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Silicon Nanopore Membrane (SNM) for Islet Encapsulation Under Convective Transport to Treat Type 1 Diabetes (T1D)

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Author
Song, Shang

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Silicon Nanopore Membrane (SNM) for Islet Encapsulation Under Convective Transport to Treat Type 1 Diabetes (T1D)

by

Shang Song

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Bioengineering

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

AND

UNIVERSITY OF CALIFORNIA, BERKELEY
This dissertation is dedicated to my grandparents, Fengxiang Wang and Zhonghao Song, and all my relatives and friends, who have always supported and encouraged me to pursue my dreams and achieve great things in life.
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I have been extremely fortunate to work with inspiring mentors and invaluable colleagues. They have continued to challenge and encourage me to pursue my interests in bioengineering research and education during my years in graduate school. This work would not be possible if it were not for the team efforts.

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ABSTRACT

Silicon Nanopore Membrane (SNM) for Islet Encapsulation Under Convective Transport to Treat Type 1 Diabetes (T1D)

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The goal of this dissertation is to develop a bioartificial pancreas device consisting of silicon nanopore membrane (SNM)-encapsulated insulin-producing pancreatic islets under convective transport to treat Type 1 Diabetes (T1D). Problems associated with islet transplantation for T1D such as shortage of donor cells, and use of immunosuppressive drugs remain as major challenges. Immune isolation using encapsulation may circumvent the use of immunosuppressants and prolong the longevity of transplanted islets. The encapsulating membrane must block the passage of host’s immune components while providing sufficient exchange of glucose, insulin and other small molecules.

This research effort investigated the feasibility of encapsulating islets with SNM to provide: (1) middle molecule selectivity against pro-inflammatory cytokines; and (2) sufficient nutrients and oxygen with convective transport to overcome the mass transfer limitations associated with diffusion through nanometer-scale pores. The selectivity analysis revealed 80% reduction in cytokines passage through SNM under convective transport. Moreover, the SNM protected encapsulated islets from infiltrating cytokines and retained islet viability and remained responsive to changes in glucose levels unlike non-encapsulated controls. The glucose-stimulated insulin response showed that membrane-encapsulation of islets with convection outperformed the diffusive conditions in terms of the magnitude of insulin secreted (1.49-fold
increase in stimulation index & 3.86-fold decrease in shut-down index) and the rate of insulin production during high (1.19-fold increase) and low glucose (6.45-fold decrease) challenges. As a result of these data, the SNM-based intravascular bioartificial pancreas (iBAP) device was presented to support high cell viability and function at clinically relevant islet densities utilizing convective mass transfer both in vitro and in vivo. The hemocompatibility of the iBAP blood flow path after intravascular implantation was also demonstrated in the porcine model. These studies address the critical challenges faced by macroencapsulation and pave the way for the development of a full-scale SNM-encapsulated iBAP to treat T1D in the future.
TABLE OF CONTENTS

**Chapter I:** Macroencapsulation for Type 1 Diabetes (T1D) treatment: cells, biomaterials, and devices................................................................. 1

1.1 Introduction........................................................................................................... 1

1.1.1 Current treatment methods ................................................................. 2

1.1.2 Alternative treatment methods.......................................................... 4

1.2 Macroencapsulation of islets .............................................................................. 13

1.2.1 Extravascular macrocapsules .............................................................. 18

1.2.2 Vascular perfusion devices ................................................................. 26

1.3 Challenges to successful macroencapsulation ........................................... 30

1.3.1 Cell source ................................................................................................. 30

1.3.2 Biomaterials ............................................................................................... 35

1.4 Immune response .............................................................................................. 37

1.5 Inflammatory response ..................................................................................... 39

1.6 Hypoxia and implantation site ......................................................................... 41

1.7 New solution for macroencapsulation ......................................................... 43

1.8 Conclusions ....................................................................................................... 44
Chapter II: Silicon Nanopore Membrane (SNM) for islet encapsulation and immunoisolation under convective transport .............................................. 46

2.1 Introduction ............................................................................................. 46

2.2 Materials and methods ............................................................................ 50

2.2.1 Substrate preparation .......................................................................... 51

2.2.2 Assessment of SNM immunoisolation in vitro ................................. 55

2.2.3 Culture of membrane-encapsulated islets in the mock-loop circuit .... 56

2.3 Results and discussion ........................................................................... 58

2.4 Conclusions ............................................................................................ 66

Chapter III: Glucose-stimulated insulin response of silicon nanopore
immunoprotected-islets under convective transport and cytokine
exposure .................................................................................................... 68

3.1 Introduction ............................................................................................. 68

3.2 Materials and methods ............................................................................ 71

3.2.1 Substrate preparation .......................................................................... 72

3.2.2 Culture of membrane-encapsulated islets in the mock-loop circuit ... 75

3.3 Results and discussion ........................................................................... 78

3.4 Conclusions ............................................................................................ 112
Chapter IV: An intravascular bioartificial pancreas device (iBAP) with Silicon Nanopore Membrane (SNM) for islet encapsulation under convection ................................................................. 114

4.1 Introduction .................................................................................................................. 114

4.2 Materials and methods ............................................................................................... 119
   4.2.1 Substrate preparation ............................................................................................... 119
   4.2.2 Assembly of the intravascular device ..................................................................... 121
   4.2.3 Testing and implantation of the intravascular device in vitro and in vivo ............ 122

4.3 Results ......................................................................................................................... 127

4.4 Discussion .................................................................................................................... 143

4.5 Conclusions .................................................................................................................. 148

Chapter V: An oxygen transport model and a device for long-term evaluation of immunoisolation in vivo ........................................................................................................ 149

5.1 Introduction .................................................................................................................. 149

5.2 Analytical modeling of the islet chamber .................................................................... 150

5.3 Design of extravascular SNM capsules for immunoisolation testing in vivo ............ 155
   5.3.1 SNM capsule preparation and implantation ......................................................... 157
   5.3.2 Future work ............................................................................................................. 160
Future directions ................................................................. 162

**Appendices**: Effect of surface topography and biochemical cues on osteogenesis and angiogenesis of human mesenchymal stem cells. 164

A.1 Introduction ........................................................................ 164

A.2 Materials and methods ............................................................. 166

A.2.1 Substrate preparation .............................................................. 167

A.2.2 Cell culture ......................................................................... 168

A.2.3 Cell culture analysis ................................................................. 169

A.2.4 Harvested scaffolds analysis .................................................. 172

A.3 Results .................................................................................. 174

A.3.1 In vitro culture analysis ......................................................... 174

A.3.2 In vivo animal study ................................................................. 181

A.4 Discussion ............................................................................ 190

A.5 Conclusions .......................................................................... 194

A.6 Future work .......................................................................... 195

References ................................................................................ 197
LIST OF TABLES

Table 1.1 Macroencapsulation used in animals and human ........................................ 9
Table 1.2 Glucose, insulin and soluble inflammatory mediators expressed by pancreatic
islets ........................................................................................................................................ 39
Table 3.1 Glucose, insulin and soluble inflammatory mediators expressed by pancreatic
islets ........................................................................................................................................ 87
Table 3.2 The rate of change in insulin secretion without cytokine exposure ............ 100
Table 3.3 The rate of change in insulin secretion .............................................................. 108
Table 4.1 Silicon nanopore membrane (SNM) hydraulic permeability as a function of
pore size ................................................................................................................................. 117
Table 5.1 The time required for molecules to travel through the islet chamber with a
thickness of 550 μm under diffusion and convection based on a physiological
pressure difference of 2 psi and a 15 nm pore sized SNM ................................. 154
Table 5.2 Experiments for assessing SNM barrier function for auto-, allo- and xeno-
immune responses ............................................................................................................ 156
Table A.1 A list of primers used in RT-PCR analysis of in vitro and in vivo microtextured
scaffolds ................................................................................................................................ 170
Table A.2 A list of primers used in RT-PCR analysis of in vivo microtextured scaffold
.............................................................................................................................................. 171
Table A.3 The number of harvested scaffolds with vascular formation based on a double-
blind observation .................................................................................................................... 181
LIST OF FIGURES

**Figure 1.1** An overview of various encapsulation methods to immunoisolation. Macro-scale encapsulation include extravascular (a), or vascular perfusion (intravascular) devices (b) which are perfused with body fluid or blood. Micro-scale encapsulation devices typically group a few number of cells into microcapsules (a). Nano-scale encapsulation directly coats surface of the islets with polymeric layers (e.g. conformal coating) (a). .............................. 7

**Figure 1.2** A schematic of the U-shaped bioartificial pancreas$^6$-$^8$. The U-shaped bioartificial pancreas consisted of islets (I) that were placed between two flat polyacrylonitrile (PAN) ultrafiltration membranes (M) separated by a gasket (G). Blood (O) circulated successively above the upper and below the lower membranes following a U-shaped circuit (L) surrounding the islet compartment. Image reproduced with copyright permission. .............................. 13

**Figure 1.3** A number of agarose macrobeads retrieved from the peritoneal cavity of a diabetic rat 145 days after implantation$^9$. None of the macrobeads showed any fibrosis but small eruptions were detected on the wall of the peritoneal cavity (indicated by black arrow). Image reproduced with copyright permission. ........................................... 19

**Figure 1.4** Diagrams of an Islet Sheet (a) and its cross-sectional view (b)$^3$,4. Islets-encapsulated alginate is enclosed between acellular immunoprotective alginate layers. A polymer mesh can be included in the sandwiched layer to
provide physical strength. Islet Sheets measure approximately 4 cm X 8 cm X 250 μm. Molecular weight cutoff depends on the modification of the alginate chemistry and its processing conditions. Images by courtesy of Dr. Scott King and reproduced with copyright permission.

**Figure 1.5** Diagrams of Sernova cell pouch™ system\(^{11}\). The pouch consisted of a multi-channel sheet inserted with an array of rods. It was first implanted under the skin for vascular integration, followed by removal of the rods (a). The cell-containing tubing could then inject islets into pre-formed cavities (b). Images by courtesy of Dr. Philip Toleikis from Sernova.

**Figure 1.6** Diagram of TheraCyte devices\(^{1,2}\). The device was a planar pouch made of bilaminar polytetrafluoroethylene (PTFE) with its inner membrane for immunoisolation, and the outer membrane for tissue integration. The inner membrane was 30 μm in thickness with 0.4 μm in pore size. The outer membrane was 15 μm in thickness with 5 μm in pore size. Image by courtesy of TheraCyte, Inc.

**Figure 1.7** A schematic of the Beta O\(_2\) device\(^{14}\). The center of the device was built as an oxygen chamber, connected to access ports for exogenous oxygen refueling. The oxygen chamber was sandwiches between two alginate-immobilized islets layers separated by gas permeable silicon membranes. The external surfaces were coved by hydrophylized polytetrafluoroethylene (PTFE) membranes of 0.4 μm in pore size. Image reproduced with copyright permission.
**Figure 1.8** An image of a nanoporous biocapsule. The nanoporous biocapsule consisted of two nanoporous silicon membranes, gaskets, and protective screens on both side of the device. The injection ports allow the sampling and replenishing of the islets (a). The dimension of the nanoporous silicon membranes, including the support ridge, was 6 X 8 mm. The active membrane area was 3.5 X 2 mm with a thickness of 5 μm (b). Images by courtesy of Dr. Tejal A. Desai.  

**Figure 1.9** An image of the Nanogland device. The Nanogland device consisted of two silicon membranes glued together with implantable silicone. The active membrane area is 6 X 6 mm. The islet chambers were microfabricated for a channel size of 20, 40, 60 μm and a pore size of 3.6, 5.7, 13, 20, and 40 nm. Image reproduced with copyright permission.  

**Figure 1.10** An image of the hybrid artificial pancreas device. The device consisted of a coiled, hollow fiber membrane on top of a disk-shaped, islet compartment. Two seeding ports allowed direct injection of islets into the compartment. The hollow fiber was connected to vascular graft. The membrane has a normal molecular weight cutoff of 50 kDa, an internal diameter of 5-6 mm, and a wall thickness of 120-140 nm. Image reproduced with copyright permission.  

**Figure 1.11** Possible causes for encapsulation failure.  

**Figure 2.1** Silicon nanoporous membranes (SNM). (a) an optical image of the SNM chip. (b) An SEM image of the surface of the membrane which illustrates nanopores with 2 μm in length. (c) An SEM image of the cross-section of the
membrane which illustrates one nanopore with 7 nm in width and 300 nm in depth. ................................................................. 48

**Figure 2.2** Conceptual illustration of the implantable intravascular bioartificial pancreas device in the arm of a T1D patient. Transplanted islets will be encapsulated between two SNM sheets mounted on as an arterio-venous (AV) graft. The arterio-venous pressure differential will generate ultrafiltrate that continuously support the islets, which will, in turn, sense glucose levels and produce insulin that will be swept into the venous blood. The small pore size of the SNM ensures appropriate immunoisolation between the transplanted islets and host. ................................................................. 48

**Figure 2.3** Schematic diagram of the mock-loop circuit for *in vitro* assessment of SNM-encapsulated islets under convective conditions. A peristaltic pump circulated liquid through the top compartment of the flow cell, a pressure transducer, a 3-way valve, the bottom compartment of the flow cell, and finally back to the original reservoir. The flow cell was composed of two membranes dividing the flow cell into three compartments, where islets were placed inside the middle chamber. Ultrafiltrate flow occurred within the middle chamber between two semipermeable membranes as the top membrane was adjacent to a high-pressure “arterial” blood channel and the second membrane was adjacent to a low-pressure “vein” blood channel. The 3-way valve was used to create a pressure difference of ~2psi between the top and the bottom compartment mimicking the physiological condition. ........................................ 49
Figure 2.4 Schematic diagram of the pressure-driven cytokine filtration testing system. A peristaltic pump circulated liquid through a flow cell that connected to a 3-way valve to establish transmembrane pressure. The permeated ultrafiltrate through the membrane was collected at various times for up to 6 hrs. 

Figure 2.5 Schematic for fabrication of silicon nanopore membranes. A) Piranha clean of double side polished Si wafer. B) Thermal oxidation growth of SiO$_2$ and low pressure chemical vapor deposition (LPCVD) of polysilicon. C) Dry-etch patterning of polysilicon. D) Thermal oxidation growth of SiO$_2$ for use as sacrificial layer defining nanopores. E) Patterning of anchor layer by wet etch. F) LPCVD of polysilicon. G) Blanket-etch of polysilicon until exposure of vertical SiO$_2$ nanopores. H) Deposition of low temperature oxide (LTO) for membrane protection and backside etch of membrane with deep reactive ion etching. I) Dry etch removal of LTO and wet etch release of SiO$_2$. 

Figure 2.6 Schematic diagram of the hydraulic permeability testing system. Air was applied through a pressure regulator into the liquid reservoir. A peristaltic pump circulated this liquid through the flow cell with enclosed membrane. The flow cell connected to a differential pressure transducer that was automatically controlled by a data acquisition laptop to adjust the transmembrane pressure. The permeated ultrafiltrate was collected into a liquid container on top of a precision mass balance. Data from the differential pressure transducer and the mass balance were automatically collected and stored in a data acquisition laptop.
Figure 2.7 Transport of various molecules through slit-pore of SNM under a pressure difference of ~2psi. Sieving coefficients (S) were expressed as the ratio of the concentration of the filtrate over the concentration of the feed (means ± SE). BSA was used as a negative control. Results showed that the sieving coefficients of TNF-α, IFN-γ, and IL-1β were 0.16, 0.27, and 0.27 after 6 hours, respectively. The sieving coefficients of glucose and insulin quickly reached 1. These data indicated that small molecules such as glucose and insulin completely passed the SNM whereas the entry of cytokines was greatly hindered under convective transport.

Figure 2.8 Comparison of relative solute size (λ). Experimental relative solute size (mean ± SE) is calculated based on the sieving coefficients for cytokines at 6 hrs. Theoretical values were determined using the Stokes-Einstein’s equation\textsuperscript{10}. 62

Figure 2.9 Assessment of solute distribution in the mock-loop system. The mock-loop circuit was composed of two membranes dividing the flow cell into the top, middle, and the bottom compartments. Concentration of solutes from each chamber was assessed at the end of the 6 hr experiment and was expressed as a percentage (mean ± SE) relative to that of the feed solution. Silicon micropore membrane (SµM) consisted of 1000 nm diameter slit pores were used as control. The data showed that the amount of TNF-α, IFN-γ, and IL-1β were significantly reduced to 30%, 35%, and 34% in the middle chamber, whereas small molecules insulin and glucose passed completely (~100%) through SNM under convective flow. However, all molecules including
cytokines passed into the middle chamber that were sandwiched between SµM. (n>3, *p < 0.05).

Figure 2.10 *In vitro* viability of mouse islets under cytokine exposure. A) Viability of SNM-encapsulated mouse islets was measured following the 6 hr experiment in which islets were subjected to culture solution circulating the mock-loop circuit at 5ml/min with a pressure difference of 2 psi. B) Viable (green) and dead (red) cells were stained for control static culture (a,b) and SNM-encapsulated mouse islets (c,d). Experiments with cytokine exposure (indicated by +Ck) consisted of media containing TNF-α, IFN-γ, and IL-1β. The viability of islets was calculated based on the ratio of dead cells (in red) over the islet area. Viabilities of islets in static cultures were evaluated as control for comparison. SNM protected encapsulated mouse islets from pro-inflammatory cytokines (SNM, +Ck), which showed similar viability to SNM-encapsulated mouse islets without cytokine exposure (SNM, -Ck) and control static culture without cytokine exposure (Control, -Ck). Control static culture with cytokine exposure (Control, +Ck) showed significantly more cell death compared with other groups. (n>3, *p < 0.05).

Figure 2.11 Glucose-stimulated insulin release of mouse islets in the SNM-encapsulation chamber and in static culture. Islets were subjected to media containing low-glucose, high-glucose, and low-glucose for 15 min each. Experiments with cytokine exposure (indicated by +Ck) consisted of culture solution containing TNF-α, IFN-γ, and IL-1β. The static culture without cytokines (Control, -Ck), mock-loop device without cytokines (SNM, -Ck), and mock-loop flow cell
device exposed with cytokines (SNM, +Ck) had a 3.0-fold, 2.6-fold, and 4.1-fold increase in the amount of insulin secreted during high glucose challenge over those secreted during low glucose phase, respectively. However, the control static culture with cytokine exposure (Control, +Ck) secreted limited amount of insulin upon high glucose challenge due to the dead cells damaged by cytokine infiltration. (n>3, *p < 0.05).

**Figure 3.1** Schematic diagram of the perfusion system for *in vitro* assessment of glucose-stimulated insulin secretion from SNM- and SµM-encapsulated mouse islets. A peristaltic pump circulated media through the upper compartment of a two-layered flow cell separated by a SµM/SNM. Islets were encapsulated in the bottom chamber. A 3-way valve was incorporated into the system to establish transmembrane pressure. A set of 75 µL perfusate samples were collected from the bottom chamber at 10-minute intervals for up to 1.5 hours. The bottom chamber was replenished with media following each collection and correction for dilution was made in calculation of the insulin concentration.

**Figure 3.2** (a) Schematic for fabrication of silicon nanopore membranes (SNM). (A) Piranha clean of double side polished Si wafer. (B) Thermal oxidation growth of SiO2 and low pressure chemical vapor deposition (LPCVD) of polysilicon. (C) Dry-etch patterning of polysilicon. (D) Thermal oxidation growth of SiO2 for use as sacrificial layer defining nanopores. (E) Patterning of anchor layer by wet etch. (F) LPCVD of polysilicon. (G) Blanket-etch of polysilicon until exposure of vertical SiO2 nanopores. (H) Deposition of low temperature oxide (LTO) for membrane protection and backside etch of membrane with
deep reactive ion etching. (I) Dry etch removal of LTO and wet etch release of SiO2. (b) An SEM image of the tilted membrane surface which depicts nanopores with 2 µm in length. (c) An SEM image of the cross-section of the membrane which depicts nanopores with 7 nm in width and 300 nm in depth. (d) An SEM image of the membrane surface which depicts micropores with 4 µm in length. (e) An SEM image of the cross-section of the membrane which depicts micropores with 1 µm in width.

