BIOMIMETIC SELF-ASSEMBLED NANOMATRIX FOR PANCREATIC ISLET TRANSPLANTATION

By

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BIOMEDICAL ENGINEERING

ABSTRACT

In an attempt to treat type 1 diabetes, pancreatic islet transplantation (PIT) had been studied, but only 10% of recipients showed maintained insulin independence after 5-year post-islet transplantation, indicating that there remains a constant demand for improving the efficacy of PIT. For a successful PIT, critical factors should be addressed: immune protection, revascularization, and ECM-microenvironment reconstitution. Among them, this study was aimed to develop an ECM mimetic scaffold that reconstitutes the ECM derived microenvironment for an isolated beta cell and islet. A growing body of studies also indicates that the pancreatic ECM is of importance in islet survival and function. Hence, inscribing the dynamic characteristics of the pancreatic ECM within a biomimetic scaffold could offer an advanced strategy for PIT. In this dissertation, biomimetic self-assembled nanomatrix scaffolds composed of peptide amphiphiles (PAs) were explored to replicate the favorable native ECM microenvironment essential for beta cell or islet survival and function. Using MIN6 β cell lines, the PA scaffold inscribed with laminin-1 derived cell adhesive ligands was successfully developed to provide an islet ECM mimic environment for supporting beta cell functionality. While incorporating cell-adhesive sequences, a nitric oxide (NO)-releasing PA scaffold was also developed to providing β cells with a nitric oxide-releasing microenvironment. The NO releasing PA scaffold improved MIN6 β cell’s survival and function, demonstrating the potential of the PA scaffold in improving the efficacy of PIT with an expectation of enhanced
revascularization by NO. In addition, the biomimetic self-assembled PA nanomatrix supported improved islet survival and function in vitro as well. Through achieving fairly good islet survival and function for 14 days, the potential of the PA scaffold was demonstrated as an intermediary scaffold. These results demonstrate that the PA scaffold can allow the host integration of islets, for which at least 10-12 days is required. Overall, this dissertation demonstrated that the biomimetic self-assembled nanomatrix provide a feasible biomaterial strategy that improves the efficacy of PIT, which has been known to be a promising treatment for type 1 diabetes.

Keywords: islet, MIN6 cell, peptide amphiphile, extracellular matrix, pancreatic islet transplantation
DEDICATION

I would like to dedicate this dissertation to my family. Without the support of my parents, Sang-Rok Lim and Sang-Sook Choi, especially, I could not have finished this long journey of life. I always thank my younger sister, Eun-Ha Lim, who gave me priceless comments that helped me reflect my own life. Because of the support of my all relatives, I never doubted that it was possible to complete this study. Without their continual support in all aspects, I would have not finished this work.
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INTRODUCTION 1, 2

Growing evidence of improving islet adhesion, β-cell proliferation, β-cell differentiation, as well as β-cell function within the pancreatic extracellular matrix (ECM) has eventually driven existing biomaterial technologies towards replicating the dynamic characteristics of the pancreatic ECM.3 Although many biomaterials have been studied, there is still a need to develop more ECM mimetic scaffolds that synthetically recreate the islet-ECM microenvironment in order to offer a clinically relevant strategy for pancreatic islet transplantation (PIT). In order to improve islet engraftment, a multi-faced approach is needed: immune protection, revascularization, and ECM-microenvironment reconstitution.4 However, most biomaterials have paid attention to protect transplanted islets from host immune responses while ignoring potential role of ECM proteins on the successful islet engraftment. Moreover, extensive follow-up studies after successful clinical trials of PIT with the Edmonton protocol have explained that impaired insulin function followed by β-cell death could be attributed to multiple reasons, including destruction of islet-ECM microenvironment.5, 6 Hence, nurturing and supportive biomaterials that replicate the major features of native ECM are necessitated to enhance the efficacy of PIT. Based on the essential requirement for the islet engraftment, rebuilding the islet-ECM microenvironment for transplanted islets has intensively been studied elsewhere. Naturally derived ECM components such as collagen, fibrin, small intestinal submucosa, and matrigel have been used to mimic the native islet-ECM microenvironment. 7-11
A microporous poly(lactide-co-glycolide) scaffold coated with collagen IV demonstrated reversal of Type I diabetes in streptozotocin-induced syngeneic mice.\textsuperscript{12} Poly(ethylene glycol) hydrogel enriched by collagen IV and laminin led to improved functionality of MIN6 insulin-producing beta-cell line.\textsuperscript{13} However, the use of ECM proteins has potential risks, such as undesirable immune responses, higher infection risks, variability in biological sources, and increased costs.\textsuperscript{14, 15} Otherwise, small peptide sequences derived from ECM proteins have been incorporated into different types of bioinert polymers for \(\beta\)-cells in order to avoid the potential risks. The survival and functionality of MIN6 \(\beta\)-cells were enhanced when encapsulated in either a PEG based thermo-reversible gel conjugated with Gly-Arg-Gly-Asp-Ser (GRGDS) or photopolymerized PEG hydrogels modified with various laminin-derived peptides or type I collagen-derived peptides.\textsuperscript{16, 17} In consideration of clinical practicality of PIT, constraints of the given encapsulating biomaterials should be accessed: formation of fibrotic processes, poor degradation of the scaffold, and local and/or systemic toxicity.\textsuperscript{18}

To provide an ECM mimetic scaffold, peptide amphiphile (PA) are studied as promising transplant intermediary scaffolds.\textsuperscript{19-21} Due to its ability to encapsulate islets, enzyme-mediated degradation property, and creating pseudo islet-ECM interactions, the PA-based nanomatrix could have potential advantages regarding PIT.\textsuperscript{2} The chemical nature of PA molecules allows for imitating the characteristic properties of the natural ECM.\textsuperscript{20} PAs also form three-dimensional nanostructures by lowering the pH or adding multivalent ions, and encapsulating isolated islets in a biocompatible manner. In this study, it was hypothesized that a natural ECM mimetic PA nanomatrix gel will support
islet survival and function during the overall process of pancreatic islet transplantation, and that a variety of cell-adhesive sequences within natural ECM proteins incorporated into the PA molecules will create a fine-tuned PA nanomatrix gel specific for islet encapsulation material. The potential of the fine-tuned PA nanomatrix gels for islet encapsulation materials will be systematically assessed by the following specific aims.
SPECIFIC AIMS

Specific Aim 1:

To evaluate MIN6 beta cell survival and function on ECM mimetic peptide amphiphiles.

Using MIN6 beta cells, the ECM mimetic peptide amphiphiles (PAs) with laminin-1 derived cell recognition sequences will be studied. Survival and function of MIN6 beta cells will be assessed to elucidate potential specific ECM mimetic PAs to improve islet encapsulation. Based on literature review, cell adhesive ligands such as Arg-Gly-Asp (RGD) found on islet ECM proteins, Ile-Lys-Leu-Leu-Ile (IKLLI) and Ile-Lys-Val-Ala-Val (IKVAV) in the α1 chain of laminin-1, and Tyr-Ile-Gly-Ser-Arg (YIGSR) peptide sequences found in the β1 chain of laminin-1 will be selected and tethered to basic PA molecules to compare the role of the ECM mimetic peptide amphiphiles on supporting beta cell survival and function.

Specific Aim 2:

To develop a nitric oxide releasing peptide amphiphile that stimulates MIN6 beta cell functionality and viability.

In this specific aim, more in-depth feasibility of the biomimetic self-assembled peptide amphiphiles will be investigated. The bioactive sequences studied in specific aim 1 will be utilized for developing a peptide amphiphile (PA) based nanomatrix containing
multifunctional bioactive cues and sustained nitric oxide (NO) release. The viability and functionality of MIN6 beta cells will be assessed. In search for providing a conducive microenvironment for transplanted islets, this study will use a novel PA-based NO donor, which attains controlled, sustained NO release in order to elucidate the role of NO in beta cells and islet cell biology. It is hypothesized that controlled NO release in synergy with multifunctional bioactive cues will promote islet cell viability and functionality. NO releasing PA nanomatrices with range from 16.25 μmol to 130 μmol will be used to analyze MIN6 cell behaviors. MIN6 functionality in response to glucose over a 7 day time point is assessed.

Specific Aim 3:
To evaluate ECM mimetic peptide amphiphiles for improving islet survival and function

\textit{in vitro}.

A systematic approach in which a variety of ECM-derived cell-adhesive ligands will be presented to intact rat islets through the PA molecules allows for suggesting a suitable ECM mimic for pancreatic islet encapsulation. Several peptide amphiphiles will be synthesized and evaluated to select ECM mimetic peptide amphiphiles served as candidates for islet encapsulation. To evaluate the effect of the ECM mimetic peptide amphiphiles on isolated rat islet function, 3, 7, and 14 days of cultivation will be performed, as revascularization is usually completed by 10 ~ 14 days after islet transplantation. Function, viability, and integrity of the islets embedded within the
nanomatrix gels will be evaluated for 14 days to validate potential of the ECM mimetic PA nanomatrices.
BACKGROUND RESEARCH AND SIGNIFICANCE

Significance of Pancreatic Islet Transplantation

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia, a status of high level of blood glucose due to either the absolute insufficiency of insulin, a peptide hormone produced by beta cells of the pancreas, or the inability of produced insulin to regulate carbohydrate and fat metabolism ineffectiveness of produced insulin. Although there was a discrepancy estimating the total number of patients, around 171 million were projected to have diabetes in 2000 and a dramatic change of the diabetes prevalence was expected in 2030, reaching up to 366 million.22, 23 While type 1 diabetes occurs when β-cells within the Islet of Langerhans fail to produce a sufficient amount of insulin, patients with type 2 diabetes are gradually subjected to increased glucose concentration of blood above normal standards and the insulin resistance, which in turn lead to defects in insulin secretion as well. Both types can also appear concurrently, which implies that both physical and functional loss of β-cell mass develop in a single patient.24

At the onset of Type 1 diabetes, hyperglycemia leads diabetic complications, which is caused by damaged hyperglycemia-susceptible cells such as capillary endothelial cells in the retina, mesangial cells in the renal glomerulus, and neurons and Schwann cells in peripheral nerves. To prevent severe diabetic complications, pancreatic islet
transplantation (PIT) had been proposed.\textsuperscript{25,26} Ideally, PIT restores sustained normoglycemia without potential risks such as hypoglycemia and procedural complications. Although recent successful clinical case reports have led scientific communities to reconsider the PIT as a cure for type 1 diabetes, a major drawback remains: the requirement for multiple islet infusions, usually obtained from 2 ~ 4 organs, to achieve insulin independence.\textsuperscript{27} While islet re-transplantation is effective, it is constrained by the shortfall of donor pancreatic tissue and sensitization to donor antigens. Thus, the disparity between islet recovery and demands of millions of diabetics mandates that improved methods for islet recovery and engraftment are needed. In the context, a major limitation for practical implementation of PIT is the substantial loss of islet cells, leading to limited engraftment after the transplantation. The disruption of the islet microenvironment results in islet cell death and dysfunction in the period of islet isolation, pre-cultivation, or post-transplantation. The islet microenvironment formed through the islet ECM, whose dynamic compositional and mechanical properties regulate survival, differentiation, and function of islets.
Biomaterial-based Strategies for Preventing Immune Responses

As an attempt to achieve normoglycemia, the state of normal level of glucose in blood, early studies had used the intravascular device which produced insulin within a microporous membrane that could be diffused into the host blood flow through the inner lumen of ultrafiltration capillaries of the membrane. This typical design of the membrane was tubular hollow fibers, whose inner pores permit delivery of metabolites including insulin while preventing leak of the transplanted cells and donor-derived proteins able to evoke immune responses. Despite the design fidelity that avoids direct contact of transplanted islet with the host blood, such devices also require additional systemic anticoagulation in order to prevent the potent thrombus formation, leading to
failure of clinical practicality. Alternatively, two types of immuno-protective scaffolds have been introduced.

The first type is macroencapsulation, in which a large quantity of islets is entrapped within permeable membranes, is implantable easily with minimal surgery and retrievable after implantation. Not only does it have a variety of shapes\textsuperscript{30-32}, but it can be implanted into a variety of implantable sites such as the peritoneal cavity, the renal capsule, and the subcutaneous site.\textsuperscript{33-35} For better retrieving islets, a combination of PVA (polyvinyl alcohol) and PET (polyethylene terephthalate) meshes was used to enhance mechanical strength. Within a Euro Collins solution, which stores organs including kidneys, liver, and pancreas, PVA and PET meshed islets exhibited reduced nonfasting blood glucose concentrations between 155 and 273 mg/dl for 30 days. However, transplanted islets within macroencapsules can be subjected to harsh conditions formed by hypoxia, impaired mass transfer, and incompatibility of used materials. In spite of providing a protective material, in an amphiphilic polymer membrane composed of crosslinked hydrophilic poly(N,N-dimethyl acrylamide) (PDMAAm) and di- or tri-methacrylate telechelic polyisobutylene (PIB) stars, for example, rat islets have been lost their viability up to around 60\% when cultured for 1.5 months.\textsuperscript{36} This study implies that simple macroencapsulation of transplanted islets is not warrant strategy to prevent a variety of cellular stresses, which cause failure of long-term survival.

In addition, its biocompatibility and geometry can affect biological performance. One study used two different types of acrylic copolymer hollow fiber: one had fenestrated
outside wall made by a conventional extruding technique, whereas the other had a smooth outer surface when fabricated in a humidified condition. Due to the insufficient biocompatibility of used materials, both hollow fibers were required additional coating with alginate polymers as internal islet protectors, as they made islets aggregated into large clumps themselves. After resolving the biocompatibility issue, the authors found that transplanted low numbers of xenogeneic rat islets within smooth outer surfaced acrylic copolymer fibers were not rejected, and maintained normoglycemia either intraperionteally or subcutaneously. It indicates that the physical structure of encapsulating materials, which are exposed to host tissue, also affect the material-derived immune rejection, even after increasing biocompatibility of used biomaterials. Moreover, macroencapsulated islets can experience limited nutrients and oxygen. In subsequent study, human islets embedded within alginate polymers were macroencapsulated with the acrylic copolymer, demonstrating only 2 weeks survival without immunosuppressive drugs in diabetic patients either type 1 or type 2.

Despite the advantageous aspects of the microencapsulation, capacity and retrievability, the macroencapsulation technique is not be practical in clinical setting, as the macrocapsules cannot hold the required large number of islets inside while compromising the loss of functional islets due to its inherent drawbacks.

The second type of immuno-protective scaffold is microencapsulation\(^{37}\), in which spherical semipermeable membrane offers better diffusion capacity, can be used for islet encapsulation. Typical materials used for macroencapsulation are chitosan, agarose,
alginate, poly (hydroxyethylmetacrylate-methyl methacrylate) (HEMA-MMA),
acrylonitrile based copolymer (AN69) and polyethyleneglycol (PEG).\textsuperscript{38-43}
Microencapsulation might protect transplanted islets from immune-related cells and
antibodies through its mechanical barrier. Transplanted islets within the microencapsules
permit the movement between inside and outside. This technique enables us to transplant
allo- and xenografts. While preventing entry of large cells and antibodies of the host
immune system, a microencapsule has sufficient mechanical strength needed for
successful xenografts. Compared to the macroencapsulation technique,
microencapsulated islets also exhibit improved mechanical stability, which is of
importance in surgical manipulation. In fact, the microencapsulation of islets has long
been studied in xeno transplantation. Reducing immunosuppressive drugs is of
importance to eliminate the potential risks such as increased chance of opportunistic
infection, side effects including mouth ulceration, anemia, leucopenia, diarrhea, headache,
neutropenia, nausea, vomiting and fatigue, and islet toxicity.

\textit{Similar to macroencapsulation, clinical application of microcapsules is far away.}
\textit{Further studies regarding microencapsulation would be required to achieve improved
long-term islet engraftment. Current usage of microencapsulation in PIT still requires
immunosuppressive drugs, whose adverse effects outweighed the risk associated with
diabetic complications.}
Figure 2. Immuno-protective devices. In general, typical types of immunoisolation are classified by physical dimensions (macro/micro) and implantation modes (intra/extravascular). For avoiding the direct contact of transplanted islets to the host blood, recent immune-protective devices are extravascular ones, and macro- or microencapsulation usually used to protect islets from the recipients’ immune system.

Biomaterials for Pancreatic Islet Transplantation

Alginate

Most common material used for microencapsulation has been introduced based on alginate, which was first employed by Lim.\textsuperscript{44} Natural alginate is composed of polysaccharides β-D-mannuronic acid (M) and 1,4-linked α-L-guluronic acid (G). Due to the structural characteristics, alginate solution allows for hydrogel beads with divalent cations, such as Ca\textsuperscript{2+} and Ba\textsuperscript{2+}. Alginate with high M contents enables to fabricate more stable gel. Additionally, high M alginate gives better binding capacity with poly-L-lysine (PLL) polymers, which are used to control porosity of capsules, thereby reducing PLL-induced fibrosis by its positive charge. In-vitro feasibility of the PLL-coated alginate in the past decade shed light on exploring a variety of synthetic materials as immunoisolating biomaterials such as polyethylene glycol, agarose, chitosan, and multicomponent capsules.\textsuperscript{45-48} When transplanted into the peritoneal cavity, one study showed a limited achievement of normoglycemia without immunosuppressive drugs. Transplantation of 10,000 human islets/kg was performed in the presence of a low dose of cyclosporine and replenished with 5,000 human islets/kg 6 months after the first implantation.\textsuperscript{49, 50} This result indicates that the major problem with microencapsulation is also that it requires multiple infusion of islets during the life span of the patients, even if using a combination of immunosuppressive drugs.

\begin{quote}
In spite of its contribution to cell encapsulation technologies, alginate is not fully biocompatible for long-term islet engraftment.\textsuperscript{54} Through a series of extensive studies, it was found that alginate contributed to allograft rejection. A gradual decrease in islet
\end{quote}
function was observed within the alginate capsule, as a gradual increase in islet necrosis was observed along with a continuous overgrowth of undesired cells around the implantation site.

Poly ethylene glycol (PEG)

As a non-ionic hydrophilic polymer, poly ethylene glycol (PEG) had paid much attention for islet encapsulation, and a broad range of PEG with different molecular weights were used to modulate the permeability of PEG-based gels. Most PEG-based hydrogels used for islet encapsulation are photopolymerized PEG hydrogels, and its cross-linked hydrogel network structures studied for selective permeability. PEG hydrogels created by dimethacrylated PEG macromers were believed to be composed of randomly coiled polymethacrylated chains and were used to predict not only protein diffusivity, but also survival and function of encapsulated islets. PEG molecules with different molecular weights have been used to exhibit varying cross-linking densities in order to improve islet survival and function. However, there were no significant changes in islet survival and islet insulin secretion, and a delayed diffusion of insulin with different cross-linking densities was observed, indicating that further study would be needed for implantation of given PEG-based hydrogel. Not only can PEG hydrogel be utilized as an immunoisolation material that controls its network structures, but also it holds ECM proteins found in the basement membrane to create a three-dimensional microenvironment. PEG hydrogels containing laminin and collagen IV resulted in increased glucose-stimulated insulin secretion. Especially, both proteins with relatively high amount of laminin demonstrated enhanced islet insulin secretion, which could be
explained by a similar percentage of the ECM proteins found in the native basement membrane. Similarly, MIN6 β-cells in ECM proteins-entrapped PEG hydrogels showed better survival over 10 days compared to those within a PEG hydrogel without ECMs. Less apoptosis of β-cells within the ECM-entrapped hydrogels was observed, indicating the role of ECM matrix interactions in pancreatic tissue engineering. Among the ECM proteins that were used, collagen IV and laminin proteins supported improved insulin secretion in respond to glucose stimulation, and synergistic improved MIN6 β-cell functions were observed when cultured within a PEG hydrogel containing collagen type IV and laminin, whose concentration ratio of collagen IV to laminin was 1:3.

