV	4.18	0.28	-	60

Table 7.2: Percentage change for binding of various targets. "AC" refers to an insulin functionalized surface sensing a non-specific antibody. "CA" refers to a blocked surface sensing a specific insulin antibody.

In Fig. 7.2, we can see the normalized capacitance data for real-time antibody detection, using sensing/stirring voltage of 350 mV at 100 kHz. Fig. 7.2A shows specific adsorption over 2 min of the antibody at concentrations of 1 and 10 μg/mL, as well as a negative control (orange line). The detection of anti-insulin was clear and immediate, leading to a 3.91% change in just 60 s for 10 μg/mL. This amount of shift is comparable to that seen in diffusion based hour-long incubations. The diluted sample of 1 μg/mL led to a 0.228% in the same timeframe. The smaller shift may be due to the concentration being outside the sensitivity range of the sensor, though looking at the first 10 s, it seems as though the target molecule concentration was exhausted after a short time.

Both 350 mV (table 7.1: 2nd row shown in figure 7.2A, 3rd row in figure 7.2B) tests confirmed the specificity of the sensor, with minimal responses resulting from the negative (figure 7.2A, orange curve) and positive (figure 7.2B, yellow curve) controls. It is promising that detection can be achieved at mV scale signals, thereby avoiding possible electrolysis and sample heating, though further exploration for resonant signals is warranted. Optimization of the flow dynamics using a specifically tailored chamber design may also lead to increased efficiency.

7.4.2 Conclusion

This work has demonstrated a proof-of-concept application of AC electrothermal mixing for enhanced antibody binding incubations, as monitored by IDE array sensors. The real-time nature of the measurement makes it a good candidate for dynamic monitoring of cell secretions in high throughput situations. Overcoming the limitations of diffusion allows for continued sensor downscaling, which leads to the need for smaller sample volumes and decreased use of reagents. As the ACET force acts upon the fluid and not the target molecule, the chemistry of functionalization can be altered to detect other hormones or pathogens. The non-invasive and label free nature of these capacitive sensors makes them optimal candidates for integration with microfluidic systems.

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Chapter 8. Global Discussion, Contributions & Perspectives

The overarching mission of this thesis project was to improve in vitro handling and cell secretion monitoring systems for pancreatic islets, addressing the need to improve transplant outcomes. Islet transplant via the Edmonton protocol offers freedom from insulin after a single infusion treatment [1]. Islet grafts, however, have limited long term survival rates, which drop by half after the first year [2]. In the first year following implant, one in five will succumb to antibody mediated rejection [3]. In a study of 255 patients receiving islet transplants by the Edmonton protocol, median graft survival was 5.9 years [4]. The most limiting challenges currently facing islet transplants are twofold: a shortage of donors, and the need for immunosuppression [5]. The instant blood mediated inflammatory response (IBMIR) and autoimmune islet insults exacerbate transplant cell scarcity, resulting in < 60% of islet survival transplant after 15 years [6, 7]. Despite potential dangers of tumorgenicity, research of embryonic stem cells (ESC) for transplant is advancing, with the first human recipient becoming insulin independent, albeit under heavy and chronic immunosuppression [5]. Just as with ESCs, the improvement of specific differentiation methods will make the use of induced pluripotent stem cells possible, which could reduce the need for immunosuppression, similar to the incorporation of accessory cells. Disrupted paracrine signaling and hypoxia following isolation, difficulty re-establishing a perfused vascular connection, immune rejection and donor scarcity are also contributing factors which lead to sub-optimal transplant lifetimes and overall success rates [8]. Tackling this challenge involves synthesizing techniques from the fields of physics, engineering, biology, chemistry, and medicine.

This thesis focused on developing 3 aspects of the field in order to facilitate islet research and improve transplant outcomes, those being: the sensing, stimulating, and regenerative cell

modelling of human pancreatic islets. This work could have useful applications in academic research, clinical practice, and industrial projects. The experimental results of this project led to published manuscripts, the main contributions of which are reviewed below.

8.1 Small Scale High-Speed Sensors

Microelectronic components are increasingly being applied for biosensing due to their small size, potential for mass production and automation. Forming a ligand-specific, biorecognition layer onto microcapacitors allows impedimetric detection of trace amounts of target analytes when immersed in solution. Biofunctionalized microcapacitor arrays are being applied for detecting pathogenic bacteria, hepatitis surface antigens, and measuring cell growth [9-12]. Here, antibody binding to insulin was measured with gold microcapacitor electrodes with specific surface functionalization. Microfabrication of interdigitated electrode array biosensors was carried out via photolithography, followed by antibody recognition element immobilization to functionalize the sensor surface. Using BSA or antibodies with off-target specificities, negative and positive controls thus validated the specificity of the anti-insulin IgG surface detecting insulin in solution [13]. An AFM study of different surface functionalization methods revealed smoother and more uniform coatings tended to correlate with improved impedance-based quantification, likely due to the maintenance of the insulating dielectric surface [14]. A study of electrodes size showed correspondence with theoretical predictions, in that the smallest electrodes actually displayed the highest sensitivity and lower measurement variability. The work resulted in a first co-author publication, not presented in detail in this dissertation.

As an alternative to microchannels in PDMS manufactured by photolithography, whose permanent binding to the glass surface limits the surface functionalization to a single chemistry, a

customized 3D printed chamber was designed. Easily attachmed over the glass chip, with an O-ring seal maintaining the fluid interface, microchips could easily be uncoupled, cleaned, refunctionalized, and recoupled. This meant that microelectrode arrays which are expensive and time consuming to produce could be saved and reused. The effect was streamlining study of the biosensor under various conditions in much lower time. Furthermore, a study of the physics underlying the electronic detection revealed that higher voltages could increase detection speed and sensitivity. Theoretical frameworks suggest this could be due to the formation of convective microstreams within an otherwise static incubation fluid, resulting from a joule heating of the electrodes [15-17]. Experimental detection of insulin antibodies vs blanks or isotype off-target specific antibodies was discernable seconds after dynamic flow injection. This work was presented orally and published in the IEEE database as a proceedings article, and is presented here in chapter 5. Finally, switching transduction mechanism from an electronic to optical signal in SPR allowed detection to be carried out directly, by use of dextran linking surface layers. Functionalized on a high-capacity amine sensor by NHS and EDC, a forest of dextran linkers increased the number of binding sites, enhancing sensitivity in detection of peptide insulin. Due to its lower molecular weight of ~ 5kDa, the insulin hormone is more difficult to detect than the absorption of much larger anti-insulin antibodies. These concepts and others are reviewed in the draft manuscript discussed in the last section of chapter 4. Further details of surface plasmon resonance experiments in insulin detection are presented in Appendix C.