Figure 3.3 Schematic diagram of the hydraulic permeability testing system. Air was applied through a pressure regulator into the liquid reservoir. A peristaltic pump circulated this liquid through the flow cell with enclosed membrane. The flow cell connected to a differential pressure transducer that was automatically controlled by a data acquisition laptop to adjust the transmembrane pressure. The permeated ultrafiltrate was collected into a liquid container on top of a precision mass balance. Data from the differential pressure transducer and the mass balance were automatically collected and stored in a data acquisition laptop.

Figure 3.4 Glucose-insulin kinetics of SNM-encapsulated islets under convection and diffusion without cytokine exposure. (a) Insulin release kinetics of SNM-encapsulated mouse islets during 90-minute low-high-low (1.6 mM, 16.6 mM, 1.6 mM) glucose stimulation under convective (2 psi) (Conv) and diffusive transport (Diff) without subjection to cytokines (-Ck). The naked islets cultured under static conditions were served as controls (Control, -Ck). The SNM-encapsulated islets under convective transport (SNM, Conv, -Ck)
exhibited higher insulin secretion following stimulation at high glucose concentration and faster insulin release kinetics in response compared to those under diffusive transport (SNM, Diff, -Ck). (Mean±SEM, n≥3) (b) The stimulation index (SI) was calculated as the ratio of (1) the first insulin collection in the high glucose phase at 40 minutes to the last insulin collection point of the previous low glucose phase at 30 minutes (Stimulation/Pre-stimulation), and (2) the highest insulin secretion in the high glucose phase to the last insulin collection point of the previous low glucose phase at 30 minutes (Highest stimulation/Pre-stimulation). The SI indicates the magnitude of insulin released as stimulated by a higher concentration of glucose. Without cytokine exposure (-Ck), SNM-encapsulated islets under convection (SNM, Conv) and diffusion (SNM, Diff) in addition to the naked islets cultured under static conditions (Control) all exhibited similar magnitude of glucose-induced insulin secretion when transitioning from low glucose to high glucose (Stimulation/Pre-stimulation). However, the SI of SNM-encapsulated islets under convection (SNM, Conv) was the highest compared to that under diffusion (SNM, Diff) and the naked islets cultured under static conditions (Control) when the highest insulin secretion in the high glucose phase was used (Highest Stimulation/Pre-stimulation). (Mean±SEM, n≥3) (c) The shut-down index (SDI) was the ratio of (1) the first insulin collection point in the subsequent low glucose phase at 70 minutes to the last insulin collection point in the high glucose phase at 60 minutes (Post-stimulation/Stimulation), and (2) the lowest insulin secretion in
the subsequent low glucose phase to the last insulin collection point in the high glucose phase at 60 minutes (Lowest Post-stimulation/Stimulation). The SDI reflects the magnitude of cessation in insulin production once glucose concentration returns to normal. Without cytokine exposure (-Ck), SNM-encapsulated islets under convection (SNM, Conv) exhibited the highest magnitude of insulin reduction compared to the diffusive condition (SNM, Diff) and the naked islet culture (Control) as glucose dropped low (Post-stimulation/Stimulation & Lowest Post-stimulation/Stimulation).

(Mean±SEM, n≥3, *p<0.05).

Figure 3.5 Glucose-insulin kinetics of SNM- and SµM-encapsulated islets under convection without cytokine exposure. (a) Insulin release kinetics of SNM- and SµM-encapsulated mouse islets during 90-minute low-high-low (1.6 mM, 16.6 mM, 1.6 mM) glucose stimulation under convective (2 psi) (Conv) without subjection to cytokines (-Ck). The naked islets cultured under static conditions were served as controls (Control, -Ck). Without cytokine exposure (-Ck), the SµM-encapsulated islets under convective transport (SµM, Conv) exhibited higher insulin secretion following stimulation at high glucose concentration and faster insulin release kinetics in response to glucose compared to the SNM-encapsulated islets under convective transport (SNM, Conv). (Mean±SEM, n≥3) (b) The stimulation index (SI) was calculated as the ratio of (1) the first insulin collection in the high glucose phase at 40 minutes to the last insulin collection point of the previous low glucose phase at 30 minutes (Stimulation/Pre-stimulation), and (2) the highest insulin
secretion in the high glucose phase to the last insulin collection point of the previous low glucose phase at 30 minutes (Highest stimulation/Pre-stimulation). The SI indicates the magnitude of insulin released as stimulated by a higher concentration of glucose. Without cytokine exposure (-Ck), the SNM- and SµM-encapsulated islets under convection (SNM, Conv & SµM, Conv) all showed a higher magnitude of secreted insulin compared to the naked islets cultured under static conditions (Control). Furthermore, the SI of SµM -encapsulated islets under convection (SµM, Conv) was the greatest compared to that for the SNM (SNM, Conv) and naked islets cultured under static conditions (Control) when the highest insulin secretion in the high glucose phase was used (Highest Stimulation/Pre-stimulation). (Mean±SEM, n≥3) (c) The shut-down index (SDI) was the ratio of (1) the first insulin collection point in the subsequent low glucose phase at 70 minutes to the last insulin collection point in the high glucose phase at 60 minutes (Post-stimulation/Stimulation), and (2) the lowest insulin secretion in the subsequent low glucose phase to the last insulin collection point in the high glucose phase at 60 minutes (Lowest Post-stimulation/Stimulation). The SDI reflects the magnitude of cessation in insulin production once glucose concentration returns to normal. Without cytokine exposure (-Ck), both SNM- and SµM-encapsulated islets under convection (SNM, Conv & SµM, Conv) exhibited significant magnitude of insulin reduction compared to the islets cultured under static conditions (Control) once glucose dropped back
Figure 3.6 Glucose-insulin kinetics of SµM-encapsulated islets under convection and diffusion without cytokine exposure. (a) Insulin release kinetics of SµM -encapsulated mouse islets during 90-minute low-high-low (1.6 mM, 16.6 mM, 1.6 mM) glucose stimulation under convective (2 psi) (Conv) and diffusive transport (Diff) without subjection to cytokines (-Ck). The naked islets cultured under static conditions served as controls (Control, -Ck). The SµM-encapsulated islets under convective transport (SµM, Conv, -Ck) exhibited higher insulin secretion following stimulation at high glucose concentration and faster insulin release kinetics in response compared to those under diffusive transport (SµM, Diff, -Ck). (Mean±SEM, n≥3) (b) The stimulation index (SI) was calculated as the ratio of (1) the first insulin collection in the high glucose phase at 40 minutes to the last insulin collection point of the previous low glucose phase at 30 minutes (Stimulation/Pre-stimulation), and (2) the highest insulin secretion in the high glucose phase to the last insulin collection point of the previous low glucose phase at 30 minutes (Highest stimulation/Pre-stimulation). The SI indicates the magnitude of insulin released as stimulated by a higher concentration of glucose. Without cytokine exposure (-Ck), SµM-encapsulated islets under convection (SµM, Conv) and diffusion (SµM, Diff) in addition to the naked islets cultured under static conditions (Control) all exhibited similar magnitude of glucose-induced insulin secretion (Stimulation/Pre-
stimulation). However, the SµM-encapsulated islets under convection (SµM, Conv) showed the highest magnitude of insulin secreted when the highest insulin secretion in the high glucose phase was used (Highest stimulation/Pre-stimulation). (Mean±SEM, n≥3) (c) The shut-down index (SDI) was the ratio of (1) the first insulin collection point in the subsequent low glucose phase at 70 minutes to the last insulin collection point in the high glucose phase at 60 minutes (Post-stimulation/Stimulation), and (2) the lowest insulin secretion in the subsequent low glucose phase to the last insulin collection point in the high glucose phase at 60 minutes (Lowest Post-stimulation/Stimulation). The SDI reflects the magnitude of cessation in insulin production once glucose concentration returns to normal. Without cytokine exposure (-Ck), SµM-encapsulated islets under convection (SµM, Conv) exhibited the highest magnitude of insulin reduction compared to the diffusive condition (SµM, Diff) and the naked islet culture (Control) as glucose dropped low (Post-stimulation/Stimulation). When the lowest insulin secretion in the low glucose phase was used, SµM-encapsulated islets under convection (SµM, Conv) also showed the largest magnitude of insulin reduction (Lowest Post-stimulation/Stimulation). (Mean±SEM, n≥3, *p<0.05). .................................................................

Figure 3.7 Glucose-insulin kinetics of SNM- and SµM-encapsulated islets under diffusion without cytokine exposure. (a) Insulin release kinetics of SNM- and SµM-encapsulated mouse islets during 90-minute low-high-low (1.6 mM, 16.6 mM, 1.6 mM) glucose stimulation under diffusion (2 psi) (Diff) without
subjection to cytokines (-Ck). The naked islets cultured under static conditions served as controls (Control, -Ck). Without cytokine exposure (-Ck), SµM-encapsulated islets under diffusive transport (SµM, Diff) exhibited higher insulin secretion that slowly plateaued following stimulation at high glucose concentration compared to the SNM-encapsulated islets under diffusive transport (SNM, Diff). (Mean ± SEM, n ≥ 3) (b) The stimulation index (SI) was calculated as the ratio of (1) the first insulin collection in the high glucose phase at 40 minutes to the last insulin collection point of the previous low glucose phase at 30 minutes (Stimulation/Pre-stimulation), and (2) the highest insulin secretion in the high glucose phase to the last insulin collection point of the previous low glucose phase at 30 minutes (Highest stimulation/Pre-stimulation). The SI indicates the magnitude of insulin released as stimulated by a higher concentration of glucose. Without cytokine exposure (-Ck), the SNM- and SµM-encapsulated islets under diffusion (SNM, Diff & SµM, Diff) all showed a similar magnitude of insulin secretion compared with the naked islets cultured under static conditions (Control) (Stimulation/Pre-stimulation). Moreover, the SNM-encapsulated islets under diffusion (SNM, Diff) and naked islets cultured under static conditions showed an increase in SI compared to the SµM-encapsulated islets under diffusion (SµM, Diff) when the highest insulin secretion in the high glucose phase was used (Highest stimulation/Pre-stimulation). (Mean ± SEM, n ≥ 3) (c) The shut-down index (SDI) was the ratio of (1) the first insulin collection point in the subsequent low glucose phase at 70 minutes to the last insulin
collection point in the high glucose phase at 60 minutes (Post-stimulation/Stimulation), and (2) the lowest insulin secretion in the subsequent low glucose phase to the last insulin collection point in the high glucose phase at 60 minutes (Lowest Post-stimulation/Stimulation). The SDI reflects the magnitude of cessation in insulin production once glucose concentration returns to normal. Without cytokine exposure (-Ck), SNM- and SµM-encapsulated islets under diffusion (SNM, Diff & SµM, Diff) exhibited similar magnitude of insulin reduction compared to the islets cultured under static conditions (Control) once glucose dropped back low (Post-stimulation/Stimulation). However, the level of shut down was more significant in SµM-encapsulated islets under diffusion (SµM, Diff) than in the other two conditions (SNM, Diff & Control) when the lowest insulin secretion was used (Lowest Post-stimulation/Stimulation). (Mean ± SEM, n ≥3, *p<0.05).

**Figure 3.8** Glucose-insulin kinetics of SNM-encapsulated islets under convection and diffusion with cytokine exposure. (a) Insulin release kinetics of SNM-encapsulated mouse islets during 90-minute low-high-low (1.6 mM, 16.6 mM, 1.6 mM) glucose stimulation under convective (2 psi) (Conv) and diffusive transport (Diff) with subjection to cytokines (+Ck). Experiments with cytokine exposure (+Ck) consisted of media containing TNF-α (2,000 U/mL), IFN-γ (1,000 U/mL), and IL-1β (10,000 U/mL). The naked islets cultured under static conditions served as controls (Control, +Ck). The SNM-encapsulated islets under convective transport (SNM, Conv, +Ck) exhibited
higher insulin secretion following stimulation at high glucose concentration and faster insulin release kinetics in response compared to those under diffusive transport (SNM, Diff, +Ck) and naked islets cultured under static conditions (Control, +Ck). (Mean±SEM, n≥3) (b) The stimulation index (SI) was calculated as the ratio of (1) the first insulin collection in the high glucose phase at 40 minutes to the last insulin collection point of the previous low glucose phase at 30 minutes (Stimulation/Pre-stimulation), and (2) the highest insulin secretion in the high glucose phase to the last insulin collection point of the previous low glucose phase at 30 minutes (Highest stimulation/Pre-stimulation). The SI indicates the magnitude of insulin released as stimulated by a higher concentration of glucose. With cytokine exposure (+Ck), all conditions including SNM-encapsulated islets under convection (SNM, Conv) and diffusion (SNM, Diff), and the naked islets cultured under static conditions (Control) all exhibited varying level of magnitude in glucose-induced insulin secretion (Stimulation/Pre-stimulation). However, when using the highest insulin secretion in the high glucose phase (Highest Stimulation/Pre-stimulation), the calculated SI was the highest for SNM-encapsulated islets under convection (SNM, Conv) compared to that under diffusion (SNM, Diff) and naked islets cultured under static conditions (Control). (Mean±SEM, n≥3, *p<0.05) (c) The shut-down index (SDI) was calculated as the ratio of (1) the first insulin collection point in the subsequent low glucose phase at 70 minutes to the last insulin collection point in the high glucose phase at 60 minutes (Post-stimulation/Stimulation), and (2) the
lowest insulin secretion in the subsequent low glucose phase to the last
insulin collection point in the high glucose phase at 60 minutes (Lowest Post-
stimulation/Stimulation). The SDI reflects the magnitude of cessation in
insulin production once glucose concentration returns to normal. With
cytokine exposure (+Ck), the SNM-encapsulated islets under convection
(SNM, Conv) exhibited the highest magnitude of insulin reduction compared
to the diffusive condition (SNM, Diff) and the naked islet culture (Control) as
glucose dropped low (Post-stimulation/Stimulation & Lowest Post-
stimulation/Stimulation). (Mean ± SEM, n⩾3, *p<0.05).

Figure 3.9 Glucose-insulin kinetics of SNM- and SμM-encapsulated islets under
convection with cytokine exposure. (a) Insulin release kinetics of SNM- and
SμM-encapsulated mouse islets during 90-minute low-high-low (1.6 mM,
16.6 mM, 1.6 mM) glucose stimulation under convective (2 psi) (Conv) with
subjection to cytokines (+Ck). The naked islets cultured under static
conditions served as controls (Control, +Ck). Experiments with cytokine
exposure (+Ck) consisted of media containing TNF-α (2,000 U/mL), IFN-γ
(1,000 U/mL), and IL-1β (10,000 U/mL). With cytokine exposure (+Ck), the
SμM-encapsulated islets under convective transport (SμM, Conv) exhibited a
continuous insulin secretion following stimulation at high glucose
concentration from 40 minutes to 60 minutes, while the SNM-encapsulated
islets under convection (SNM, Conv) showed a plateau in insulin production
during this period of challenge. (Mean ± SEM, n⩾3) (b) The stimulation
index (SI) was calculated as the ratio of (1) the first insulin collection in the
high glucose phase at 40 minutes to the last insulin collection point of the previous low glucose phase at 30 minutes (Stimulation/Pre-stimulation), and (2) the highest insulin secretion in the high glucose phase to the last insulin collection point of the previous low glucose phase at 30 minutes (Highest stimulation/Pre-stimulation). The SI indicates the magnitude of insulin released as stimulated by a higher concentration of glucose. With cytokine exposure (+Ck), the SNM- and SμM-encapsulated islets under convection (SNM, Conv & SμM, Conv) and the naked islet culture under static conditions (Control) all showed a significant difference in the magnitude of insulin secreted upon high glucose challenge (Stimulation/Pre-stimulation). However, the SNM- and SμM-encapsulated islets under convection (SNM, Conv & SμM, Conv) showed greater difference in the magnitude of insulin secreted upon high glucose challenge when the highest insulin secretion was used (Highest stimulation/Pre-stimulation). (Mean ± SEM, n≥3, *p<0.05)
(c) The shut-down index (SDI) was the ratio of (1) the first insulin collection point in the subsequent low glucose phase at 70 minutes to the last insulin collection point in the high glucose phase at 60 minutes (Post-stimulation/Stimulation), and (2) the lowest insulin secretion in the subsequent low glucose phase to the last insulin collection point in the high glucose phase at 60 minutes (Lowest Post-stimulation/Stimulation). The SDI reflects the magnitude of cessation in insulin production once glucose concentration returns to normal. With cytokine exposure (+Ck), the SNM- and SμM-encapsulated islets under convection (SNM, Conv & SμM, Conv)
exhibited the highest magnitude of insulin reduction compared to the naked islet culture (Control) as glucose dropped low (Post-stimulation/Stimulation & Lowest Post-stimulation/Stimulation). (Mean ± SEM, n ≥ 3, *p < 0.05). 

**Figure 3.10** Glucose-insulin kinetics of SµM-encapsulated islets under convection and diffusion with cytokine exposure. (a) Insulin release kinetics of SµM-encapsulated mouse islets during 90-minute low-high-low (1.6 mM, 16.6 mM, 1.6 mM) glucose stimulation under convective (2 psi) (Conv) and diffusive transport (Diff) with subjection to cytokines (+Ck). Experiments with cytokine exposure (+Ck) consisted of media containing TNF-α (2,000 U/mL), IFN-γ (1,000 U/mL), and IL-1β (10,000 U/mL). The naked islets cultured under static conditions served as controls (Control, +Ck). The SµM-encapsulated islets under convective transport (SµM, Conv, +Ck) exhibited higher insulin secretion and faster insulin release kinetics in response to stimulation at high glucose concentration compared to those under diffusive transport (SµM, Diff, +Ck) and naked islets cultured under static conditions (Control, +Ck). (Mean ± SEM, n ≥ 3) (b) The stimulation index (SI) was calculated as the ratio of (1) the first insulin collection in the high glucose phase at 40 minutes to the last insulin collection point of the previous low glucose phase at 30 minutes (Stimulation/Pre-stimulation), and (2) the highest insulin secretion in the high glucose phase to the last insulin collection point of the previous low glucose phase at 30 minutes (Highest stimulation/Pre-stimulation). The SI indicates the magnitude of insulin released as stimulated by a higher concentration of glucose. With cytokine
exposure (+Ck), all conditions including SµM-encapsulated islets under convection (SµM, Conv) and diffusion (SµM, Diff), and the naked islets cultured under static conditions (Control) all exhibited varying level of magnitude in glucose-induced insulin secretion (Stimulation/Pre-stimulation). The SµM-encapsulated islets under convection (SµM, Conv) and naked islets cultured under static conditions (Control) showed an increase in the magnitude of insulin secretion when the highest insulin secretion in the high glucose phase was used (Highest stimulation/Pre-stimulation). (Mean ± SEM, n ≥ 3, *p<0.05) (c) The shut-down index (SDI) was the ratio of (1) the first insulin collection point in the subsequent low glucose phase at 70 minutes to the last insulin collection point in the high glucose phase at 60 minutes (Post-stimulation/Stimulation), and (2) the lowest insulin secretion in the subsequent low glucose phase to the last insulin collection point in the high glucose phase at 60 minutes (Lowest Post-stimulation/Stimulation). The SDI reflects the magnitude of cessation in insulin production once glucose concentration returns to normal. With cytokine exposure (+Ck), the SµM-encapsulated islets under convection (SµM, Conv) and under diffusion (SµM, Diff) both exhibited the highest magnitude of insulin reduction compared to the naked islet culture (Control) as glucose dropped low (Post-stimulation/Stimulation & Lowest Post-stimulation/Stimulation). (Mean ± SEM, n ≥ 3, *p<0.05).