The structural and functional flexibility of PEG molecules allows for PEG hydrogel formation and subsequent modification to make biologically functionalized PEG hydrogels. The meshed PEG hydrogel networks are able to protect islets from host-derived antibodies and cells involved in immune responses, whereas protecting transplanted islets from cytokines and radicals is limited due to the relatively small size of these molecules. To address one of drawbacks in hydrogel-based biomaterials for cell encapsulation, pro-inflammatory cytokine tumor necrosis factor-α (TNFα, 25.6kDa) was sequestered in a PEG hydrogel functionalized with a TNFα binding peptide, WP9QY. Under TNFα challenges, mouse islets within the PEG-WP9QY hydrogel showed a decreased caspase 3/7 activity, an increased metabolic activity measured by adenosine triphosphate (ATP) concentration, and an improved insulin response, indicating that PEG hydrogels capable of blocking pro-inflammatory cytokines could extend survival and function of transplanted islets. The blocking of MIN6 β-cell surface IL-1 receptors (IL-
1R) with interleukin-1 beta (IL-1β, 17kDa) proteins was studied to provide the immunoisolating property onto PEG hydrogels. When cultured with islet-specific CD4+ T-lymphocyte cells, MIN6 β-cells encapsulated within a PEG hydrogel presenting 1% GRGDSPG and 1% IL-1R inhibitory peptide sequences (FEWTPGWYQPY-NH₂, IL-1RIP) showed significant improved survival compared to the co-culture of both cells without PEG hydrogel.

Beyond the primary reason of utilizing PEG hydrogels in immune protection, peptide-functionalized PEG hydrogel has been studied to mimic the cell-ECM matrix interactions. Synthetic ECM mimics composed of peptide sequences found in laminin and collagen I were created with poly(ethylene glycol) dimethacrylates and used to encapsulate MIN6 β-cells. MIN6 β-cells encapsulated within IKLLI or IKVAV-tethered PEG hydrogel showed good viability compared to those of the non-functionalized PEG hydrogel. Except the collagen I DGEA peptide sequences, the laminin sequences LRE, PDSGR, RGD, and YIGSR supported improved viability within the functionalized PEG hydrogel. However, both IKLLI and IKVAV sequences represented significantly enhanced functionality. The amounts of normalized secreted insulin by ATP content in each sample demonstrated that two sequences, IKLLI and IKVAV, have beneficial role on function of MIN6 β-cells, and that combinational effects of synthetic ECM mimics are present.

However, potential risks occurring from used bulk synthetic biomaterials still remain; the physical or chemical properties of bulk biomaterials could induce a non-specific foreign
body reaction, resulting in graft failure. While PEG biomaterial has widely used for PIT both macroscopically and microscopically and is regarded as a biocompatible, some studies also pointed out that the biocompatibility of PEG is dependent on both transplanted sites and duration of implantation. In intra-epididymis (IE) of Lewis rats, more fibrotic growth was observed around PEG capsules than ones of alginate-poly-L-lysine (PLL), which are unable to be used for clinical purposes due to relative poor biocompatibility. Although PEG hydrogels are still a good encapsulation material, excluding non-specific binding of host cells as well as biomolecules, the chemical nature of this biomaterial exhibits no biological activity. In contrast, peptide- or protein-based materials bear these essential features as clinically acceptable scaffolds: biocompatibility, bioactivity, and biodegradability. As one of the peptide-based scaffolds, the PA molecules could offer a biochemical and biophysical tenability for creating specific cellular microenvironment.
Peptide Amphiphiles

Peptide amphiphiles (PAs) are peptide-based molecules that create an extracellular matrix (ECM)-like nanostructured scaffold capable of supporting cells and tissues. Due to the amphiphilic nature of these materials, cylindrical micelles are created in an aqueous solution, where the outer region of hydrophilic segment of PAs is displayed and the hydrophobic segment made by alkyl tails goes toward inside. PAs can be used to confer biological cues and utilized for developing a scaffold for tissue engineering applications. Incorporating specific amino acid compositions allows for adapting PAs to a variety of applications, including replicating the nature ECM. In the early work by Stupp and coworkers, both the hydrophilic peptide segment and hydrophobic alkyl tail were modified to explore the structural versatility. The role of alkyl tail in self-assembled PA was studied using up to 22 carbon fatty acids, forming the cylindrical packing of PAs. C6 hydrophobic tails attached to a same hydrophilic peptide segment explained that a certain length of the alkyl tail is critical to make elongated cylindrical micelles, which are considered to be nanofibers characterized by nanometers in diameter and micrometers in length. Aggregation of PAs in water forms cylindrical micellar nanofibers. Several factors are thought to determine the aggregate morphology. A critical packing parameter, $\frac{\nu}{l_c a_o}$, can be used to predict the formed structures, where $a_o$ is the surface area of the polar head group located in the upper part of hydrophilic peptide segment, $\nu$ is the volume of the alkyl tail, and $l_c$ is the critical chain length of the alkyl tail. The truncated cone shape has about $1/3 \sim 1/2$ of the critical packing parameter. PAs are one-dimensional assemblies, which elongate towards a single axis with a 100 ~ 1000 fold increase compared to other axes, thereby acting like linear polymers in water.
indicates that PAs have unique features contributing to formation of a viscoelastic gel. The 1D assemblies are capable of creating a network structure with an aid of several non-covalent interactions such as hydrogen bonding and π-π stacking, exhibiting viscoelastic behaviors that emulates one of the essential ECM properties.

The interwoven network of PAs can mimic the native microenvironment of native ECM, in which specific peptide-derived epitopes are displayed. A peptide-derived epitope composed of 6 ~ 12 amino acid sequentially can be attached to fatty acids, and exposed towards aqueous environment, presenting dense epitopes. While maintaining high epitope density by a bundle of PA nanofibers, a degree of mobile epitopes within the PA nanofibers can be modulated by using PAs with structural variations: linear, branched, and cyclic architectures. Accessible epitopes in different PA architectures have been quantified in vitro, confirming the role of PA architectures on the focal adhesion formation. The peripheral region of PAs consists of β-sheet secondary structures. The β-sheet secondary structures, assessed previously by infrared spectroscopy (IR) and circular dichroism (CD), were confirmed by Jiang and coworkers using transmission infrared spectroscopy (transmission IR) and polarized modulation-infrared reflection-absorption spectroscopy (PM-IRRAS). Interestingly, the structural variations of PAs reflect the extent of the internal structures of PAs. More disorganized β-sheet secondary structures were observed in both PAs containing IKVAV (PA-IKVAVs) and branched PAs. It explains that both hydrophobic and β-sheet disrupting amino acids within PA-IKVAVs led to a disorganized internal β-sheet formation of PA molecules, whereas branched architectures of PAs hinders close packing of peptide segments in the PA nanofibers.
There are different modes available to trigger self-assembly of PA molecules into nanostructured fibers: pH control, divalent ions-mediated induction, and solvent evaporation.\textsuperscript{20} Using a number of metal ions, Beniash and coworkers demonstrated that polyvalent metal ions induced the formation of PA gels, as negatively charged PA molecules formed a self-assembled gel-like structure in the presence of polyvalent metal ions including Ca\textsuperscript{2+}, Mg\textsuperscript{2+}, and Cu\textsuperscript{2+}. When placed on a oscillating rheometry with 20 mM of each ion salt, the formed PA gels indicated much more storage moduli (G’) than the loss moduli (G’’) under 3\% oscillatory strain ranging from 100 to 0.1 rad/s. It means that alkaline earth metals created an elastic gel of PAs.\textsuperscript{56} As an interesting route to induce self-assembly, an aqueous PA solution can be placed onto a surface, drying out solvent while inducing self-assembled PA coating on the surface. This technique is able to form bioinspired self-assembled coatings on biologically inert materials, meaning that traditional materials with attractive properties to biomedical fields could be utilized not only for expanding their applicability, but also for offering improved strategy in the biological applications. For example, nitric oxide releasing PA nanomatrix treated onto traditional stent materials enhanced selective adhesion and proliferation of endothelial cells due to the sustained released nitric oxide, thereby overcoming several limitations of traditional cardiovascular implants that might lead to incomplete re-endothelization, restenosis, and late-thrombosis.\textsuperscript{59}

The biocompatibility of PA-derived ECM mimics enables cells entrapped within PAs to function as part of the cellular-fate processes. Not only can the PA-derived ECM mimics present biologically functional cues that are usually inscribed within the native ECM, but
they could also provide a physically supportive scaffold for the cells. One interesting fact is that each PA molecule can be internalized into a cell and metabolized into nutrients. Based on biocompatible PA-derived ECM mimics, a number of examples applying the PA-derived ECM mimics on a variety of tissue engineering fields have been documented. One study demonstrated that PAs bearing RGDS sequences showed adhesion of bone marrow mononuclear cells, while branched RGDS-presenting PAs incorporated within a conventional polymer scaffold, poly(glycolic acid) (PGA), enhanced adhesion and proliferation properties for primary human bladder cells, implying that PAs could be a useful biomaterial to functionalize biologically inactive materials for tissue engineering. In a similar manner, selective differentiation of neural progenitor cells was observed, and the IKVAV containing PAs showed significant results that would open an attractive strategy for treating spine cord injury, reducing cell death at the injury site when injected into mouse spinal cord injury models. Increasing oligodendroglia cells responsible for the neural regeneration was correlated with histological observation showing regeneration of descending motor neuron, which result in improved hind limb function in treated groups. Functionalized with PA nanofibers, titanium-based implants could be used for better bone replacement, where RGDS sequences presented by nanofibers enhanced adhesion of preosteoblastic cells exhibiting osteogenic differentiations. Similarly, RGDS containing PA nanofibers supported ameloblast-like cells as well as primary enamel organ epithelial cells, demonstrating a potential bioinspired scaffold for hard tissue replacement and regeneration. In an attempt to construct vascularized scaffold, a heparin-binding domain, the Carcin-Weintraub heparin-binding domain, was
inscribed into heparin-binding PA (HBPA) designed for binding heparin sulfate-like glycosaminoglycans (HSGAG).  

**Figure 3.** Schematic representation of β-sheets within PA nanofibers. As depicted in the inset, β-sheets are oriented parallel to the long axis of the nanofibers (inter-β-strand hydrogen bonds are represented as yellow lines; carbon, oxygen, hydrogen, and nitrogen atoms are colored grey, red, light blue, and blue, respectively). Reprinted with permission from ref. 64. Copyright 2006 American Chemical Society.
Pancreatic Islet Extracellular Matrix

The low success rate of pancreatic islet transplantation continues to hamper promising treatments for diabetes. One of the main reasons attributed for this low success rate is the loss of the ECM microenvironment incurred during the pancreatic islet isolation process. This is because the isolation process involved in islet procurement not only blocks the blood supply, but also leads to disruption of islet-ECM microenvironment. Several factors are attributed to the poor islet engraftment success rate, which remains below 10%, compared to 86% for whole pancreatic transplant surgery. The role that the ECM plays in determining islet graft success has been confirmed by the observation of reduced glucose response, increased islet death, and impaired differentiation when the ECM disrupted. The ECM could be considered as a complex scaffold composed of a variety of proteins and polysaccharides that provide essential molecular cues that regulate cell behavior. In other words, the native ECM is a dynamic three-dimensional scaffold capable of supporting cell behaviors based on the composition of its microenvironment and serves as the medium by which most biological and cellular activities are regulated.

A growing number of studies involving the function of islet tissues indicate that the ECM is involved in a variety of biological features such as providing for adequate islet development, homeostasis, and recovery from stress or injury. It is apparent that there is a great need for understanding the roles the ECM plays in islet function. The ECM is a physically organized micro-structure according to the specific requirement of functional tissues, and has a unique mechanical feature according to its varied composition. The calcified matrix could be found in teeth and bones, while the cornea has a gel-matrix
that meets its functional requirement. Similarly, the islet base membrane consists of thin sheets of ECM fibers, in which cells talk mutually through cell to cell signaling that regulates proliferation, migration, function, and even development of islet of Langerhans. It represents that understanding the nature of islet ECM interacting with resident cells is of importance to direct transplanted islet function. The major ECM proteins are collagen, laminin, fibronectin, and elastin, all of which are woven together with polysaccharides. Not only can the ECM support the specific tissues with biochemical cues, it also interacts with biologically relevant molecules such as growth factors, cytokines, and enzymes.

Collagen: Significant tensile strength of collagen explains supportive properties of collagen internally and externally. Collagen I, II, III, IV, V, and VI are founded in the islet ECM. Collagen IV promoted the intact islet survival, while purified β-cells showed a reduced insulin secretion in response to glucose. Integrins, which are of importance in cellular signal transduction, can interact with collagen, and α1β1, α2β1, α10β1, and α11β1 integrins interact with the GFOGER sequence in domain I of the alpha subunit of collagen. Islet cells express β1 integrin, and α1β1-mediated cell attachment, migration, and function was reported in human fetal β-cells. In addition to well-defined interaction through integrins, nonintegrin receptors could be involved in regulating cell adhesion, migration, differentiation, and ECM production. Discoidin domain receptors found only in islet cells are an excellent example of nonintegrin receptors.

Laminin: Among major proteins of the basement membrane, laminin can form a stabilized networks bounded together, and nidogen and the glycosaminoglycans (GAGs)
of perlecan help to make those networks. Cross-shaped trimeric laminin proteins are able to bind to other laminin molecules through their short arms, forming sheets. While building its self-assembled structures, the long arm binds cell membranes. Laminin is prevalent in the islet ECM, and interacts with pancreatic endocrine cells. Interaction of laminin with cells is allowed either by integrin or by non-integrin pathways. The integrin receptors associated with laminin are varied: the β1 subfamily (α1β1, α2β1, α3β1, α6β1, α7β1, and α9β1), the αv subfamily (αvβ3, αvβ5, and αvβ8), and the α6β4.69 Also non-integrin receptors are involved in cellular communications, whose names are the dystroglycan protein complex, the Lutheran blood group glycoprotein (Lu), and laminin receptor-1. In the islets of Langerhans, the α3 and β1 integrin and dystroglycan were founded.70

Fibronectin3,65: Fibronectin is a dimeric glycoprotein, forming fibrillar forms able to interact with other ECM components such as collagen, heparin, and chondroitin sulfate proteoglycans (CSPGs). The RGD sequence within the fibronectin proteins is recognizable through a range of integrins, including α3β1, α4β1, α5β1, α4β7, α8β1, αvβ1, αvβ3, αvβ5, αvβ6, and αIIbβ3. Nonintegrin receptors in fibronectin are dystroglycan and syndecan. Integrin α3 and α5 are found in developing pancreatic tissues, and are involved in interactions with fibronectin during morphogenesis. Fibronectin has been immunolocalized in the adult islet periphery, islet ductal pole, and perivascular area, and is often associated with collagen I, III, IV and laminin. The α5β1 integrin has been implicated to signal cell survival through the transcriptional upregulation of the
antiapoptotic protein, Bcl-2, thus suggesting that fibronectin may play a role in preventing anoikis.

Nidogen/entactin\textsuperscript{65}: Nidogen is a globular glycoprotein able to bind to the protein core of perlecan, collagen IV, and the short arms of the laminin trimer. Islet cells adhere to the central domain of nidogen by an RGD sequences and $\alpha3\beta1$ and $\alpha\nu\beta3$ integrins. It was reported that two isoforms are observed in the peri-islet basement membrane.

Vitronectin\textsuperscript{65}: Vitronectin, one of the glycoproteins in islet basement membrane, is only found in human fetal islet tissue, and upregulation of the vitronectin receptor, $\alpha\nu\beta1$, was observed in the fetal islet tissue. The $\alpha\nu\beta1$ is of importance to spread and migrate fetal $\beta$-cells grown on the vitronectin.

Proteoglycans\textsuperscript{65}: Proteoglycans are glycosylated glycoproteins found in ECM. A core protein of the proteoglycan has long carbohydrate side chains that are posttranslationally attached. Under physiological conditions, the carbohydrate chains are negatively charged, leading to serve as molecular sieve that binds and sequesters growth factors and cytokines. The major proteoglycans consisted of the islet ECM are heparin sulfate proteoglycans (HSPGs) and chondroitin 4- and 6-sulfate proteoglycans (CSPGs). Long and linear polymer chains of N-acetylglucosamine and glucuronic acid in the HSPG allow for multiple binding domains for various ECM components and cell surface receptors. Also, the negatively charged sugar residues can bind to growth factors and modulate their distribution within the ECM. HSPGs include perlecan, syndecan, glypican,
and betaglycan. In the case of CSPGs, the protein core has covalently bounded sugar chains composed of alternating N-acetylgalactosamine and glucuronic acid. CSPGs are found abundantly in cartilage and in the islet basement membrane.

**Figure 4.** Pancreatic islet extracellular matrix. The islet ECM is a basement membrane composed of collagen IV (major), fibronectin, laminin, perlecan, and other components, and a dynamic natural scaffold able to modulate islet survival and function in vivo. From ref. 65
Innovation and Significance of the Proposed Study

This dissertation proposal is aimed at developing a natural extracellular matrix (ECM)-like nanomatrix gel that supports islet survival and function by replicating the essential features of natural ECM microenvironment.

To achieve the aims of proposed study, a novel peptide-based biomaterial that can offer a great deal of design flexibility for creating an islet-specific ECM microenvironment will be utilized. In this study, the peptide amphiphiles (PAs), which have successfully studied in regenerative tissue engineering, will be used to create an islet-specific ECM mimic for improving pancreatic islet transplantation.

Islet encapsulation has long been studied as one such promising approach that permits allo or xeno islet transplantation, which could solve the shortage of islet supply. In spite of the efforts to improve the efficacy of islet engraftment in immune protection, clinical usage of the islet encapsulation is still challenging because the failure of islet engraftment is attributed to several factors including: incomplete immunoprotection, inadequate biocompatibility of used materials, and destructed ECM microenvironment. The need for improved biomimetic character of islet scaffolds is supported by recent studies demonstrating that substantial β-cell loss during the peritransplant period is detrimental to the efficacy of islet transplantation. Specifically, the loss of β-cell function during islet isolation is believed to result from the destruction of the native islet microenvironment. In addition, the disruption of islet-ECM interactions exposes the islets to a variety of cellular
stresses that further contribute to loss of biological functions. Therefore, biomimetic materials that create more ECM mimetic microenvironments are required to better maintain islet function and survival during the intermediate stage between implantation and restored host integration.