8.2 The Effect of Neogenesis Associated Proteins on Human Islets

To investigate means of enhancing insulin secretion and the impact of smaller islet populations, peptide agents were added to high glucose stimulation buffers and incubated with human donor islets. Similar to the nanobodies used to increase the effective signal in SPR sensing,

short peptide sequences thought to be bioactive fragments of endogenous beta cell promoting proteins were tested. Measurement of the insulin content in the stimulation wells was done by ELISA, then recovered islets were lysed and sampled for gene expressions of nuclear mRNA. RT-qPCR measurements revealed an interplay between opposing islet genes Glut2 and Glucagon. When concurrently active, Glut2 and PDX1 upregulation correlated the overall amplitude of insulin response to glucose. The cause of and age at the time of death likely impact the performance of human islets obtained through isolation. Variation among donors hinders the usefulness of standard screening methods for islet transplant. Further in-depth testing should explore peptide dosage concentration and exposure time. Improved computing algorithms will allow for greater simulation power in predicting favorable conformations across libraries of peptide sequences and administration conditions. Future studies could uncover useful insight by integrating multiplexed biosensors with dynamically perfused cell culture chambers. Here, surgical transplant protocols for characterizing islets were used to evaluate the effects of specifically designed and synthesized peptides on islet biology. This work resulted in a first author publication, which is presented here in chapter 6. A discussion of donor islet isolation and culture in vitro can be found in Appendix A. Supplementary information for this publication is included in Appendix B.

8.3 Establishing a new 3D tissue model for research

Building on the techniques and knowledge gained from peptide screening studies, a subsequent tissue culture project was motivated by the need for consistent and available cell models for human islet research. In that spirit, endothelial cells were chosen for coculture with beta cells in 3D spheroids, then tested for insulin production, cell receptor expression, and metabolic respiration. This work has been accepted for publication in the Journal of Diabetes

Research and is presented in chapter 7. The supplemental information for this article is included in Appendix D.

It was hypothesized, based off prior studies, that incorporating endothelial cells would promote spheroid structure and stability, while playing a secondary role supporting insulin secretion from beta cells [18-20]. Three spheroid types were tested and compared based on their cellular composition. A monoculture EndoC-βH5 group was compared to 1:1 and 1:3 cocultures with human umbilical vein endothelial cells. All cell groups tested were amenable to robust spheroid formation with good viability. Immunofluorescent confocal imaging confirmed beta cell/HUVEC integration, within a spheroid, by receptor specific staining. Immersing the spheroids in elevated glucose media prompted a strong and highly significant increase in insulin output. These results, taken collectively with metabolic activity (monitored over 8 days), show HUVECs had no detriment, in fact some benefits to beta cell performance. Further replicates should be tested to determine the true significance of these merits.

8.4 Limitations and Future Directions

Although multiple facets of in vitro islet research have herein been incrementally advanced, there are many areas in need of development. Luckily, the field is also rife with opportunity. Progress in bioengineering technologies has opened many doors and uncovered just as many challenges. The most critical limitations and proposed directions are given below.

The detection of human insulin in solution can be cumbersome with standard methods, however next generation sensors require improvements in sensitivity and throughput capabilities to justify wider implementation. It is now known that beta cells in the healthy state display an oscillatory, pulsatile insulin response to glucose [21, 22]. A deviation from this waveform can be

an early indicator of dysfunction. Strides have been made in time resolution of affinity biosensors, however their complex design and limited reusability remain hurdles to their full implementation. Development of real time, label free detection methods such as SPR will help to uncover the nature of cellular communication in healthy islets, thereby elucidating disease onset and possibilities of mediation. Establishing a standardized 'secretory fingerprint' in the healthy condition, across 3 to 5 signalling proteins would provide a benchmark in the evaluation of stem cell derived islets and contribute to improved diabetes classification and personalized treatment. Quantifying hormonal release of a single peptide remains a challenge, however, no less resolving time-dependent multiplexed secretion dynamics of the complex micro-organ. If these data were widely gathered across a meta study, it would help to establish a gold standard of the non-diseased state, deviation from which could help to alert clinicians of dysfunction. Only 5-10% of those in a prediabetic state go on to develop diabetes each year, a disease projected to affect nearly have a billion people in the next ten years [23]. Overt symptoms may not appear until near total destruction of the pancreatic beta cells has occurred [24]. Greater insight into the process of disease onset therefore is crucial to early or even preventative treatment. Compounding the issue is that each particular patient has their own individual medical history and disease characteristics. Having a more informative picture of a patients insulin, glucagon, Hb1Ac, c-peptide and other relevant biomarker levels would be instrumental to developing personalized medicine. Individually tailored and adjustable treatment plans would naturally allow better disease management.

Experimental costs and accessibility remain a bottleneck for researchers attempting to unravel the causes and cures of diabetes. Future efforts will make fundamental discoveries in biology through use of cutting edge technology, though also continue to advance translational

engineering projects. Improving access and facilitating research for wider demographics will only increase our collective chances of conquering this devastating disease.

Primary research limitations involved with screening human islets also include the inherent variability across individual donors, cost, and intermittent access to donors based on availability. The study of islet biology is hindered by access to native cells or relevant tissue models, compounded by inadequate technologies for interrogating their behaviour. Obtaining a suitable immortalized cell model for screening islet secretagogues would lead to greater data reproducibility, essentially unlimited access and continuous availability. Future experiments should investigate the immunogenic nature of multicellular constructs, and how to best protect them from attack. If endothelial cells could be isolated from the recipient, coculturing them with a beta cell graft could reduce the overall immune footprint and lower the risk of foreign body rejection. Longer spheroid culture periods with the addition of either proangiogenic factors or ECM proteins could lead to highly stable and functional pseudoislets [25]. Culturing within a scaffold could help recreate the islet community within the pancreas, promoting longevity of a graft [26]. Decellularized mammal organs provide excellent scaffold housing for islet and multi-cell cocultures. The increasing resolution of 3D bioprinters will soon lead to synthetic alternatives for explanted tissue-based scaffolds. Beneficial molecular factors can even be incorporated in structures of controlled size and geometry. Advancements in gene editing and stem cell differentiation protocols will be crucial to obtaining a continuous supply of islets for research and transplant.

No doubt the interplay of biological, electronic and materials sciences will develop innovative and elegant solutions to many hurdles faced today. Artificial intelligence and machine learning methods will help researchers identify patterns in massive datasets in a high throughput

manner. As biotechnologies become more finely tuned, the resulting understanding and deepening of knowledge will in turn provide insight to guide further advancement of in vitro research techniques.

8.5 Conclusions

This work represents important advances in multiple aspects that challenge the understanding and success of islet transplantation. Diabetes is a complex and chronic autoimmune dysfunction, and the suitable handling of cells for patient transplant incorporates techniques and experience from a vast set of fields. Certainly there is much advancement still to be made, but encouraging results have so far be seen. Cellular transplant therapies have to potential end the need for insulin administration daily, which would lead to much safer and healthier lives for millions. Aside from the knowledge of diabetes and pancreatic biology, the device and material aspects along with cell culture techniques could be applied to a wide variety of research questions for other diseases or conditions. Lastly, the streamlining and increase to access of the in vitro assays discussed herein moves us towards involving more human minds to partake in the field of work. Hopefully, as technology becomes more advanced, cheaper, faster and smaller, we will soon make a discovery that could truly impact the world.