Figure 3.11 Glucose-insulin kinetics of SNM- and SµM-encapsulated islets under diffusion with cytokine exposure. (a) Insulin release kinetics of SNM- and
SµM-encapsulated mouse islets during 90-minute low-high-low (1.6 mM, 16.6 mM, 1.6 mM) glucose stimulation under diffusion (Diff) with subjection to cytokines (+Ck). The naked islets cultured under static conditions served as controls (Control, +Ck). Experiments with cytokine exposure (+Ck) consisted of media containing TNF-α (2,000 U/mL), IFN-γ (1,000 U/mL), and IL-1β (10,000 U/mL). With cytokine exposure (+Ck), the SµM-encapsulated islets under diffusive transport (SµM, Diff) exhibited the fastest insulin secretion at high glucose concentration from 40 minutes to 60 minutes followed by the SNM-encapsulated islets under diffusion (SNM, Diff). The level of glucose-induced insulin secretion from the naked islets cultured under static conditions (Control) was not as significant as the other two groups. (Mean ± SEM, n ≥3) (b) The stimulation index (SI) was calculated as the ratio of (1) the first insulin collection in the high glucose phase at 40 minutes to the last insulin collection point of the previous low glucose phase at 30 minutes (Stimulation/Pre-stimulation), and (2) the highest insulin secretion in the high glucose phase to the last insulin collection point of the previous low glucose phase at 30 minutes (Highest stimulation/Pre-stimulation). The SI indicates the magnitude of insulin released as stimulated by a higher concentration of glucose. With cytokine exposure (+Ck), the SNM- and SµM-encapsulated islets under diffusion (SNM,Diff & SµM, Diff) and the naked islet culture under static conditions (Control) all showed a significant difference in the magnitude of insulin secreted upon high glucose challenge (Stimulation/Pre-stimulation). The SNM-encapsulated islets under
diffusion (SNM, Diff) and naked islets cultured under static conditions (Control) showed an increase in the magnitude of insulin secretion when the highest insulin secretion in the high glucose phase was used (Highest stimulation/Pre-stimulation). (Mean ± SEM, n ≥ 3, *p<0.05) (c) The shut-down index (SDI) was the ratio of (1) the first insulin collection point in the subsequent low glucose phase at 70 minutes to the last insulin collection point in the high glucose phase at 60 minutes (Post-stimulation/Stimulation), and (2) the lowest insulin secretion in the subsequent low glucose phase to the last insulin collection point in the high glucose phase at 60 minutes (Lowest Post-stimulation/Stimulation). The SDI reflects the magnitude of cessation in insulin production once glucose concentration returns to normal. With cytokine exposure (+Ck), the SµM-encapsulated islets under diffusion (SµM, Diff) exhibited the highest magnitude of insulin reduction compared to the SNM-encapsulated islets under diffusion (SNM, Diff) and naked islet culture (Control) as glucose dropped low (Post-stimulation/Stimulation & Lowest Post-stimulation/Stimulation). (Mean ± SEM, n ≥ 3, *p<0.05). ..... 106

Figure 3.12 In vitro viability of mouse islets. (a) Viability of mouse islets was measured following the 90-minute low-high-low (1.6 mM, 16.6 mM, 1.6 mM) glucose stimulation in which islets were subjected to the mock-loop circuit with or without cytokine exposure (+Ck vs -Ck) for SNM- and SµM-encapsulation under convection (SNM, C & SµM, C). The naked islet culture under static culture with cytokine exposure (Control, +Ck) showed significantly less viability compared to all other conditions. (Mean ± SEM, n ≥ 3, *p<0.05) (b)
Viable (green) and dead (red) cells were stained for control static culture without cytokines (A: Control, -Ck), control static culture with cytokines (B: Control, +Ck), SNM-encapsulated mouse islets under convection without cytokines (C: SNM, C, -Ck), SNM-encapsulated mouse islets under convection with cytokines (D: SNM, C, +Ck), SµM-encapsulated mouse islets under convection without cytokines (E: SµM, C, -Ck), and SµM-encapsulated mouse islets under convection with cytokines (F: SµM, C, +Ck). Experiments with cytokine exposure (indicated by +Ck) consisted of media containing TNF-α, IFN-γ, and IL-1β. Both control static culture with cytokines (B: Control, +Ck) and SµM-encapsulated mouse islets under convection with cytokines (F: SµM, C, +Ck) showed a higher level of islet damage compared to other groups, however, the viability of SµM-encapsulated mouse islets under convection with cytokines (F: SµM, C, +Ck) was not statistically significant (Fig. 3.12,a). .........................................................

**Figure 3.13 In vitro viability of mouse islets.** (a) Viability of mouse islets was measured following the 90-minute low-high-low (1.6 mM, 16.6 mM, 1.6 mM) glucose stimulation in which islets were subjected to the mock-loop circuit with or without cytokine exposure (+Ck vs -Ck) for SNM- and SµM-encapsulation under diffusion (SNM, D & SµM, D). The naked islet culture under static culture with cytokine exposure (Control, +Ck) showed significantly less viability compared to all other conditions. (Mean ± SEM, n ≥ 3, *p<0.05) (b) Viable (green) and dead (red) cells were stained for control static culture without cytokines (A: Control, -Ck), control static culture with cytokines (B:
Control, +Ck), SNM-encapsulated mouse islets under diffusion without cytokines (C: SNM, D, -Ck), SNM-encapsulated mouse islets under diffusion with cytokines (D: SNM, D, +Ck), SµM-encapsulated mouse islets under diffusion without cytokines (E: SµM, D, -Ck), and SµM-encapsulated mouse islets under diffusion with cytokines (F: SµM, D, +Ck). Experiments with cytokine exposure (indicated by +Ck) consisted of media containing TNF-α, IFN-γ, and IL-1β. The control static culture with cytokines (B: Control, +Ck) showed significant level of islet damage compared to all other conditions.

**Figure 4.1** The assembly of intravascular bioartificial pancreas device (iBAP). (a,i) An exploded view of the iBAP components: ultrafiltrate outlet (blue), polycarbonate backside (PC Backside), silicon nanopore membrane (SNM) (green), islet chamber (IC), and polycarbonate piece with flow path (Flow Path). (a,ii) An assembled iBAP used for *in vitro* and *in vivo* convective experiments. (b,i) An optical image of the SNM membrane. (b,ii) A top view SEM image illustrating the rectangular pore-containing regions surrounded by solid silicon regions, which provide mechanical support. (b,iii) A further magnified top view SEM image of the pore region showing individual 10 nm-wide pores. (c,i) An illustration of the acrylic islet chamber (IC) where there are eight ultrafiltrate channels within the islet-agarose gel region (purple). (c,ii) A gross image of islets and agarose mixture inside the IC in which the maximum diameter surrounding each ultrafiltrate channel is 800 µm.
**Figure 4.2** (a) Illustration of the full-scale iBAP connected to arterial-venous grafts and an Ultrafiltrate Outlet catheter delivering insulin rich ultrafiltrate to the ultrafiltrate vein. Blood flows into the iBAP and a looped blood channel transports blood to a vein. The SNM encapsulated IC is placed directly above and below the blood channel. (b) A cross-sectional view perpendicular to blood flow illustrating the blood channel surrounded by the SNM (green) encapsulated IC (blue). Ultrafiltrate (black arrows) crosses the SNM encapsulated IC into ultrafiltrate channels (side) and exits the Ultrafiltrate Outlet catheter into the ultrafiltrate vein.

**Figure 4.3** *In vitro* testing of the intravascular bioartificial pancreas device (iBAP) with 10% or 20% islet density encapsulated with 10 nm-pore size SNM. (a) Glucose-insulin kinetics of the SNM-encapsulated iBAP with 10% (i) or 20% (ii) islet densities under convection was measured from exposing them to a series of low, high, and low glucose conditions. (b) The SNM-encapsulated iBAP with 10% islet density under convection (10% convection) showed significantly higher viability compared to that of 10% islet density under diffusion (10% diffusion), and 20% islet density under both diffusion (20% diffusion) and convection (20% convection) after 3 days. (n > 3, *p < 0.05). Viabilities of islets that were immediately encapsulated in agarose and dispensed into the islet chamber (IC) without further testing were evaluated as the *in vitro* positive control. (c) Viable (green) and dead (red) cells were stained for *in vitro* positive control (i), 10% islet density under diffusion (ii), 10% islet density under convection (iii), 20% islet density under diffusion
(iv), and 20% islet density under convection (v) (scale bar = 50 µm). The SNM-encapsulated iBAP with 10% islet density under convection (iii) showed higher viability than that of 10% islet density under diffusion (ii), and 20% islet density under both diffusion (iv) and convection (v). The 10% islet density under diffusion (ii), and 20% islet density under both diffusion (iv) and convection (v) showed similar viability with significant amount of cell death. 

Figure 4.4 In vitro testing of the intravascular bioartificial pancreas device (iBAP) with 10% or 20% islet density encapsulated with 40 nm-pore size SNM. (a) Glucose-insulin kinetics of the SNM-encapsulated iBAP with 10% (i) or 20% (ii) islet densities under convection was measured from exposing them to a series of low, high, and low glucose conditions. (b) The SNM-encapsulated iBAP with 10% and 20% islet density under convection (10% & 20% convection) showed significantly higher viability compared to that of 10% and 20% islet density under diffusion (10% & 20% diffusion) after 3 days (n > 3, *p < 0.05). Viabilities of islets that were immediately encapsulated in agarose and dispensed into the islet chamber (IC) without further testing were evaluated as the in vitro positive control. (c) Viable (green) and dead (red) cells were stained for in vitro positive control (i), 10% islet density under diffusion (ii), 10% islet density under convection (iii), 20% islet density under diffusion (iv), and 20% islet density under convection (v) (scale bar = 50 µm). The SNM-encapsulated iBAP with 10% and 20% islet density under convection (iii & v) showed higher viability than those under diffusion (ii &
iv). In particular, the 20% islet density under diffusion (iv) showed significant amount of cell death.

**Figure 4.5** *In vivo* testing of the intravascular bioartificial pancreas device (iBAP) with 5% islet density encapsulated with 10 nm-pore size SNM for 3 days. (a) An image of the explanted diffusion-based iBAP (i). The angiogram showed no obstruction of flow after injecting the contrast agent into the device (ii). An SEM image of the implanted membrane showing attachment of red blood cells and platelets (iii) (scale bar = 10 µm). (b) Immunofluorescence staining of platelet adhesion CD41 marker (green) and platelet activation CD62p marker (red). The rectangular pore-containing regions surrounded by solid silicon regions were shown in the bright field image (i). The platelet adhesion (green) mostly occurred in the window regions where pores reside, whereas minimal platelet activation (red) was detected (ii) (scale bar = 20 µm). (c) The SNM-encapsulated iBAP with 5% islet density under diffusion both *in vitro* (*in vitro* 5% diffusion) and *in vivo* (*in vivo* 5% diffusion) showed significantly higher viability compared to the *in vitro* negative control (n >3, *p < 0.05). The *in vitro* negative control was those islets that were assembled in the iBAP with no medium circulation for 3 days. Viabilities of islets that were immediately encapsulated in agarose and dispensed into the islet chamber (IC) without further testing were evaluated as the *in vitro* positive control. (d) Viable (green) and dead (red) cells were stained for *in vitro* positive control (i), *in vitro* negative control (ii), *in vitro* 5% islet density under diffusion (iii), *in vivo* 5% islet density under diffusion (iv) (scale bar =
The SNM-encapsulated iBAP with 5% islet density under convection (iii & v) showed similar viability to the in vitro positive control.

Figure 4.6 Daily measurement of the systematic cytokine concentration in the pig. The intravascular bioartificial pancreas (iBAP) with 5% islet density encapsulated with 10 nm-pore size SNM. Cytokines namely granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor-alpha (TNF-α), interleukin 1-alpha (IL-1α), interleukin 1-beta (IL-1β), interleukin 8 (IL-8), interleukin 12 (IL-12), interleukin 18 (IL-18), interleukin-1 receptor antagonist (IL-1Ra), interleukin 4 (IL-4), and interleukin 10 (IL-10) were analyzed. Interferon gamma (IFN-ϒ) was not detected. About 35.89 pg/ml of interleukin 2 (IL-2) was detected post-implantation on Day 0 only.

Figure 4.7 In vivo testing of the intravascular bioartificial pancreas device (iBAP) with 10% islet density encapsulated with 10 nm-pore size SNM under either diffusion or convection for 3 days. (a) An image of the explanted iBAP with diffusion (back) and convection (front) of the device (i). An SEM image of the diffusion-side implanted membrane showed a patent surface (ii, top) (scale bar = 100 µm) and an SEM image of the convection-side membrane presented coverage of proteins and cells on the surface (ii) (scale bar = 10 µm). The angiogram indicated no obstruction of fluid flow inside the device after 3 days in pig (iii). (b) Immunofluorescence staining of platelet adhesion CD41 marker (green) and platelet activation CD62p marker (red). The rectangular pore-containing regions surrounded by solid silicon regions were
shown in the bright field image for diffusion-side membrane (i) and convection-side membrane (iii). The platelet adhesion (green) was minimal on the diffusion-side membrane (ii), whereas more platelet adhesion (green) and activation (red) was detected on the convection-side membrane (iv) (scale bar = 20 µm). (c) The SNM-encapsulated iBAP with 10% islet density under convection both in vitro (in vitro 10% convection) and in vivo (in vivo 10% convection) showed higher cell viability compared to that under diffusion in vitro (in vitro 10% diffusion) and in vivo (in vivo 10% diffusion). (n > 3, *p < 0.05). The in vitro negative control was those islets that were assembled in the iBAP with no medium circulation for 3 days. Viabilities of islets that were immediately encapsulated in agarose and dispensed into the islet chamber (IC) without further testing were evaluated as the in vitro positive control. (d) Viable (green) and dead (red) cells were stained for in vitro positive control (i), in vitro negative control (ii), in vitro 10% islet density under diffusion (iii), in vitro 10% islet density under convection (iv), in vivo 10% islet density under diffusion (v), in vivo 10% islet density under convection (vi) (scale bar = 50 µm). The SNM-encapsulated iBAP with 10% islet density under convection in vivo (vi) showed similar viability to the in vitro positive control.

**Figure 4.8** Daily measurement of the systematic cytokine concentration in the pig. The intravascular bioartificial pancreas (iBAP) with 10% islet density encapsulated with 10 nm-pore size SNM. Cytokines namely Interferon gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), interleukin 1-alpha
(IL-1α), interleukin 1-beta (IL-1β), interleukin 2 (IL-2), interleukin 6 (IL-6), interleukin 12 (IL-12), interleukin 18 (IL-18), interleukin-1 receptor antagonist (IL-1Ra), interleukin 4 (IL-4), and interleukin 10 (IL-10) were analyzed. Granulocyte-macrophage colony-stimulating factor (GM-CSF) was not detected. About 25.44 pg/ml of interleukin 8 (IL-8) was detected on Day 2 only.

Figure 5.1 Conceptual illustration of the implantable intravascular bioartificial pancreas device in the arm of a T1D patient (left). Transplanted islets will be encapsulated between two SNM sheets (black) mounted on as an arterio-venous (AV) graft. The arterio-venous (red-blue) pressure differential will generate ultrafiltrate that continuously support the islets inside the cell chamber (green), which will, in turn, sense glucose levels and produce insulin that will be swept into the venous blood (right).

Figure 5.2 An illustration of the arterial (red) and venous (blue) blood sweeping the SNM surfaces (black) (adapted from Fig. 6.1). The entire thickness of the chamber is denoted as “L” with x-direction indicating the distance into the islet chamber. At the arterial blood and arterial SNM interface (x = 0), the solute concentration is the same as the initial arterial concentration (C = C₀). At the venous blood and venous SNM interface (x = L), the solute concentration is the same as the initial venous concentration (C = C₀).

Figure 5.3 The concentration of oxygen as a function of distance inside the islet chamber with 100% cell density (c.d.) (blue). When the oxygen concentration drops under 50 mmHg, hypoxia can occur leading to massive cell death inside the
islet chamber (orange). The oxygen concentration inside the islet chamber was above the hypoxia limit with a maximum thickness of 550 μm. Figure 5.4 The SNM macrocapsule with 10-15 μl volume. (a) A top view of SNM macrocapsule consisted of two SNM chips and PDMS rings (scale bar = 1 cm) (b) A side view of SNM macrocapsule consisted of two SNM chips and PDMS rings (scale bar = 600 μm).

Figure 5.5 The SNM macrocapsule implanted subcutaneously in mice after 45 days. (a) An image of the implanted SNM macrocapsule with blood vessels in the surrounding tissue (b) An image of the retrieved SNM macrocapsule with intact PDMS and SNM components.

Figure A.1 Fabrication of PDMS post microtextures by soft lithography. Briefly, 10 μm thick SU-8 2010 photoresist was spin coated on top of silicon wafers. The post microtexture pattern with 10 μm inter-space was transferred from a photomask onto the photoresist under UV exposure. The liquid PDMS and curing photocatalyst were mixed at a ratio of 10:1, degassed for 20 min, and then poured uniformly on top of the patterned mold. The PDMS substrates were cured at 85 °C for 2 h.

Figure A.2 Number of cells on PDMS microtextured substrates in basal medium (BM) and osteogenic medium (OM) for 6 weeks. The positive control mouse pre-osteoblast cell lines calB2T3 and MC3T3 and hMSCs were cultured on microtextured substrates. Proliferation study showed that hMSCs have less proliferative capacity than the positive controls in both BM and OM conditions. The number of hMSCs on microtextured surfaces increased

xliii
significantly in BM compared to OM. (n>3, *p<0.05, **p<0.005, 
****p<0.0001). Figure A3 SEM images of hMSCs on PDMS microtextured substrates for 6 weeks. (a) PDMS post microtexture. (b) hMSCs on PDMS microtextured substrates in BM. (c) hMSCs on PDMS microtextured substrates in OM. On microtextured substrates in BM (d), hMSCs mostly tended to attach next to the posts and spread their processes towards posts and other cells (white arrows). hMSCs on microtextured substrates in OM (e) showed cell spreading over the top of the microposts and coverage with ECM (black arrows) on the substrate surfaces.

Figure A4 RT-PCR analysis of hMSCs on microtextured substrates in BM and OM. Relative gene expression of hMSCs on microtextured substrates in BM and OM compared to the negative controls, hMSCs cultured in BM (a) and hMSCs cultured in OM (b) on smooth surface. Relative gene expression of hMSCs on microtextured substrates in BM and OM compared to the positive control, human osteosarcoma cell line Saos-2 on smooth surface (c). The PDMS microtextured substrates enhanced the osteogenic differentiation of hMSCs in the proliferative basal medium (BM) condition as indicated by the high levels of expression in osteogenic markers (COL I, ALP, BSP, and OC) (a,b). The osteogenic differentiation of hMSCs on microtextured substrates was further elevated by culturing under the differentiative osteogenic medium (OM) (a,b). The expression of BSP, an early marker of osteoblast differentiation, and OC, a marker of late-stage differentiation, were
significantly enhanced for hMSCs on microtextured substrates in BM and OM compared to the positive control human osteosarcoma cell line Saos-2 (c), affirming the effectiveness of using microtextured substrates and biochemical supplements to promote late-stage bone differentiation. (n>3, *p<0.05).

**Figure A.5** Immunofluorescence staining of osteogenic markers COL I (red, b,f,j) and OC (green, c,g,k) on microtextured scaffolds. Nuclei were stained with DAPI (d,h,l). *(Note: the original color images were converted to grayscale and reversed to provide visual clarity.)* (a-d) hMSCs on smooth PDMS substrates. (e-h) hMSCs on microtextured surfaces in BM. (i-l) hMSCs on microtextured surfaces in OM. hMSCs on microtextured surfaces in BM (f,g) and OM (j,k) revealed high intensity of COL I and OC compared to the negative control, undifferentiated hMSCs on smooth surface (b,c), indicating the differentiation of hMSCs toward osteogenic lineage.