In this dissertation proposal, it is hypothesized that a natural ECM mimetic peptide amphiphile (PA) nanomatrix can enhance the efficacy of pancreatic islet transplantation as the PA nanomatrix offers essential engineering parameters: a rapid gel-like 3D network formed by self-assembly at physiological conditions for islet encapsulation, versatility to incorporate various cell adhesive moieties for bioactivity, cell-mediated degradable sites (matrix metalloproteinase-2, MMP-2) for progressive scaffold degradation, thereby preventing long-term islet dysfunction, and an ability to release growth factors in a highly controlled manner to promote revascularization. Moreover, the PA nanomatrix has an ability to deliver nitric oxide (NO), exhibiting beneficial effects of NO including insulin production and enhanced revascularization, which is a critical factor for successful islet transplantation.\textsuperscript{71, 72}
BIOLOGICAL SENSITIVITY TO SELF-ASSEMBLED NANOMATRIX
PLATFORMS DEPENDS ON THE PHENOTYPE OF MIN6 β-CELLS

by

LIM DJ, ANTIPENKO SV, ANDUKURI A, CORBETT JA, JUN HW
Abstract

Pancreatic beta cell lines that possess the same native characteristics as insulin producing \( \beta \) cells within islets of Langerhans have widely been used to evaluate newly developed biomaterials for the ultimate purpose of improving pancreatic islet transplantation (PIT). Although a number of extracellular matrix (ECM)-mimicking platforms have been introduced and tested with some pancreatic beta cell lines, the change of properties of those cell lines over time are often overlooked. Aged pancreatic cell lines experience alterations in morphology and function, and altered cellular characteristics can lead to poor evaluation of the ECM-mimicking platforms. To study the influence of aged pancreatic beta cell lines on evaluating the potential of ECM-mimicking platforms for PIT, self-assembled nanomatrix platforms formed by peptide amphiphiles (PAs) that mimic native ECM were used. After preparing self-assembled nanomatrix platforms presenting ECM-mimicking ligands, MIN-6 cells of different subcultures were seeded onto the platforms and cultured for up to 7 days. Compared to young MIN-6 cells, old MIN-6 cells showed reduced functional response to ECM-mimicking ligands presented by the self-assembled nanomatrix platforms. Normalized glucose-stimulated insulin secretion results demonstrated that old MIN-6 cells have less sensitivity to ECM-mimicking ligands than young MIN-6 cells. In the self-assembled nanomatrix platform providing RGDS sequences, young MIN-6 cells displayed higher normalized insulin values than old MIN-6 cells, indicating that the functional characteristics of ECM-mimicking platforms depends not only on ECM-mimicking ligands present but also on the passage number of the pancreatic beta cell line. Moreover, morphological differences were also observed, which correlated with previous literatures describing reduced
function as well as altered morphology. Overall, this study describes the importance of using suitable pancreatic beta cell lines not only for evaluating an ECM-mimicking platform for PIT but also for choosing ECM-mimicking ligands that make the ECM-mimicking platform closer to the native islet microenvironment.

Keywords: MIN-6 cells, peptide amphiphiles, self-assembly, ECM
1. Introduction

Reignited by the initial success of the Edmonton protocol in 2000, pancreatic islet cell transplantation (PIT) has gained much attention as a viable option for type 1 diabetes, and many biomaterial strategies have been studied to attain even more successful PIT. [1, 2] Naturally derived or synthetic biomaterials that provide an extracellular matrix (ECM) mimicking microenvironment have been designed and studied for the potential as suitable platforms with insulin-producing cell lines such as MIN-6, RINm5F, and INS-1 (832/13). [3-5] From these studies, it is well-documented that the ECM has significant roles in regulating proliferation, survival, and insulin secretion function; therefore, mimicking islet-ECM interactions has become one of the critical parameters in developing effective ECM-mimicking platforms for PIT. [6] However, the assessment of these potential platforms is likely to be affected by the inherent problems of pancreatic cell lines, such as low glucose-stimulated response in some insulin-producing cell lines and altered cellular characteristics over time due to tumor origin. [7] In this regard, we speculated that not only does long-term culture of pancreatic β cell lines result in their loss of function, but also that validating the efficacy of ECM-mimicking platforms for PIT can be affected by this loss of function. To investigate the biological responses of MIN-6 cells with different subcultures to ECM-mimicking platforms, self-assembled nanomatrix platforms formed by peptide amphiphiles (PAs) were used. These PA molecules have an amphiphilic nature due to a peptide sequence covalently attached to a hydrophobic alkyl chain and are self-assembled into cylindrical nanofibers. [8-10] Without any organic solvents or chemicals, the PA can form not only two-dimensional platforms via a simple evaporation
method, but also three-dimensional ECM-mimicking platforms by changing pH or adding calcium ions. The PA represents the essential characteristics of native ECM, including self-assembly, enzyme-mediated degradability, and versatility to incorporate cell adhesive ligands. [11] Our recent works have highlighted the potential of the self-assembled nanomatrix platforms not only in osteogenic, cardiovascular, and pancreatic islet transplantation applications, but also in fabricating a hybrid biomimetic scaffold and developing a drug delivery system. [12-18] Therefore, the self-assembled nanomatrix platform that exhibits consistent multi-layered nanofibers, while also presenting ECM-mimicking ligands would be a useful ECM-mimicking platform for assessing cellular behavior of pancreatic beta cell lines. With old and young MIN-6 insulin-producing cells, the aim of study was to compare any morphological and functional differences, resulting from changed sensitivity to ECM-mimicking ligands. MIN-6 insulin-producing cells, established by the simian virus 40T antigen gene in transgenic mice, have been used to study the cellular mechanisms of islet β-cells. [19] MIN-6 cells not only show functional activities similar to those of normal islets, but they also have similarities in glucose-stimulated insulin secretion and glucose metabolism. [20] In developing ECM-mimicking platforms, these MIN-6 cells have widely used, but few consideration has been made on the passage number of MIN-6 cells. Repeated subcultures of MIN-6 cells can lead to alterations in morphology, gene expression, and response to glucose. [7] Therefore, we hypothesized that the biological responses of MIN-6 insulin-producing cells to ECM-mimicking ligands can be affected by phenotypic difference between old and young MIN-6 cells. For creating self-assembled PA nanomatix platforms, two different peptide sequences were chosen and incorporated into the PAs, which consist of a cell adhesive
ligand, an enzyme degradable site specific for matrix metalloproteinase-2 (MMP-2), and a hydrophobic alkyl tail linked to the N-terminus of the peptide portion. In both cases, the amino acid sequence GTAGLIGQ (Gly-Thr-Ala-Gly-Leu-Ile-Gly-Gln), which is sensitive to MMP-2 were included, as proteolytic degradation of the PAs allows for cell migration and remodeling with natural ECM produced by cells. [9] One had the RGDS (Arg-Gly-Asp-Ser) sequence, and the other had no bioactive sequence, ending in a serine which served as a control. The RGD sequence has been known to reduce apoptosis of both islets and MIN-6 cells. [21, 22] However, there are conflicting glucose-stimulated insulin secretion results of MIN-6 cells grown on RGDS functionalized hydrogels. [3, 22] It seems that some design parameters required for making the ECM-mimicking platforms might affect the bioactivity of the platforms bestowed by the selected ECM-mimicking ligands. This study used a two-dimensional platform created by self-assembled PA nanomatrix to eliminate the confounding factors associated with three-dimensional ECM-mimicking platforms. Moreover, a DNA normalization technique that explains glucose-stimulated insulin secretions quantitatively was used to assess MIN-6 functional activity more accurately. [23] Overall, this study was to observe how different MIN-6 cell phenotypes respond to a self-assembled nanomatrix platform providing ECM- mimicking ligands.
2. Materials and Methods

2.1. Synthesis of peptide amphiphiles

Using standard Fmoc-chemistry on an Advanced Chemtech Apex 396 peptide synthesizer (AAPPTec, Louisville, KY), two peptides were synthesized at a 0.30 mmol scale and subsequent procedures were performed as previously described to synthesis two PAs: PA-S (C16-GTAGLIGQS) consisting only of a MMP-2 sensitive (GTAGLIGQ) sequence, and PA-RGDS (C16-GTAGLIGQRGDS) consisting of a MMP-2 sensitive sequence and a cell adhesive ligand (RGDS) sequence. [11, 12] The synthesized PAs were confirmed by matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry.

2.2. Cell Culture on self-assembled nanomatrix platforms

Two different passages of MIN-6 cells were used: MIN-6 cells at passage 20 were received from Dr. Donald F. Steiner (Howard Hughes Medical Institute, Department of Biochemistry, University of Chicago, Chicago, IL) under the permission of Dr. Jun-Ichi Miyazaki (Division of Stem Cell Regulation Research (G6), Osaka University Graduate School of Medicine, Osaka, Japan) and MIN-6 cells at passage 45 were obtained from Dr. John A. Corbett (Department of Biochemistry, Medical College of Wisconsin, Milwaukee, WI). Both high and low passage cells were cultured in Dulbecco’s modified Eagle’s medium containing 25 mmol/l glucose supplemented with 15% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA), 100 units/ml penicillin (Invitrogen, Carlsbad, CA), 100 µg/ml streptomycin (Invitrogen), 100 µg/ml L-glutamine (Invitrogen),
and 5 µl/l β-mercaptoethanol (Gibco, Carlsbad, CA). Cells were routinely expanded in 75 cm² tissue culture treated flasks at 37°C in an atmosphere of 95% air and 5% CO₂. PA-S and PA-RGDS were coated onto tissue culture plastic (TCP) of 48-well tissue culture plates (BD Biosciences, San Jose, CA). In brief, after preparing 0.1% wt stock solution of each PA (pH 7.4), 200 µl per well were dispensed into the 48-well tissue culture plates, and the plates were placed in an incubator to evaporate water spontaneously. All plates were sterilized under ultraviolet lamps for 4 hrs, each coated well was seeded with 40,000 cells/well and all cell cultures were incubated for 3 and 7 days with media changes every 3-4 days.

2.3. Glucose-stimulated insulin secretion assay

Glucose-stimulated insulin secretion was assessed at 3, 7 days after cultivation. At each time point, each well was washed twice with PBS, and then incubated with 22 mmol/L Krebs-Ringer bicarbonate buffer (KRB) (25 mmol/L HEPES, 115 mmol/L NaCl, 24 mmol/L NaHCO₃, 5 mmol/L KCl, 1 mmol/L MgCl₂, 2.5 mmol/L CaCl₂, and 0.1% bovine serum albumin, 22 mmol/L D-glucose, pH 7.4). After 1 hour, the samples were collected, and secreted insulin amounts were assayed by the ELISA method using an Ultra Sensitive Rat Insulin ELISA kit (Crystal Chem Inc., Downers Grove, IL). To normalize the secreted insulin data, DNA content of each sample was assayed according to the manufacturer’s protocol with a fluorometric PicoGreen DNA kit (Molecular Probes, Eugene, OR) using a microplate fluorescent reader (Synergy HT, BIO-TEK Instrument, Winooski, VT).
2.4. Evaluation of cellular behavior on self-assembled nanomatrix platforms

In order to evaluate cellular behavior, MIN-6 cells with different passage numbers were seeded on the two different PA coatings and cultured for 3 and 7 days. After cultivation, medium in each well was aspirated, and rinsed with PBS. To assess MIN-6 cell viability for each condition, cells were then assayed with a Live-Dead Assay Kit (Molecular Probes Inc., OR) consisting of calcein AM and ethidium homodimer-1. Due to the enzymatic activity of cytosolic esterases within live cells, calcein AM is converted to a green fluorescent product, whereas ethidium homodimer-1, a red fluorescent compound, accumulates in dead cells due to damaged membranes of dead cells. In addition, morphological differences for each condition were evaluated by treatment with rhodamine-phalloidin (Molecular Probes, OR), which specifically stains actin filaments. For treating with rhodamine-phalloidin, cells were fixed by incubation in 10% neutral buffered formalin solution for 10 minutes and 0.1% Triton-X100 in PBS was used to permeabilize cells for 20 minutes. Rhodamine-phalloidin treatment was performed for 30 minutes in a dark and humid environment. DAPI (1:40,000 in DI water) was used to counterstain the nuclei of the cells. All images were taken using a Nikon Eclipse TE2000-S fluorescent microscope.

2.5. Statistical analysis

All experiments were performed at least three independent times, and all values were expressed as means ± standard deviations. To examine statistical significances, one-way analysis of variance was used. Also, Turkey multiple comparisons test was conducted to
determine significant differences between pairs. SPSS 15.0 software (SPSS Inc., IL) was used to perform statistical analysis. A p < 0.05 was considered statistically significant.

3. Results and Discussions

3.1. Characterization of self-assembled nanomatrix platforms

To investigate morphological and functional responses of MIN-6 cells with different passage numbers to ECM-mimicking ligands presented by self-assembled nanomatrix platforms generated by PAs, a solvent evaporating method was applied to induce the self-assembly of PAs onto the surface of tissue culture plate. Two self-assembled nanomatrix platforms were used: RGDS nanomatrix for a RGDS presenting self-assembled nanomatrix platform and PA-S nanomatrix for a control group. Figure 1 represents the experimental design for this study. The self-assembled nanomatrix platforms successfully formed, and each nanofiber with a uniform diameter of 6-10 nm and at least 50-fold greater size in length was observed as previously reported. [12]

3.2. Morphological differences between young and old MIN-6 cells cultured on self-assembled nanomatrix platforms

Despite exposure to the same self-assembled nanomatrix platforms, young and old MIN-6 cells showed significantly morphological differences. (Figure 2) After 3 days of cultivation, young MIN-6 cells (low passage) tended to form clusters like intact islets, whereas less uniformity was found in old MIN-6 cells (high passage). Moreover, long-term cultivation (7 days) showed even more distinctive results. Old MIN-6 cells grown
on both PA-S and PA-RGDS nanomatrix showed no well-defined cell-clusters, and multi-layered cells seemed to exhibit fibroblast-like morphology. These findings correlated with previous studies showing that subcultures of MIN-6 cells lead to the formation of monolayers. [7] Meanwhile, young MIN-6 cells maintained distinctively organized clusters on either self-assembled nanomatrix platform even after long-term cultivation. Interestingly, the PA-RGDS nanomatrix promoted the creation of islet-like clusters with fair sensitivity to glucose stimulation. All MIN-6 cells cultured on the PA-RGDS nanomatrix showed relatively more aggregated cells than those on the PA-S nanomatrix. Furthermore, it was clear that the PA-RGDS nanomatrix caused young MIN-6 cells to form pseudo-islets, even after 7 days. In rhodamine-phallloidin staining results, more stained actin filaments were observed in all young MIN-6 cells, and more clustered young MIN-6 cells with dense actin filaments were found in the PA-RGDS nanomatrix compared to others. (Figure 3)

3.3. Viability assessment of MIN-6 cells cultured onto self-assembled nanomatrix platforms

Viability of old and young MIN-6 cells cultured on each self-assembled nanomatrix platform was assessed using a Live/Dead assay kit. (Figure 4) Overall, there was no significant difference of viability between old and young MIN-6 cells. MIN-6 cells both of low and high passage number demonstrated similar viability. Representative images after 3 days cultivation represented good viability, whereas much more dead cells were found in every condition after 7 days. Also, there was no significant difference between different self-assembled nanomatrix platforms: PA-S and PA-RGDS nanomatrix. These
findings were interesting because of early studies, where RGD tethering poly (ethylene glycol) (PEG) hydrogel showed an improved viability compared to without RGD. [3] The different observations on MIN-6 cell might result from different characteristics between ECM-mimicking platforms. Compared to the fabrication method to create PEG hydrogel using reactive chemicals, self-assembled nanomatrix platforms can be created by biocompatible means such as lowering pH, adding multivalent ions, or evaporating water. These methods would make the self-assembled nanomatrix platform suitable for ECM-mimicking platform to improve PIT.

3.4. Functional differences between old and young MIN-6 cells cultured onto self-assembled nanomatrix platforms

To evaluate glucose-stimulated insulin secretion with different passages of MIN-6 cells, 22 mmol/L glucose KBR solution was used. All secreted insulin amounts normalized by DNA were shown in Figure 5. The normalized values of old MIN-6 cells on either PA-S or PA-RGDS nanomatrix indicated that that secretion of insulin was diminished due to the loss of responsiveness to glucose. On both substrates, the old MIN-6 cells showed slightly lower normalized insulin secretion than young MIN-6 cells after 3 days of cultivation. Moreover, the difference in normalized values between old and young MIN-6 cells was even more distinctive after 7 days on both PA-S and PA-RGDS nanomatrix. The difference in normalized insulin secretion values between 3 and 7 days of cultivation was insignificant for old MIN-6 cultured on all substrates, whereas young MIN-6 cells cultured on each substrate showed an increase in normalized insulin secretion after 7 days
of cultivation. These reduced functional responses in old MIN-6 cells may result from altered mechanisms involved in the regulated secretory pathway. [24]

There was clear evidence that the self-assembled nanomatrix platform inscribed with RGDS showed higher normalized insulin secretion in both old and young MIN-6 cells than the PA-S nanomatrix (served as a control platform). However, the functional response to the PA-RGDS nanomatrix was more significant for young MIN-6 cells, suggesting that the functional response of MIN-6 cells to ECM-mimicking ligands is dependent on phenotypic differences between old and young MIN-6 cells. In consideration of the controversial issue of whether small peptide sequences tethered to various biomaterials can improve β-cell survival and function, these findings clearly demonstrated that RGDS has a beneficial role in the development of a ECM-mimicking platform for PIT. Our results also indicated that using pancreatic beta cell lines with altered characteristics would cause misunderstandings in the exact roles of ECM-mimicking ligands. In the simplistic design approach, in which ECM-mimicking ligands are selected for tissue-specific ECM microenvironment, there is a susceptibility to engineer less cellular responsive biomaterials, as their usefulness is validated by the cellular activities of the specific cell types used. Our results showed that young MIN-6 cells are more responsive to PA-RGDS nanomatrix platforms, suggesting that long-term cultured pancreatic cell beta cell line have altered functional responses to the external stimulus provided by ECM-mimicking platforms. As many studies use pancreatic beta cell lines in the early phase of evaluating biomaterials being considered for clinical application, it is of importance to evaluate new ECM-mimicking platforms with well-
characterized cells for imitating the essential characteristics of native ECM microenvironment.