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Appendix A

Isolating Donor Islets

Islet transplantation is a procedure in which living pancreatic cells from recently deceased donors are isolated and implanted to type 1 diabetic patients to replace insulin. Endocrine hormone producing Islets of Langerhans are separated from fatty exocrine tissue, cultured, and subsequently implanted into suitable recipients through a minimally invasive laparoscopic procedure. Patients can be released from the hospital the same day, as compared to the weeks of recovery and burden on the healthcare system involved with total pancreatic transplant. Whereas the current standard of replacing insulin directly requires multiple daily injections, islet transplant offers a one-time procedure that would remove insulin dependence as successfully grafted islets dynamically sense glucose and release insulin in their natural response. Longer term success rates are still limited however, with most grafts lasting a few years at best [32]. The majority of cells lost are in the first hours following

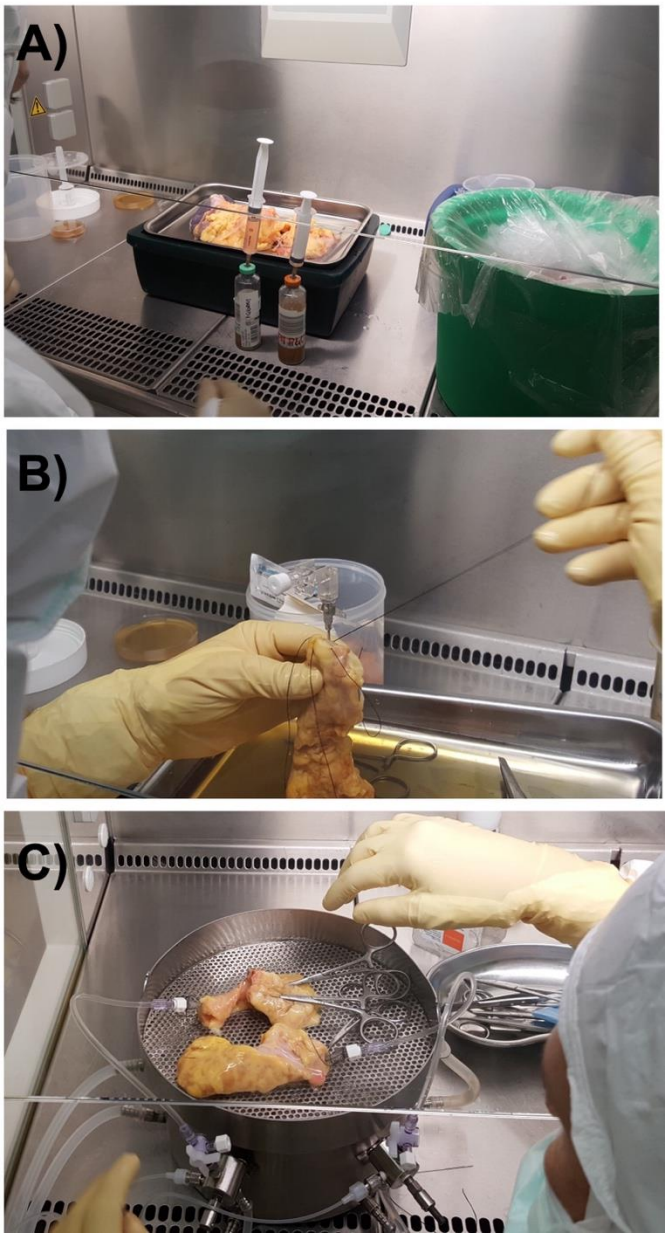

Figure SA1: (A) Collagenase/Protease (~2:1) enzyme solution for digestion of excised donor pancreas (B) Sutured cannulation for enzyme perfusion (C) Warm collagenase actively breaks down pancreatic ECM, while switch to cold perfusion stops the digestion (captured by J. M. Porter at IRCCS Milan)

transplant due to innate immune response, with subsequent autoimmune destruction by T-cell attack [24]. The situation is exacerbated by the difficulty islets have reintegrating with local vasculature after being disrupted from their native basement membrane which housed them in a connective network.

During the window of *in vitro* culture, following isolation and before transplant, there is an opportunity to screen cells for health and function, though extended study of islet mechanisms is limited by short culture lifetimes and donor availability.

Figure SA2: A) Digested pancreatic tissue sections are placed into a Ricordi's chamber with metal ball bearings for mechanical homogenization, B) Specialized radial centrifugation system for separating islets from fatty exocrine tissue by mass density, C) Separated fractions of varying purity, D) Selected fractions chosen for highest islet content and quality, insulin stained red by dithizone. (captured by J. M. Porter at IRCCS Milan)

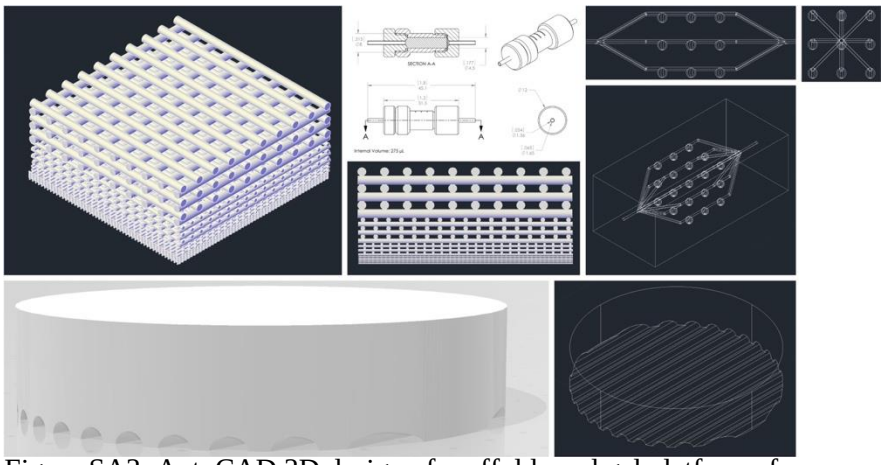

Figure SA3: AutoCAD 3D design of scaffolds and gel platforms for embedding with islets

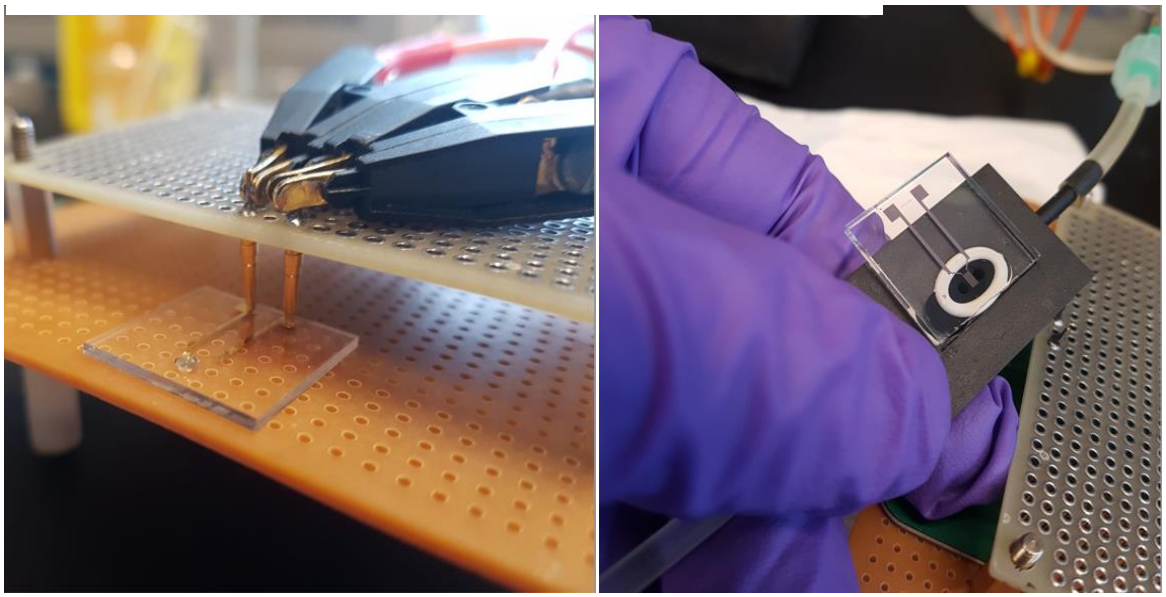

Figure SA4: (A) Source (cathode) and drain (anode) electrodes attach high-frequency signal generator to the interdigitated electrode array, (B) 3D printed chamber with custom O-ring coupling to fluid inlet/outlet to the microchip sensing area. (J. M. Porter, 2018)