**Figure A.6** Alkaline phosphatase levels were normalized for cells cultured on microtextured surfaces in basal medium (BM) and osteogenic medium (OM). The level of mineralization enhanced significantly for hMSCs on microtextured substrates in OM compared to BM. The positive control pre-osteoblast cell lines calB2T3 and MC3T3 showed a greater level of mineralization in both BM and OM. (n>3, **p<0.05).

**Figure A.7** Alizarin red staining of cells cultured on microtextured surfaces in basal medium (BM) (a-d) and osteogenic medium (OM) (e-h). (a,e) microtextured substrates. (b,f) calB2T3 on microtextured surfaces. (c,g) MC3T3 on
microtextured surfaces. (d,h) hMSCs on microtextured surfaces. The OM condition (f-h) enhanced calcium deposition of cells on microtextured surfaces compared to the BM condition (b-d). hMSCs on microtextured substrates in both BM (d) and OM (h) showed significant amount of calcium deposition................................................................. 180

**Figure A.8** Vasculature observed from harvested scaffolds after a 6-week implantation in mice. (a) microtextured substrates without hMSCs. (b) hMSCs on microtextured surfaces in BM. (c,d) hMSCs on microtextured surfaces in OM. Microtextured substrates without hMSCs (a) that were implanted in mice lacked any microvasculature and integration with the surrounding host tissues. hMSCs on microtextured surfaces in BM (b) exhibited micro-vessel growth surrounding the edge of the substrates, whereas hMSCs on microtextured surfaces in OM (c,d) showed a more perfused and extensive network of blood vessels in all directions................................................................. 182

**Figure A.9** SEM images of harvested scaffolds after a 6-week implantation in mice. (a,b) microtextured substrates without hMSCs. (c,d) hMSCs on microtextured surfaces in BM. (e,f) hMSCs on microtextured surfaces in OM. All implanted scaffolds developed ECM rich capsules with uniform shape and thickness around the entire microtextured substrates. However, the sprouting of new blood vessels\(^{15}\) in tissue surrounding the implants was significantly higher for hMSCs on microtextured surfaces in OM (e,f). Red blood cells (black arrows) were observed for implants of hMSCs on microtextured surfaces in OM (e,f). ............................................................................................................. 182
Figure A.10 RT-PCR analysis of harvested scaffolds after a 6-week implantation in mice. (a) The expression of osteogenic markers (COL I, COL X, ALP, BSP, and OC) were determined for blank microtextured substrates (PDMS) without hMSCs, hMSCs on microtextured surface in basal medium (hMSCs + PDMS in BM), and hMSCs on microtextured surface in osteogenic medium (hMSCs + PDMS in OM). (b) The expression of angiogenic markers (PECAM and VEGF) were measured for blank microtextured substrates (PDMS), hMSCs on microtextured surface in basal medium (hMSCs + PDMS in BM), and hMSCs on microtextured surface in osteogenic medium (hMSCs + PDMS in OM). Gene analysis of harvested implants demonstrated that hMSCs on microtextured surfaces in OM (hMSCs + PDMS in OM) provided the optimal condition to accelerate the early and late stage of hMSCs osteogenesis in vivo. hMSCs on microtextured surfaces in OM (hMSCs + PDMS in OM) also promoted vascularization based on the increased expression of angiogenic makers, indicating successful integration of vascularized osteogenic bone grafts. (n>3, *p<0.05, ***p<0.001).

Figure A.11 Immunofluorescence staining of osteogenic markers COL I (red, b,f,j) and OC (green, c,g,k) for harvested scaffolds after a 6-week implantation. Nuclei were stained with DAPI (d,h,l). (Note: the original color images were converted to grayscale and reversed to provide visual clarity.) (a-d) microtextured substrates without hMSCs. (e-h) hMSCs on microtextured surfaces in BM. (i-l) hMSCs on microtextured surfaces in OM (i-l). Blank microtextured substrates showed minimal cell attachment on the surface (d).
and exhibited no COL I and OC stainings (b,c). hMSCs on microtextured surfaces in BM (f,g) and OM (j,k) revealed high intensity of COL I and OC compared to the negative control blank microtextured substrates (b,c), indicating the differentiation of hMSCs toward osteogenic lineage on microtextured surfaces \textit{in vivo}.

\textbf{Figure A.12} Immunofluorescence staining of human mitochondria (green, e,d,g) and mouse PECAM (red, b,e,h) for harvested scaffolds after a 6-week implantation. Nuclei were stained with DAPI (c,f,i). (Note: the original color images were converted to grayscale and reversed to provide visual clarity.) (a-c) microtextured substrates without hMSCs. (d-f) hMSCs on microtextured surfaces in BM. (g-i) hMSCs on microtextured surfaces in OM. Blank microtextured substrates without hMSCs showed minimal cell attachment on the surface (c) and exhibited neither human nor mouse markers (a,b). hMSCs on microtextured surfaces in BM (d,e) and OM (g,h) revealed elevated signals of human mitochondria and mouse PECAM compared to the negative control blank microtextured substrates without hMSCs (a,b), which confirmed the presence of hMSCs on the microtextured surfaces and identified mouse blood vessels attracted to the scaffolds.

\textbf{Figure A.13} Alkaline phosphatase levels were determined in harvested scaffolds after a 6-week implantation. hMSCs on microtextured surfaces in basal medium (BM) and osteogenic medium (OM) demonstrated bone matrix mineralization characterized by an increased level of alkaline phosphatase activity \textit{in vivo}. (n=3, **p<0.005, *p<0.05).
Figure A.14 Alizarin red staining of harvested scaffolds after a 6-week implantation. (a,d) microtextured substrates without hMSCs. (b,e) hMSCs on microtextured surfaces in BM. (c,f) hMSCs on microtextured surfaces in OM. The microtextured substrates showed minimal calcium deposition on top of the substrate surfaces. hMSCs on microtextured surfaces in BM (b,e) and OM (c,f) showed significant amount of calcium deposition characterized by the amount of red staining, indicating the successful differentiation of hMSCs toward osteogenic lineage.

Figure A.15 Cross-sectional images of HE staining for harvested scaffolds after a 6-week implantation. (a) microtextured substrates without hMSCs. (b) hMSCs on microtextured substrates in BM. (c-e) hMSCs on microtextured substrates in OM. Microtextured substrates were shredded into PDMS pieces during HE sectioning (a). No stained tissue debris detected for microtextured substrates (a). hMSCs on microtextured substrates in BM (b) showed densely nucleated epithelial layers surrounding the substrates, whereas hMSCs on microtextured substrates in OM (c-e) exhibited very loose tissues accompanied by extensive blood vessels (black arrows). Blood (red arrows) was also observed in the vascular lumen for hMSCs on microtextured substrates in OM (c-e).

Figure A.16 Cross-sectional HE staining of harvest scaffolds after a 6-week implantation. (a) hMSCs on microtextured substrates in BM. (b) hMSCs on microtextured substrates in OM. hMSCs on microtextured substrates in BM (a) exhibited dense epithelial-like tissue surrounding the scaffolds, while
hMSCs on microtextured substrates in OM (b) showed loose tissue structure and blood vessels.
CHAPTER I

MACROENCAPSULATION FOR TYPE 1 DIABETES (T1D) TREATMENT: CELLS, BIOMATERIALS, AND DEVICES

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1.1 INTRODUCTION

Type 1 diabetes (T1D) mellitus results from autoimmune destruction of insulin-producing β cells in the islets of Langerhans of the endocrine pancreas, causing reduction in β cell mass and dysfunction. Of the more than 366 million people worldwide affected by diabetes today, it is estimated that as many as 40 million patients have T1D\textsuperscript{16}. The global incidence of T1D doubles approximately every 20 years\textsuperscript{17,18}, increasing up to 5\% per year\textsuperscript{19}.

As the prevalence of T1D increases worldwide, the associated chronic complications are the main cause of morbidity and mortality, which adversely affect the quality of T1D patients’ lives\textsuperscript{20}. Specifically, complications of diabetes have been classified as either microvascular (e.g. retinopathy, nephropathy, and neuropathy) or macrovascular (e.g. cardiovascular disease and peripheral vascular disease)\textsuperscript{21,22}. Macrovascular complications in T1D show significant morbidity and mortality in comparison to individuals with Type 2 diabetes. For T1D patients under age 40, the onset of macrovascular complications occur much earlier in life, exacerbate throughout the course of disease, and result in a higher mortality compared to the general population\textsuperscript{22}. The total estimated financial burden for T1D is $14.9 billion in health care costs in the U.S. each year, including medical costs of $10.5 billion and indirect costs of $4.4 billion\textsuperscript{23}. 

1
The economic burden per case of diabetes is greater for T1D than type 2 diabetes and the difference increases with age\textsuperscript{23}. This trend will only continue given the escalation in global incidence and worsen as the T1D population ages and disease progresses, especially for patients in low-resource settings.

1.1.1 CURRENT TREATMENT METHODS

There are currently two dominant paradigms associated with the treatment of T1D: insulin infusion therapy and whole organ transplantation.

1.1.1.1 Insulin Infusion

Insulin therapy is administered with multiple daily injections or subcutaneous infusion using an insulin pump\textsuperscript{24-26}. To survive, T1D patients must measure their blood glucose levels and administer insulin in response to those glucose levels multiple times per day for the rest of their lives. Even in the most compliant patients, tight glucose control is difficult to maintain. For example, patients must calculate insulin dose at mealtimes by taking in account of several factors, such as blood glucose levels, insulin/carbohydrate ratio, carbohydrate intake, intensity of physical exercise after injection, and individual insulin sensitivity. Any small miscalculation can result in episodes of hypo- and hyperglycemia, causing life-threatening conditions. These dangerous fluctuations in glucose levels are the primary cause of diabetic complications\textsuperscript{25,27}. Hypoglycemia can result in cognitive impairment, unconsciousness, seizures, and death\textsuperscript{27}. Hyperglycemia leads to similarly devastating complications, such as kidney failure, heart attack, stroke, blindness, nerve damage, and many other diseases\textsuperscript{28}. The elevated levels of glucose may induce glycation of various structural and functional proteins that leads to advanced glycation end products (AGES), which are thought to be the major causes of different diabetic complications\textsuperscript{29}. 
Although use of insulin injections and insulin pumps are life-prolonging technologies, they do not mimic real-time secretory patterns of pancreatic β cells nor do they prevent long-term complications\textsuperscript{30,31}. Medtronic has recently designed a new algorithm, Predictive Low Glucose Management (PLGM), which automatically stops the delivery of insulin when a sensor detects a predetermined low glucose level\textsuperscript{32}. However, designing algorithms to make therapeutic decisions with accurate and instantaneous regulation of blood sugar level with minimal human input remains a challenge\textsuperscript{33}.

1.1.1.2 Pancreas Transplantation

Whole pancreas transplantation presents an alternative intervention for T1D by re-establishing normoglycemia without the excessive need for insulin therapy. From 2004 to 2008, the most common pancreas transplant category was a combined pancreas/kidney transplant (SPK) (~73%) where immunosuppressives were used for both transplants, followed by a kidney transplant before undergoing a pancreas transplant (PAK) (~18%), and pancreas transplants alone (PTA) (~9%)\textsuperscript{34}. Prior to 2000, PTA and PAK transplant categories had experienced more graft loss comparing with SPK\textsuperscript{35,36}. Graft loss happens frequently in PTA and PAK patients because these patients do not suffer from the uremia associated with renal failure, hence their healthy platelet function places them at a higher risk for thrombosis in the low-flow state of the pancreas graft\textsuperscript{37,38}. Treatment with anti-coagulant agents like heparin and dipyridamole are required during the perioperative period to decrease the likelihood of graft thrombosis, but they increase bleeding risk\textsuperscript{39}. With advances in immunosuppression since 2000, the use of antibody induction and steroid avoidance-based maintenance protocols (Tacrolimus/Mycophenolate Mofetil or Sirolimus) in all transplant categories has been shown to improve the outcome of pancreas transplantation extensively, as illustrated by the pancreas graft survival rates of 85%
and 52% in PTA, 81% and 55% in PAK, and 87% and 72% in SPK at one year and five years, respectively.

Although pancreas transplantation can achieve insulin-independence with a greater than 80% graft survival rates in all categories after one year, this approach has many drawbacks. First, whole organ transplantation is constrained by the number of donors. Of 8,000 available donors in the US, just around 1,400 donors (16%) are potentially suitable for whole organ transplantation annually. Second, T1D patients with accelerated course of cardiovascular complications are not recommended for such a complex operation due to their underlying cardiovascular disease and increased risk of perioperative complications. The combination of perioperative cardiac risk coupled with surgical complications places whole pancreas transplantation as the procedure with highest morbidity among all routinely performed abdominal solid organ transplantation surgeries.

1.1.2 ALTERNATIVE TREATMENT METHODS

1.1.2.1 Cell Therapy

Intraportal alloislet transplantation, as described by The Edmonton Protocol, has shown promise in becoming a viable T1D treatment after demonstrating that a cohort of seven patients remained insulin-independent with an average of 12 months under steroid-free immunosuppressive drugs during the last decade. The Clinical Islet Transplantation Consortium (CIT) created by the US National Institute of Diabetes & Digestive & Kidney Diseases (NIDDK) and the US National Institute of Allergy and Infectious Diseases (NIAID) is a network consisting of 13 clinical centers to conduct studies of islet transplantation in T1D patients to improve the safety and long-term success of intraportal islet transplantation. The CIT initiated two Phase III clinical trials to demonstrate that islet transplantation could improve glycemic control in T1D
patients with severe hypoglycemia and extreme glycemic lability (CIT-07; NCT00434811) and in T1D patients who had received a kidney transplant (CIT-06; NCT00468117). The CIT-07 protocol markedly improved the beta-cell mass and secretory capacity compared to the Edmonton protocol\textsuperscript{47}. Islet graft function and insulin independence were achieved for 94\% and 52.1\% of all participants a year after the first islet transplant\textsuperscript{48}. The CIT-07 protocol showed a favorable safety profile and patients experienced substantially reduced insulin use and glycemic lability. Human islet product release can be prepared at multiple manufacturing centers using this standardized protocol. The current islet transplant procedure involves catheter delivery of islets into the liver via the hepatic portal vein under radiological guidance. Once the catheter is in place, a suspension containing islet tissue is infused. Unlike whole organ transplant, the clinical procedure of islet transplant is less invasive and patients require minimal time to recover. The main advantage of using the portal vein is that it allows rapid delivery of insulin to the hepatic portal circulation in response to post-prandial glucose delivery to portal vein from intestine\textsuperscript{49}. Other benefits of islet transplantation include reduced need for exogenous insulin administration among recipients, improved blood glucose control, and greatly reduced risk of severe hypoglycemic episodes\textsuperscript{50}.

Islet transplantation based on the Edmonton Protocol possesses limitations. A long-term follow up revealed that only 10\% of patients were free from exogenous insulin use after five years\textsuperscript{51}. This technology also faces the donor shortage problem. Islet transplantation requires infusion of high-quality islets isolated from a total of two to four donors to treat one recipient (Markmann et al. 2003). Multiple donors are needed because islet attrition occurs during the islet isolation process where uncontrollable factors such as donor’s body mass index, pancreas size, chemical digestion, and pancreatic surface integrity could all damage islet quality\textsuperscript{52}. This could
also happen during the post transplantation where 50% to 70% of islets die due to the immediate hypoxia and inflammatory response\textsuperscript{53}. Similar to whole pancreas transplants, islet transplants require chronic immunosuppression to ensure long-term performance of the grafts\textsuperscript{54}. Detrimental side effects of chronic high-dose immunosuppressive regimens can also lead to nephrotoxicity and kidney dysfunction in T1D patients who are already at heightened risk\textsuperscript{55,56}. The high metabolite concentrations in the liver may cause graft failure over time\textsuperscript{57}. Hence, the applicability of this type of procedure is still greatly constrained by the limited supply of human donor tissue, graft damage from long-term usage of immunosuppressive regimens, and inadequate implantation sites that result in graft failure from hypoxia.

\textbf{1.1.2.2 Insulin Therapy via Cell Encapsulation}

Given the aforementioned deficiencies associated with current transplantation methods in T1D treatment, researchers need to tackle the challenge of immunosuppressives, along with the issues of donor shortage and physiological mimicry of a functional pancreas. An attractive strategy for the development of a bioartificial pancreas that would eliminate the need for immunosuppressives is encapsulation of insulin-producing islets within a semipermeable membrane. The properties of the suitable semipermeable membrane are such that it protects islets from the host’s immune system, while allowing the exchange of nutrients and small molecules (including glucose and insulin) between the encapsulated islets and their external environment\textsuperscript{58}. Successful immunoisolation would potentially allow cells from xenogeneic and stem cell sources to be used as alternatives to standard human pancreatic islets or β cells, thereby significantly easing the donor shortage problem. Stem-derived cell sources or xenotransplantation are, however, highly challenging with respect to safety and immunologic
perspectives. The implantable bioartificial pancreas would mimic physiological responses by functioning autonomously and dynamically to the varying state of the human body.

To date, existing semipermeable membranes can be categorized into ultra-thin coatings, microcapsules, and macrocapsules based on differences in diffusion distance (Fig. 1.1, a). Ultra-thin coatings using conformal or Layer-by-Layer (LbL) assembly directly modify the surface of islets to enhance transport and mechanical properties\textsuperscript{59-63}. The conformal coatings aim to cover each islet with a uniform thickness rather than controlling the overall capsule diameter like the microencapsulation. These techniques create polyelectrolyte multilayer thin films based on

\textbf{Figure 1.1} An overview of various encapsulation methods to immunoisolation. Macro-scale encapsulation include extravascular (a), or vascular perfusion (intravascular) devices (b) which are perfused with body fluid or blood. Micro-scale encapsulation devices typically group a few number of cells into microcapsules (a). Nano-scale encapsulation directly coats surface of the islets with polymeric layers (e.g. conformal coating) (a).
sequential adsorption of oppositely charged components. However, cytotoxic byproducts released during the manufacturing process could disrupt the integrity of cell membrane, causing cell death\textsuperscript{64-67}. Microencapsulated islets ranging 400-800 μm in diameter rely on diffusive nutrient transport and require minor surgery for implantation in the peritoneal cavity, subcutaneously, or under the renal capsule due to their relatively small size\textsuperscript{68}. In particular, Calafiore's Minimal Volume Capsules which are small alginate micro-capsules with 300-400 μm in diameter were implanted intraperitoneally in patients under echography guidance and local anesthesia and showed clinical relevance with reduced exogenous insulin requirements\textsuperscript{69-71}. The large surface area to volume ratio is advantageous for mass transport in microcapsules; however, limitations of this technology include the need for a large transplantation site that accommodates the necessary number of capsules, a favorable microvascular bed that provides immediate nutrient access, difficulty in microcapsule removal if required, and insufficient long-term survival rates for functional islets to adequately address the daily insulin requirement\textsuperscript{72-75}. Though notable applications of microcapsules have been attempted in large animals\textsuperscript{76,77} and human subjects\textsuperscript{78-83}, the challenges such as cell sources, implant location, mass transfer, and vascularization remain unsolved.

Unlike microencapsulation where only few islets are grouped together, the large capsule size of the macroencapsulation strategy requires assembling a greater number of islets, and hence, it imposes an even greater mass transport limitations and challenges conventional sites such as renal capsules for implantation. Specifically, vascular perfusion macrocapsules may cause life-threatening blood coagulation and thrombosis in vascular surgery and surface-induced thrombosis\textsuperscript{84}. Nonetheless, macrocapsules have successfully demonstrated the feasibility of implantation and retrieval\textsuperscript{85,86}. While far from a comprehensive review of islet encapsulation,
Table 1.1 summarizes some notable macroencapsulation methods to restore normoglycemia in animals and human.