4. Conclusions

Following the growing trends of endowing biomaterials with ECM-mimicking ligands for improving pancreatic β-cell survival and function, many ECM-mimicking platforms have been designed and evaluated using several pancreatic beta cell lines such as INS-1, INS-1 (832/13), and MIN-6. However, there is often little consideration of the disadvantages involved in using pancreatic beta cell lines to evaluate bio-inspired platforms. Not using fully characterized cell lines could lead to development of suboptimal ECM-mimicking platform for pancreatic islet transplantation due to the inability to imitate the complexity of ECM. In this study, two MIN-6 beta cell lines of different subcultures were used, and a self-assembled nanomatrix platform formed by PAs were used to determine whether cellular sensitivity to ECM-mimicking ligands depends on passage number. Young MIN-6 cells presented a better response to glucose stimulation than old MIN-6 cells after both 3 days and 7 days of culture. Especially, increased normalized glucose-stimulated insulin secretion was observed on PA-RGDS nanomatrix. Young MIN-6 cells were also found to have cluster-like morphology similar to islets unlike that of old MIN-6 cells. These results demonstrate that pancreatic beta cell lines sensitive to ECM-mimicking ligands are required to determine the best ECM-mimicking ligands to design an ECM-mimicking platform for pancreatic islet transplantation.
Acknowledgements

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References


Figures

**Figure 1.** Schematic drawing of (A) old MIN-6 cultured on PA-RGDS nanomatrix, (B) young MIN-6 cultured on PA-RGDS nanomatrix, (C) old MIN-6 cultured on PA-S nanomatrix, and (D) young MIN-6 cultured on PA-S nanomatrix.
Figure 2. Bright-field images of (A) 3 days cultivation of old MIN-6 cells on PA-S nanomatrix, (B) 3 days cultivation of young MIN-6 cells on PA-S nanomatrix, (C) 3 days cultivation of old MIN-6 cells on PA-RGDS nanomatrix, (D) 3 days cultivation of young MIN-6 cells on PA-RGDS nanomatrix, (E) 7 days cultivation of old MIN-6 cells on PA-S nanomatrix, (F) 7 days cultivation of young MIN-6 cells on PA-S nanomatrix, (G) 7 days cultivation of old MIN-6 cells on PA-RGDS nanomatrix, and (H) 7 days cultivation of young MIN-6 cells on PA-RGDS nanomatrix. All scale bars indicate 20 µm.
Figure 3. Rhodamine-phallodin staining images of (A) 3 days cultivation of old MIN-6 cells on PA-S nanomatrix, (B) 3 days cultivation of young MIN-6 cells on PA-S nanomatrix, (C) 3 days cultivation of old MIN-6 cells on PA-RGDS nanomatrix, (D) 3 days cultivation of young MIN-6 cells on PA-RGDS nanomatrix, (E) 7 days cultivation of old MIN-6 cells on PA-S nanomatrix, (F) 7 days cultivation of young MIN-6 cells on PA-S nanomatrix, (G) 7 days cultivation of old MIN-6 cells on PA-RGDS nanomatrix, and (H) 7 days cultivation of young MIN-6 cells on PA-RGDS nanomatrix. Blue color represents DAPI (4′,6-diamidino-2-phenylindole) staining as a counterstain. All scale bars indicate 50 µm.
Figure 4. Live/Dead assay images of (A) 3 days cultivation of old MIN-6 cells on PA-S nanomatrix, (B) 3 days cultivation of young MIN-6 cells on PA-S nanomatrix, (C) 3 days cultivation of old MIN-6 cells on PA-RGDS nanomatrix, (D) 3 days cultivation of young MIN-6 cells on PA-RGDS nanomatrix, (E) 7 days cultivation of old MIN-6 cells on PA-S nanomatrix, (F) 7 days cultivation of young MIN-6 cells on PA-S nanomatrix, (G) 7 days cultivation of old MIN-6 cells on PA-RGDS nanomatrix, and (H) 7 days cultivation of young MIN-6 cells on PA-RGDS nanomatrix. Green represents live cells, and red represents dead cell. All scale bars indicate 20 µm.
Figure 5. Normalized glucose-stimulated insulin secretion with Krebs-Ringer bicarbonate buffer (22 mmol/L glucose) of both old and young MIN-6 cells after 3 days and 7 days of cultures on either PA-S or PA-RGDS nanomatrix. (*) PA-RGDS nanomatrix exhibited significantly higher normalized insulin secretion than PA-S nanomatrix after 3 days of old MIN-6 cultivation (p < 0.05). (#) PA-RGDS nanomatrix showed significantly higher normalized insulin secretion than PA-S nanomatrix after 3 days of young MIN-6 cultivation (p < 0.05). (§) PA-RGDS nanomatrix expressed significantly higher normalized insulin secretion than PA-S nanomatrix after 7 days of old MIN-6 cultivation (p < 0.05). (***) In the PA-S nanomatrix, young MIN-6 exhibited significantly higher normalized secretion than old MIN-6 after 3 days of cultivation (p < 0.05). (##) young MIN-6 showed significant higher normalized insulin secretion than old MIN-6 after 7 days of cultivation (p < 0.05). (§§) In the PA-S nanomatrix, young MIN-6 after 7 days of cultivation showed significantly higher normalized insulin secretion than young MIN-6 after 3 days of cultivation (p < 0.05). Errors bars represent standard deviation for n = 3.
IMPROVED MIN6 β-CELL FUNCTION ON SELF-ASSEMBLED PEPTIDE AMPHIPHILE NANOMATRIX INSCRIBED WITH EXTRACELLULAR MATRIX-DERIVED CELL ADHESIVE LIGANDS

by

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Format adapted for dissertation
Abstract

Understanding the role of the pancreatic extracellular matrix (ECM) in supporting islet survival and function drives the pursuit to create biomaterials that imitate and restore the pancreatic ECM microenvironment. To create an ECM mimic holding bioinductive cues for β-cells, self-assembled peptide amphiphiles (PAs) inscribed with four selected ECM-derived cell adhesive ligands were synthesized. After 7 days, compared to control groups cultured on biologically inert substrates, MIN6 β-cells cultured on PAs functionalized with YIGSR and RGDS cell adhesive ligands exhibit elevated insulin secretion in responses to glucose and also form β-cell clusters. These findings suggest that the self-assembled PA nanomatrix may be utilized to improve pancreatic islet transplantation for treating type 1 diabetes.
1. Introduction

Growing awareness of the pancreatic extracellular matrix (ECM) in improving islet adhesion, β-cell proliferation, β-cell differentiation, and β-cell function has driven existing biomaterial technologies towards replicating the dynamic characteristics of the pancreatic ECM. [1] A variety of biomaterials that synthetically recreate the islet-ECM microenvironment have been studied to offer a clinically relevant strategy for pancreatic islet transplantation (PIT). [2, 3] Developing an ECM mimic for improving islet engraftment requires a multi-faceted approach in which the following factors should be considered: immune protection, revascularization, and ECM-microenvironment reconstitution.[4] Moreover, extensive follow-up studies after a clinical trial of PIT based on the Edmonton protocol implied that impaired insulin function followed by β-cell death could be attributed to the destruction of the islet-ECM microenvironment, thereby requiring an alternative approach to overcome the loss of β-cell mass and function. [5, 6] Thus, such nurturing and supportive biomaterials that replicate the major features of native ECM are necessitated to maintain proximity to islet-ECM microenvironment.

While natural ECM proteins have been used to enhance islet survival and function, it is still challenging to utilize them as supplements and bulk biomaterials for clinical usage due to potential pathogen transmissions from animal resources. [7, 8] Moreover, natural ECM proteins are very difficult to be specifically designed to direct cellular behaviors such as cell adhesion, migration, proliferation, and differentiation. As an alternative approach, a recently emerged class of peptide-based or protein-based biomaterials can
offer a great deal of design flexibility in creating a specific ECM microenvironment. [9-11]

Peptide amphiphiles (PAs) are peptide-based molecules that create an extracellular matrix (ECM)-like nanostructured scaffold capable of supporting cells and tissues. [12, 13] Due to the amphiphilic nature of these materials, cylindrical micelles are created in an aqueous solution, where the outer region of hydrophilic segment of PAs is displayed and the hydrophobic segment made by alkyl tails goes toward inside. [14] PAs have been studied as biomaterials, as incorporating specific amino acid compositions allows for adapting PAs to a variety of applications, including replicating the nature ECM. [10, 15] Offering intrinsic biocompatibility, biochemical versatility, tunable degradability, and mechanical flexibility, peptide amphiphiles (PAs) can be utilized to improve pancreatic islet transplantation as well. [16] Covalently linked to a hydrophobic alkyl chain, the hydrophilic peptide segment of PAs can serve not only as a recognizable domain for specific cell types but also as a degradable site for specific enzymes. [17] The PAs can be self-assembled into nanofibers, exposing the hydrophilic bioactive peptide segments. [18] A simple means to induce the self-assembly of PAs into nanofibers involves adding multivalent ions, leading to a viscoelastic three-dimensional hydrogel formed through interwoven nanofiber networks. In addition, a simple evaporation method, where a PA solution is dispensed onto a desired surface and allowed to evaporate, can be used to create a PA nanomatrix coating that provides bioactivity suitable to study cellular responses. [13] Our recent works have also demonstrated the potential of using PAs as biomaterials in osteogenic, cardiovascular, and pancreatic islet transplantation.
applications, as well as in the fabrication of a hybrid biomimetic scaffold, and the
development of a drug delivery system. [16, 19-29] Hence, the self-assembled
nanomatrices created by PAs could provide a synthetic ECM microenvironment for
transplanted islets, and the versatility of PAs can fully mimic the essential characteristics
of the pancreatic ECM.

To confer bioactivity on self-assembled PA nanomatrices, four cell adhesive ligands were
chosen: Arg-Gly-Asp (RGD) found collagen IV and laminin-1, Ile-Lys-Leu-Leu-Ile
(IKLLI), Ile-Lys-Val-Ala-Val (IKVAV) in the α1 chain of laminin-1, and Tyr-Ile-Gly-
Ser-Arg (YIGSR) peptide sequences found in the β1 chain of laminin-1 (Figure 1).
Laminin and collagen IV are major components of the islet basement membrane, and
each ECM protein regulates islet survival, differentiation, and function. [30] Laminin, a
heterotrimetric glycoprotein, is one of the most abundant proteins in the vascular
basement membrane of pancreatic islets, and assembly of an α-, β-, and γ- chain has been
identified to form fifteen different laminin trimer structures. [31] Laminin has been
known to play a crucial role in β-cell proliferation, differentiation, and insulin secretion.
[32] Laminin-1, in which several different cell-adhesion sites are identified in the α1, β1
and γ1 chains, induces β-cell differentiation during pancreatic β-cell development. [33,
34] As one of the ECM proteins in the islet basement membrane, collagen IV is thought
to be involved in the spatial organization of β-cells for the morphogenesis of islets. [35]
Also, the combination of laminin and collagen IV has been demonstrated to improve islet
function in vitro. [36]
Endowed with a specific cell adhesive ligand, each PA is composed of a cell adhesive ligand, an enzymatic cleavable sequence (Gly-Thr-Ala-Gly-Leu-Ile-Gly-Gln, GTAGLIGQ) specific for matrix metalloproteinase-2 (MMP-2), and a hydrophobic C16 alkyl tail. For a control, a serine amino acid directly attached to the end of the enzymatically cleavable sequence, instead of any adhesion peptide sequence, was used (PA-S). Each of the functionalized PAs was coated onto tissue culture plates and MIN6 cells were cultured for up to 7 days to study effects of cell-adhesive ligands on cellular behaviors of MIN6 cells. MIN6 cells, established by the simian virus 40T antigen gene in transgenic mice, were used in this study because it has been used for a number of β-cell studies that need similar physiological characteristics with primary β-cells. [37, 38] The self-assembled PA nanomatrices inscribed with extracellular matrix-derived cell adhesive ligands were characterized by transmission electron microscopy (TEM) and subsequently, both functional and morphological responses on these substrates were evaluated. Also, expression of related genes was assessed to confirm the potential ability of the functionalized PA nanomatrices. It was demonstrated that self-assembled PA nanomatrix could be utilized to direct adhesion, survival and function of β-cells.
2. Experimental Section

2.1. Preparation of peptide amphiphiles

As described in our previous studies, a total of five different peptide amphiphiles (PAs) were prepared using standard Fmoc-chemistry on an Advanced Chemtech Apex 396 peptide synthesizer (AAPPTec, Louisville, KY) and subsequently were alkylated at the N-termini with palmitic acid by a manual coupling reaction for 24 hours at room temperature using a mixture of o-benzotriazole-N,N,N,N’-tetramethyluroniumhexafluorophosphate (HBTU), diisopropylethylamine (DiEA), and dimethylformamide (DMF). Cleavage and deprotection were immediately performed with a mixture of trifluoroacetic acid (TFA), deionized (DI) water, triisopropylsilane, and anisole (40:1:1:1) for 3 hours at room temperature. [16, 27, 39] After removing excess TFA, the PAs were precipitated in cold ether followed by lyophilization. Matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry was used for characterization. The molecular formula of all PAs are as follows: PA-S \( [\text{CH}_3(\text{C}_2)_{14}\text{CONH-GTAGLIGQ-S}] \), PA-IKLLI \( [\text{CH}_3(\text{C}_2)_{14}\text{CONH-GTAGLIGQ-IKLLI}] \), PA-IKVAV \( [\text{CH}_3(\text{C}_2)_{14}\text{CONH-GTAGLIGQ-IKVAV}] \), PA-RGDS \( [\text{CH}_3(\text{C}_2)_{14}\text{CONH-GTAGLIGQ-RGDS}] \), and PA-YIGSR \( [\text{CH}_3(\text{C}_2)_{14}\text{CONH-GTAGLIGQ-YIGSR}] \). Except previously characterized PAs, newly synthesized PA-IKLLI and PA-IKVAV were characterized using transmission electron microscope (TEM). In brief, 5 µl of each 0.1 wt % PA-IKLLI and PA-IKVAV solutions were cast on a carbon coated formvar copper grid (400 mesh), and dried overnight. The dried samples were negatively stained with 10 µl of 20% phosphotungstic acid (PTA) for 30s, and a
FEI Tecnai T12 TEM microscope at 60 kV accelerating voltage was used to obtain TEM images.

2.2 MIN6 cell culture on self-assembled peptide amphiphile nanomatrices inscribed with extracellular matrix-derived cell adhesive ligands

MIN6 cells were received from Dr. Donald F. Steiner (Howard Hughes Medical Institute, Department of Biochemistry, University of Chicago, Chicago, IL) under the permission of Dr. Jun-Ichi Miyazaki (Division of Stem Cell Regulation Research (G6), Osaka University Graduate School of Medicine, Osaka, Japan) and routinely expanded in 75 cm² tissue culture treated flasks at 37 °C in an atmosphere of 95% air and 5% CO₂. Dulbecco’s modified Eagle’s medium (DMEM) containing 25 mmol/l glucose supplemented with 15% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA), 100 units/ml penicillin (Invitrogen, Carlsbad, CA), 100 µg/ml streptomycin (Invitrogen), 100 µg/ml L-glutamine (Invitrogen), and 5 µl/l β-mercaptoethanol (Gibco, Carlsbad, CA) was used. All PAs were coated onto tissue culture plastic (TCP) of 48-well tissue culture plates (BD Biosciences, San Jose, CA) as previously described. [23] In brief, after preparing 0.1% wt stock solution of each PA (pH 7.4), 200 µl per well were dispensed into the 48-well tissue culture plates, and the plates were placed in an incubator to evaporate water spontaneously. All plates were sterilized under ultraviolet lamps for 3 hr, each coated well was seeded with 42,000 cells/cm² and all cell cultures were incubated for 3 and 7 days with media changes every 3-4 days.

2.3 Glucose-stimulated insulin secretion assay
At 3 and 7 days, glucose-stimulated insulin secretion was measured. After the wells were washed with phosphate-buffered saline, 22 and 2.2 mmol/L Krebs-Ringer bicarbonate buffer (KRB) solutions (25 mmol/L HEPES, 115 mmol/L NaCl, 24 mmol/L NaHCO₃, 5 mmol/L KCl, 1 mmol/L MgCl₂, 2.5 mmol/L CaCl₂, and 0.1% bovine serum albumin, 22 or 2.2 mmol/L D-glucose, pH 7.4) were used to measure secreted insulin amounts in each condition. After 2 hours of incubation, the samples were collected and quantified using an Ultra Sensitive Rat Insulin ELISA kit (Crystal Chem Inc., Downers Grove, IL). To normalize the secreted insulin data, a fluorometric PicoGreen DNA kit (Molecular Probes, Eugene, OR) was used, and DNA content of each sample was quantified according to the manufacturer’s instruction with using a microplate fluorescent reader (Synergy HT, BIO-TEK Instrument, Winooski, VT).

2.4 Capase 3/7 activity of MIN6 cell on self-assembled peptide amphiphile nanomatrices inscribed with extracellular matrix-derived cell adhesive ligands

To quantify the caspase activities of MIN6 cells on the five different PA coatings, the Caspase-Glo 3/7 assay kit (Promega) was used. Briefly, each PA was coated onto a white, opaque 96-well plate, and MIN6 cells (42,000 cells/cm²) were cultured for 3 and 7 days. At each time period, cells were washed with PBS and 100 μl of fresh media and 100 μl of the buffered Caspase-Glo substrate were added. After 3 hrs, the amount of caspase activity was evaluated by luminescence (Synergy HT, BIO-TEK Instrument).

2.5 Evaluation of cellular behaviors on self-assembled peptide amphiphile nanomatrices inscribed with extracellular matrix-derived cell adhesive ligands
MIN6 cells were cultured on the five different PA coatings and cultured for 3 and 7 days. After cultivation, the medium in each well was aspirated, and rinsed with PBS. MIN6 cell viability for each condition was assessed using the Live/Dead Assay Kit (Molecular Probes Inc., OR) consisting of calcein AM and ethidium homodimer-1. Within live cells, calcein AM is converted to a green fluorescent product due to the enzymatic activity of cytosolic esterases, whereas ethidium homodimer-1, a red fluorescent compound, accumulates in dead cells due to damaged membranes of dead cells.

2.6 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Using the Trizol regent (Invitrogen, Carlsbad, CA), total RNA was collected for all samples in each condition at days 3 and 7. All five different PA coatings were cultured at specific time points, and then isolated based on the manufacturer instructions. For measurement of ins1 and ins2 expression, all samples were incubated for 2 hours in the presence of glucose (22mM) and then collected for RNA extraction. The isolated samples were pelleted, dried in ethanol, and re-suspended in nuclease-free water. DNase treatments were carried out (TURBO DNase, Ambion, Austin, TX) in order to destroy genomic DNA before performing RT-PCR. RNA concentration of each sample was measured with a ND-1000 UV spectrophotometer (Nanodrop, Wilmington, DE), and 1 µg of isolated RNA was then reverse-transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) according to manufacturer’s instructions. All RT-PCR reactions were carried out using an iCycler iQ Real-Time PCR machine (Bio-Rad) with the iQ SYBR Green Supermix (Bio-Rad). To obtain best results, different PCR amplification conditions were adopted:  1) 95°C for 3 mins, 40 cycles of 95 °C for 20 s,
57 °C for 13 s, and 72°C for 13 s (ins1); 2) 95°C for 3 mins, 40 cycles of 95 °C for 20 s, 58 °C for 11 s, and 72°C for 10 s (ins2); 3) 95°C for 3 mins, 40 cycles of 95 °C for 10 s, 60 °C for 7 s, and 72°C for 12 s (connexin 36); 4) 95°C for 3 mins, 40 cycles of 95 °C for 20 s, 56 °C for 10 s, and 72°C for 25 s (E-cadherin). Considered primers were described in Table 1, and 18S rRNA was used as the internal standard (18s RNA forward: 5’-CAT TCG AAC GTC TGC CCT ATC-3’, reverse: 5’-CCT GCT GCC TTC CTT GGA-3’).

The measured values were normalized to the internal standard and calculated using the 2^ΔΔCt method. [40] After 18S rRNA normalization, gene expression levels were presented as the fold ratio relative to those of control group (tissue culture plate; TCP).

2.7 Statistical Analysis

All experiments were performed in quadruplicate, and the normalized values for glucose-stimulated insulin secretion were expressed as means ± standard deviations. To examine statistical significances, one-way analysis of variance was used. Also, Tukey multiple comparison test was used to determine significant differences between pairs. SPSS 15.0 software (SPSS Inc., IL) was used to perform statistical analysis. A p < 0.05 was considered statistically significant.
3. Results and Discussion

3.1 Characterization of self-assembled peptide amphiphile nanomatrices inscribed with extracellular matrix-derived cell adhesive ligands

To investigate the biological responses of MIN6 cells on self-assembled PA nanomatrices, four adhesion sequences were selected and incorporated into PA molecules (Figure 1). Using a solvent evaporating technique, four different islet ECM mimetic PA nanomatrices were created onto the surface of the 48 well tissue culture plates. Four adhesion sequences, namely PA-IKLLI, PA-IKVAV, PA-YIGSR, and PA-RGDS were used as experimental groups, while the PA without any cell-adhesive sequences served as a PA control group (PA-S). The PA-S, PA-YIGSR, and PA-RGDS nanomatrices have previously been characterized, while newly synthesized PA-IKLLI and PA-IKVAV were characterized by TEM (see Supporting Information, Figure S1). [19, 39] All PAs had similar nanostructures under TEM imaging, exhibiting uniform nanofiber bundles of 6-10 nanometers in diameter and several micrometers in length.

3.2 Assessment of MIN6 cell morphology and viability on self-assembled peptide amphiphile nanomatrices inscribed with extracellular matrix-derived cell adhesive ligands

On each of the biocompatible self-assembled PA nanomatrices, the morphological behavior of MIN6 cells in response to self-assembled islet ECM mimetic PA nanomatrices was observed under a bright-field inverted microscope (see Supporting Information, Figure S2 and S3). The viability of MIN6 cells cultured on each PA was
assessed using a Live/Dead assay (Figure 2). There were no differences in viability between the self-assembled islet ECM mimetic PAs (Figure 2C ~ 2L) and each PA demonstrated similar cellular viability compared to MIN6 cells cultured on TCP, indicating that the use of the PA nanomatrices does not alter the viability of the cultured MIN6 cells and that the PAs are able to support β-cell function and viability. Hence, it was speculated that functional differences in β-cell secretion between the different PAs were not due to a variation in MIN6 cell viability.