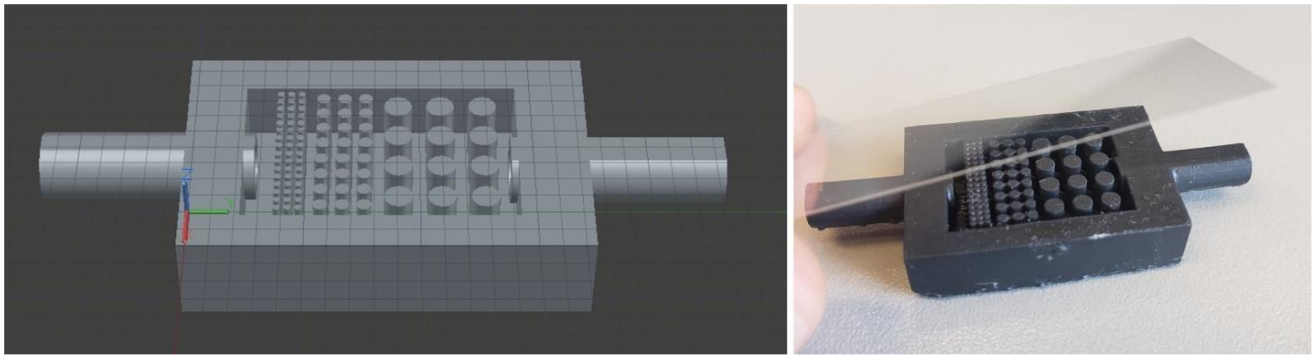

Figure SA5: (A) CAD design of islet-capture chamber for perfused secretion studies, (B) 3D-printed device couples with glass coverslip for optical interrogation (J.M. Porter, 2019)

Appendix B

Supporting Information

INGAP-Peptide Variants as a Novel Therapy for Type 1 Diabetes: Effect on Human Islet Insulin Secretion and Gene Expression

James M. Porter[a], *Léa Guerassimoff*[b,d], *Francisco Rafael Castiello*[a], *André Charette*[d] *and Maryam Tabrizian*[a,b*]

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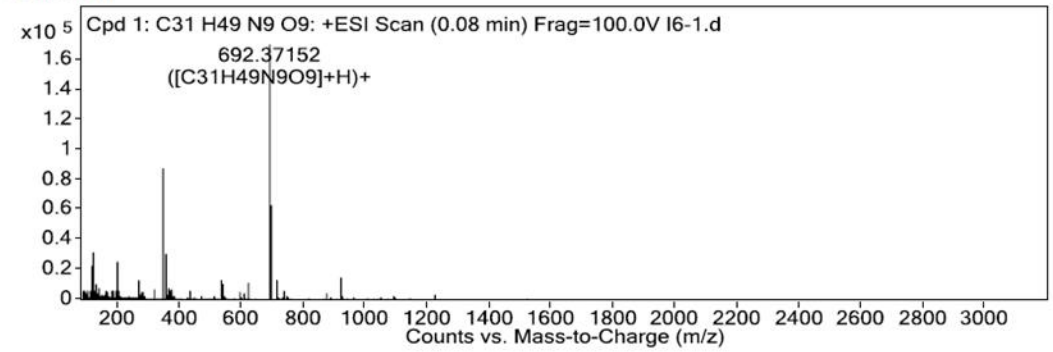
* Corresponding Author: maryam.tabrizian@mcgill.ca

Sample Mass Spectrometry Data: I6 exact mass isomer

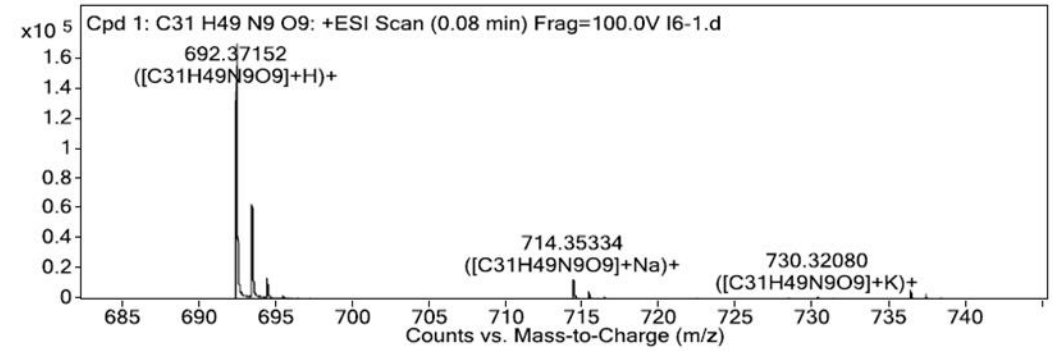
Exact Mass Report – I6

Data File	I6-1.d	**Sample Name**	I6-1
Sample Type	Sample	**Position**	P1-B1
Analysis Date	7/11/2019 9:50:04 AM	**User Name**	KG
Acq Method	ESI_POS_DI.m	**InstrumentName**	TOF 6224
Comment	MPA:H20+0.1%FA MPB:MeOH		