**Table 1.1** Macroencapsulation used in animals and human.

<table>
<thead>
<tr>
<th>Macro-encapsulation</th>
<th>Type of transplant</th>
<th>Device Description</th>
<th>Location</th>
<th>No. of Encapsulated Islets (IEQ)</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extravascular</td>
<td>Human to mice (xeno)</td>
<td>TheraCyte (PTFE) device with an inner semipermeable membrane (pore size of 0.4 µm) laminated to an outer membrane and covered by a loose polyester mesh. Device is 2-cm long with inner lumen volume of 4.5 µL</td>
<td>Intraperitoneal</td>
<td>70-216</td>
<td>C-peptide and responsiveness to glucose changes were not observed in the first 12 weeks of transplantation but were detected after 5 months(^7)</td>
</tr>
<tr>
<td></td>
<td>Human to mice (xeno)</td>
<td>Nanogland (silicon membrane: 6 mm × 6 mm) with channel sizes: 3.6, 5.7, 13, 20 and 40 nm nanochannels and 20, 40, and 60 µm microchannels. The membrane has a circular pattern of 161 square islets chambers (200 µm width by 200 µm height)</td>
<td>Subcutaneous</td>
<td>1000</td>
<td>Detectable insulin production for at least 120 days(^2)</td>
</tr>
<tr>
<td>Source of Organ</td>
<td>Description</td>
<td>Site</td>
<td>Duration</td>
<td>Remarks</td>
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<tr>
<td>Rat to rat (allo)</td>
<td>Meshed PVA tubings</td>
<td>Peritoneal</td>
<td>2500-3000</td>
<td>Normoglycemia was maintained for 1 month&lt;sup&gt;88&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Dog, cow, pig to rat (xeno)</td>
<td>Tubular, permselective acrylic-copolymer membrane chambers (2-3 cm long, 1.8-4.8 mm in inner diameter, 69-105 µm in wall thickness)</td>
<td>Intraperitoneal</td>
<td>4-6/mm³ or 20,000</td>
<td>Insulin independence for an average of 138+/−16 days, and two animals showed 260 days with higher dose&lt;sup&gt;89&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Pig to rat (xeno)</td>
<td>Agarose macrobeads</td>
<td>Intraperitoneal</td>
<td>1000-1500</td>
<td>Macrobeads remained functional for 199+ days&lt;sup&gt;9&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Human to rats (xeno)</td>
<td>Alginate sheets</td>
<td>Subcutaneous</td>
<td>1000</td>
<td>Encapsulated islets were viable and functional after 8 weeks&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Dog to dog (allo)</td>
<td>Wider-bore tubular membrane made of acrylic-copolymer with a 1.7-4.8 mm in inner diameter and a wall thickness of 69-105 µm</td>
<td>Peritoneal</td>
<td>300,000</td>
<td>70 days insulin free&lt;sup&gt;90&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Extravascular</td>
<td>Pig to monkey (xeno)</td>
<td>A monolayer of islet-seeded human acellular collagen matrix covered in alginate</td>
<td>Subcutaneous</td>
<td>30,000/kg</td>
<td>Diabetes was corrected for a maximum of 6 months in 5 animals$^{91}$</td>
</tr>
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<tr>
<td>Human to human (allo)</td>
<td>Acrylic-copolymer hollow fiber (1.5-cm length, 800 µm in inner diameter, and 100 µm in wall thickness)</td>
<td>Subcutaneous</td>
<td>150-200</td>
<td>14 days with 90% viability$^{92}$</td>
<td></td>
</tr>
<tr>
<td>Intravascular</td>
<td>Rat to rat (allo)</td>
<td>Hollow-fiber setup with 100 11-cm capillary fibers of polyacrylonitrile-e-PVC copolymer sealed into cylindrical glass with 0.5 cm inner diameter. Islets were seeded at the outside surface of the fiber bundles</td>
<td>Silastic iliac</td>
<td>180 neonatal rats</td>
<td>90-min decrease in plasma blood glucose$^{93}$</td>
</tr>
<tr>
<td>Dog, cow, pig to dog (allo &amp; xeno)</td>
<td>A single-coiled tubular membrane made of acrylic-copolymer. The coil membrane contained disk-shaped acrylic islet housing. The membrane has a normal molecular</td>
<td>External iliac artery and common iliac vein</td>
<td>220,000-320,000</td>
<td>Zero or minimal insulin required for ~50 weeks$^{94-96}$</td>
<td>Reduced exogenous insulin after 284 days and 106 days$^{5}$</td>
</tr>
<tr>
<td>Intravascular</td>
<td>Rabbit to dog (xeno)</td>
<td>Polyamide or nylon capsules with 1-2 mm pore dimension, 20 mm in length, and 5 mm in diameter</td>
<td>Abdominal Aortic lumen</td>
<td>500,000</td>
<td>Minimal insulin required for 57-366 days$^{97}$</td>
</tr>
<tr>
<td>Rabbit to human (xeno)</td>
<td>Nylon macrocapsule with 1-2 µm in pore size, 30-40 mm in length, and 3-4 mm in diameter</td>
<td>Deep femoral artery or forearm cubital vein</td>
<td>6000/kg</td>
<td>2 years with reduced insulin requirement for 73.7% recipients$^{100}$</td>
<td></td>
</tr>
</tbody>
</table>

The goal of this chapter is to present the development of macroencapsulation devices with an emphasis on its challenges and limitations, progress in generating alternative sources for pancreatic β cells, and the variety of biomaterials used for cell encapsulation. With the great
potential of a bioartificial pancreas in mind, a successful macroencapsulation device will depend on the availability of cell sources, the physical and chemical properties of the underlying semipermeable membranes, and the mass transport between cells and their outside environment.

1.2 MACROENCAPSULATION OF ISLETS

The early development of macroencapsulation dated from the 1950s (Fig. 1.2). Macroencapsulation devices may possess different geometries such as hollow fibers\textsuperscript{5,101}, bag-like structures\textsuperscript{11,102,103}, polymeric hydrogel sheets\textsuperscript{4,104}, or planar membranes\textsuperscript{2} for high flow rate or reduced surface area. They can be categorized as either extravascular or vascular perfusion based on their mechanism of transport.

\textbf{Figure 1.2} A schematic of the U-shaped bioartificial pancreas\textsuperscript{6-8}. The U-shaped bioartificial pancreas consisted of islets (I) that were placed between two flat polyacrylonitrile (PAN) ultrafiltration membranes (M) separated by a gasket (G). Blood (O) circulated successively above the upper and below the lower membranes following a U-shaped circuit (L) surrounding the islet compartment. Image reproduced with copyright permission.
Extravascular Macrocapsules

Prior to 1980s

In the 1950s, Algire and co-workers transplanted cells enclosed in Millipore diffusion chambers made of cellulose esters into mice to study the mechanisms of cellular immune rejection\textsuperscript{105-107}. This approach initiated vast interest in using planar diffusion devices consisting of parallel, flat-sheet microporous membranes with different materials (e.g. polycarbonate, acrylic copolymer) to study islet transplantation in small animals. Throughout the 1970s and early 1980s, studies showed that only partial or transient normoglycemia was achieved in some studies, but islet allografts and xenografts in rodents were severely compromised as a result of fibrous overgrowth of the extracapillary-situated chambers\textsuperscript{108-115}. Using diffusion chambers made of polytetrafluoroethylene (PTFE), Brauker and co-workers showed that transplanted tissues survived in an allogeneic model for up to a year, whereas xenogeneic tissues died within a few weeks even when implanted with intact membranes\textsuperscript{103}. This concluded that the inner PTFE membrane with 0.4-μm pore size was effective in preventing allogeneic immune response. The outer PTFE membrane with 5-μm pore size utilized the foreign-body reaction to enable the implant to be vascularized\textsuperscript{103}.

From 1980s to early 1990s

Attention soon shifted from planar chambers to hollow fibers as the latter elicited a smaller foreign body response due to its geometry\textsuperscript{116}. Experiments and mathematical models designed to understand how tissue viability varies with diffusion distance suggested that oxygen concentration as well as time response to insulin decreased radially within cylindrical or spherical devices\textsuperscript{117-120}. The time for diffusion (t) is characterized by $R^2/D$, where R is the radius of the sphere or cylinder and D is the diffusivity of the solute through the encapsulated
membrane. Although an increase in diameter can result in shorter overall length of the hollow fiber, diffusion would be greatly impeded. Lack of oxygen transport and the accumulation of waste products would therefore lead to necrosis in the central core of cell-encapsulated fibers. In contrast, a small fiber diameter, while improving the transport properties, requires a much longer fiber to contain the same quantity of cells. Longer fibers are mechanically fragile and increases the likelihood of leakage while making implantation of procedure more difficult. Hence, hollow fibers, because of their shape, tend to break when forced to bend under physiological stress.

Rupture of the membrane wall will cause immediate rejection of the implanted islet tissue and loss of function.

Among hollow fiber devices, narrow-bore tubes with an inner diameter of less than 1 mm were assessed for their potential in restoring normoglycemia in diabetic rodents when implanted intraperitoneally. Histologic analysis showed an intense fibrous tissue layer surrounding the membranes. When implanted in pigs, a similar tissue reaction was observed with a greater intensity of several layers of fibroblasts, collagen, macrophages, leukocytes, and other immune cells. Further studies with subcutaneous transplants of 150-200 human islet equivalent (IEQ) using hollow fibers of 800 μm in inner diameter into T1D and T2D patients showed greater than 90% islet viability after two weeks.

Wide-bore hollow fibers made of polyacrylonitrile-polyvinyl chloride (PAN-PVC) with 1-5 mm inner diameters showed minimal reaction with nearby tissues. Semipermeable hollow fiber membranes that were 2-3 cm in length, 1.8-4.8 mm in diameter, 69-105 μm in wall thickness, and nominal molecular weight cutoff of 50,000-80,000 Da have been used in rats and dogs, employing a variety of sources for the islets. After peritoneal implantation of 9-50 fibers with 20,000 IEQ enclosed in each rat, normoglycemia was maintained in some cases for one
year, whereas in others, membrane breakage occurred as a result of the thin membrane wall\textsuperscript{89}. To further test these hollow fibers in a large animal model, 155-248 fibers containing 300,000 IEQ were peritoneally implanted in pancreatectomized dogs\textsuperscript{130}. Two dogs were insulin-independent for at least 70 days after receiving islet allografts within hollow fibers. However, cell death or dysfunction frequently occurred in wide-bore membrane chambers due to oxygen supply limitations and accumulation of wastes. The passive diffusion is predictably less efficient than transport possessed in normal, vascularized islets\textsuperscript{131,132}. Because hypoxic conditions can occur for cells that are further than 150-200 μm away from the nearest blood vessels\textsuperscript{133}, membranes with larger diameter ranging 4.5-4.8 mm indeed exhibited necrotic islet core due to oxygen limitations, while viable islets were only observed within 0.5-1 mm layer along the membrane wall\textsuperscript{89}.

**Intravascular Macrocapsules**

**During 1970s**

Reach and co-workers also confirmed that blood ultrafiltrate generated \textit{in vivo} from normal rats could support the normal islet function\textsuperscript{134}. More importantly, the advantage of devices based on ultrafiltration is the ability to respond to minute changes in blood glucose concentration, thereby achieving a physiological feedback control system of glucose-stimulated insulin secretion. Pillarella and Zydney discovered that convective recirculation flow could dramatically improve insulin response by accounting the following factors: (1) diffusion and convection of glucose and insulin in both the axial and radial directions, (2) the time-dependent insulin secretion kinetics, and (3) the spatial distribution of islets in the peripheral compartment based on modeling of the flow transport in lumen, matrix, and shell\textsuperscript{135}. This prediction was further validated by Ramirez and co-workers that the solute radial convection and islet spatial distribution could have a profound impact on insulin response\textsuperscript{136}.
In 1980s

During 1980s, numerous studies with implanted ex vivo and in vivo hollow-fiber devices showed islet immunoisolation, and restoration and maintenance of normoglycemia for short periods of time in induced diabetic rats, dogs, and monkeys\textsuperscript{93,137,138}. Important parameters influencing the dynamic response of intravascular devices depend on many factors such as whether the device is AV or arterio-arterio (AA) connected, diffusion distance in the islet compartment, membrane matrix thickness, and flow rate of the circulating medium\textsuperscript{139-142}. Reach and co-workers attempted to use ultrafiltration-induced convective transport to improve the dynamic response of the intravascular hollow-fiber device\textsuperscript{143}. Their results showed that ultrafiltration flux was proportional to the flow rate of the medium circulating through the fiber\textsuperscript{144}. They further concluded that the original islet extra-capillary volume of 2.6 ml had to be reduced to smaller than 0.2 ml in order for this type of hollow fiber systems to work. Hence, a new type of ultrafiltration device was designed as a U-shaped bioartificial pancreas (Fig. 1.2), which consists of two flat membranes with blood circulation first above the upper membrane and then below the bottom membranes in the reverse direction\textsuperscript{6,7}. Ultrafiltration-reabsorption flux was generated directly perpendicular to the surface of the semipermeable membrane. The U-shaped design reduced the volume of the islet compartment to 0.15 ml, 17 times smaller compared to the hollow fiber design. It accelerated insulin secretion in response to glucose and shortened the lag time (<5 min) irrespective of the patterns of glucose stimulus (square-wave or progressive rise)\textsuperscript{7}. Additional tests in normal rats and dogs demonstrated that U-shaped design provided acute increase of secreted insulin in response to glucose\textsuperscript{7,145,146}. 
1.2.1 EXTRAVASCULAR MACROCAPSULES

The concept of extravascular macrocapsules is based on the principle of diffusive transport. Because extravascular devices do not require vascular anastomoses, the corresponding surgical risks are much lower than vascular perfusion devices.

1.2.1.1 Hydrogels

During the past two decades, there has been a growing interest in using hydrogels as a means to achieve greater biocompatibility for macroencapsulation. Due to the hydrophilic nature of the material, almost no interfacial tension is created with surrounding fluids and tissues, hence, minimizing protein adsorption and cell adhesion. Furthermore, the mechanical properties of hydrogels can be easily controlled via crosslinking to obtain desired selectivity and permeability, allowing the passage of low molecular weight nutrients and metabolites entering the encased cells.

AN69, a copolymer of acrylonitrile and sodium methallyl sulphonate, was one of the early hydrogels studied as a macroencapsulation material. Early studies reported that AN69 induced only minimal fibrosis in the peritoneal cavity of rats with low permeability for insulin. Application of corona discharge on AN69 caused a more hydrophobic surface with less molecular adhesion. This approach improved both the insulin permeability and the long-term biocompatibility. Poly(vinyl alcohol) (PVA) hydrogel macrocapsules reinforced with mechanically strong support also restored normoglycemia a month after implantation into the abdominal cavity of diabetic rats. Other supportive results from Jain and co-workers demonstrated that macrobeads made of agarose contained functional porcine islets for almost 200 days after intraperitoneal transplantation into rats.
One of the most serious problems associated with macroencapsulated hydrogels is the loss of viability of the transplanted islets due to central necrosis of the tissue clusters. The islet aggregation has been reported to cause hypoxia and result in gradual loss of tissue viability within 1-2 weeks\textsuperscript{152}. To prevent undesired clustering, islets are usually immobilized in gel matrices prior to encapsulation, as demonstrated by previous studies that cell-matrix interaction could also enhance islet viability\textsuperscript{104}.

**Figure 1.3** A number of agarose macrobeads retrieved from the peritoneal cavity of a diabetic rat 145 days after implantation\textsuperscript{9}. None of the macrobeads showed any fibrosis but small eruptions were detected on the wall of the peritoneal cavity (indicated by black arrow). Image reproduced with copyright permission.

1.2.1.2 Sheets and Pouches

Advances in technology and knowledge from studies conducted in the 80s and 90s have pushed the development of extravascular macroencapsulation forward. Islet sheets, developed by Hanuman Medical Foundation, are composed of highly purified alginate (12-45 kDa) with a reinforcing mesh that encapsulates islets (Fig. 1.4)\textsuperscript{4}. Lamb and co-workers reported that alginate encapsulated human islets remained both viable and functional after 8 weeks in culture and in the
subcutaneous space of rats\textsuperscript{3}. The team has since moved to large-animal efficacy studies using a canine model. Dufrane and co-workers developed a similar system where porcine islets were seeded on a human acellular collagen matrix to create a cell monolayer before covering with high mannnuronic acid alginate\textsuperscript{91}. Alginate macrocapsules that were then transplanted into abdominal subcutaneous tissue of cynomolgus monkey showed metabolic control over the glucose course with an acute stimulation\textsuperscript{91}. Encapsulated adult pig islets corrected streptozotocin-induced diabetes up to a maximum of 6 months in five animals, in spite of the strong humoral response that was elicited\textsuperscript{91}. The authors concluded that failure at 6 months was possibly due to the lifespan of adult pig islets\textsuperscript{91}.

Sernova’s Cell Pouch\textsuperscript{TM}, made from FDA approved materials, consists of a multi-channel sheet inserted with an array of rods (Fig. 1.5)\textsuperscript{11}. The pouch creates a favorable pre-vascularized environment but it does not offer an immune-barrier to protect cells from the host immune

\textbf{Figure 1.4} Diagrams of an Islet Sheet (a) and its cross-sectional view (b)\textsuperscript{3,4}. Islets-encapsulated alginate is enclosed between acellular immunoprotective alginate layers. A polymer mesh can be included in the sandwiched layer to provide physical strength. Islet Sheets measure approximately 4 cm X 8 cm X 250 μm. Molecular weight cutoff depends on the modification of the alginate chemistry and its processing conditions. Images by courtesy of Dr. Scott King and reproduced with copyright permission.
This device is first placed under the skin for better vascular integration with the surrounding tissues for a month. Once microvasculature is developed around the device, the rods are removed to expose channels that allow the infusion of transplanted islets into the device. Sernova devices were pre-implanted in mice four weeks before diabetes induction and transplantation. The implanted mouse islets restored glycemic control and maintained normoglycemia until graft explantation after 100 days. Although there was a modest delay, the implants responded well to glucose challenge and islets within the cell pouch stained positively for insulin, glucagon, and microvessels. Glycemic control by cell pouch encapsulated islets was also demonstrated in pigs for two months, along with high infiltration of blood vessels to promote survival of encapsulated islet autografts. Currently, this device has moved to a three-year Phase I/II clinical study with human patients with standard immunosuppressive regimes from the Edmonton Protocol.

Figure 1.5 Diagrams of Sernova cell pouch™ system. The pouch consisted of a multi-channel sheet inserted with an array of rods. It was first implanted under the skin for vascular integration, followed by removal of the rods (a). The cell-containing tubing could then inject islets into pre-formed cavities (b). Images by courtesy of Dr. Philip Toleikis from Sernova.
The TheraCyte device (Fig. 1.6) produced by Baxter Healthcare is an example of pouch encapsulation that aims to promote angiogenesis and immunoisolation\textsuperscript{103}. Theracyte is a planar pouch featuring a double membrane of polytetrafluoroethylene (PTFE) with its inner 30-µm thick membrane of 0.4 µm in pore size for selectivity and immunoisolation, and its outer 15-µm thick membrane of 5 µm in pore size for angiogenesis through an alternative foreign body response\textsuperscript{1,2}. Undoubtedly, enhancing microvasculature plays an essential role in promoting survival of macroencapsulated islets\textsuperscript{133}. The outer membrane consisted of 5-µm pores showed a 80 to 100-fold more vascular structure with much improved biocompatibility compared with the 0.02-µm pores\textsuperscript{103}. It is also possible to integrate angiogenic factors to further enhance vascularization of the device. For example, the infusion of VEGF greatly enhanced angiogenesis by increasing the number of surrounding blood vessels and promoting insulin diffusion from the TheraCyte device\textsuperscript{2}. Lee and co-workers reported that transplant of TheraCyte with human fetal pancreatic islet-like cell clusters led to maturation of β-cells and correction of diabetes for at

\textbf{Figure 1.6} Diagram of TheraCyte devices\textsuperscript{1,2}. The device was a planar pouch made of bilaminar polytetrafluoroethylene (PTFE) with its inner membrane for immunoisolation, and the outer membrane for tissue integration. The inner membrane was 30 µm in thickness with 0.4 µm in pore size. The outer membrane was 15 µm in thickness with 5 µm in pore size. Image by courtesy of TheraCyte, Inc.
least 10 days in immunodeficient mice\textsuperscript{1}. ViaCyte has developed an encapsulation system called Enkaptra that is designed to be implanted subcutaneously. This device (based on the TheraCyte) is also a planar pouch with a single layer of membrane which will protect transplanted cells from host’s immune system while allowing oxygen and nutrients freely transport through the membrane. This system will allow the encapsulation of stem cells or pancreatic progenitor cells to differentiate into functioning insulin-producing cells\textsuperscript{153,154}. ViaCyte has announced a Phase I/II clinical trial using Encaptra with stem cell-derived cell sources to assess safety and efficacy of the system with an expected completion in 2017.