3.3 Functional Assessment of glucose-stimulated insulin secretion

The role of laminin proteins on MIN6 cell proliferation and up-regulation of insulin gene expression were studied. [32] To evaluate MIN6 cell function in each PA substrate, glucose-stimulated insulin secretion responses to glucose were evaluated after 3 and 7 days of cultivations (Figure 3; Supporting Information, Figure S4 and S5). Both PA-IKLLI and PA-IKVAV nanomatrices showed slightly increased responses to high glucose KBR buffer solution (22mM) at 3 and 7 days of cultivations when compared to the control groups, TCP and PA-S. Meanwhile, the PA-YIGSR and PA-RGDS showed better insulin secretion responses than the other PAs and, more significantly, the control groups. Overall, PA-YIGSR exhibited the highest insulin secretion response after both 3 and 7 days. Compared to other PAs, statistically significant differences in MIN6 cell function of both PA-YIGSR and PA-RGDS nanomatrices could be related to morphological differences. Of the self-assembled PA nanomatrices, our results exhibited the formation of beta-cell clusters on PA-YIGSR and PA-RGDS, which also correlated to greater glucose-stimulated insulin secretion. Improved responses after 7 days would be
explained by the creation of native ECM within the beta-cell clusters, which would provide improved cell-cell interactions and create a more favorable microenvironment for the function of MIN6 cells. [41] The greater glucose-stimulated insulin secretion of the PA-YIGSR and PA-RGDS indicates that the PAs are capable of creating pancreatic ECM mimics, consequently providing benefit to MIN6 cell survival, viability, and function.

3.4 Quantitative Real-Time PCR Gene Expressions
Elevated functionality of self-assembled islet ECM mimetic PA nanomatrices was confirmed using qRT-PCR techniques, and several genes of interest related to MIN6 cell configurations were investigated. Expression of such genes is believed to change dynamically according to the morphological features. It has been documented that E-cadherin (ECAD) and connexin 36 (CX36) are directly related to the formation of beta-cell clusters. [42-44] Early studies demonstrated ECAD expressed in MIN6 cells would up-regulate in beta-cell clusters and described ECAD as a distinctive marker for beta-cell clusters. [43] CX36, a marker solely expressed in β-cells, plays an important role in communication between adjacent β-cells and appears at a high level in pseudo islet formation. [45] Considering the fact that the insulin content of monolayer and pseudo islet is unchanged, up-regulated CX36 gene expressions might explain the increased insulin secretion responses in beta-cell clusters. Moreover, ins1 mRNA is up-regulated in pseudo islet, whereas no significant difference in the expression level of ins2 gene is observed between monolayer and pseudo islet structures. [46]
To determine levels of insulin gene expression on each substrate, preproinsulins of MIN6 cells were measured. Two genes consisting of preproinsulin 1 (ins1) and preproinsulin 2
(ins2) were studied, as the morphological features affecting cell-cell contacts have been led to changes in ins1 and ins2 gene expressions. As shown in Figure 4, MIN6 cells on both PA-YIGSR and PA-RGDS, in response to high glucose (22mM), exhibited increased ins1 gene expression when compared to control and PA-S, indicating that the significantly enhanced functionality on both PA-YIGSR and PA-RGDS substrates might result from up-regulated ins1 gene expressions. Although there were no significant differences among all PA substrates on ins1 gene expressions at 3 days, after 7 days of cultivation, greater gene expression of ins1 was observed, reflecting the increased secretory responses on both PA-YIGSR and PA-RGDS substrates. While levels of ins2 gene expression were relatively less changed over 7 days cultivations (see Supporting Information, Figure S6), our results demonstrated that the formation of beta-cell clusters either in PA-YIGSR or PA-RGDS nanomatrix was supported by the fact that cell-cell contacts on beta-cell clusters increase ins1 mRNA expression. It is reported that enhanced levels of ins1 gene expression are highly related to morphological characteristic of MIN6 cells. [46] Although ins2 gene expression is not significantly changed when configured as beta-cell clusters, increased cell-cell contact within the aggregates led to enhanced ins1 gene expression, which might be due either to interaction of cell adhesion molecules or to released insulin that positively up-regulates insulin gene expression through the β-cell insulin receptors. Elevated ins1 mRNA in MIN6 cells may be attributed to increased cell-cell contact, resulting from promoted pro-insulin biosynthesis through insulin receptor activations. Because it has been proven that both glucose and insulin are able to induce proinsulin biosynthesis, the possibility of enhanced proinsulin
mRNA could be reliable in beta-cell clusters, where each β-cell responds in a positive autocrine feedback. [47, 48]

Connexin 36 gene expression was measured in order to elucidate the mechanism by which MIN6 cells on PA-YIGSR and PA-RGDS substrates demonstrated elevated functionality (Figure 5). In the both substrates, marked increased levels of CX36 were observed at 3 days, indicating that both morphological changes and enhanced functionality of MIN6 cells cultured onto PA-RGDS and PA-YIGSR were linked to the up-regulated CX36 gene expression. [45] However, ECAD gene expression levels were not up-regulated at 3 days (see Supporting Information, Figure S7). Rather, gene expression of ECAD was significantly down-regulated by both substrates at 7 days. Using CX36 absent MIN6 cells, it was found that CX36 is a critical molecule for enhancing insulin secretion, and ECAD is not directly related in regulating insulin secretion. [49] Therefore, it is speculated that elevated normalized insulin secretions in response to glucose on the PA-YIGSR and PA-RGDS substrates were attributable to up-regulated CX36 gene expression by formation of beta-cell clusters during MIN6 cells cultivations.

3.5 Discussions

In this study, a peptide-based biomimetic scaffold capable of replicating the native β-cell microenvironment was explored as an advanced islet encapsulation material for treating type I diabetes. Several extracellular matrix-derived cell adhesive ligands on the islet basement membrane have been documented to improve survival, differentiation, and function of β-cells. In order to provide a favorable β-cell microenvironment, self-
assembled islet ECM mimetic PA nanomatrices were created by incorporating cell adhesive ligands from ECM proteins found in the islet basement membrane (Figure 1).

Recent clinical experiences demonstrating reduced function in transplanted islets are instructive in highlighting the importance of islet-ECM to improve transplanted islet survival and function. It has been progressively shown that destruction of the native islet microenvironment is one of the reasons for the decreased efficacy of pancreatic islet transplantation. [6, 50] In native vascular basement membranes composed of collagen IV, laminin, and sulfate proteoglycan, islet cells communicate mutually via cell-surface interactions. [32] Based on these molecular findings, recent efforts to develop a suitable scaffold for islet transplantation have shifted from protecting islets within biologically inert encapsulation materials to recreating the native microenvironment by either natural ECM proteins or functionalized synthetic polymers. [1] Enhanced islet function has been reported in natural ECM proteins embedded within synthetic polymers, but there are several potential limitations to clinical potency. [7, 51] These limitations include batch-to-batch variations, and safety concerns associated with origins. For instance, different cellular activities of fibroblasts were observed in type I collagen gels extracted from young and aged mice tails. [52] In this sense, peptide- or protein-based materials bear these essential features as clinically acceptable scaffolds: biocompatibility, bioactivity, and biodegradability. As one of the peptide-based scaffolds, the peptide amphiphiles could offer tunable biochemical and biophysical features that create a protecting and nurturing microenvironment for transplanted islets. Specifically, a variety of biomimetic
PA scaffolds could be fabricated for PIT by different cell adhesive ligands and enzyme mediated degradation sequences.

Initiated by self-assembled heterotrimeric laminins, the interwoven basement membrane typically composed of the laminin family, type IV collagens, entactin/ridogen and heparin sulfate proteoglycan is created. [31] The ECM membrane has a role in developing the matured pancreatic islet, in which a variety of endocrine cell types are organized into a proper islet cluster. [34] Mediated by either laminin-binding integrins including α3β1, α6β1, α7β1, and α6β4 or non-integrin receptor proteins such as α-dystroglycan and 67-kDa laminin receptor, the laminin family is involved in pancreatic β cell development. At least 15 different laminins are found in combination of several α (α1-α5), β (β1-β3), and γ (γ1-γ3) subunits, and the expression of different laminin isoforms is dependent on both the type and developmental stage of tissue. In addition, laminins increase insulin gene expression in β cells of islets, and MIN6 cells cultured onto several laminin isoforms showed an increase in both insulin gene expression and proliferation. The best results on insulin gene expression and proliferation were observed in laminin-1. [32] Similarly, collagen IV is not only involved in supporting the islet microenvironment but also in regulating the spatial organization of islet morphogenesis. [35] Based on these studies, it was hypothesized that specific cell adhesive ligands on islet ECMs would have an effect on functional responses of MIN6 cells, and that an integrated approach to selecting suitable islet ECM-derived cues for peptide-based ECM mimics could be a useful strategy for creating a biomimetic scaffold able to improve islet survival and function.
As previously described in the results section, there was a tendency of MIN6 cells to form beta-cell clusters in PA-YIGSR and PA-RGDS, whereas PA-IKVAV and PA-IKLLI showed monolayered clusters of MIN6 cells. Based on the fact that both laminin-5 and collagen IV are expressed much more in the beta-cell clusters than monolayered beta cells, it was speculated that YIGSR and RGD cell adhesive ligands presented by PAs could promote the formation of the beta-cell clusters, resulting in improved insulin responses to glucose. [35] Additionally, RGD cell adhesive ligand found in collagen IV inhibits apoptosis of islets. [53, 54] Similarly, PA-RGDS demonstrated the reduced activities of caspases 3 and 7 that mediate mitochondrial events of apoptosis (Figure 6). Along with these morphological differences, a similar pattern in function was observed; both PA-YIGSR and PA-RGDS supported improved normalized insulin secretion values. The more aggregation, the better MIN6 cells respond to high glucose concentrations (22mM). Because formation of the beta-cell structure is highly associated with connexin36 which facilitates insulin releasing mechanisms, increased cell-cell interactions within beta-cell clusters of MIN6 cells formed by PA-YIGSR and PA-RGDS may lead to improved responses. The beneficial effect of PA-YIGSR and PA-RGDS on MIN6 cells was also confirmed by insulin-related gene expressions, resulting in increased ins1 and CX36 gene expressions due to the induced beta-cell clusters on both substrates.

Despite the advanced technologies and protocols associated with clinical pancreatic islet transplantation, two major challenges—the required number of islets and the shortage of islets—have constantly necessitated an ideal scaffold that replicates the native islet microenvironment not only for protecting transplanted islets but also for constructing
insulin-producing cells from non β-cell origins. In this study, self-assembled islet ECM mimetic PAs were investigated as a pancreatic ECM mimic for pancreatic islet transplantation. Encouraged by specific ECM-derived adhesive peptide sequences that enhanced MIN6 cells function, it was demonstrated that self-assembled islet ECM mimetic PA nanomatrices are supportive of β-cell functionality, and thus, have a great potential for further improving the efficacy of pancreatic islet transplantation.

4. Conclusion

Recent biomaterial strategies for pancreatic islet encapsulation have been aimed towards the ECM mimics that provide a favorable microenvironment to maintain islet survival and function. In this study, self-assembled islet ECM mimetic PA nanomatrices were created by incorporating cell adhesive ligands isolated from islet ECM proteins and utilized to assess MIN6 β-cell function. It was observed that the functionalized PA nanomatrices lead to enhanced insulin secretion function from glucose stimulation. Compared to the control groups, tissue culture plates and non-bioactive PA nanomatrix, PAs inscribed with ECM-derived cell adhesive ligands supported increased insulin secretion responses of MIN6 cells. These findings indicate that ECM-derived cell adhesive ligands may be beneficial in supporting MIN6 cell survival and function. In particular, when tethered by YIGSR and RGDS peptide sequences, the significantly enhanced insulin responses to glucose were observed and the elevated functionality was confirmed by up-regulated gene expressions related to formation of beta-cell clusters.
The self-assembled islet ECM mimetic PA nanomatrix allows MIN6 cells to better respond to glucose, demonstrating the potential of peptide amphiphiles to provide a pancreatic ECM mimic that would improve the survival and function of transplanted islets used in treating type 1 diabetes.

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Figure 1. General schematic drawing of (A) extracellular matrix-derived adhesion peptides selection and incorporation to peptide amphiphiles (PAs), and (B) MIN6 cell cultivations on the self-assembled PA nanomatrices.
Figure 2. Representative Live/Dead assay images of (A) 3 days cultivation of MIN6 cells on tissue culture plate (TCP), (B) 7 days cultivation of MIN6 cells on TCP, (C) 3 days cultivation of MIN6 cells on PA-S nanomatrix, (D) 7 days cultivation of MIN6 cells on PA-S nanomatrix, (E) 3 days cultivation of MIN6 cells on PA-IKLLI nanomatrix, (F) 7 days cultivation of MIN6 cells on PA-IKLLI nanomatrix, (G) 3 days cultivation of MIN6 cells on PA-IKVAV nanomatrix, (H) 7 days cultivation of MIN6 cells on PA-IKVAV nanomatrix, (I) 3 days cultivation of MIN6 cells on PA-YIGSR nanomatrix, (J) 7 days cultivation of MIN6 cells on PA-YIGSR nanomatrix, (K) 3 days cultivation of MIN6 cells on PA-RGDS nanomatrix, and (L) 7 days cultivation of MIN6 cells on PA-RGDS nanomatrix. All scale bars indicate 20 µm.
Figure 3. Glucose-stimulated insulin secretion with Krebs-Ringer bicarbonate buffer (22 mmol/L glucose) of MIN6 cells on different PA nanomatrices. Results were expressed as secreted insulin values per DNA. Error bar represents mean ± standard deviations (§: p < 0.05 compared to day 3 control, †: p < 0.05 compared to day 3 PA-S, #: p < 0.05 compared to day 7 control, ‡: p < 0.05 compared to day 7 PA-S).
Figure 4. Gene expression profile for ins1 over 7 days. Each value expressed as mean ± standard deviation relative to tissue culture plate (TCP) (dashed line) for all time points. At 7 days, both PA-YIGSR and PA-RGDS exhibited significant gene expression than day 7 PA-S (#) and day 7 TCP control (§) (p < 0.05).
Figure 5. Gene expression profile for connexin 36 (CX36) over 7 days. Each value expressed as mean ± standard deviation relative to tissue culture plate (TCP) (dashed line) for all time points. At 3 days, connexin 36 gene expressions were increased significantly on both PA-YIGSR and PA-RGDS nanomatrices than day3 PA-S (†) and day 3 TCP control (§) (p < 0.05). At 7 days, PA-IKLLI exhibited slightly promoted gene expression than day 7 PA-S (#) and day 7 TCP control (§) (p < 0.05).
Figure 6. Effect of the self-assembled peptide amphiphile nanomatrices inscribed with extracellular matrix-derived adhesive ligands on apoptosis of MIN6 cells. Each percentage of the caspase 3/7 activity was expressed compared to tissue culture plate (TCP) served as control (§: p < 0.05 compared to other groups at day 3, †: p < 0.05 compared to other groups at day 7).
Table 1. Primer Sequences for Real-Time PCR

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Supporting Information

**Supporting Figure S1.** TEM images of two newly synthesized peptide amphiphiles (PAs): (A) PA-IKLLI and (B) PA-IKVAV. All scale bars represent 20 nm.
Supporting Figure S2. MIN6 cell viability on self-assembled peptide amphiphile nanomatrices inscribed with extracellular matrix-derived cell adhesive ligands cultured for 24 hours. To assess MIN6 cell viability on self-assembled islet ECM mimetic PA nanomatrices, the MultiTox-Fluor Multiplex Cytotoxicity Assay kit (Promega, Madison, WI) was used. In each PA coated surface of 96-well plates, cells were seeded at a density of 42,000 cells/cm². After 24 hrs, a mixture of substrates for live/dead cells’ protease activities was added and incubated for 2 hrs. Protease activities for live/dead cells were measured in a microplate fluorescent reader at 400 Ex/505 Em for living cells and at 485 Ex/520 Em for dead cells.
Morphological responses of MIN6 cells cultured onto self-assembled peptide amphiphile nanomatrices inscribed with extracellular matrix-derived cell adhesive ligands

After 3 days of cultivations, MIN6 cells on most functionalized PAs were monolayered cells (Figure S3E, S3G, and S3I). The cultured MIN6 cells on PA-IKLLI and PA-IKVAV nanomatrices remained monolayered cells after 7 days (Figure S3F and S3H), and these morphological behaviors were similar with MIN6 cells cultured on both tissue culture plate (TCP) and PA-S (Figure S3B and S3D). MIN6 cells on both PA-YIGSR and PA-RGDS nanomatrices were observed to form a combination of monolayered cells and islet-like clusters after 3 days, and predominantly islet-like clusters after 7 days (Figure S3I, S3J, S3K and S3L). In the case of PA-RGDS nanomatrix, islet-like clusters were more extended islet-like clusters compared to PA-YIGSR nanomatrix at 7 days (Figure S3J and S3L).
Supporting Figure S3. Representative bright-field images of (A) 3 days cultivation of MIN6 cells on tissue culture plate (TCP), (B) 7 days cultivation of MIN6 cells on TCP, (C) 3 days cultivation of MIN6 cells on PA-S nanomatrix, (D) 7 days cultivation of MIN6 cells on PA-S nanomatrix, (E) 3 days cultivation of MIN6 cells on PA-IKLLI nanomatrix, (F) 7 days cultivation of MIN6 cells on PA-IKLLI nanomatrix, (G) 3 days
cultivation of MIN6 cells on PA-IKVAV nanomatrix, (H) 7 days cultivation of MIN6 cells on PA-IKVAV nanomatrix, (I) 3 days cultivation of MIN6 cells on PA-YIGSR nanomatrix, (J) 7 days cultivation of MIN6 cells on PA-YIGSR nanomatrix, (K) 3 days cultivation of MIN6 cells on PA-RGDS nanomatrix, and (L) 7 days cultivation of MIN6 cells on PA-RGDS nanomatrix. All scale bars indicate 20 μm.
**Supporting Figure S4.** Glucose-stimulated insulin secretion with Krebs-Ringer bicarbonate buffers (2.2 and 22 mmol/L glucose) of MIN6 cells on different PA nanomatrices after 3 days of culture. Results are expressed as secreted insulin values per DNA. Error bar represents mean ± standard deviations (‡: p < 0.05).
**Supporting Figure S5.** Glucose-stimulated insulin secretion with Krebs-Ringer bicarbonate buffers (2.2 and 22 mmol/L glucose) of MIN6 cells on different PA nanomatrices after 7 days of culture. Results are expressed as secreted insulin values per DNA. Error bar represents mean ± standard deviations (‡: p < 0.05).
Supporting Figure S6. Gene expression profile for ins2 over 7 days. Each value expressed as mean ± standard deviation relative to tissue culture plate (TCP) (dashed line) for all time points. On the PA-RGDS substrate, MIN6 cells showed relatively greater expression of ins2 compared to day 3 TCP control (§). At 7 days, PA-RGDS exhibited significantly lower gene expression than day 7 PA-S (#) and day 7 TCP control (§) (p < 0.05).
Supporting Figure S7. Gene expression profile for E-cadherin (ECAD) over 7 days. Values expressed as $2^{-\Delta\Delta Ct}$ normalized to 18s RNA and represented as normalized gene expression ratio relative to tissue culture plate (TCP). Error bars represent mean ± standard deviation. ECAD gene expression value on the PA-YIGSR nanomatrix was relatively higher than PA-S on day 3 (†). Compared to day 3 TCP control, both PA-IKLLI and PA-YIGSR nanomatrices expressed greater ECAD gene expressions (§). Compared to day 7 PA-S, day 7 ECAD gene expression on PA-IKVAV nanomatrix was slightly promoted (#), whereas lowered ECAD gene expressions were observed on both PA-YIGSR and PA-RGDS nanomatrices (§) (p < 0.05).
Abstract

Innovative biomaterial strategies are required to improve islet cell retention, viability, and functionality, and therefore obtain clinically successful outcomes from pancreatic islet cell transplantation (PIT). To address this need, we have developed a peptide amphiphile (PA) based nanomatrix that incorporates multifunctional bioactive cues and sustained nitric oxide (NO) release. The goal of this study is to evaluate the effect of this PA nanomatrix on the viability and functionality of MIN-6 islet cells. Additionally, this study provides insight into the role of NO in islet cell biology, as conventional NO donors are unable to release NO in a controlled, sustained manner, leading to ambiguous results. It was hypothesized that controlled NO release in synergy with multifunctional bioactive cues would promote islet cell viability and functionality. NO releasing PA nanomatrices with range from 16.25 μmol to 130 μmol were used to analyze MIN6 cell behaviors. Both 32.5 and 65 μmol PAs showed improved MIN-6 functionality in response to glucose over a 7 day time point, and the elevated functionality was correlated with both PDX-1 and insulin gene expression. The results demonstrate that NO has a beneficial effect on MIN6 cells in a concentration dependent manner.