MS Spectrum



MS Zoomed Spectrum



MS Spectrum Peak List

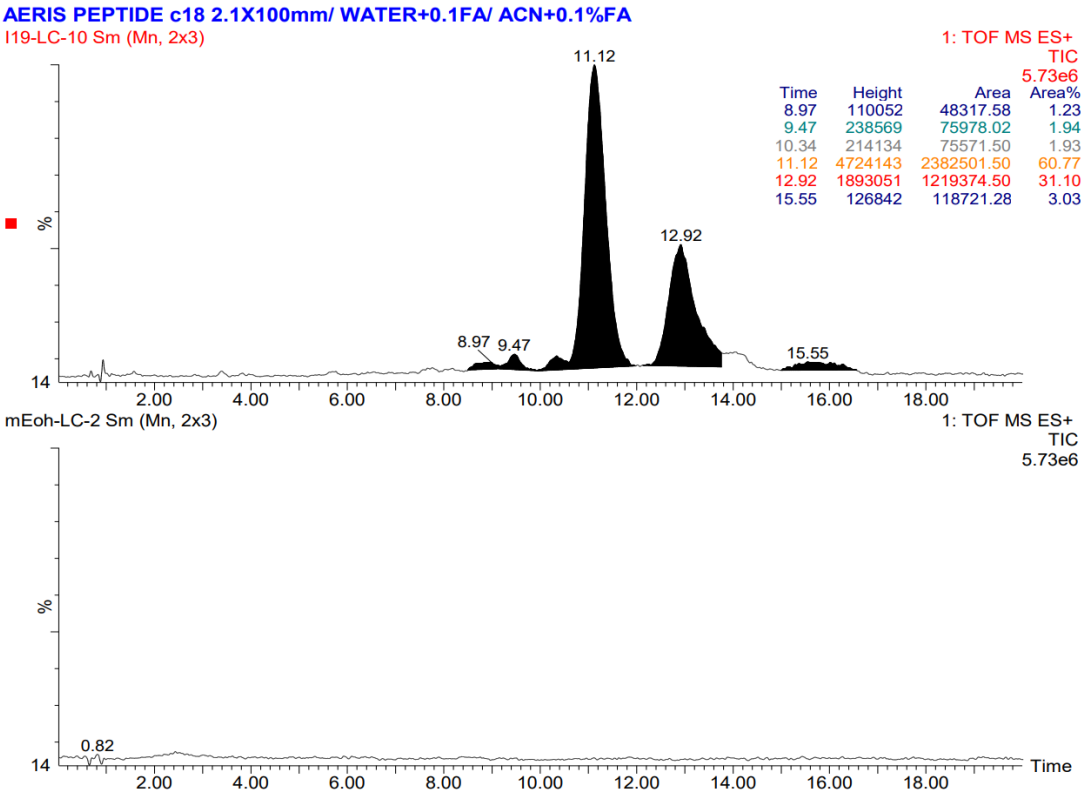
Ion	Formula	Abund	*Expe. m/z*	*Calc. m/z*	Diff(ppm)
(M+H)+	C31H49N9O9	171227.52	692.37152	692.3726	1.56
(M+Na)+	C31H49N9O9	13114.12	714.35334	714.35455	1.69

Sample Purity Analysis: I19 (after purification)

Purity

Data File	I19	**Sample Name**	I19
Folder	2019I_sep	**User Name**	LOUIZA
Acq Method	LC_ISO23_20min_col2	**DA Method**	
Instrument	synapt		
Date	15-Sep-19		

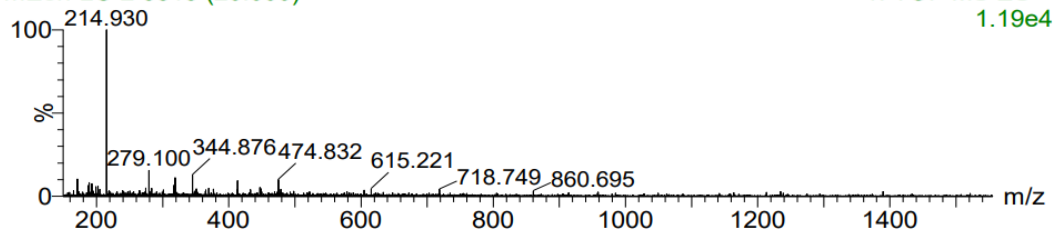
Chromatogram



MS spectrum

AERIS PEPTIDE c18 2.1X100mm/ WATER+0.1FA/ ACN+0.1%FA

mEoh-LC-2 3815 (20.000) 1: TOF MS ES+ 1.19e4



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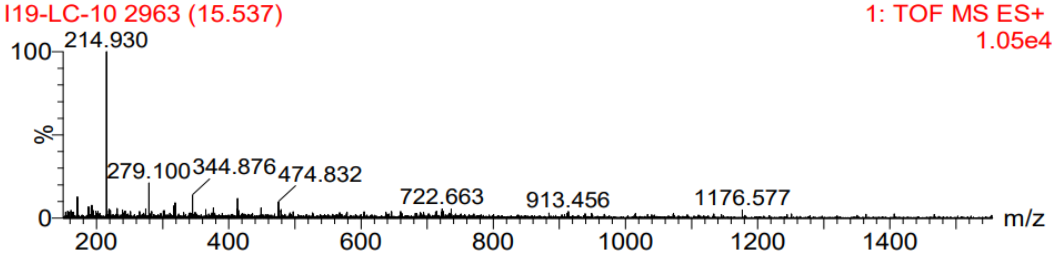

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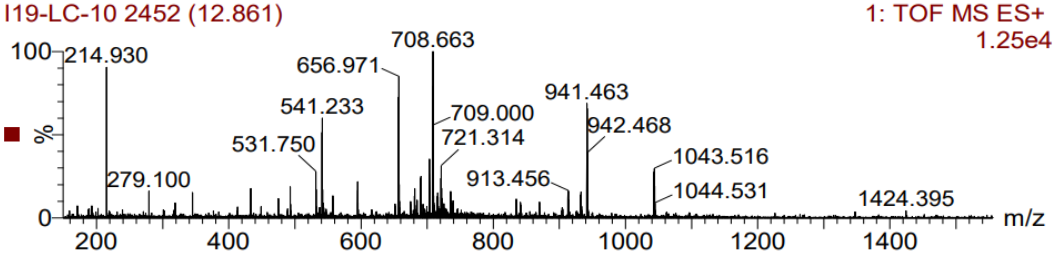
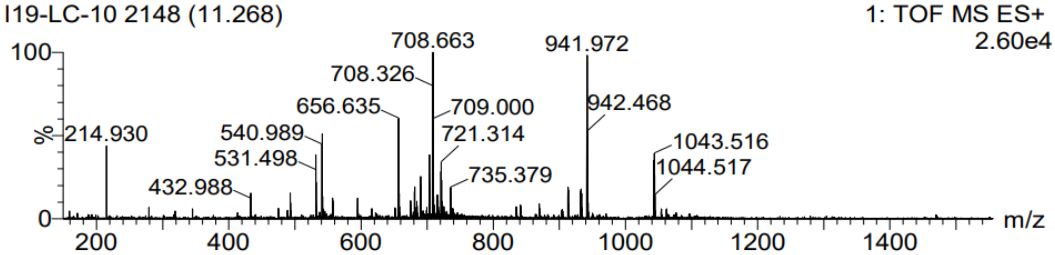
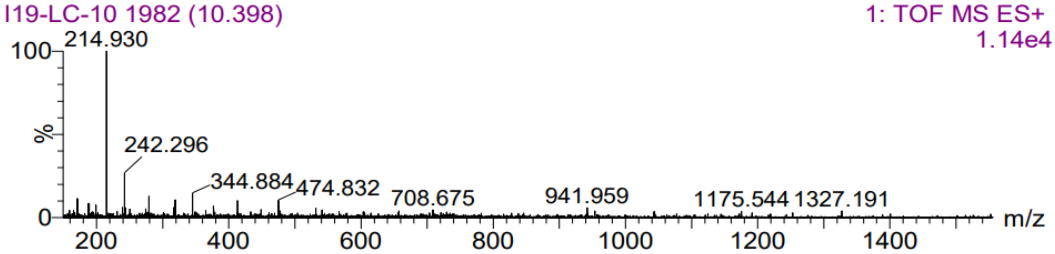