Beta-O\textsubscript{2} is an implantable device composed of a central gas cavity that connects to access ports for refueling oxygen from the outside (Fig. 1.7)\textsuperscript{14}. Surrounding the central gas cavity are

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{beta-o2-device.png}
\caption{A schematic of the Beta O\textsubscript{2} device\textsuperscript{14}. The center of the device was built as an oxygen chamber, connected to access ports for exogenous oxygen refueling. The oxygen chamber was sandwiches between two alginate-immobilized islets layers separated by gas permeable silicon membranes. The external surfaces were coved by hydrophylized polytetrafluoroethylene (PTFE) membranes of 0.4 \textmu m in pore size. Image reproduced with copyright permission.}
\end{figure}
the gas permeable membranes that house alginate-immobilized pancreatic islets with 0.4 µm PTFE porous membranes covering the external surfaces. After implantation of the device in the abdomen, oxygen is pumped daily to support islet viability. Results showed that rat islets corrected glucose levels in diabetic mini-pigs for 90 days with no signs of islet disintegration. One human case study using this device reported that encapsulated islets were functional over the entire period of 10 months with moderate improvement in reduction of insulin requirement.

Although remarkable progress has been made in autologous and allogeneic cell transplantation without immunosuppression, overcoming immunologic rejection of xenogeneic cells still remains as a great obstacle due to the mechanical rupture of the membranes, biochemical instability, islet cell heterogeneity, and the broad distribution of pore sizes in the encapsulation materials. It is known that cross-linking macrocapsules does not provide precise control over molecular weight cutoff, so antibodies and cytokines cannot be sufficiently excluded in many hydrogel macrocapsules. For example, large PEG microbeads that were manufactured with poor porosity exhibited hindered molecular transport. These issues have brought interest from scientists and engineers to apply a more controlled, fabrication technology, associated with the production of Micro-Electro-Mechanical Systems (MEMS), to produce biocompatible materials with features in the range of micro- and nanometers for biomedical applications. Desai and co-workers utilized bulk and surface micro-machining to produce biocapsules with uniform and well-controlled pore sizes, channel lengths, and surface properties. The nanoporous biocapsule consisted of two permeable silicon membranes with specific pore sizes, which can be as small as 7nm (Fig. 1.8). They reported that upon transplantation in peritoneal cavity, encapsulated insulinoma cells reversed
diabetes in rats for at least 14 days using this nanoporous biocapsule\textsuperscript{13}. Capsules made of 20-nm barriers maintained secretory output whereas 66-nm capsules led to loss of cell function. They also developed nanoporous alumina capsules with nominal pore size of 75 nm where diffusion of glucose was undisturbed but the transport of immunoglobulin G (IgG) was impeded\textsuperscript{182,183}. Nanoporous alumina encapsulated MIN6 cells secreted insulin with dosage-dependent response\textsuperscript{182}. Nanogland, another device produced using MEMS technology, consists of parallel nanochannels and perpendicular microchannels to the islet chamber (Fig. 1.9)\textsuperscript{12}. The membranes present a 6 mm by 6 mm surface area with channel size ranges from 3.6-40 nm for nanochannels and 20-60 μm for microchannels. All membranes have a circular pattern of 161 square islet chambers (200 μm x 200 μm; W x H) separated from each other by 50 μm walls. Subcutaneous implantation of the Nanogland with human islets in mice showed survival of implants over 120 days with endothelial cell infiltration, suggesting potential vascularization of the device\textsuperscript{12}. These

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{nanoporous_biocapsule.png}
\caption{An image of a nanoporous biocapsule\textsuperscript{13}. The nanoporous biocapsule consisted of two nanoporous silicon membranes, gaskets, and protective screens on both side of the device. The injection ports allow the sampling and replenishing of the islets (a). The dimension of the nanoporous silicon membranes, including the support ridge, was 6 X 8 mm. The active membrane area was 3.5 X 2 mm with a thickness of 5 μm (b). Images by courtesy of Dr. Tejal A. Desai.}
\end{figure}
Encouraging results demonstrated the feasibility of using MEMS technology to precisely control pore dimension to achieve immunoisolation and sustain cell viability.

In summary, a major advantage of extravascular macro-devices is the feasibility of replenishing islets without removing the device in case of experimental analysis or surgical complications. Because of their large sizes, extravascular macrocapsules face difficulties in maintaining high permeability to diffusive transport necessary to support encased islets while ensuring mechanical strength of the membrane to prevent graft failure.

1.2.2 VASCULAR PERFUSION DEVICES

To circumvent hypoxia and necrosis of cells located at the center of extravascular devices, the development of vascular perfusion devices struggled to overcome issues with diffusive transport but achieved limited success. Islet density of the extravascular macrocapsules is suggested to be 5-10% of the volume fraction in order to ensure the adequate exchange of nutrients and waste of the islets. As a result, large volume of macrocapsules is proposed to support sufficient masses of insulin-producing islets. This requirement challenges...
the conventional sites such as the limited space in renal capsules, and even the relatively large space in the peritoneal cavity may not satisfy the volume required for the long-term function of macroencapsulated islets.  

It is well recognized that delay of insulin secretion in response to glucose (>20 min) has been a common problem encountered in the early extravascular hollow-fiber systems. One of the crucial tasks is to minimize the volume of islet encapsulating compartment to reduce the lag in insulin release. To satisfy this requirement, thinner and longer hollow fibers are needed to accommodate a large number of cells to maintain normoglycemia in the body. However, thin fibers are prone to rupture under physical stress and demand enormous area for implantation, making implantation of these types of fibers impractical. The delay in glucose-insulin response further prompted scientists to design vascular perfusion devices directly connected with blood circulation (Fig. 1.1, b) relying on convective movements of glucose and insulin carried by ultrafiltration, instead of passive diffusion in the case of extravascular devices, to provide a faster glucose-insulin response.

In the 1970s, Chick and co-workers first reported diabetic rats connected ex vivo to an intravascular hollow fiber device consisting of neonatal islets. When implanted ex vivo as arteriovenous (AV) shunts, the plasma glucose decreased to normal range (100-130 mg/100mL). This approach provides the encapsulated islets in close contact with the blood circulation. It also allows fast exchange of glucose and insulin to correct blood glucose levels in almost real time. Similar studies using this approach ex vivo or in vivo also restored short-term normoglycemia in chemically or surgically induced diabetic animals. In contrast to a pure diffusion process under extravascular conditions, this device utilized the physiological pressure difference between the artery and vein to reduce the overall insulin response time. The unidirectional blood flow
causes the pressure of the first part of the lumen to be greater than the pressure in the periphery of the islet compartment, and therefore, ultrafiltrate crosses from the bloodstream to the islet graft. Because the hydrostatic pressure drops as a function of flow distance within the lumen, hydrostatic pressure becomes lower in the second half of the fiber, which creates an equal, reverse flux where ultrafiltrate moves from the islet compartment to the bloodstream. The resulting effect is that the ultrafiltrate in the shell compartment first stimulates islets to release insulin in response to glucose challenge, and then carries insulin back to the bloodstream. Specifically, vascular perfusion devices are developed as shunts that connect to the systemic circulation and allow blood perfusion through the devices. Despite the promise and potential of vascular perfusion devices, anticoagulant requirements have limited the utility of this approach, especially for pediatric patients.

First demonstration of the long-term use of a bioartificial pancreas in a large animal using a vascular perfusion device was demonstrated in the 1990s. Maki and co-workers developed a hybrid pancreas device, which contains an acrylic housing with islets separated from the common iliac artery and vein in dogs through the semipermeable membrane (80kDa) (Fig. 1.10). They showed that allogeneic islets could control diabetes induced by total pancreatectomy for up to 1 year with zero or minimal exogenous insulin in dogs. Devices retrieved from two recipient dogs showed a 50-70% viability of islets after 1 year. They also observed that insulin requirements were greatly reduced by 50% in the allogeneic and xenogeneic recipients after 284 days and 106 days, respectively. No gross fibrosis observed throughout the membrane, except for thin layers of fibrin-like material adhered to the luminal surface of the membrane. Importantly, clotting occurred at either the anastomosis sites or the junction of the PTFE graft and tubular acrylic copolymer membrane. These findings were considered to be
remarkable because of the difficulty in maintaining the patency of vascular device in dogs\textsuperscript{188}. Additional modifications to devices such as increasing the length of semipermeable membrane coil and the size of acrylic housing to accommodate more islets showed improvement in glycemic control and reduced insulin requirements for up to 9 months in pancreatectomized diabetic dogs without immunosuppression\textsuperscript{189}. Specifically, nine out of 17 dogs had a marked reduction in exogenous insulin requirements by porcine islet xenograft. Monaco and co-workers found out that only 4-16 IU insulin per day was needed for 57-366 days in half of the pancreatectomized dogs that received between 114,000-341,000 IEQ.

These studies demonstrated the feasibility and clinical applicability of the intravascular hybrid artificial pancreas; however, it did not move to the clinical stage due to potential risks associated with thrombosis and hemorrhage. Other blood contacting devices such as the

\textbf{Figure 1.10} An image of the hybrid artificial pancreas device\textsuperscript{5}. The device consisted of a coiled, hollow fiber membrane on top of a disk-shaped, islet compartment. Two seeding ports allowed direct injection of islets into the compartment. The hollow fiber was connected to vascular graft. The membrane has a normal molecular weight cutoff of 50 kDa, an internal diameter of 5-6 mm, and a wall thickness of 120-140 nm. Image reproduced with copyright permission.
polyacrylonitrile and polyvinylchloride copolymer (PAN-PVC) ultrafiltration capillary design, a hollow-fiber shaped tube with islets at the outside of the artificial capillaries, also failed due to excess clotting of the blood in the lumen of those small diameter artificial capillaries, in spite of anticoagulant medication in massive doses\(^{190}\). As a summary, the advantages of vascular perfusion devices include the high oxygen tension of the arterial blood exposed to islets. The disadvantages of this type of system are risks associated with the surgery required for creating AV or AA shunts, vascular thrombosis, and potential risks in cardiac stress and diversion of large volume of blood from the distal extremity.

1.3 CHALLENGES TO SUCCESSFUL MACROENCAPSULATION

1.3.1 CELL SOURCE

The clinical application of encapsulated islets requires an inexhaustible source of cells or tissues capable of delivering therapeutic agents in response to physiological changes. Insufficient number of human donors and long-term immunosuppression are the major motives for scientists to focus on alternative sources of insulin-producing cells for future transplants. New advances in the field of stem cell differentiation and regeneration therapy suggest use of xenogenic islets, immortalized β cell lines\(^{191}\), embryonic stem cells (ESC), adult stem cells (ASC), and progenitor cells that reside in the pancreas for generating insulin-producing cells. Cells from allogenic or xenogenic sources will require protection from the host immune system, and thus, efforts directed toward an encapsulation method will prove highly valuable as various cell sources gain significance in clinical relevance. Another feature of encapsulation is the improved safety of cell-based therapies since cells can be readily retrieved and separated from patients in the case of malignancy.
1.3.1.1 Xenogenic Porcine Islets

Using islets from non-human sources for transplantation has been explored to supplement the insufficient supply of donor tissue. The porcine islet is the most popular animal candidate because porcine insulin differs from human insulin by one amino acid, a higher islet yield per animal, and hypoxia tolerance observed in different age groups of pig islets with neonatal porcine islets being the most resilient\textsuperscript{192}. If not immune-protected, porcine islets in non-immunosuppressed nonhuman primates can be rejected by both humoral and cellular immune reactions\textsuperscript{193-195}. After a 72 hour transplant of fetal pig islets under the kidney capsule of primates, a large number of macrophages and T cells were observed at the periphery of and within transplanted islets\textsuperscript{193}. Infiltration of neutrophils caused tissue damage by releasing enzymes and producing chemokines that directed T cells and dendritic cells\textsuperscript{193}.

In addition, the risks associated with porcine tissues include endogenous virus transfer from porcine cells to human. These may impede the use of this porcine islets in clinical applications. However, a prospective pig-to-primate islet xenotransplantation study consisting of gene expression and serology for potentially xenotic viruses such as porcine cytomegalovirus (PCMV), porcine endogenous retrovirus (PERV), porcine lymphotropic herpesvirus (PLHV) and porcine circovirus (PCV) showed no evidence of pig virus transmission to primate recipients\textsuperscript{196}.

1.3.1.2 Embryonic Stem Cells (ESCs)

Stem cells have the ability to self-regenerate and differentiate into specialized cell types under appropriate external niche and signaling cues\textsuperscript{197}. They have the potential to provide a sufficient supply of insulin-producing source\textsuperscript{198}. ESCs derived from the inner cell mass of pre-implantation blastocysts are self-renewing and have the intrinsic capacity to generate all types of differentiated cells\textsuperscript{199}. Due to the pluripotent nature in ESCs, several groups have attempted to
direct differentiation of ESC into functional β cells\textsuperscript{153,200-203}. Insulin-expressing cells were initially produced from murine\textsuperscript{204} and human\textsuperscript{205} ESCs through the formation of an embryoid body, but this method was insufficient to generate a large amount of insulin-positive cell formation\textsuperscript{204}. It is now understood that β cells are derived from controlled formation of the definitive endoderm\textsuperscript{202} followed by a sequential and transient activation of specific transcription factors like Pdx1, NeuroD/Beta 2, Isl1, Nkx6.1, Nkx2.2, Mafa, Pax4, and Pax6\textsuperscript{153}. However, these derived cells were not very responsive to glucose \textit{in vitro}, but they could mature and restore euglycemia after being transplanted into diabetic animals\textsuperscript{153,200-205}. In addition to the need for stem-cell differentiation into insulin-producing cells, the ability of insulin-producing cells to replicate is also very important as observed during human normal growth\textsuperscript{206}, pregnancy\textsuperscript{207,208}, and obesity\textsuperscript{209}. High-throughput screens of chemical libraries could potentially identify small molecules that can stimulate the propagation of such cells \textit{in vitro} or \textit{in vivo}\textsuperscript{210,211}.

Despite the versatility of ESCs, ethical concerns and possible teratoma formation limit the usage of ESCs\textsuperscript{212,213}. To address the ethical concerns with ESCs, induced pluripotent stem cells (iPS) were generated by reprogramming adult somatic cells after ectopic expression of stem cell transcription factors Oct4, Sox3, c-myc and Klf4\textsuperscript{214-217}. The initial mouse and human fibroblast reprogramming has now extended to other somatic cells including stomach\textsuperscript{218} and pancreatic epithelium\textsuperscript{219}. Furthermore, mature exocrine pancreas was transformed into functional β cells through expression of endocrine transcription factors (Ngn 3, Pdx1 and Mafa)\textsuperscript{220}. Although the transformed cells secreted insulin and relieved diabetes in animals, an underlying mechanism study showed that the cells lacked glucose-sensitive insulin secretion and critical aspects of the β cell phenotype\textsuperscript{221}. In addition, transplantation of undifferentiated iPS containing derived insulin-producing cells could also result in teratoma formation.
In 2014, ViaCyte launched a Phase I/II clinical trial using a pouch approach with human embryonic stem cell-derived, encapsulated cell replacement therapy\textsuperscript{153,154}. It is known that insulin producing cells previously generated from human stem cells lack many functional characteristics of beta cells. In the same year, Kieffer’s group published a seven-stage protocol that described embryonic stem cell-derived insulin-producing cells not only responded to glucose challenge \textit{in vitro}, but also reversed diabetes in mice within 40 days, roughly four times faster than pancreatic progenitors\textsuperscript{222}. Melton’s group also reported a human stem cell differentiation protocol that could generate glucose-responsive beta-cells to treat hyperglycemia in diabetic mice\textsuperscript{223}. Hebrok’s group used a pancreatic differentiation protocol that enables temporal activation of endocrine differentiation in the progenitor cells to produce glucose-responsive beta-like cells, which reduced blood glucose levels in diabetic mice after short-term transplantation\textsuperscript{224}. Successful differentiation of stem cells into functional insulin-producing cells has significant clinical relevance and could potentially solve the shortage of donor tissues. However, safety issues such as the propensity of cells to form tumors have to be addressed before using ESCs and iPS in the clinical setting. Again, encapsulation could accelerate the acceptance of cell-based therapies because devices can be readily removed without spreading of tumors to the host.

1.3.1.3 Adult stem cells (ASCs)

ASCs are multipotent cells that are capable of self-renewal but limited in their pluripotent potential. ASCs can be differentiated to specialized cell types under appropriate signaling cues and microenvironment\textsuperscript{225,226}. The relative ease of isolation and expansion of ASCs makes them a potential cell based therapy for T1D treatment.

The ability to control growth and differentiation of pancreatic stem cells provides an attractive islet source for beta cell reconstitution. Human ductal structures of the adult pancreas
contain stem cells that differentiate into islets of Langerhans. Propagation and differentiation of these islet-like cells demonstrated insulin production in vitro to normalize blood glucose levels for more than 3 months in diabetic mice\textsuperscript{227}. Nestin positive derived islet cell clusters expressed pancreatic endocrine markers like Glut2, glucagon, Pdx1, and exocrine genes\textsuperscript{228}. Exocrine pancreatic tissue\textsuperscript{229} and neurogenin 3 (ngn 3) positive cells\textsuperscript{230} could also be used as an alternative source to β cells. However, the harvest procedure of pancreatic stem cells from the pancreas is very invasive. The number of isolated precursor cells is few and heterogeneously distributed in the body, thus, restricting the actual application in the clinical setting.

There have been controversies regarding the origin of neonatal β cells during the normal pancreatic tissue maintenance, the role of the regenerating cells after injury, and the signaling mechanism by which they regenerate\textsuperscript{231,232}. It is known that the adult pancreas has a capacity to respond to changing physiological needs. Lineage tracing experiments after partial pancreatectomy suggested that the majority of new β cells is from the proliferation of pre-existing β cells rather than stem cell differentiation\textsuperscript{233,234}. However, other evidence demonstrated that stem cells or progenitor cells expressing markers cytokeratin-19 (CK) give rise to new β cells after 90\% pancreatectomy or treatment with streptozotocin\textsuperscript{235-237}. Despite the recent debates, there is little doubt that both replication and neogenesis (the differentiation of new islet cells form progenitors or stem cells) pathways play an important role in maintaining an adequate β cell mass after birth. However, the degree of replication of β cells or differentiation of progenitors/stem cells in a particular model system depends on the species (e.g. human vs. mice, transgenic vs non-transgenic animals), the pathophysiological conditions, and the physiological states\textsuperscript{238}. 
1.3.2 BIOMATERIALS

To date, a variety of polymeric and inorganic materials have been utilized to create the semipermeable membranes with immunoprotective barriers characteristics. The materials required for encapsulation must demonstrate adequate permselectivity - high selectivity excludes immune components and high permeability supports the metabolic needs of encapsulated cells. Some of the commonly used biomaterials for macroencapsulation are alginate, agarose, nitro-cellulose acetate, 2-hydroxy-ethyl methacrylate (HEMA), acrylonitrile and sodium-methallylsulfonate, and PTFE. Unlike materials used for microencapsulation, polymers for macroencapsulation are mechanically more stable with thicker capsule walls. However, thicker walls can actually impair diffusion across the membrane, threatening the viability of transplant tissue. Many techniques have been used to improve the survival rate of macroencapsulated islets such as smoothened capsule surface and hydrophilic materials with low interfacial energy to reduce protein adsorption, cell adhesion and fibrosis.