Keywords: nitric oxide; peptide amphiphile; biomimetic; diabetes; beta cells
1. Introduction

Pancreatic islet transplantation (PIT) is a promising new mode of treatment for type 1 diabetes (T1D).\textsuperscript{1} Successful outcomes from PIT could lead to the resolution of the complications associated with T1D that lead to mortality and morbidity.\textsuperscript{2,3} However, the potential advantages and clinical successes of PIT are dependent on the development of innovative biomaterial strategies. These strategies are required for fulfilling the critical needs for success of PIT: islet cell retention, viability, and functionality. In response to these needs, peptide amphiphile (PA) based biomaterials that closely mimic the islet cell microenvironment and extracellular matrix (ECM) proteins have been garnering increasing attention.\textsuperscript{4-7} Peptide amphiphiles are molecules that consist of a hydrophilic, functional peptide sequence coupled to a hydrophobic alkyl tail.\textsuperscript{8-10} The amphiphilic nature of the molecule drives the self-assembly of PAs into a three dimensional nanomatrix, and the inclusion of multifunctional bioactive cues in the functional peptide sequence regulate islet cell behavior.

For this study, we have developed a peptide amphiphile nanomatrix that incorporates a laminin derived cell adhesive ligand (Tyr-Ile-Gly-Ser-Arg), a matrix metalloprotease-2 (MMP-2) degradable site (Gly-Thr-Ala-Gly-Leu-Ile-Gly-Gln)\textsuperscript{9}, and a nitric oxide (NO) donor (Lys-Lys-Lys-Lys-Lys)\textsuperscript{11-14} to provide the cells with the requisite bioactive cues. Laminin is a major component of the islet cell ECM, and laminin derived sequences have been previously investigated with favorable outcomes.\textsuperscript{15} MMP-2 degradable site allows for cell mediated degradation of the nanomatrix, thereby promoting cell migration and
native ECM production. Tunable NO release from this nanomatrix has been shown to occur in a controlled biphasic manner, with a burst release followed by sustained release over a 30 day period. Previous studies with this PA nanomatrix have shown great potential for applications in cardiovascular implant devices such as stents and vascular grafts. The nanomatrix can be self-assembled in two dimensional coatings or three dimensional nanomatrix gels. It has been shown to promote endothelial cell adhesion and proliferation, and endothelial progenitor cell (EPC) adhesion and differentiation. Therefore, the nanomatrix has the potential to stimulate and support angiogenesis.

Thus, the goal of this work was to study the effect of the NO releasing PA nanomatrix on the viability and functionality of MIN6 β-cells which have similar physiological features to islets. The PA nanomatrix is composed of two PAs: one PA containing the laminin derived cell adhesive ligand (YIGSR) linked to the MMP-2 degradable site (GTAGLIGQ) to form PA-YIGSR, and the second PA containing a polylysine NO donor linked to the MMP-2 degradable site to form PA-KKKKK. PA-YIGSR and PA-KKKKK were mixed in a 9:1 molar ratio based on previous studies, and reacted with pure NO to form the NO releasing PA, called PA-YK-NO. Self-assembly of PA-YK-NO into uniform nanofibers and controlled, biphasic NO release have been previously demonstrated. However, the role of NO in islet cell biology remains a source of ambiguity and controversy. The unavailability of NO donors that can release NO in a controlled, sustained manner can lead to results that cannot be reliably interpreted. Some studies
suggest a detrimental role, where NO mediates the inflammatory reaction in response to the engrafted islet cells.\textsuperscript{23-27} This has been evidenced by elevated expression of IL-1\(\beta\) and inducible nitric oxide synthase (iNOS) in regions of pancreatic islet allotransplantation.\textsuperscript{28,29}

Some studies, however suggest that NO stimulates insulin production and cell viability.\textsuperscript{30-33} This would suggest that the role of NO in islet cell biology may be concentration dependent. Furthermore, conventional NO donors do not provide the islet cells with other bioactive cues that promote cell viability and functionality. This study therefore provides the added opportunity of studying the effect of controlled NO release on islet cells. We hypothesized that controlled NO release in synergy with multifunctional bioactive cues incorporated into the PA nanomatrix will improve islet cell viability and functionality.
2. Material and methods

Preparation of peptide amphiphiles (PAs)

Two peptide amphiphiles – PA-YIGSR [CH$_3$(CH$_2$)$_{14}$CONH-GTAGLIGQ-YIGSR] and PA-KKKKK [CH$_3$(CH$_2$)$_{14}$CONH- GTAGLIGQ-KKKKK] were prepared using the Fmoc chemistry in Advanced Chemtech Apex 396 peptide synthesizer (AAPPTec, Louisville, KY) and subsequently were alkylated at the N-termini with palmitic acid by a manual coupling reaction for 24 hours at room temperature.$^{18,20}$ To alkylate with palmitic acid, a mixture of o-benzotriazole-N,N,N,N¢-tetramethyluroniumhexafluorophosphate (HBTU), diisopropylethylamine (DiEA), and dimethylformamide (DMF) was used, and cleavage and deprotection were achieved using a mixture of trifluoroacetic acid (TFA), deionized (DI) water, triisopropylsilane, and anisole (40:1:1:1) for 3 hours at room temperature. The peptide amphiphiles precipitated in cold ether were lyophilized and characterized by matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry.

Preparation of nitric oxide releasing PA nanomatrices (PA-YK-NOs)

As documented in previous works, a mixture of PA-YIGSR and PA-KKKKK at 9 to 1 molar ratio was reacted with NO gas to generate the NO releasing PA nanomatrix (PA-YK-NO).$^{11}$ In order to make NO releasing PA nanomatrices, 120 µl of a 0.1% wt stock PA-YK-NO solution (pH 7.4) was prepared and dispensed into each of the 48-well tissue culture plates (BD Biosciences, San Jose, CA) with serial dilutions. After drying under a
chemical hood overnight, four different NO releasing donors were created: 130 µmol PA-YK-NO, 65 µmol PA-YK-NO, 32.5 µmol PA-YK-NO, 12.5 µmol PA-YK-NO.

**MIN6 cell culture on nitric oxide releasing PA nanomatrices (PA-YK-NOs)**

MIN6 cells were received from Dr. Donald F. Steiner (Howard Hughes Medical Institute, Department of Biochemistry, University of Chicago, Chicago, IL) under the permission of Dr. Jun-Ichi Miyazaki (Division of Stem Cell Regulation Research (G6), Osaka University Graduate School of Medicine, Osaka, Japan). MIN6 cells were routinely expanded in 75 cm$^2$ tissue culture treated flasks at 37 °C in an atmosphere of 95% air and 5% CO$_2$. Dulbecco’s modified Eagle’s medium (DMEM) containing 25 mmol/l glucose supplemented with 15% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA), 100 units/ml penicillin (Invitrogen, Carlsbad, CA), 100 µg/ml streptomycin (Invitrogen), 100 µg/ml L-glutamine (Invitrogen), and 5 µl/l β-mercaptoethanol (Gibco, Carlsbad, CA) was used to culture MIN6 cells. MIN6 cells were seeded at the density of 42,000 cells/cm$^2$ and incubated for 3 and 7 days, of which the plates of 7 day cultivations were refilled with fresh media at 3 days.

**Glucose-stimulated insulin secretion assay**

To assess MIN6 functionality in response to glucose, glucose-stimulated insulin secretions at 3 and 7 days were performed in each condition with 22 mmol/L Krebs-Ringer bicarbonate buffer (KRB) solutions (25 mmol/L HEPES, 115 mmol/L NaCl, 24 mmol/L NaHCO$_3$, 5 mmol/L KCl, 1 mmol/L MgCl$_2$, 2.5 mmol/L CaCl$_2$, and 0.1% bovine serum albumin, 22 mmol/L D-glucose, pH 7.4). Using an Ultra Sensitive Rat Insulin
ELISA kit (Crystal Chem Inc., Downers Grove, IL), the collected samples were quantified, and normalized by DNA content to obtain the secreted insulin values. The DNA content was quantified by a fluorometric PicoGreen DNA kit (Molecular Probes, Eugene, OR) with a microplate fluorescent reader (Synergy HT, BIO-TEK Instrument, Winooski, VT).

**Caspase 3/7 activity in cultured MIN6 cells on nitric oxide releasing PA nanomatrices (PA-YK-NOs)**

Cultured onto four different PA-YK-NO nanomatrices, the caspase activities in MIN6 cells were assayed using the Caspase-Glo 3/7 assay kit (Promega) to assess cellular apoptosis. Briefly, each PA was coated onto a white, opaque 96-well plate, and MIN6 cells (42,000 cells/cm²) were cultured for 3 and 7 days. At each time period, cells were washed with PBS and 100 μl of fresh media and 100 μl of the buffered Caspase-Glo substrate were added. After 3 hrs, the amount of caspase activity was evaluated by luminescence (Synergy HT, BIO-TEK Instrument).

**Evaluation of cellular behaviors on nitric oxide releasing PA nanomatrices (PA-YK-NOs)**

MIN6 cells were cultured on five different PA-YK-NO nanomatrices for 3 and 7 days. At each time point, the media was aspirated, and the cells were rinsed with PBS. MIN6 cell viability for each condition was assessed using the Live/Dead Assay Kit (Molecular Probes Inc., OR) consisting of calcein AM and ethidium homodimer-1.

**Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)**
Using the Trizol reagent (Invitrogen, Carlsbad, CA), total RNA was collected for all samples in each condition at days 3 and 7. For measurement of ins1 and PDX-1 gene expression, all samples were incubated for 2 hours in the presence of glucose (22mM) and then subjected to RNA extraction. The isolated samples were pelleted, dried in ethanol, and re-suspended in nuclease-free water. DNase treatments were carried out (TURBO DNase, Ambion, Austin, TX) before performing qRT-PCR. RNA concentration of each sample was measured with a ND-1000 UV spectrophotometer (Nanodrop, Wilmington, DE), and 1 µg of isolated RNA was then reverse-transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) according to manufacturer’s instructions. All qRT-PCR reactions were carried out using a Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) with the iQ SYBR Green Supermix (Bio-Rad). The primers used in this study are described in Table 1, and 18S rRNA was used as the internal standard (18s RNA forward: 5’-AGT CCC TGC CCT TTG TAC ACA-3’, reverse: 5’-GAT CCG AGG GCC TCA CTA AAC-3’). The measured values were normalized to the internal standard and calculated using the 2-ΔΔCt method. After 18S rRNA normalization, gene expression levels were presented as the fold ratio relative to those of control group (tissue culture plate; TCP).

Statistical Analysis

All experiments were performed in quadruplicate, and the normalized values for glucose-stimulated insulin secretion were expressed as mean ± standard deviation. To examine statistical significance, one-way analysis of variance was used. Also, Tukey multiple comparison test was used to determine significant differences between pairs. SPSS 15.0
software (SPSS Inc., IL) was used to perform statistical analysis. p < 0.05 was considered statistically significant.

3. Results and discussion

Evaluation of cellular behaviors on the four different nitric oxide releasing peptide amphiphile (PA) nanomatrices

Four different NO releasing PA nanomatrices (PA-YK-NOs) were used for this study: 130 μmol, 65 μmol, 32.5 μmol, and 16.25 μmol (Figure 1). A PA-YK-NO was composed of 2 PAs: an ECM-mimetic PA and a NO releasing PA combined in a 9 to 1 molar ratio. The ECM mimetic PA, referred to as PA-YIGSR, contained a cell-adhesive sequence Tyr-Ile-Gly-Ser-Arg (YIGSR), which has been shown to improve MIN6 cell survival as well as function. Similarly, the NO releasing PA had NO donating sequence Lys-Lys-Lys-Lys-Lys (KKKKK). Dispensing different concentrations of the PA-YK-NO solution, four different PA-YK-NO nanomatrices were created and used to evaluate MIN6 cellular behaviors in response to NO. All PA-YK-NOs were characterized and confirmed to release NO in a physiologically acceptable concentration over 1 month. MIN6 cells were attached and grown with similar cellular behaviors in each PA-YK-NO nanomatrices (Table 2). Although the average number of cells was slightly much more in the control group at 7 days, all PA-YK-NO substrates showed similar growth behaviors with comparable cell viability and cell concentrations. The MIN6 cell viability on the four different PA-YK-NO nanomatrices was similar, indicating that all PA-YK-NO nanomatrices were biocompatible (Figure 2).
Evaluation of functional behavior on the four different nitric oxide releasing peptide amphiphile (PA) nanomatrices

Despite the negative effect on beta-cells, recent studies also indicate that physiologically acceptable NO exhibits positive effects as well.22,34 Enhancing several pathways involving in insulin gene expressions, exogenous NO has been shown to stimulate insulin secretion. When cultured on PA-YK-NOs, glucose-stimulated insulin secretion responses (22mM) for both 3 and 7 days of cultivations were either better or at the same level of the control, indicating that nitric oxide in a low concentration may have beneficial effect on the functionality of MIN6 cells. Excluding the highest NO dose, 130 μmol PA-YK-NO, all NO releasing PA nanomatrices have beneficial effects on MIN6 functionality. Additionally, in the 65 μmol PA-YK-NO, MIN6 cells demonstrated the highest level of insulin secretion response with 32.5 μmol PA-YK-NO having the second best effect over 7 days (Figure 3). At 3 days, on three out of the four PA-YK-NO nanomatrices, MIN6 cells showed significant improved insulin functionality, whereas the 16.25 μmol of PA-YK-NO did not show initial improved response to glucose. This demonstrates that initial release of NO by the majority of the PA-YK-NO nanomatrices stimulates MIN6 cells to respond better to glucose, and indicates that the sustained low NO concentrations have a positive effect. Increasing exposure to NO over time allowed MIN6 cells to respond to sustained nitric oxide dose. 16.25 μmol of PA-YK-NO showed an increase in insulin secretion at 7 days, whereas the 130 μmol of PA-YK-NO did not exhibit the positive effect on MIN6 functionality. Overall, both 32.5 and 65 μmol of PA-YK-NO had improved functionality over time, demonstrating that the middle range of PA-YK-NO nanomatrices showed best results regarding MIN6 functionality.
Quantitative Real-Time PCR Gene Expressions

Previous studies indicate that a stimulatory effect of exogenous nitric oxide relates to the gene transcription factor PDX-1, which regulates insulin gene expression. Campbell et al found that PDX-1 upregulated by NO improved insulin gene expression, concluding that nitric oxide at low levels could play a regulatory role on the insulin secretion of MIN6 cells. At 3 days, relatively higher amounts of PA-YK-NO (130 μmol PA-YK-NO and 65 μmol PA-YK-NO) exhibited increased PDX-1 gene expression when compared to control and 16.25 μmol of PA-YK-NO (Figure 4). This upregulation of insulin genes in the higher PA-YK-NOs at 3 days indicates that an initial sustained release of NO by the PA-YK-NOs could have short-term beneficial effects for MIN6 cells (Figure 5).

Following 7 days, 16.25 and 32.5 μmol PA-YK-NO nanomatrices respectively showed improved PDX-1 gene expression, resulting in the significant increase in insulin gene expression (Figure 6 and 7). These results demonstrated that the beneficial functional role of NO exerted by the biomimetic NO releasing PAs resulted from the upregulation of the PDX-1 gene, which corresponded to the increase in insulin gene expression.

Seven-day observation of PDX-1 gene and insulin gene expression showed a shift from up-regulation in the higher dose of PA-YK-NOs to up-regulation in the lower dose of PA-YK-NOs. The expression pattern of both PDX-1 and insulin gene explained the functional behaviors of MIN6 cells on the biomimetic NO releasing PA nanomatrices. While being less responsive to relatively high dose of PA-YK-NO (130 μmol PA-YK-NO) over a prolonged period of time, the MIN6 cells cultured on the 32.5 and 65 μmol PA-YK-NO nanomatrices, over a longer period of time could have beneficial effects in
terms of insulin-related gene expression as well as functionality. Overall, this study demonstrated enhanced functionality of MIN-6 beta cells in response to NO delivered by an NO-releasing peptide amphiphile (PA) molecule that mimics the beneficial cellular microenvironment conducive to successful islet transplantation. Our findings indicate that PA-YK-NO can release NO in a sustained manner over time, while promoting cell adhesion onto the extracellular matrix mimetic surface of the PA-YK-NO. This mechanism potentially has a dual effect as indicated by the fact that the nanofibrous matrix could be utilized to both enhance islet functionality and improve islet engraftment by promoting NO-mediated angiogenesis post transplantation, thus enhancing islet survival.\textsuperscript{35,36} NO is responsible for increasing endothelial cell proliferation, inhibiting apoptosis, and stimulating endothelial cell migration, thereby inducing the revascularization essential for successful islet transplantation.\textsuperscript{37}
4. Conclusion

Based on the recent findings on the beneficial role of the NO, the aim of this study was to investigate the effect of biomimetic NO donors, which not only provide the native β-cell microenvironment through laminin-1 mimetic peptides but also release NO in a controlled manner over time. Nitric oxide releasing peptide amphiphile (PA) nanomatrices, PA-YK-NOs, exhibited a beneficial effect on glucose-stimulated insulin secretion, implying that the NO producing scaffold could be utilized for improving pancreatic islet transplantation. Specifically, this study showed there is the range of NO that presents a beneficial effect (Figure 8). Creating the biomimetic NO releasing scaffold that releases NO continuously within a physiologically acceptable concentration resulted in an improved response to glucose in MIN6 beta cells. It was demonstrated that the sustained release of NO by the PA-YK-NO between 32.5 and 65 μmol nanomatrices upregulates both PDX-1 and insulin gene, leading to improved functional responses of MIN6 cells to glucose (22 mM). These findings are thought to be due to the sustained NO release because similar level of apoptosis between PA-YK-NOs were observed, implying that the cell viability and survival rate were comparable when compared with the control (Figure 9).