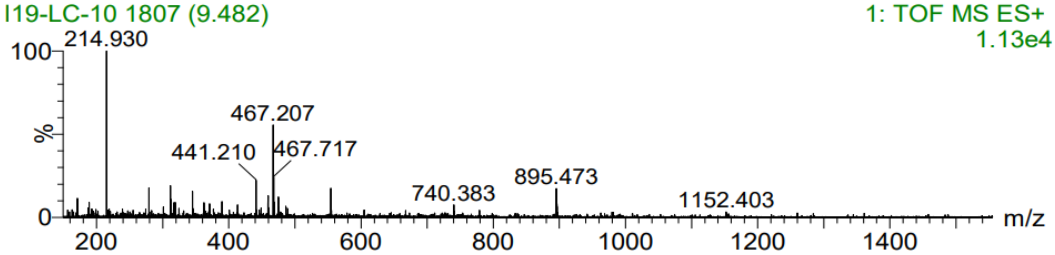
I19-LC-10 2148 (11.268) 1: TOF MS ES+ 2.60e4



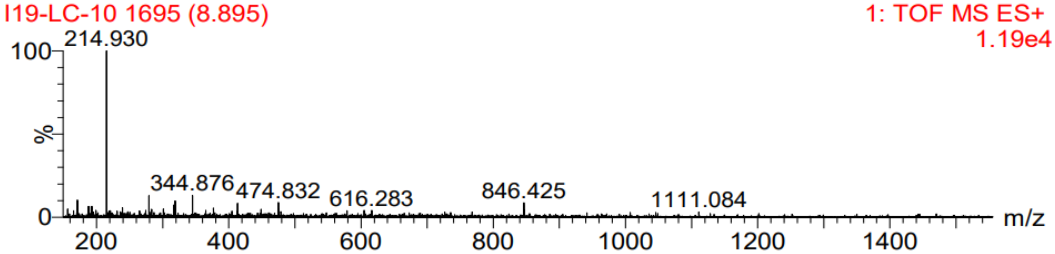
I19-LC-10 1982 (10.398) 1: TOF MS ES+ 1.14e4



I19-LC-10 1807 (9.482) 1: TOF MS ES+ 1.13e4



I19-LC-10 1695 (8.895) 1: TOF MS ES+ 1.19e4



Preliminary perifusion tests of human islets

Figure S1 displays very much similar line shapes of human islets in response to stimuli despite varying degrees of insulin released. Based off these initial observations, we focused on quantifying the amount of insulin released during the primary response as a gauge for evaluation. This figure has been added in the supporting information.

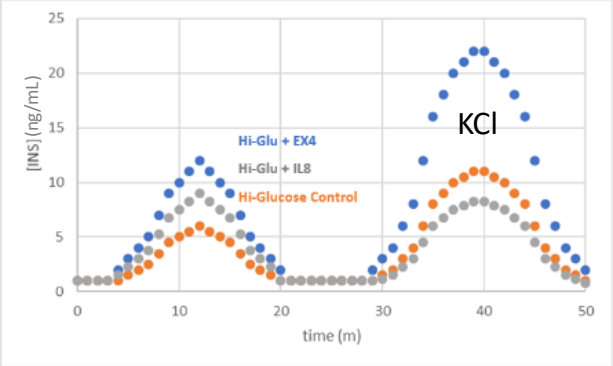


Figure SB1: Dynamic stimulation of human islets in response to stimuli. Primary stimulation (5-20m) is followed by membrane depolarization by KCl (30-50m).

Gene Expression

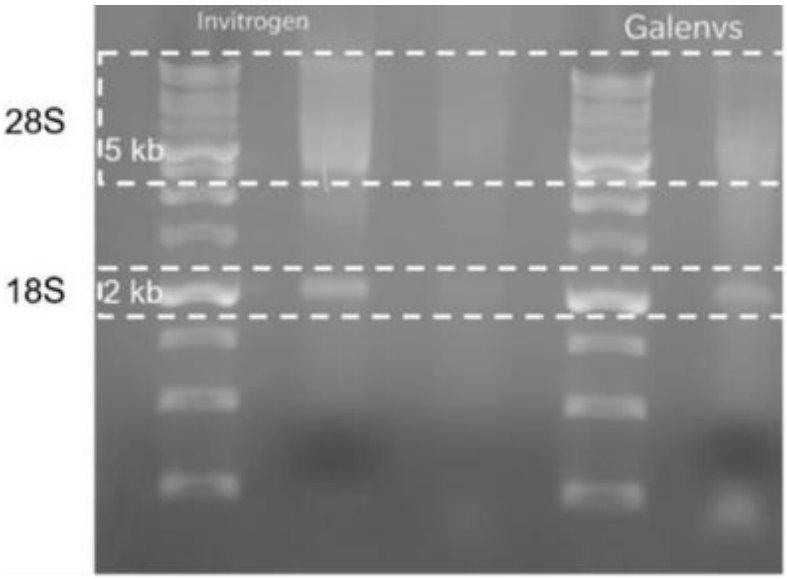


Figure SB2: Gel electrophoresis of purified RNA using Invitrogen kit

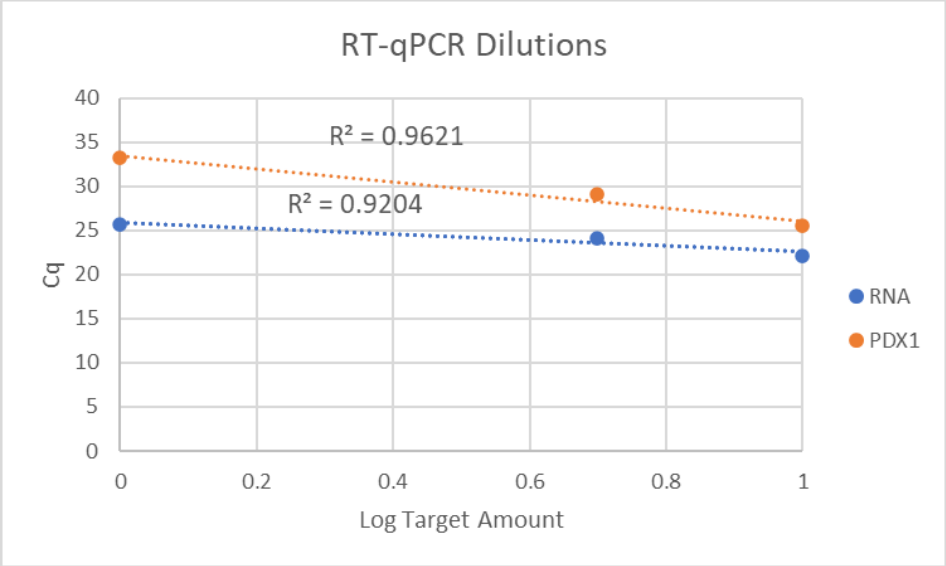


Figure SB3: Linear dependence of threshold amplification validates qPCR primers and RNA extraction

Appendix C

Supporting Information:

Renewable Human Cell Model for Type 1 Diabetes Research:

EndoC-βH5/HUVEC Coculture Spheroids

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[b]*Faculty of Dental Medicine and Oral Health Sciences, McGill University, Montreal, QC H3A 1G1, Canada*
**Corresponding Author*

Cell Types	Cell A (%)	Cell B (%)
MIN6	100	0
MIN6/HUVEC	50	50
EndoC-βH5	100	0
EndoC-βH5/HUVEC	50	50
EndoC-βH5/HUVEC	25	75