1.3.2.1 Alginate

Alginate is a polysaccharide whose biocompatibility and gelling properties make it the most popular choice for encapsulation. The final gelled polymers form many non-uniform alginate strands that serve as a barrier to the movement of molecules passing from the outside of the capsule to the tissue within or vice versa. These non-uniform alginate strands create a wide distribution of pore sizes, which can greatly affect the diffusion of molecules. Therefore, rigorous purification of the naturally occurring compound is required. Otherwise, inadequate alginate purification can cause increased alginate immunogenicity and splenocyte proliferation, and decreased encapsulated islet viability. Highly purified alginate does not interfere with islet
function and shows good stability\textsuperscript{250}. Moreover, various biomaterials such as polyethylene glycol (PEG) and poly-L-lysine (PLL) have been used to improve the permeability and selectivity of alginate with reduced plasma absorption. Cui et al. demonstrated that grafting PEG chains onto alginate capsules increased \textit{in vivo} viability of islet cells (Cui et al. 2004). The Stabler group further improved the cross-linking process of alginate/PEG by Staudinger ligation\textsuperscript{251}. Goosen et al. reported alginate/PLL/alginate capsules blocked diffusion of serum immunoglobulin, albumin, and hemoglobin\textsuperscript{252}. The Anderson group showed that larger alginate capsules of 1.5-mm in size restored blood-glucose control for up to 180 days in diabetic C57BL/6 mice, which was five times longer than the conventionally sized 0.5-mm alginate capsules\textsuperscript{253}. They reported that alternating the spherical dimension of implanted devices can significantly improve the device biocompatibility \textit{in vivo}.

\subsection*{1.3.2.2 Agarose}

Agarose is a thermo-sensitive, linear polymer made of repeating monomeric unit of agarobiose. The gelling temperature of agarose used in encapsulation is 15-30 °C, but it is dependent on the concentration of agarose used. Often, droplet extrusion followed by hardening with reduction in temperature is used to create an encapsulation capsule. Agarose has been shown to be biocompatible and prevent extrusion of cells\textsuperscript{254}. Selectivity increases as the concentration of agarose rises\textsuperscript{255}. Although it has not been studied intensively as alginate, agarose-macroencapsulated porcine islets remained viable and functional for almost 200 days after intraperitoneal transplantation in rat recipients\textsuperscript{9,86}.

\subsection*{1.3.2.3 PTFE}

PTFE is a fluorocarbon-based polymer which is very stable both thermally and chemically. This polymer is hydrophobic, biologically inert, and non-biodegradable which
gained popularity as a vascular graft material in the expanded form (ePTFE), due to greater porosity, better tissue adhesion, and improved pliability\textsuperscript{256,257}. Microporosity of this material can be controlled through processing techniques including mixing resin with a solvent binder, cold extrusion of a billet, and mechanical expansion and stretching followed by sintering\textsuperscript{258}. A notable example of using this material to encapsulate cells is the Baxter TheraCyte System (section 1.2.1), which was also an expanded PTFE structure that enhanced blood vessel formation with 5 days of implantation\textsuperscript{103} and prevented allograft rejection in non-immunized recipients for 6 months\textsuperscript{259} and xenograft rejection for up to 8 weeks in a porcine-to-cynomolgus monkey model\textsuperscript{76}.

\section*{1.4 IMMUNE RESPONSE}

The principle of immunoisolation is based on physical separation of graft cells from the host immune system to prevent direct cell-to-cell contact, thereby circumventing the direct antigen pathway that causes activation of cytotoxic CD8\textsuperscript{+} T cells by donor major histocompatibility complex (MHC)-peptide complexes expressed on the surface of antigen presenting cells\textsuperscript{260,261}. Allograft rejection is primarily mediated by such aforementioned process through cellular immunity. To prevent the macrophage and T cell reaction to allografts, allotransplantation of islets with testicular Sertoli cells or genetically engineered cells induced with Fas ligand (FasL) have been shown to protect islets\textsuperscript{262-264}. Antigens shredded from encapsulated cells could also trigger T cell activation resulting in a series of cytotoxic granules and cytokine release. Therefore, it is important to consider using size exclusion to prevent graft rejection. For example, TheraCyte device with 0.4 \textmu m in pore size protected against allograft rejection in non-immunized recipients for 6 months\textsuperscript{259} and provided effective immunoisolation.
that allowed neonatal porcine islets to survive in cynomolgus monkeys for up to 8 weeks\textsuperscript{76}. The pore size of this membrane is insufficient for long-term xenograft protection.

Rejection of non-vascularized xenografts includes both the humoral immunity (involving IgG and IgM antibodies and complement) and cellular immunity (involving T-cells and macrophages, cytokines, free radicals, and NO)\textsuperscript{265-269}. For the humoral immune system, complement reaction is initiated either by the classic pathway that involves binding of the complement component C1q to an IgM or IgG molecule, or by the alternate pathway which involves C3\textsuperscript{270}. The final result of the cascade of events is the lysis of cells. In the cellular immunity system, host antigen presenting cells display peptides from donor proteins to engage CD4+ helper T cells which develop into Th2 cells. These cells produce cytokines that stimulate the maturation of B cells into plasma cells, which secrete xenoantigen-specific antibodies\textsuperscript{260,261}. CD4+ T cells also induce production of additional cytokines (IFN-γ, IL-2, IL-5) and pro-inflammatory molecules by macrophages (TNF-α, IL-1β, histamine), which can be highly destructive to encapsulated cells through oxidative and endoplasmic reticulum stress pathways\textsuperscript{271-274}. The effects of indirect antigen pathway with humoral and cellular responses on the transport characteristics of immunobarriers have been discussed in many studies\textsuperscript{166,190}. Complement reaction can be prevented by using a membrane with a maximum effective pore diameter of 30 nm to hinder passage of complement and antibodies to islets\textsuperscript{190}, or using molecules that inhibit a step in the formation of the membrane attack complex on encapsulated tissue\textsuperscript{270,275}. However, the physical nature of immunocellular components presents an enormous challenge to the size-selective based immunoisolation techniques, as shown by dimensions of glucose, insulin and other inflammatory mediators in Table 1.2. While some immunoisolation membranes have managed to protect cells from IL-1β and/or TNF-α\textsuperscript{276,277}, blockade of free
radical diffusion is very unlikely as demonstrated by Wiegand et al.\textsuperscript{278} and Chae at al.\textsuperscript{279} that, despite its short half-life, nitric oxide (NO) can still destroy encapsulated islets. This observation was also supported by a mathematical model of free radical diffusion through a spherical matrix containing pancreatic islets\textsuperscript{280}. New paradigms in the development of immunoisolation barriers must be explored in the case of indirect antigen presentation for the use of xenografts. As ESC-based cell therapies develop, immunoprotection of xenogeneic cells may not be as critical. Alloprotection may offer sufficient immunoisolation and improved nutrient transport given the pore size used for this type of immunoisolation.

Table 1.2 Glucose, insulin and soluble inflammatory mediators expressed by pancreatic islets.

<table>
<thead>
<tr>
<th>Molecule (MIF)</th>
<th>Molecular weight (Da)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>150,000</td>
<td>281</td>
</tr>
<tr>
<td>IL-6</td>
<td>21,500-28,000</td>
<td>282,283</td>
</tr>
<tr>
<td>IL-1β</td>
<td>17,500</td>
<td>282,284-286</td>
</tr>
<tr>
<td>TNF-α</td>
<td>17,300</td>
<td>287,288</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>15,500-25,000</td>
<td>289</td>
</tr>
<tr>
<td>Macrophage migration inhibitory factor (MIF)</td>
<td>12,000</td>
<td>286</td>
</tr>
<tr>
<td>CXCL9 (MIG)</td>
<td>11,700</td>
<td>290</td>
</tr>
<tr>
<td>CXCL10</td>
<td>10,000</td>
<td>290</td>
</tr>
<tr>
<td>IL-8</td>
<td>8,000</td>
<td>283,286</td>
</tr>
<tr>
<td>CCL5 (RANTES)</td>
<td>8,000</td>
<td>290</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>7800</td>
<td>291</td>
</tr>
<tr>
<td>MCP-1</td>
<td>6000-7000</td>
<td>283,285,286,292,293</td>
</tr>
<tr>
<td>CXCL2 (MIP-2α)</td>
<td>6000</td>
<td>290</td>
</tr>
<tr>
<td>Insulin</td>
<td>5,087</td>
<td>294</td>
</tr>
<tr>
<td>Glucose</td>
<td>180</td>
<td>295</td>
</tr>
<tr>
<td>Nitric oxide (NO)</td>
<td>30</td>
<td>284,296</td>
</tr>
</tbody>
</table>

1.5 INFLAMMATORY RESPONSE

1.5.1 Acute Response

Non-specific inflammatory responses occur at the transplant site immediately after implantation of immunoisolated or naked islets, mediated by activated macrophages that produce
cytokines, free radicals, and NO to damage islet cells. The inflammatory milieu is very metabolically active, and therefore, oxygen is rapidly consumed, which further reduces the amount of oxygen available to islets, resulting in hypoxia and death. Not surprisingly, reduction of these effects and prolonged engraftment of both encapsulated and non-encapsulated cells were observed following macrophage depletion\textsuperscript{297}.

1.5.2 Foreign Body Response

Foreign body response to implanted biomaterials can be described as initial recruitment of neutrophils and macrophages by non-specific adsorption of proteins on the material surface, which leads to subsequent attachment and overgrowth of the device by macrophages, foreign body giant cells, and fibroblasts\textsuperscript{298}. The severity of foreign body responses to immunoisolation devices depends on transplantation site and material properties, such as surface charge and chemistry, porosity, roughness, and implant size\textsuperscript{299}. Capsular overgrowth of the implanted devices inhibits nutrient transport to the islets and cause hypoxia and necrosis that lead to islet destruction and graft failure\textsuperscript{300}. The biocompatibility of immunoisolating materials could be improved by using highly purified materials\textsuperscript{249} or by engendering an alternative foreign body response as illustrated by the TheraCyte device, which encourages blood vessel growth at the capsule surface\textsuperscript{103}.

1.5.3 Instant Blood-Mediated Inflammation Reaction

In addition to the challenge associated with complex immune responses aforementioned, vascular perfusion macrocapsules face another big hurdle because implantation of intravascular devices injures vessel walls, which, in turn, induces significant platelet adhesion and activation, and blood coagulation. Instant blood mediated inflammation reaction (IBMIR) is another mechanism for acute graft rejection that involves platelet consumption, complement activation
and the initiation of the coagulation cascade. To prevent acute rejection as a result of direct contact with the blood, conjugation of thrombomodulin and anti-coagulation agents such as heparin and warfarin, use of low-molecular weight dextran sulfate, and genetic modifications of encapsulated islets have been investigated.

1.6 HYPOXIA AND IMPLANTATION SITE

Progressive islet graft dysfunction and loss occurs due to many reasons as summarized in Fig. 1.11: absent re-innervations, chronic hypoxia due to poor vascularization, premature apoptosis, lack of regeneration in insulin-producing cells, pro-inflammatory milieu, coagulation and thrombosis in vascular perfusion devices, and mechanical failure of the encapsulation.
membrane. When islets are transplanted, many of them die in the first few days due to hypoxic death before vascularization develops\textsuperscript{312}. Cell necrosis occurs when islets are placed beyond the diffusion limit of tissue (>150-200 μm away from the nearest blood vessels)\textsuperscript{133}. Vascularization only occurs in 7-10 days after transplant\textsuperscript{72,313}. This delayed and insufficient vascularization creates low oxygen tension, resulting in cell death and graft failure\textsuperscript{314}. The Sernova and Theracyte devices (section 1.2.1) have the capability to pre-vascularize the system prior to cell insertion to overcome the low oxygen tension.

To avoid hypoxia, intravascular devices can be connected by vascular anastomoses to the vessels of the host with either AV or AA connection\textsuperscript{76,315}, where oxygen and nutrients directly passed to the cells. Common iliac artery and the common iliac vein are the popular sites to use\textsuperscript{94-97} in addition to aortic lumen\textsuperscript{98,99} in canine models. Intravascular devices were also grafted into the deep femoral artery or the forearm cubital vein after performing AV anastomosis in patients\textsuperscript{100}. The method of islet transplantation into the forearm cubital vein with AVA formation was less traumatic and more physiological such that patients showed a faster and larger decrease of insulin demand, and euglycemia was maintained for two years with 14 recipients (73.7\%)\textsuperscript{100}.

Extravascular macrocapsules require large volumes to accommodate sufficient masses of insulin-producing islets, and therefore, locations with established vascular beds are preferred. Peritoneal cavity offers less restriction on the volume of encapsulated islets that can be transplanted, and the procedure can be invasive compared to subcutaneous implantation\textsuperscript{316,317}. However, the lack of vascularization and gravity-induced clumping of islets on the pelvic floor in upright primates, if not immobilized within macrocapsules, are the main issues concerning this location\textsuperscript{318}. Kidney subcapsular space also offers good vascular network, but the space is quite limited\textsuperscript{319,320}. Subcutaneous tissue such as epididymal fat pad is close to vasculature, but a large
surface area may be required for transplantation\textsuperscript{58,93,321}. Other possible locations include omentum\textsuperscript{322,323}, muscle\textsuperscript{324}, and intraocular sites\textsuperscript{325}, but vascularization and space limitation make implantation of extravascular macrocapsules impractical.

1.7 NEW SOLUTION FOR MACROENCAPSULATION

There are two major areas to be considered for making the successful design of next generation of islet macroencapsulation devices, namely providing effective immunoisolation and presenting sufficient mass transfer between the outside environment and the encased islets. As previously discussed, semipermeable membranes must exhibit precisely controlled pore size to separate soluble inflammatory mediators (Table 1.2) that are on a scale of nanometer in size while exhibiting exceptional uniformity in pore size distribution to provide suitable immunoisolation. Microfabricated silicon membranes can be used to achieve such level of high precision control over pore sizes, as illustrated by examples like nanoporous biocapsule\textsuperscript{13,180,181} (Fig. 1.8) and Nanogland\textsuperscript{12} (Fig. 1.9). The nanoporous biocapsule and Nanogland were designed with L-shaped pore paths with perpendicular microchannels and parallel nanochannels to the membrane surface. This L-shaped design effectively prevented diffusion of larger immune components, but hindered diffusion of small molecules due to the indirect, long diffusion distance. This effect was observed in the Nanogland device where nanochannels with 3.6 and 5.7 nm pore sizes showed a reduction in glucose diffusivity by 40\% and 25\% compared with the molecule in the bulk medium\textsuperscript{12}. Besides the long diffusion distance, solutes also face reduced diffusion as their size approach the molecular dimension of the pores. Dechadilok and Deen reviewed hindered transport theory for both diffusive and convective hindrance factors in which uncharged, spherical particles travel in the long cylindrical and slit pores of uniform cross-
Depending on the mode of transport, it is crucial to design immunoisolating membranes with size exclusion properties for the larger immune components (e.g. cytokines, antibodies) while still permit the passage of smaller molecules (e.g. glucose, insulin). The surfaces of silicon membranes can also be selectively grafted with biocompatible polymer thin films to ensure functional performance over extended time periods, making them suitable for biological applications\textsuperscript{327-329}. Although nano-sized pores are ideal to restrict the passage of immune components, encased islet functions and viability could be greatly impacted under the diffusive transport approach. A faster mass transfer of oxygen and nutrients to the encapsulated islets is needed given the size constraints on the pores. To monitor vessel stenosis and prevent thrombosis in vascular devices, advances including the use of intra-access blood flow and pressure measurements and duplex ultrasound\textsuperscript{330} could further shed light on the optimal intravascular design (e.g. pressure drop, blood flow path) for macroencapsulation devices.

1.8 CONCLUSIONS

To date, the pursuit of bioartificial pancreas devices that restore glucose homeostasis without the need for immunosuppression still remains one of the most challenging goals within the field of regenerative medicine and tissue engineering. With more than 50 years of intensive research directed at developing encapsulation methods for immunoisolation of transplanted cells, both promise and inherent challenges, particularly related to macroencapsulation, have been discussed within this chapter. The progress on macroencapsulation has been limited due to inefficient mass transport of oxygen and nutrients under extravascular setting and problematic blood coagulation and thrombosis under intravascular environment in large animals. But advances in membrane development such as use of MEMS technology have the potential to
improve macroencapsulation. In addition to the maintenance of adequate oxygen and nutrient transport, inflammatory response also plays a pivotal role in reacting with any implanted cell-material composite, and therefore, a proper control of membrane transport properties to prevent host responses is of paramount importance. Given the promising potential of encapsulation based bioartificial pancreas, successful devices will depend on merging knowledge from cell-based therapeutics with advanced engineering approaches to overcome several major obstacles: first, the development of a renewable, alternative insulin-producing cell source to solve the current donor organ shortage; second, enhanced biocompatibility and permselectivity of immunobarriers to reduce deleterious host immune response; and thirdly, improved mass transport characteristics of existing encapsulation techniques using advanced engineering approach. Therefore, a synergistic effort between biological and physical scientists, physician-scientists, and engineers will be essential for the development of novel life-saving technologies in the field of T1D research.
CHAPTER II

SILICON NANOPOR Membrane (SNM) FOR ISLET ENCAPSULATION AND IMMUNOISOLATION UNDER CONVECTIVE TRANSPORT

Substantial portions of this Chapter have been published in Scientific Reports 2016; (6):23679.

2.1 INTRODUCTION

Type 1 diabetes (T1D) results from autoimmune destruction of the insulin-producing β-cells within the pancreatic islets of Langerhans. Islet transplantation by direct infusion of cadaveric islets into the portal vein of the recipient’s liver offers a non-invasive cure for patients with T1D mellitus\textsuperscript{46}. However, donor availability, poor engraftment, and side effects from global immunosuppression remain as obstacles for wider application of this approach\textsuperscript{55,57,331}. Moreover, up to 60% of the infused islets become non-viable within a few days after surgical delivery\textsuperscript{319} and the long-term insulin independence is frequently lost by 5 years of transplantation\textsuperscript{332}. The activation of innate and the adaptive immune responses are among the main causes of islet graft failure\textsuperscript{333,334}.

The idea of encapsulating islets using selective semi-permeable membranes to protect islets from the host’s immune system has generated tremendous interest\textsuperscript{58}. The immunoisolating membranes would prevent the passage of the host’s immune factors, while allowing the exchange of glucose, insulin, nutrients and small molecules to sustain the function and viability of the graft. Although membranes with pores smaller than 1 µm can easily block immune cells (\textasciitilde 10 µm), the blockage of molecules such as antibodies and cytokines proves to be a significant
challenge. Previous studies showed that large antibody (IgM) and complement (C1q) were hindered using membranes with a maximum pore diameter of 30 nm\textsuperscript{190}. For cytokines, the membranes must selectively discriminate between molecules on the scale of few nanometers, as shown by the molecular weights and Stokes diameters in Tumor Necrosis Factor-alpha (TNF-\( \alpha \)) (17,300 Da; 3.80 nm)\textsuperscript{10,287}, and Interferon-gamma (IFN-\( \gamma \)) (15,600 Da; 3.67 nm)\textsuperscript{10,335}, and Interleukin-1 beta (IL-1\( \beta \)) (17,500 Da; 3.81 nm)\textsuperscript{282,285} compared to glucose (180 Da; 0.82 nm)\textsuperscript{10,336} and insulin (5,800 Da; 2.64 nm)\textsuperscript{10,337}. These cytokines are known to be synergistically cytotoxic to islets through a cascade of inflammatory events such as production of nitric oxide (NO) and chemokines, and trigger of endoplasmic reticulum stress\textsuperscript{274,338}. Conventional polymeric membranes face enormous challenge for size-dependent separation of these cytokines as polymeric membranes frequently exhibit pore sizes with relatively broad distributions (30\%\textsuperscript{339}).