In conclusion, the sustained NO release at low concentrations from the NO releasing PA nanomatrices exhibits an increase in MIN6 functionality, implying that NO has a multifaceted role on beta cells in a concentration-dependent manner.
Acknowledgments

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Disclosure

The author reports no conflicts of interest in this work.
References


31. Laffranchi R, Gogvadze V, Richter C, Spinas GA. Nitric oxide (nitrogen monoxide, NO) stimulates insulin secretion by inducing calcium release from


Figures

Figure 1. General schematic summary of the study.
Figure 2. Representative Live/Dead assay images of (A) 3 days cultivation of MIN6 cells on tissue culture plate (TCP), (B) 7 days cultivation of MIN6 cells on TCP, (C) 3 days cultivation of MIN6 cells on 16.25 µmol PA-YK-NO nanomatrix, (D) 7 days cultivation of MIN6 cells on 16.25 µmol PA-YK-NO nanomatrix, (E) 3 days cultivation of MIN6 cells on 32.5 µmol PA-YK-NO nanomatrix, (F) 7 days cultivation of MIN6 cells on 32.5 µmol PA-YK-NO nanomatrix, (G) 3 days cultivation of MIN6 cells on 65 µmol PA-YK-NO nanomatrix, (H) 7 days cultivation of MIN6 cells on 65 µmol PA-YK-NO nanomatrix, (I) 3 days cultivation of MIN6 cells on 130 µmol PA-YK-NO nanomatrix, (J) 7 days cultivation of MIN6 cells on 130 µmol PA-YK-NO nanomatrix. All scale bars indicate 200 µm.
Figure 3. Glucose-stimulated insulin secretion with Krebs-Ringer bicarbonate buffer (22 mmol/L glucose) of MIN6 cells on different PA-YK-NO nanomatrices. Results were expressed as secreted insulin values per DNA. Error bars represent mean ± standard deviation (‡: p < 0.05 compared to day 3 secreted value in each condition, §: p < 0.05 compared to day 3 control, #: p < 0.05 compared to day 7 control, even after 7 days cultivations).
Figure 4. Gene expression profile for PDX-1 over 3 days. Each value is expressed as mean ± standard deviation relative to tissue culture plate (TCP) (black bar). Significant gene expression was represented compared to TCP control (‡) and 16.25 μmol PA-YK-NO (†) (p < 0.05).
Figure 5. Gene expression profile for insulin (*ins1*) over 3 days. Each value is expressed as mean ± standard deviation relative to tissue culture plate (TCP) (black bar). Significant gene expression was represented compared to TCP control (§), 16.25 μmol PA-YK-NO (†), and 32.5 μmol PA-YK-NO (#) (p < 0.05).
Figure 6. Gene expression profile for PDX-1 over 7 days. Each value is expressed as mean ± standard deviation relative to tissue culture plate (TCP) (black bar). Significant gene expression was represented compared to TCP control (§), 65 μmol PA-YK-NO (‡), and 130 μmol PA-YK-NO (#) (p < 0.05).
Figure 7. Gene expression profile for insulin (ins1) over 7 days. Each value is expressed as mean ± standard deviation relative to tissue culture plate (TCP) (black bar). Significant gene expression was represented compared to TCP control (§), 65 mol PA-YK-NO (‡), and 130 mol PA-YK-NO (#) (p < 0.05).
Figure 8. Beneficial role of NO by the nitric oxide releasing peptide amphiphile nanomatrix on the MIN6 β-cell functionality.
**Figure 9.** Effect of the nitric oxide releasing peptide amphiphile nanomatrices on apoptosis of MIN6 cells. Each percentage of the caspase 3/7 activity was expressed compared to tissue culture plate (TCP) served as control (dashed bar) (†: p < 0.05 compared to day 3 control, §: p < 0.05 compared to day 7 control).
**Table 1.** Primer Sequences for Real-Time PCR

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Table 2. Growth characterization of cultured MIN6 cells on different PA-YK-NO nanomatries

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ENHANCED RAT ISLET FUNCTION AND SURVIVAL IN VITRO USING A BIOIMETIC SELF-ASSEMBLED NANOMATRIX GEL

by

LIM DJ, ANTIPENKO SV, ANDERSON JM, JAimes KF, VIERA L, STEPHEN B, BRYANT SMJ, YANCEY BD, CUI W, THOMPSON JA, CORBETT JA, JUN HW

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Format adapted for dissertation
Abstract

Peptide amphiphile (PA) is a peptide-based biomaterial that can self-assemble into a nanostructured gel-like scaffold, mimicking the chemical and biological complexity of natural extracellular matrix (ECM). To evaluate the capacity of the PA scaffold to improve islet function and survival in vitro, rat islets were cultured in three different groups: 1) bare group: isolated rat islets cultured in a 12 well non-tissue culture treated plate, 2) insert group: isolated rat islets cultured in modified insert chambers, and 3) nanomatrix group: isolated rat islets encapsulated within the PA nanomatrix gel and cultured in modified insert chambers. Over 14 days, both the bare and insert groups showed a marked decrease in insulin secretion, whereas the nanomatrix group maintained glucose-stimulated insulin secretion. Moreover, entire islets in the nanomatrix gel stained positive for dithizone up to 14 days, indicating better maintained glucose-stimulated insulin production. Fluorescein diacetate/propidium iodide staining results also verified necrosis in the bare and insert groups after 7 days, whereas the PA nanomatrix gel maintained islet viability after 14 days. Thus, these results demonstrate the potential of PAs as an intermediary scaffold for increasing the efficacy of pancreatic islet transplantation.
Introduction

The ongoing investigation of treatment options for human type I diabetes mellitus requires the continual development of innovative strategies that more effectively restore long-term physiological function, while still maintaining a simple approach with minimal invasiveness. In the past decade, islet engraftment has been heavily investigated as one such promising treatment for type I diabetic patients, offering a less invasive alternative to full pancreas replacement. In spite of the numerous efforts to improve islet engraftment, clinical efficacy is still lacking because the primary focus has been on avoiding host immune response via the development of semipermeable and biocompatible membranes, while relatively ignoring the potential benefits of a more biomimetic engraftment material. The need for improving the biomimetic character of islet scaffolds is supported by recent literature demonstrating that substantial \(\beta\)-cell loss during the peritransplant period is detrimental to the efficacy of islet transplantation. Specifically, the loss of \(\beta\)-cell function during islet isolation is believed to occur from the destruction of the native islet microenvironment, thereby triggering islet death. In addition, the disruption of islet-extracellular matrix (ECM) interactions exposes the islets to a variety of cellular stresses that further contribute to loss of biological functions. Therefore, biomimetic materials that create ECM-mimicking environments are needed to better maintain islet function and survival during the intermediate stage between implantation and fully restored host integration.

The importance of islet-ECM interactions are well-documented by a number of studies that demonstrate improved islet survival and function facilitated through the use of
scaffolds containing isolated ECM proteins. For example, Nagata et al. demonstrated that a mixture of different types of collagen increased both rat islet β-cell viability and glucose-stimulated insulin secretion. Human pancreatic β-cells grown on a bovine corneal endothelial cell matrix have also been found to maintain glucose-stimulated insulin secretion. Additionally, laminin-5-rich ECM substrates derived from rat bladder carcinoma cells have been shown to enhance the spreading of rat islet β-cells and improve glucose-stimulated insulin secretion. Thus, many research groups have begun incorporating ECM proteins into synthetic biomaterials for the encapsulation of islets or β-cells. To this effect, collagen IV absorbed in a poly(lactide-co-glycolide) (PLG) scaffold showed enhanced functions of transplanted islets. Similarly, MIN6 β-cells showed reduced apoptosis and maintained insulin secretion when cultured in poly(ethylene glycol) (PEG) hydrogels containing ECM proteins compared to control hydrogels alone.

However, for clinical applications, the use of ECM proteins has some potential problems, such as undesirable immune responses, higher infection risks, variability in biological sources, and increased costs. To overcome these limitations, small peptide sequences derived from ECM proteins have been employed to modify the different types of polymers used for islet engraftment. For example, the growth and function of MIN6 cells were enhanced when encapsulated in either a PEG based thermo-reversible gel conjugated with Gly-Arg-Gly-Asp-Ser (GRGDS) or photopolymerized PEG hydrogels modified with various laminin-derived peptides or type I collagen-derived peptides. Despite the benefits of the isolated ECM peptides, limitations of encapsulating biomaterials still need to be given consideration. In particular, the entrapment of cells in
photopolymerized biomaterials can lead to many potential problems after implantation, such as the formation of fibrotic processes, poor degradation of the scaffold, and local and/or systemic toxicity.\textsuperscript{13} Differing compositions and concentrations in alginate have also been found to affect the cellular overgrowth of implanted capsules, as metabolic barriers to nutrient diffusion can form around the implant if non-optimal levels of the material are used, despite the established biocompatibility of alginate.\textsuperscript{14}

As a promising solution to overcome these drawbacks, peptide amphiphile (PA) nanomatrix gels offer a promising solution for improved efficacy in pancreatic islet engraftment. The use of the PA nanomatrix gel as a transplant intermediary for islets is potentially advantageous because they meet the essential design criteria for synthetically recapitulating the extracellular matrix: rapid gel-like 3D network formation by self-assembly, versatility to incorporate various cell adhesive moieties, and cell-mediated degradable sites (matrix metalloproteinase-2) for progressive scaffold degradation and eventual replacement by host-ECM.\textsuperscript{15} Structurally, the PA consists of a hydrophilic functional peptide sequence attached to a hydrophobic alkyl tail, and the internal peptide structure can be adapted to mimic the characteristic properties of the natural ECM.\textsuperscript{16-20} Furthermore, PAs self-assemble into long cylindrical structures that are 8-10 nm in diameter with a length up to several microns in length, and the self-assembly process is initiated by lowering the pH or adding multivalent ions, providing biocompatible means to encapsulate islets for engraftment.\textsuperscript{16,17}

In this study, isolated rodent islets were incorporated into the PA nanomatrix gel containing a cell adhesive ligand isolated from ECM proteins, RGD (arginine-glycine-aspartic acid), as well as a matrix metalloproteinase-2 (MMP-2) sensitive sequence. The
interactions between islets and ECM are known to be important for β-cell viability and function, especially integrin signaling via RGD, which has been shown to decrease apoptosis of islets. Moreover, the MMP-2 enzyme is activated during rat pancreatic development, enabling cell migration of pancreatic endocrine cells throughout the ECM during islet morphogenesis. Thus, it is believed that the PA nanomatrix gel will facilitate progressive degradation and replacement by host ECM after transplantation in vivo. Importantly, the PA forms a rapid, viscoelastic three-dimensional microenvironment without any organic solvents or chemicals, providing the islets with the needed ECM mimicking protective and nurturing environment to promote islet survival and function. Hence, we hypothesize that the PA nanomatrix gel will provide an ECM-mimicking microenvironment that imitates the native ECM microenvironment between islets and ECM, thereby improving islet survival and function. To evaluate this hypothesis, rat islets encapsulated within the PA nanomatrix gel were studied over 14 days of cultivation.

Since traditional islet culturing methods cause variability in the results due to physical loss during cultivation, we have also developed a new in-vitro culture method consisting of a modified insert chamber with a 5 µm nylon mesh sheet. This system allows long-term cultivation with a minimal loss of islets. Thus, we were able to assess the islet survival and function more consistently and precisely. Overall, our results demonstrated that islets encapsulated in the PA nanomatrix maintained their function with increased glucose-stimulated insulin secretion throughout the 14 days compared to the negative control groups. This demonstrates the potential of the PA nanomatrix gel to restore islet-ECM interactions and provide more precise evaluations of islet responses using the
devised culture method. Therefore, this study proposes an innovative strategy to tackle some of limitations facing the clinical practice of islet transplantation.
Materials and Methods

Materials and animals

CMRL-1066 tissue culture medium, L-glutamine, penicillin, and streptomycin were purchased from Gibco (Grand Island, NY). Fetal bovine serum was obtained from Hyclone (Logan, UT). Male Sprague-Dawley rats (250 to 300 g) were purchased from Harlan Laboratories (Indianapolis, IN). Collagenase type XI was purchased from Sigma Chemical Co. (St. Louis, MO). Rat insulin ELISA kit was obtained from Crystal Chem Inc. (Downers Grove, IL). All other reagents were from commercially available sources.

Islet Isolation and culture

Islets were isolated from male Sprague-Dawley rats (250 to 300 g) by collagenase digestion as described previously. After digestion, islets were isolated by density gradient purification and individually hand-picked under a dissection microscope. Fifty hand-picked islets were used per sample for each condition group. All islet samples were cultured at 37°C in an atmosphere of 95% air and 5% CO₂ in CMRL-1066 tissue culture medium supplemented with 2 mmol/L L-glutamine, 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin.

Synthesis of peptide amphiphile

Using standard Fmoc-chemistry, the peptide sequence, CH₃(CH₂)₁₄CONH-GTAGLIGQ-ERGDS, was synthesized on an Advanced Chemtech Apex 396 peptide synthesizer as described previously. After synthesis, the peptide was alkylated at the N-termini with
palmitic acid by a manual coupling reaction. Alkylation was performed for 24 hours at room temperature in a mixture of o-benzotriazole-N,N,N',N'-tetramethyluroniumhexafluorophosphate (HBTU), diisopropylethylamine (DiEA), and dimethylformamide (DMF). Cleavage and deprotection were performed with a mixture of trifluoroacetic acid (TFA), deionized (DI) water, triisopropylsilane, and anisole (40:1:1:1) for 3 hours at room temperature. The PA solution was precipitated in cold ether after removing excess TFA and lyophilized. Matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry was used for PA characterization.

Islet encapsulation within PA self-assembled nanomatrix gel

PA stock solution (2% weight/volume) was prepared and buffered to neutral pH (~7) with NaOH. Self-assembly of the PA with rat islets was induced by combining 50 µL of PA solution with 50 µL of complete CMRL-1066 medium and 15 µL 0.1 M CaCl$_2$ in 12-well silicone flexiPERM cell-culture chambers (Sigma-Aldrich, St. Louis, MO) attached to glass coverslips. The molar ratio between PA and calcium ion (Mr = Ca$^{2+}$/PA) was held constant at Mr = 2, as previously described. The PA self-assembled nanomatrix gel was formed as a sphere-shaped hydrogel with an approximate 7.2 mm diameter. After encapsulating, the PA self-assembled nanomatrix gel containing 50 hand-picked rat islets (nanomatrix group) was transferred into the fabricated insert with 5 µm nylon mesh and cultured in a 12 well non-tissue culture treated plate (Corning Costar, Corning, NY) (Figure 1A).

Glucose-stimulated insulin secretion

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Glucose-stimulated insulin secretion was assessed at 3, 7, and 14 days after encapsulation. To eliminate any residual insulin, all samples were pre-incubated for 1 hour in low glucose Krebs-Ringer bicarbonate buffer (low glucose KRB) (25 mmol/L HEPES, 115 mmol/L NaCl, 24 mmol/L NaHCO₃, 5 mmol/L KCl, 1 mmol/L MgCl₂, 2.5 mmol/L CaCl₂, and 0.1% bovine serum albumin, 3 mmol/L D-glucose, pH 7.4). Then, each sample was placed in 1 mL low glucose KRB for 1 hour, followed by incubation in 1 mL high glucose KRB (20 mmol/L D-glucose) for 1 hour. The supernatant was withdrawn, and insulin was measured by the ELISA method. To normalize the secreted insulin data, a fluorometric PicoGreen DNA kit (Molecular Probes, Eugene, OR) was used to measure DNA content of each sample using a microplate fluorescent reader (Synergy HT, BIO-TEK Instrument, Winooski, VT).¹⁷

Islet viability assessment

Islet cell viability in each group was assessed by microscopic examination using fluorescein diacetate/propidium iodide (FDA/PI) staining at 3, 7, and 14 days after encapsulation. A fluorescein diacetate stock solution was prepared by dissolving 10 mg FDA into 2 ml of acetone. The FDA stock solution was stored at -20°C. When in use, 10 µL of FDA stock solution were diluted with 990 µL of phosphate buffered saline (PBS). Propidium iodide (1mg/ml, from Invitrogen, Eugene, OR) was prepared each time to be used immediately, as 50 µL of solution were diluted with 450 µL of PBS. For viability staining, each sample was immersed in a mixture of 2 mL PBS, 10 µL of diluted PI, and 20 µL of diluted FDA. Stained islets were observed with a Nikon TE2000-S fluorescence microscope (Nikon Inc., Tokyo, Japan) equipped with a Nikon high pressure mercury arc
lamp. Fluorescein dyes that de-acetylated from FDA through non-specific esterases in the cytoplasm of cells were shown under a fluorescent green filter, whereas propidium iodide (PI) dyes, staining nucleic acids of dead cells, were viewed under red fluorescence.

_Evaluation of insulin-producing β-cells using dithizone staining_

To identify insulin-producing beta cells from each group, dithizone (DTZ) staining was used after 3, 7, and 14 days post encapsulation. DTZ forms a red-colored complex when reacted with zinc, indicating positive staining for insulin production. Making the DTZ stock solution, 50 mg of DTZ were dissolved in 5 mL of dimethyl sulfoxide and diluted with 30 mL of PBS. The stock solution was filtered with a 0.45 µm filter. DTZ solution was added to each group for microscopic examination at the pre-determined time points.

_Statistical analysis_

All experiments were performed at least three independent times in quadruplicate. All values were denoted as means ± standard deviation. Statistical analysis was performed using the SPSS 15.0 software (SPSS Inc., Chicago, IL). One-way analysis of variance (ANOVA) was used for statistical comparison. A level p < 0.05 was considered to be statistically significance.
Results

Experimental design with a modified insert system

To evaluate the effect of the PA self-assembled nanomatrix gel on isolated rat islet function, three groups were designed: 1) Bare group: isolated rat islets cultured in a 12 well non-tissue culture treated plate, 2) Insert group: isolated rat islets cultured in the modified insert chamber, and 3) Nanomatrix group: isolated rat islets encapsulated within the PA self-assembled nanomatrix gel and cultured in the modified insert chambers. In the experimental design, two key factors were emphasized: (a) maintaining the islet number throughout the culture period because periodic medium change could lead to loss of islets and (b) accurately quantifying the secreted insulin of each experimental group with little variance. From past experience using traditional culture methods, we found that most rat islets remained weakly attached to the culture surface, leading to some physical loss of islets when replacing medium over long-term culture. As a result, relative variations in the remaining islets during the cultivation could affect the assessment of islet function. Thus, in this study, we developed a modified insert chamber in which a 5 µm nylon mesh sheet was placed into a commercial insert chamber to retain free-floating islets, thereby preventing physical loss of islets (Figure 1A).

Encapsulating rat islets with the peptide amphiphile nanomatrix gel

To encapsulate rat islets, the PA was designed with the following peptide sequence attached to a hydrophobic alkyl tail: \( \text{CH}_3(\text{CH}_2)_{14}\text{CONH-GTAGLIGQERGDS} \). By adding calcium ions to the mixture of PA solution and isolated rat islets suspended in CRML
media, we were able to encapsulate the islets under physiological conditions without any organic solvents or chemicals. Thus, the addition of calcium ions triggered self-assembly of the PA into a nanofibrous gel, encapsulating the rat islets within the PA nanomatrix (Figure 1B).