Table S1. Spheroid Groups

Gene	Function	Forward Sequence	Reverse Sequence
GAPDH	Catalyzes glycolysis, can activate transcription	CAC-CCA-CTC-CTC-CAC-CTT-TG	CCA-CCA-CCC-TGT-TGC-TGT-AG
Insulin	Encodes for insulin hormone production	GAA-CGA-GGC-TTC-TTC-TAC-AC	ACA-ATG-CCA-CGC-TTC-TG
Glut2	Transmembrane glucose transporter	CTC-TCC-TTG-CTC-CTC-CTC-CT	TTG-GGA-GTC-CTG-TCA-ATT-CC
PDX1	Transcription Activator	ATG-GAT-GAA-GTC-TAC-CAA-AGC	CGT-GAG-ATG-TAC-TTG-TTG-AAT-AG
MafA	Regulator of insulin gene	ATTCTGGAGAGCGAGAAGTGCCAA	CGCCAGCTTCTCGTATTTCTCCTT

Table S2. RT-qPCR β-cell gene targets

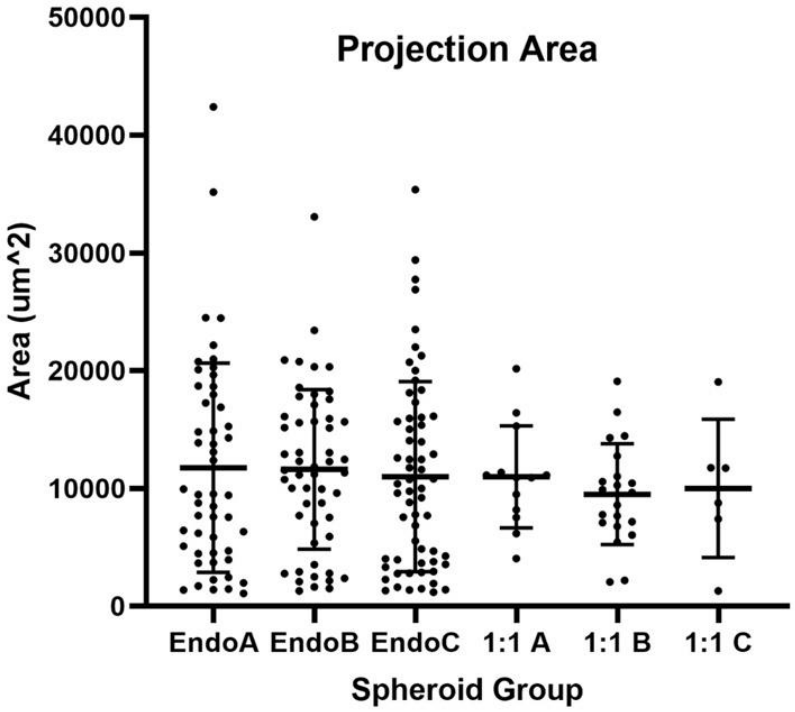

Figure SC1: ImageJ quantification of spheroid area, for individual aggregates in each of 3 batches for EndoC-βH5 and 1:1 EndoC-βH5/HUVEC coculture spheroids

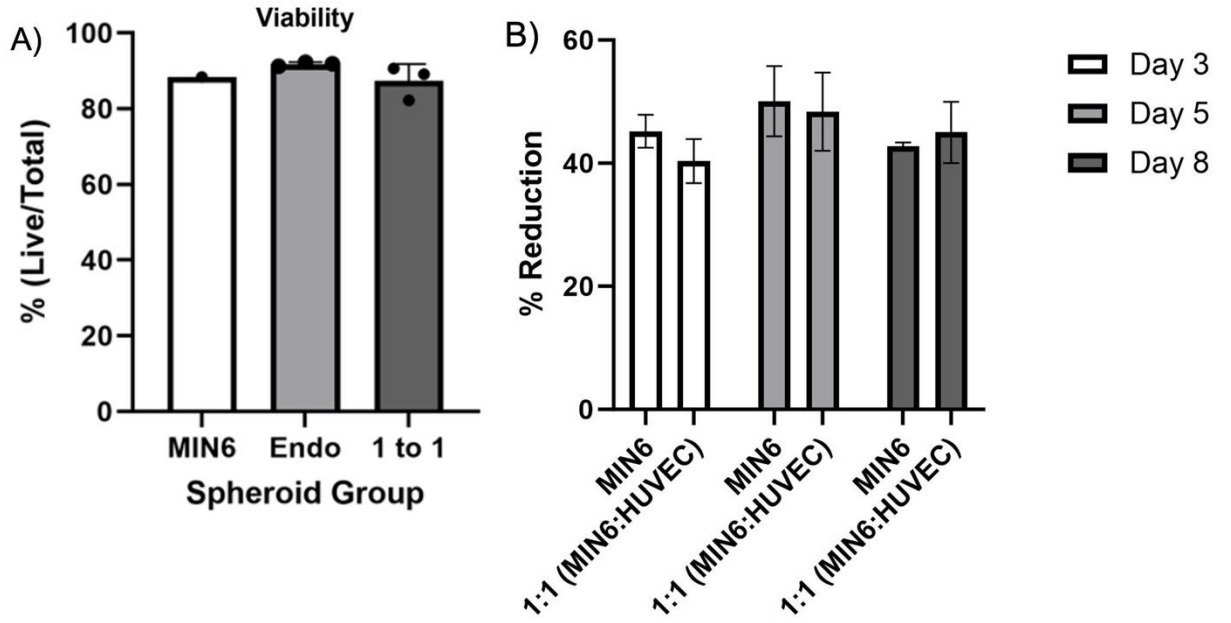

Figure SC2: (A) Mean viability shows statistically similar values for MIN6 and EndoC-βH5 monoculture with 1:1 βH5/HUVEC coculture spheroids, by one way ANOVA. (B) Metabolic activity of MIN6 and 1:1 MIN6:HUVEC spheroids at days 3, 5 and 8 after formation, as measured by AlamarBlue assay.

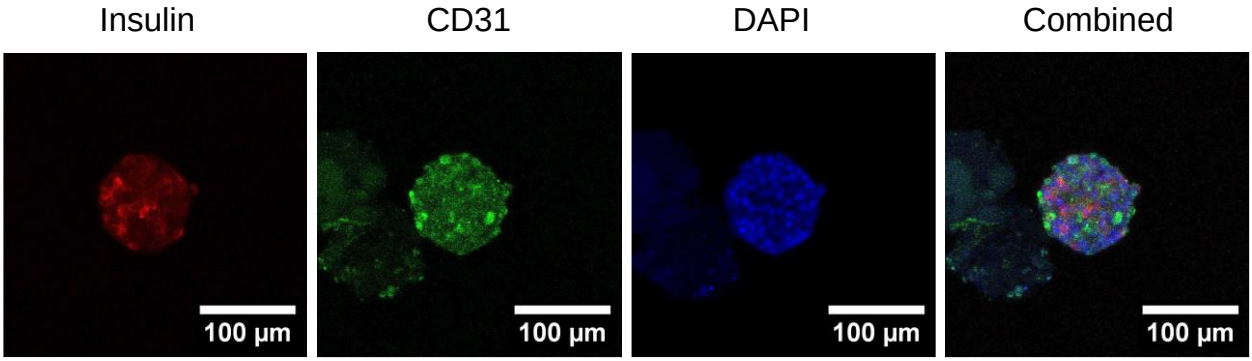

Figure SC3: Cell-specific staining of 1:1 (EndoC-βH5:HUVEC) spheroid including nuclear staining with DAPI