Assessment of glucose-stimulated insulin secretion

After a period of cultivation, glucose-stimulated insulin secretion responses were measured to evaluate the function of encapsulated rat islets. Over the 14 days of cultivation, both the bare and insert groups showed a marked decrease in insulin secretion, whereas the nanomatrix group maintained glucose-stimulated insulin secretion, even after 14 days (Figure 2). Additionally, islets in the bare group were not responsive to elevated levels of glucose after 14 days, as most of the islets not only lost their functionality, but were found to be completely missing due to the periodic medium changes. In contrast, the response of β-cells to the high-glucose condition was maintained throughout for the nanomatrix group. Furthermore, over the entire cultivation period, there was a significant statistical difference in glucose stimulated insulin responses for the nanomatrix group. After 3, 7, and 14 days, the low glucose response values were 8.7 ± 9.6, 19.9 ± 11.2, and 7.0 ± 1.9 ng, whereas the high glucose response values were 145.1 ± 49.8, 118.3 ± 71.0, and 105.6 ± 52.5 ng, respectively. These results were validated by the stimulation index data observed. To compare the insulin secretion values between groups, we calculated each average stimulation index obtained by dividing average high glucose response value with average low glucose response value of each group. The average SI values were 16.7, 5.9, and 15.0 for islets in the nanomatrix group after 3, 7, and 14 days, respectively.
However, the average SI values over the same time points were 2.0, 1.8, and 1.6 for the insert groups and 1.6, 1.9 and 1.0 for the bare groups, respectively. Thus, the average SI values after 14 days for the nanomatrix group were approximately seven-fold more than the insert group and almost nine-fold greater than the bare group.

**FDA/PI staining to determine islet viability**

FDA/PI staining was used to assess the viability of the rat islets. After 3 days, islets in all three groups displayed maintained viability (Figure 3). After 7 days, the cores of the islets in the bare and insert groups developed necrotic cores (dark centers localized with red fluorescence), whereas the islets in the nanomatrix group still retained most of their viability (Figure 4). After 14 days in the insert or bare groups, there were fewer remaining islets in general, and out of the retained islets, many were fragmented opaque clusters of islets or simply cellular debris lacking in viability. Conversely, the islets in the nanomatrix group still maintained islet integrity and almost all remained viable (Figure 5). In summary, these FDA/PI staining results represent a marked improvement in maintained islet viability for the nanomatrix group over the entire incubation period compared to the other two groups, which began to display necrosis and reduced viability after 7 days.

**Dithizone staining to evaluate insulin-producing β-cells**

Dithizone (DTZ) staining was used to qualitatively assess the function of the islets, appearing as a crimson red positive stain in the insulin-producing β-cells within the islets. After three days, all three groups showed positive DTZ staining (Figure 6A,D,G).
After seven days, the bare and insert groups had significantly reduced positive DTZ staining, whereas the nanomatrix group still maintained high positive staining (Figure 6B, E, H). After 14 days, the bare group had no intact islets left to stain, and the insert group only retained positive staining in the peripheral areas of a few disintegrated islets (Figure 6C, F). In contrast, the islets in the nanomatrix group still maintained integrity and DTZ positive staining throughout the core of the islets (Figure 6I). Hence, these results indicate that the nanomatrix group maintained function throughout the 14 days.
Discussion

To improve the success of pancreatic islet transplantation for human type I diabetes mellitus, our studies investigated the PA nanomatrix gel as an islet engraftment material that expands on earlier studies primarily focused on the avoidance of the host immune response. Pancreatic islet transplantation has gained lots of attention to treat type I diabetes mellitus. However, about 40-60% of islet grafts transplanted failed during the peritransplant period. One of the main reasons for this failure includes the disruption of the islet-ECM microenvironments during the islet isolation, which leads to reduced islet function and loss of islet viability.

To create an ECM-mimicking microenvironment, we used a PA nanomatrix gel, which offers inherent biocompatibility, versatility within the internal peptide structure, enzyme-mediated degradability, easily accessible bioactivity, and durability for clinical practice. The PA nanomatrix gel contains RGDS cell adhesive ligands and enzyme mediated degradable sites within an ECM-mimicking viscoelastic environment, thereby endowing the needed characteristics for enhanced islet survival and function.

From our investigations, the nanomatrix group demonstrated the best results, maintaining prolonged survival and enhanced function of islets. Over all the experiments, the glucose-stimulated insulin secretion of nanomatrix group was significantly maintained for up to 14 days, while the bare and insert groups showed a much lower level of insulin secretion (Figure 2). Additionally, most of the islets were retained in the nanomatrix gel with positive DTZ staining throughout the 14 days of cultivation, demonstrating that the
nanomatrix gel provides the functional support needed to maintain the oxidative capacity for insulin secretion and granule density for prolonged incubation.

For the insert group, however, slightly fewer islets were observed with reduced viability and function, while the bare group lost most of its islets over the same period and could not be accurately measured. These findings were consistent with the FDA/PI staining results for all three groups. Thus, throughout the entire cultivation period, the nanomatrix group consistently displayed intact islet integrity with enhanced function, whereas fewer islets remained in the bare and insert groups with reduced utility. Moreover, the nanomatrix gels began to degrade as intended after 14 days due to the inclusion of MMP-2 sensitive sequence. Based on the fact that revascularization begins 2-4 days after islet transplantation and is completed by 10-14 days, these results demonstrate the potential of the nanomatrix gel as a useful intermediary scaffold that bridges the gap between implantation and fully restored host integration.26

In contrast to the traditional islet culturing methods, which lead to variability in the observed results due to physical loss of islets during cultivation, we devised a modified insert chamber to more accurately measure islet function in this study. Specifically, in traditional culture methods, islets are susceptible to necrotic death within their central cores due to islet size, non-proliferative nature, and variability of culture conditions.27 Therefore, the devised insert chamber provided a consistent islet culturing method that not only measured islet function more accurately, but also provided a better system for quantifying the viability of the remaining islets over long-term cultivation.

The normalization of islet quantification data is critical to more accurately reflecting the overall islet performance. However, the traditional normalization methods do not account
for variations in the viability and number of islets, which can both be altered due to the physical loss that occurs during the cultivation. Thus, traditional methods frequently lead to misinterpretation of the data, especially in long-term studies. Consequently, we normalized the glucose-stimulated insulin secretion values in this study by the amount of DNA per sample to reduce variations and account for the different numbers of remaining islets in each condition. It was found that not only were islets in the nanomatrix group producing significantly more total insulin, but when normalized by total islet DNA, the islets in the nanomatrix group showed more insulin per DNA than the bare and insert groups (Figure 7). In contrast, there were marked decreases in the bare and insert groups when normalized by DNA. Moreover, it is negligible that β cell proliferation during cultivation affected the normalized islet function data, as cultured β cells vary rarely proliferate in a normal culture condition.\textsuperscript{28-29} Thus, these results indicate that not only were more islets retained in the nanomatrix group, but also that the individually remaining islets demonstrated enhanced survival and function per DNA.

In conclusion, this study demonstrates the importance of developing an ECM-mimicking microenvironment for promoting the long-term survival of encapsulated islets in vitro. Our findings suggest that the ECM-mimicking PA nanomatrix gel may provide a protective and nurturing microenvironment that enhances islet cell survival, and most importantly, increases function in the β-cell mass in-vivo. It is striking that the islets encapsulated in the PA nanomatrix gel maintained normal glucose-stimulated insulin secretion throughout the 14 days of in vitro culture at a sufficiently high level that could not even be maintained for up to 3 days in the islets cultured under the free floating conditions of the bare and insert groups. These findings provide experimental evidences
that support the need to continue developing this PA nanomatrix for islet transplantation, especially for further testing under in vivo conditions. Overall, these results demonstrate the potential of the PA nanomatrix gel as an intermediary scaffold for increasing the efficacy of pancreatic islet transplantation.
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The authors are indebted to the many contributions of Dr. Juan L. Contreras that include assistance with the experimental design, islet isolation, and methodologies for assessment of islet viability and function. Many thanks to Eun-Ha Lim as well for expert graphical assistance. This study was supported in part by the UAB Diabetes Research Training Center Pilot Grant and the Innovation Award from the American Diabetes Association for HWJ, a NIH T32 predoctoral training grant (NIBIB #EB004312-01) for JMA, and NIH grants DK 52194 and AI 44458 for JAC.
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Figure 1. Schematic drawing of (A) the modified insert culture system and (B) islet encapsulation within a PA nanomatrix gel
Figure 2. Glucose-stimulated insulin secretion for 14 days of cultivations (* indicates significant differences in insulin release between low glucose incubation (3mM) and high glucose incubation (20mM), p<0.05) (n=4).
Figure 3. Morphology and viability of rat islets in different culture conditions after 3 days of cultivations: (A) brightfield image of islets in the bare group, (B) FDA/PI staining of islets in the bare group, (C) brightfield image of islets in the insert group, (D) FDA/PI staining of islets in the insert group, (E) brightfield image of islets in the nanomatrix group, and (F) FDA/PI staining of islets in the nanomatrix group. All scale bars indicate 100 µm.
Figure 4. Morphology and viability of rat islets in different culture conditions after 7 days of cultivations: (A) brightfield image of islets in the bare group, (B) FDA/PI staining of islets in the bare group, (C) brightfield image of islets in the insert group, (D) FDA/PI staining of islets in the insert group, (E) brightfield image of islets in the nanomatrix group, and (F) FDA/PI staining of islets in the nanomatrix group. All scale bars indicate 100 µm.
Figure 5. Morphology and viability of rat islets in different culture conditions after 14 days of cultivations: (A) brightfield image of islets in the bare group, (B) FDA/PI staining of islets in the bare group, (C) brightfield image of islets in the insert group, (D) FDA/PI staining of islets in the insert group, (E) brightfield image of islets in the nanomatrix group, and (F) FDA/PI staining of islets in the nanomatrix group. All scale bars indicate 100 µm.
Figure 6. Evaluation of insulin-producing β-cells using dithizone staining: (A) after 3 days cultivation in the bare group, (B) after 7 days cultivation in the bare group, (C) after 14 days cultivation in the bare group, (D) after 3 days cultivation in the insert group, (E) after 7 days cultivation in the insert group, (F) after 14 days cultivation in the insert group, (G) after 3 days cultivation in the nanomatrix group, (H) after 7 days cultivation in the nanomatrix group, and (I) after 14 days cultivation in the nanomatrix group. All scale bars indicate 100 µm.
Figure 7A. Glucose-stimulated insulin secretions normalized by DNA in the bare group.

(*, p<0.05) (n=4)
Figure 7B. Glucose-stimulated insulin secretions normalized by DNA in the insert group.

(*, p<0.05) (n=4)
Figure 7C. Glucose-stimulated insulin secretions normalized by DNA in the nanomatrix group. (*, p<0.05) (n=4)
Table 1. Design of experimental groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Descriptions</th>
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<tbody>
<tr>
<td>Bare</td>
<td>Cultured in a 12 well non-tissue culture treated plate</td>
</tr>
<tr>
<td>Insert</td>
<td>Cultured in the modified insert chamber</td>
</tr>
<tr>
<td>Nanomatrix</td>
<td>Encapsulated within the PA nanomatrix gel and cultured in the modified insert chamber</td>
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CONCLUSIONS

This dissertation studied a biomimetic peptide amphiphile (PA) nanomatrix capable of supporting beta cells and islets for the purpose of providing a better synthetic extracellular matrix (ECM) for pancreatic islet transplantation (PIT). Developing an ECM mimic has been thought as one of the critical challenges in improving islet engraftment, and a constant demand to develop better specific ECM analogs remains. This study utilized low passage MIN6 β-cells as these MIN6 β-cells showed better sensitivity to cellular ligands tethered onto PA nanomatrices. Based on the fact that the laminin protein is one of major proteins in islet ECM, laminin-1 mimetic self-assembled PAs were successfully synthesized and characterized. When cultured with MIN6 β-cells, two laminin-1 derived PA scaffolds, PA-YIGSR and PA-RGDS, showed significantly improved functionality, implying that the ECM mimics are capable of supporting beta cell survival and function in the absence of native ECM. The improved functionality of MIN6 β-cells on both substrates was observed and confirmed by checking insulin-related gene expressions. In addition, the substrates were shown to form beta cell clusters similar to those found in the natural environment. Thus, it was concluded that the laminin-1 mimetic self-assembled PA was capable of providing a nurturing microenvironment for beta cells. Considering the importance of the ECM on islet survival and function post transplantation, our findings could be utilized to tackle the significant loss of the islet ECM that occurs during islet isolation, which is known as one of the factors that limit a
successful PIT. In addition to imitating beta cell and ECM interactions with multifunctional bioactive cues, the capability of the PA scaffolds to provide a nitric oxide (NO)-releasing microenvironment that would promote angiogenesis of transplanted islets was studied. When cultured onto NO-releasing PA nanomatrices (called as PA-YK-NO), improved MIN6 β-cells’ functionality was observed. This study indicates that a NO-releasing PA nanomatrix has beneficial effects in not only on supporting beta cells, but also on developing a multifunctional scaffold that facilitates revascularization of transplanted islets while restoring islet-ECM interactions. In order to evaluate the PA scaffold, in specific aim 3, isolated rat islets were embedded in a PA scaffold (nanomatrix group), and compared with the other groups: bare group and insert group. Isolated rat islets were cultured without any manipulation (bare group), while isolated rat islets were cultured in an insert chamber to prevent physical loss of islets, which were subjected to periodical media changes (insert group). Isolated rat islets in the nanomatrix group were observed to retain their functionality and viability over 14 days of cultivation in vitro. This implies that a PA scaffold could potentially support survival and function of transplanted islets during the process of revascularization because the revascularization process takes 10-12 days.

In summary, this biomimetic PA nanomatrix has shown potential to be utilized as an intermediary scaffold to improve the practicality of the pancreatic islet transplantation. It provided a bioinducive environment to improve beta cell functionality over time, while providing a three-dimensional scaffold that preserves islet functionality as well. In consideration of an in vivo setting for PIT, future work should be aimed at not only
developing an immune-protective scaffold but also exploring an innovative strategy that would make extrahepatic transplantation sites available for long-term success of PIT.
FUTURE WORK

To develop an innovative strategy to improve the practicality of the pancreatic islet transplantation, three major factors must be considered with high priority: 1) the recovery of the islet microenvironment in order to prevent the substantial loss of islet survival caused by disruption of the islet microenvironment during the peritransplant period, 2) an immune protecting technique that prevents the entry of immune cells and host antibodies while reducing the proinflammatory responses on transplanted islets, and 3) an alternative islet transplantation site with enhanced revascularization to overcome limitations of current intrahepatic implantation sites. Intrahepatic PIT has potential risks such as procedural hazards, islet damage by instant blood-mediated inflammatory reaction (IBMIR), progressive attrition of islet function, exposure to the toxic effects of immunosuppressive drugs, and toxic products from the gastrointestinal tract. Hence, future work should be aimed to deal with the following specific aims.

Specific aim 1: To develop an immune-protecting biomaterial, the PA scaffold will be evaluated and improved. The semi-permeability of the PA scaffold will be studied to determine whether it allows for the blocking immune cells and antibodies, while facilitating the movement of metabolites, including insulin and glucose. Moreover, a practical strategy that blocks proinflammatory cytokines will be also studied. Endowing PA scaffolds with immune protective properties could help us to make a clinically
applicable biomaterial, as immune response is a significant and crucial component in clinical PIT. To confirm the potential of the developed PA scaffold, animal models including diabetic rodents and diabetic primates will be used as well.

Specific aim 2: The omentum, which is a promising extrahepatic site, will be an alternative site for better islet engraftment. In order to use the omentum, the PA scaffold will be incorporated into electrospinning poly (ε-caprolactone) nanofibers with crater-like structures. Due to the potential of PA scaffolds demonstrated in this study, the hybrid material is expected to exhibit following unique properties: a) transplantation of islets encapsulated in the PA nanomatrix gel, b) a controlled release of angiogenic growth factors in a multi-stage manner, c) suitable mechanical properties for surgical manipulation, and d) enhanced infiltration of blood vessels through porous structures called crater-like structures. This study will demonstrate that this hybrid material is able not only to reconstruct an islet ECM microenvironment but also to induce rapid revasculazation in the omentum.

With the demonstrated results in this study, further studies will provide an in-depth insight on developing a clinically applicable biomaterial that treats type 1 diabetes.
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Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

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FROM:
   Robert A. Kesterson, Ph.D., Chair
   Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: A Hybrid Nanosack for the Enhanced Islet Engraftment in the Omentum
   Sponsor: NIH
   Animal Project Number: 13060986

As of June 11, 2013 the animal use proposed in the above referenced application is approved. The
University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves
the use of the following species and number of animals:

<table>
<thead>
<tr>
<th>Species</th>
<th>Use Category</th>
<th>Number In Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rats</td>
<td>B</td>
<td>444</td>
</tr>
</tbody>
</table>

Animal use must be renewed by June 10, 2014. Approval from the IACUC must be obtained before
implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting
agency.

Refer to Animal Protocol Number (APN) 13060986 when ordering animals or in any correspondence
with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns
or questions regarding this notice, please call the IACUC office at (205) 934-7692.
FOR OFFICE USE ONLY

Date Received: Date Approved: Animal Project Number (APN):

PROVIDE A COPY OF THE CORRESPONDING GRANT, CONTRACT APPLICATION, OR STUDY PLAN (PDF format is preferred), which should be consistent with the IACUC but do not refer to it instead of responding to the questions below.

SECTION I: GENERAL INFORMATION (Questions 1-4)

1. Principal Investigator

   Name: How-Wook Jun
   Blazer ID: Jwjun
   Department: Biomedical Engineering
   Division:
   Office Address: BSE 224, 1325 University Blvd.
   Office Phone: 205-996-9933
   Email: hswjun@bham.edu
   Fax Number:
   Email FAX Number:
   Contact who should receive copies of IACUC correspondence (Optional):

2. Application

   Project Title: A hybrid nanosensor for the enhanced gut engraftment of the intestinal
   Sponsor: NIH R01 grant
   Project Period (start date & end date): 05/01/2013 – 04/30/2015
   Previous APNs: 11/2007
   If part of a Program Project, SCORE, Center, etc.:
   Name of PI:
   Title:

3. Will personnel other than the PI (e.g., faculty, staff, students, or fellows) be involved in the animal work being proposed? [ ] NO [ ] YES

   If the response is YES, provide the names below:

   Name: Patrick Heang
   Blazer ID: phheang
   For Nonhuman Primate Users: Date of Confirmation of Negative TB Status: NA

   Name: Donglin Lim
   Blazer ID: dlim
   Date of Confirmation of Negative TB Status: NA

   Name: Shalen Gribert
   Blazer ID: shlang
   Date of Confirmation of Negative TB Status: N/A

   Name: Jiquan Tan
   Blazer ID: jtan
   Date of Confirmation of Negative TB Status: N/A

4. Certification

   By submission of this form, I certify that the information provided in this Animal Use Request (AUR) completely and accurately describes the work to be performed and all work proposed in the associated grant application, contract application, or study plan.

   I further certify that:
   - No personnel working under my direction will perform any animal procedures until their experience and training has been reviewed and approved by the IACUC.
   - I will submit to the IACUC the names and qualifications of new or additional personnel including students and visiting faculty before they become involved in these studies.
   - I will ensure that all personnel are enrolled in the Institutional Occupational Health Program prior to their contact with animals or their entry into the animal facilities.
   - I will comply with the procedures described in the NIH Guide for the Care and Use of Laboratory Animals, the PHS Policy on Humane Care and Use of Laboratory Animals, the USDA Animal Welfare Act Regulations, applicable UAB policies, and Standard Operating Procedures as described by the Animal Resources Program and IACUC.
   - I acknowledge responsibility for this project and all faculty, staff, and students who participate in it.

   I also understand that I must submit a modification to an approved protocol and update IACUC approval before:
   - Use additional animal species, increase the number of animals to be used, or increase the number of procedures performed on individual animals.
   - Change procedures that in any way increase the pain/stress an animal might experience or that might be considered a significant departure from those described in this AUR.
   - Perform procedures not described in this AUR.
   - Use or allow to be used for other studies animals purchased, produced, or otherwise acquired for this project.

   I understand that I must request renewal of IACUC approval annually and on the third anniversary date of the original IACUC approval. I am required to submit an AUR reflecting the work to be performed for the remainder of the grant/contract award.