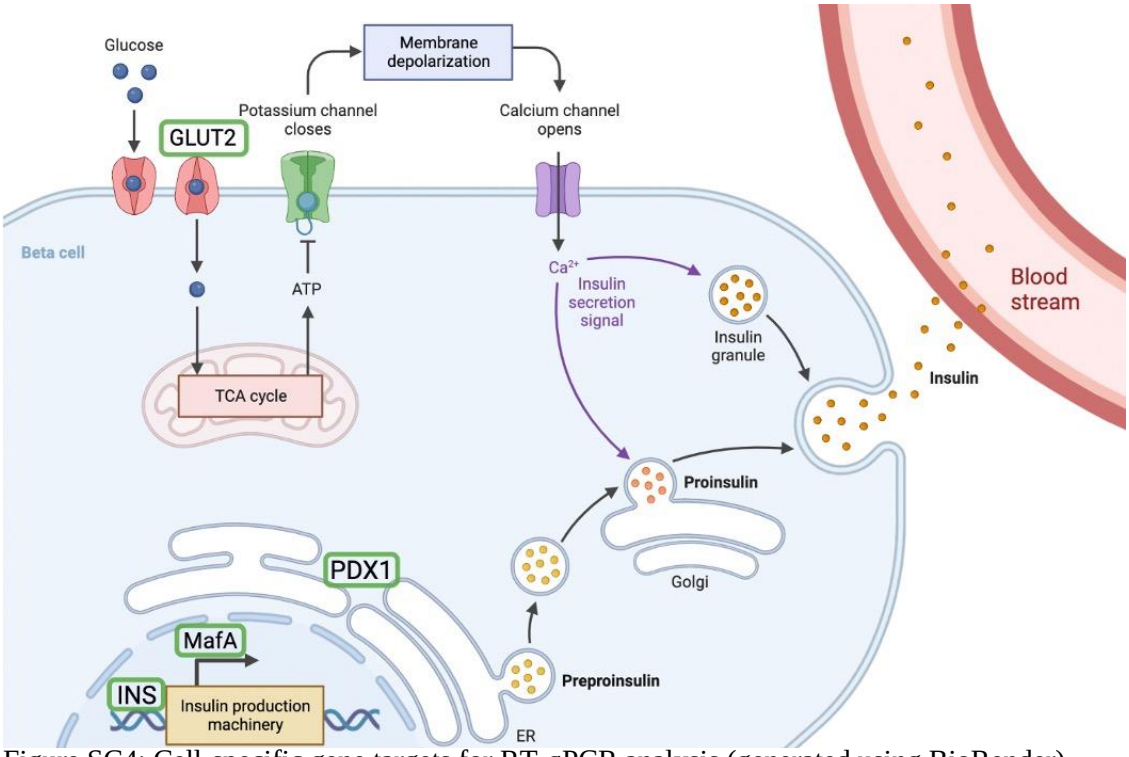

Figure SC4: Cell-specific gene targets for RT-qPCR analysis (generated using BioRender)

Appendix D

Surface Plasmon Resonance Biosensing for Type 1 Diabetes Research

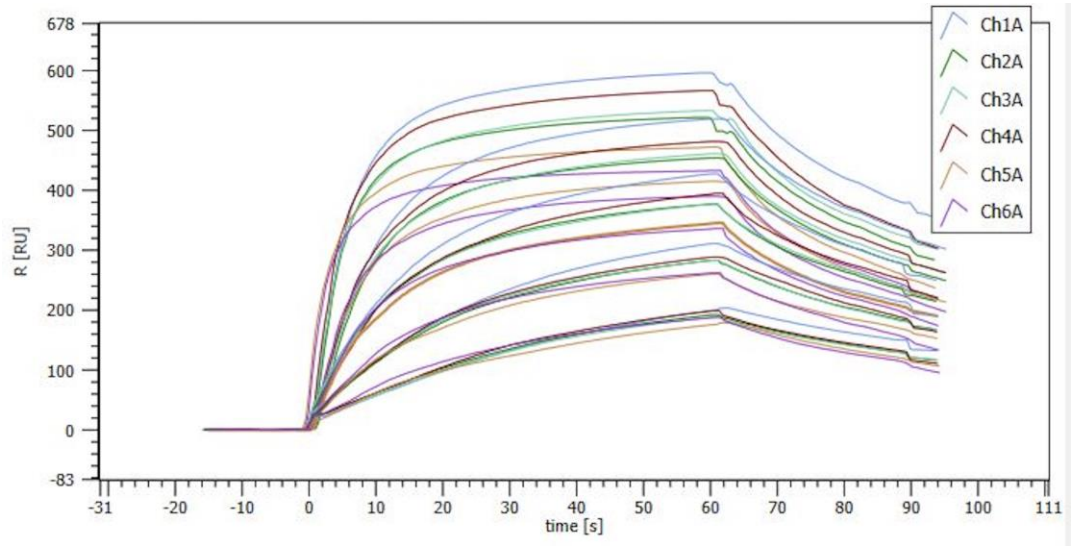

Fig. SD1: Human insulin (recombinant) vs. IgG quantification capture

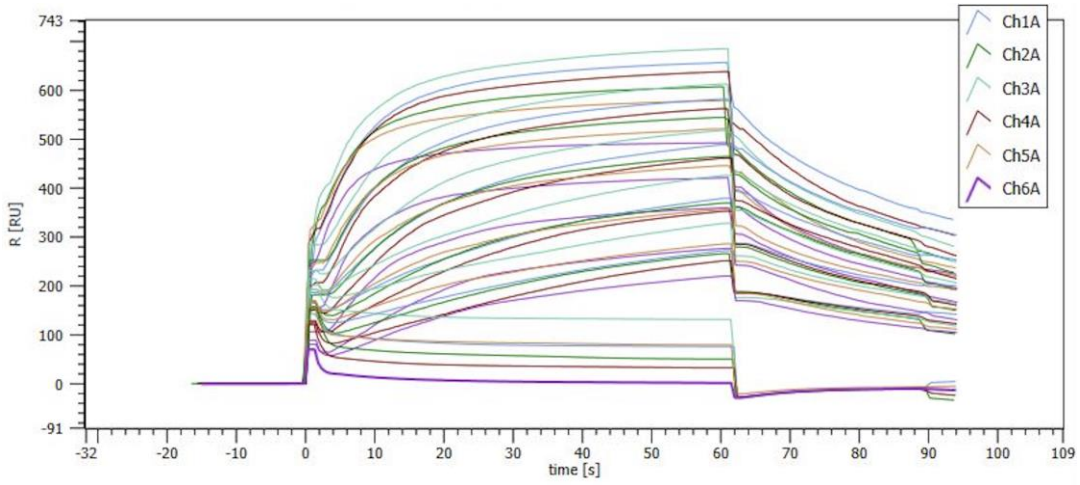

Fig. SD2: Human insulin (recombinant) in B20 Krebs vs. IgG quantification capture