Our lab has developed a new generation of encapsulating membranes for immunoisolation of transplanted islets based on microelectromechanical systems (MEMS) technology initially pioneered by Ferrari and colleagues\textsuperscript{340,341} to create more uniform pore sizes at nanometer scale. These semipermeable filtration membranes, termed silicon nanopore membranes (SNM), can be engineered with precise pore sizes down to 5 nm (Fig. 2.1)\textsuperscript{342} and a monodisperse pore size distribution (\~{}1\%) for superior selectivity\textsuperscript{339,342-344}. The ability to engineer precise pore dimensions in a uniform manner enables SNM to discriminate larger immune components from smaller molecules that will pass into the encapsulated cells. When pore dimensions are of the same order as those of a solute molecule\textsuperscript{345}, the slow diffusion significantly hinders transport of nutrients and oxygen. In contrast, convective transport is attractive as it offers a more efficient mass transfer where solutes actively move along with solvent flux due to applied pressure gradient. Our overall objective is an implantable bioartificial
pancreas where transplanted islets are encapsulated between two SNM sheets in a device that will be mounted similarly to an arterio-venous (AV) graft (Fig. 2.2). The concept involves using

Figure 2.1 Silicon nanoporous membranes (SNM). (a) an optical image of the SNM chip. (b) An SEM image of the surface of the membrane which illustrates nanopores with 2 µm in length. (c) An SEM image of the cross-section of the membrane which illustrates one nanopore with 7 nm in width and 300 nm in depth.

Figure 2.2 Conceptual illustration of the implantable intravascular bioartificial pancreas device in the arm of a T1D patient. Transplanted islets will be encapsulated between two SNM sheets mounted on as an arterio-venous (AV) graft. The arterio-venous pressure differential will generate ultrafiltrate that continuously support the islets, which will, in turn, sense glucose levels and produce insulin that will be swept into the venous blood. The small pore size of the SNM ensures appropriate immunoisolation between the transplanted islets and host.
the pressure difference between the artery and vein to generate ultrafiltrate and drive transport of glucose, insulin, and other small molecules through the SNM to support function of encased islets while preventing passage of immune components.

In this study, we focused on SNM design and fabrication, followed by characterization of its immunobarrier properties under cytokine challenge with convective transport, and assessment of SNM-encapsulated islet viability and glucose-insulin response. Specifically, hydraulic permeability measurement and solute selectivity for SNM were determined. Mouse islets were encapsulated between SNM in a closed mock-loop fluid circuit (Fig. 2.3) under simulated physiological pressure difference in the presence of a cocktail of pro-inflammatory cytokines including TNF-α, IL-1β, and IFN-γ. Islet viability and glucose stimulated insulin production

Figure 2.3 Schematic diagram of the mock-loop circuit for in vitro assessment of SNM-encapsulated islets under convective conditions. A peristaltic pump circulated liquid through the top compartment of the flow cell, a pressure transducer, a 3-way valve, the bottom compartment of the flow cell, and finally back to the original reservoir. The flow cell was composed of two membranes dividing the flow cell into three compartments, where islets were placed inside the middle chamber. Ultrafiltrate flow occurred within the middle compartment.
were evaluated to demonstrate the potential of SNM as an encapsulation material for islet immunoisolation under convective transport.

### 2.2 MATERIALS AND METHODS

SNM were fabricated to produce an active membrane area (6X6 mm) consisting of $\sim 10^6$ rectangular slit pores with $\sim 7$ nm in width, 300 nm in depth, and 2 $\mu$m in length (Fig. 2.1). The surface of SNM was subsequently modified with polyethylene glycol (PEG) to minimize protein fouling\textsuperscript{346}. All SNM membranes in this study were tested with an average pore size of $\sim 7$ nm.

![Figure 2.4 Schematic diagram of the pressure-driven cytokine filtration testing system. A peristaltic pump circulated liquid through a flow cell that connected to a 3-way valve to establish transmembrane pressure. The permeated ultrafiltrate through the membrane was collected at various time for up to 6 hrs.](image)

We first analyzed the transport of small solutes including cytokines across a single SNM using a pressure-driven filtration assembly (Fig. 2.4). To mimic the proposed bioartificial pancreas device with convective ultrafiltration under physiological pressure, we constructed a benchtop mock-loop circuit consisting of a three-layer flow cell with two enclosed SNM (Fig. 2.3), where the top, middle, and bottom compartments recapitulated the “artery”, “encapsulated islet
chamber”, and “vein”, respectively. We subsequently characterized the percentage of cytokines, glucose, and insulin within the different locations of the mock-loop device. Finally, we tested the viability and glucose-insulin response of the SNM-encapsulated mouse islets in the mock-loop circuit with circulating cytokines.

2.2.1 SUBSTRATE PREPARATION

Silicon Nanopore Membranes (SNM) architecture and fabrication

Silicon nanopore membranes (SNM) have been prototyped from silicon substrates by MEMS technology as previously reported with some modifications (Fig. 2.5). Briefly, the process used the growth of a thin SiO$_2$ (oxide) layer on 400 µm-thick double side polished (DSP) silicon wafers followed by a low pressure chemical vapor deposition (LPCVD) of polysilicon (~500 nm). The wafers were then specifically patterned, dry oxidized, wet etched, deposited with a second polysilicon layer, and finally blanket etched until 400 nm of polysilicon remained and the underlying vertical oxide layer was exposed. The vertical sacrificial oxide layer defined the critical nanoscale pore size of the membranes. The low temperature oxide (LTO) (~1 µm) was deposited onto polysilicon of the wafers to serve as the hard mask for membrane protection. Deep reactive ion etching (DRIE) removed the backside of each window until membranes were disclosed. Eventually, the sacrificial oxide was etched away in 49% hydrofluoric acid (HF) during the final step of the fabrication process to leave behind open nanoscale slit pores. The wafers were subsequently cut into 1×1 cm chips with an effective area of 6X6 mm$^2$ containing 1500 windows each, with a total of 10$^6$ pores per membrane. Each rectangular pore was 7 nm in width, 300 nm in depth, and 2 µm in length. All membranes were cleaned using a conventional “piranha” clean procedure, which involved a 20 min-immersion in 3:1 sulfuric acid (H$_2$SO$_4$)/hydrogen peroxide (H$_2$O$_2$) mixture, followed by thorough rinses in
deionized (DI) water. Images of SNM were obtained using scanning electron microscope (SEM) (Leo 1550) (Fig. 2.1).

**Surface modification of SNM with poly(ethylene glycol) (PEG)**

SNM were covalently modified with PEG using a previously reported protocol\textsuperscript{149} with some modifications to prevent protein fouling on the membrane surface. The technique used for PEG attachment involved a single reaction step which covalently couples silicon surface silanol
group (Si-OH) to a chain of PEG polymer through a trimethoxysilane group forming a Si-O-Si-PEG sequence. Briefly, SNM were immersed in a solution of 3 mM 2-[methoxy(polyethyleneoxy)propyl]trimethoxysilane (PEG-silane) (Gelest: SIM6492.7) in toluene for 2 hr at 70 °C. A series of extensive washing steps involving toluene, ethanol, and DI water were used to rinse away unbounded PEG residue.

**Hydraulic permeability for SNM pore size characterization**

An automated mass and pressure measurement system was utilized for characterizing liquid flow through the SNM under a tangential-flow filtration operation\(^3\). The pore size of the SNM can be related to filtration flow parameters using \( h = \left( \frac{12 \mu Q}{nw \Delta P} \right) \) (Eq. 3), where \( h \) is pore width, \( \mu \) is the viscosity, \( l \) is the membrane thickness, \( Q \) is the volumetric flow rate, \( n \) is the number of pores per membrane, \( w \) is the pore length, and \( \Delta P \) is the transmembrane pressure\(^3\). To assemble the overall system for SNM pore size characterization (Fig. 2.6), air was applied through a syringe pump (Sigma: Z675709) into a water reservoir. Water was circulated by a peristaltic pump (Masterflex: 07551-00) through a differential pressure transducer (Omega: PX429 015GI), a flow cell with enclosed membrane, and returned to the original water reservoir. The flow cell was assembled with the SNM submerged under water to remove air bubbles from all compartments. Specifically, a membrane was positioned with the polysilicon interface facing down with a customized silicone gasket positioned on top of the membrane, followed by the final placement of a filtrate chamber on top of the gasket. All sections were fastened together and secured to the base with hand-tightened hex bolts until gasket was visibly compressed. The ultrafiltrate permeated through the membrane was routed to a liquid collection container that rested on a precision mass balance (Mettler Toledo: XS205). Measurements from the differential pressure transducer and the mass balance were automatically collected with a data acquisition
A typical membrane hydraulic permeability test consisted of 5 ml/min flow rate and 4 pressure cycles (5, 1, 5, and 1 psi) for durations of 150 s each. Using the specifications for pore length, membrane thickness, and total number of pores provided based on individual wafer designs, the average pore size of SNM was calculated using Equation 1. All SNM membranes in this study were surface-modified with PEG and exhibited an average pore size of ~7 nm.

Figure 2.6 Schematic diagram of the hydraulic permeability testing system. Air was applied through a pressure regulator into the liquid reservoir. A peristaltic pump circulated this liquid through the flow cell with enclosed membrane. The flow cell connected to a differential pressure transducer that was automatically controlled by a data acquisition laptop to adjust the transmembrane pressure. The permeated ultrafiltrate was collected into a liquid container on top of a precision mass balance. Data from the differential pressure transducer and the mass balance were automatically collected and stored in a data acquisition laptop.
2.2.2 ASSESSMENT OF SNM IMMUNOISOLATION IN VITRO

Membrane sieving coefficients under pressure-driven filtration

Fluid was circulated by a peristaltic pump through a circuit that consisted of a differential pressure transducer, a polycarbonate flow cell with enclosed SNM, a three-way valve, and a fluid reservoir (Fig. 2.4). The flow cell consisted of two separate flow cell compartments sandwiching a single SNM and silicone gasket. The top filtrate chamber routed permeated ultrafiltrate to a liquid collection container, whereas the base chamber was connected to a three-way valve. A solution of 3% bovine serum albumin (BSA) (Sigma: A-7030) was used to flush the entire loop prior to the experiment. Solution consisting of mouse cytokines TNF-α (1000 U/ml) (Peprotech: 315-01A), IFN-γ (1000 U/ml) (Peprotech: 315-05), IL-1β (50 U/ml) (Peprotech: 211-11B)\textsuperscript{351}, glucose (400 mg/dL) (Sigma-Aldrich: G8270), and insulin (150 mU/L) (Novo Nordisk: 0169-1833-11) in a 3% BSA solution was then switched to the circuit at 5 ml/min with a physiological pressure difference \(\sim 2\) psi\textsuperscript{352}. Ultrafiltrate that permeated through the SNM was collected at various time points for up to 6 hrs and analyzed with the enzyme-linked immunosorbent assays (ELISA) (BD Biosciences: 560478 & 558258; Thermo Pierce: EM2IL1B). The sieving coefficients of solutes across SNM were calculated using \(S = \frac{c_f}{c_b}\) (Eq. 1)\textsuperscript{353}, where \(S\) is the sieving coefficient, \(c_f\) is the concentration of the solute in the filtrate, and \(c_b\) is the molecule concentration in the bulk retentate solution.

Solute distribution in the mock-loop circuit

We assembled a mock-loop circuit with three flow cell components without cells (Fig. 2.3) to mimic the architecture of the final bioartificial pancreas device. Briefly, two SNM with customized silicone gasket frames were sandwiched in between three flow cell components. The middle flow cell was the encapsulation chamber comprised of a cylindrical chamber separating
the two membranes. A peristaltic pump drove the fluid through the top of the flow cell mimicking the “artery”, then over the bottom of the flow cell resembling “vein”, and finally back to the original reservoir. For convective experiments, a three-way valve was used to create flow resistance for a physiological pressure difference ~2 psi between the top and the bottom compartments of the flow cell. Ultrafiltration occurred in the middle encapsulation chamber at this pressure difference. To study the transport of cytokines through the three-layered bioartificial pancreas device, solution consisting of mouse cytokines TNF-α (1000 U/ml), IFN-γ (1000 U/ml), and IL-1β (50 U/ml), glucose (400 mg/dL), insulin (150 mU/L) in 3% BSA was circulated through the circuit at a flow rate of 5ml/min. Silicon membranes with 1000 nm-wide slit pores (SμM) were used as the control. Solutions were collected and analyzed with ELISA at the end of 6-hr experiments for the top, middle, and bottom chambers.

2.2.3 CULTURE OF MEMBRANE-ENCAPSULATED ISLETS IN THE MOCK-LOOP CIRCUIT

All procedures described involving isolation of mouse islets were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, San Francisco (UCSF). Mouse islets were isolated from 8 to 10-week-old male B6 mice (Jackson Laboratories) based on previously described protocols. Harvested islets were maintained in suspension culture with RPMI 1640 with L-glutamine and 11.1 mM glucose (Gibco: 11875-093), 10% fetal bovine serum (FBS) (Gibco: 16000), and 1% penicillin-streptomycin (P/S) (UCSF Cell Culture Facility: CCFGK003). A group of 500 mouse islets were introduced into the middle encapsulation chamber of the mock-loop device (Fig. 2.3). To evaluate cell performance with cytokine exposure, the circuit reservoir was replaced with culture medium added with TNF-α (1000 U/ml), IFN-γ (1000 U/ml), and IL-
1β (50 U/ml) for 6 hrs. Static culture conditions with or without cytokine exposure were used as the controls. Mouse islets were subsequently isolated for viability testing (2.3.4) and glucose challenge (2.3.5).

**Islet viability**

Islet viability was assessed by double staining with fluorescein diacetate (FDA) (Sigma: F7378) and propidium iodide (PI) (Sigma: 287075) as described by protocol (SOP Document: 3104, A02) from National Institute of Allergy and Infectious Diseases (NIAID). Briefly, mouse islets were incubated in phosphate buffered saline (PBS) containing 0.067 µM FDA and 4.0 µM PI for 30 min and extensively washed in PBS to remove excess staining. Images of mouse islets were obtained using laser scanning Nikon Spectral C1si confocal microscope (Nikon Instruments). Viability of islets was calculated based on the ratio between the number of live cells in the islet and the area of that islet.

**Glucose stimulated insulin secretion assay**

Mouse islets retrieved from the middle chamber of the mock-loop circuit were rested in RPMI 1640 containing 30 mg/dL glucose (Gibco: 11879) for 15 minutes before exposed to medium containing 300 mg/dL glucose for 15 minutes. After glucose stimulation, the islets were then returned to medium containing 30 mg/dL glucose. Supernatant was collected every 5 minutes during the series of incubations and insulin content was measured with mouse insulin ELISA kits (Mercodia:10-1247-01) and normalized by extracted total protein concentration (Thermo: 78505; 23225).

**Statistical analysis**

Sample pairs were analyzed using Student’s t-test. Multiple samples were evaluated with one-way or two-way analysis of variance (ANOVA) followed by Bonferroni and multiple
comparison using Graphpad Prism software (San Diego, CA). A p value of <0.05 was accepted as statistically significant for all analyses.

2.3 RESULTS AND DISCUSSION

MEMS fabrication technologies offers unprecedented potential in reproducibility and precision to engineer controlled pore dimensions that can selectively block the passage of immune components while allowing transport of small molecules (e.g. glucose and insulin) to sustain the viability of the encased cells. In the present study, we characterized the permeability and selectivity of the SNM to prevent cytokine infiltration and assessed the functional performance of SNM-encapsulated mouse islets in a mock-loop device under convective transport.

SNM design and fabrication

Previously, Desai et al. reported silicon-based micromachined nanochannels that consisted of L-shaped pore paths with nanochannels running parallel to the membrane surface. Although the design was effective in preventing diffusion of larger immunogenic molecules, the L-shaped path drastically reduced diffusion of smaller molecules of interest because of the long, indirect flow path and the less optimal pore density stemming from the large area per pore. The L-shaped pore design was also utilized in the islet-encapsulating Nanogland device, in which laterally positioned 3.6 and 5.7 nm nanochannels produced a reduction in glucose diffusivity by 40% and 25% respectively compared to the diffusivity in bulk medium.

We have engineered a new generation of semipermeable membranes, SNM, with slit-pore designs initially investigated by Desai et al. The SNM exhibit a pore size distribution
of ~1%\textsuperscript{13,16-18} and a consistent pore size control in the range of 5-15 nm\textsuperscript{342} (Fig. 2.1). The slit pore microarchitecture of SNM was achieved by dry oxidation of polysilicon for the growth of silicon dioxide (SiO$_2$) (Fig. 2.5,D) and through backside patterning with deep ion-reactive etching (DRIE) which resulted in vertical sidewalls in each membrane window (Fig. 2.5,H). This process allows for fabrication of membranes with greater number of exposed nanopores per area compared to those with v-shaped sidewalls achieved by anisotropic KOH etching used by Desai \textit{et al.}\textsuperscript{355}. SNM were produced with an active membrane area (6X6 mm) consisting of ~10\textsuperscript{6} rectangular slit pores with ~7 nm in width, 300 nm in depth, and 2 µm in length (Fig. 2.1). The travel path could be further optimized by lowering the thickness of the membrane which can easily be controlled by the thin film low-pressure chemical vapor deposition (LPCVD) (Fig. 2.5,B) or dry etch process (Fig. 2.5,G). The utilization of a sacrificial layer to define the nanopores resulted in a membrane with a straight slit-pore path that presents a shorter distance for molecules to travel compared to the previous “L” pore design. The pore geometry could further influence the trade-off between selectivity and permeability of the membranes. The permeability – selectivity analysis for ultrafiltration demonstrated that membranes with slit-shaped pores showed higher performance and greater selectivity at a given value of permeability, than membranes with cylindrical pores for pore size below 100 nm\textsuperscript{343}. To circumvent the slow concentration-dependent diffusion occurred in size-restricted nanoporous membranes, the concept of using convection-dominated transport is more advantageous in terms of creating faster solvent movement under transmembrane pressure gradient\textsuperscript{359}, which efficiently drags small molecules such as glucose and insulin across membranes to the encapsulated cells.
SNM permeability and selectivity characterization

Permeability and selectivity of the SNM were characterized with the hydraulic permeability testing setup (Fig. 2.6), which uses liquid flow through planar nanoporous membranes under tangential-flow filtration operation. We demonstrated that SNM with pore sizes of 7 nm generated a hydraulic permeability of 130 ml/hr/m²/mmHg, which is much greater compared with conventional polymer membranes (~40 ml/hr/m²/mmHg) used in previous bioartificial pancreas devices. To further demonstrate the feasibility of SNM for immunoisolation, we then characterized the membrane selectivity against transport of cytokines and small molecules using the pressure-driven ultrafiltration system (Fig. 2.4). Solute transport was evaluated at ~2 psi driving pressure to mimic the typical physiological pressure difference between artery and vein, which results in an ultrafiltration rate of ~4 ul/min. The membrane Peclet number (Pe) for the pressure-driven ultrafiltration system was significantly greater than 1, suggesting that convective transport dominates. The observed sieving coefficients (calculated using Eq. 1) should reflect the rejection characteristics of the membrane. After 6 hours, the sieving coefficients of TNF-α, IFN-γ, and IL-1β were 0.16, 0.27, and 0.27, respectively (Fig. 2.7). In contrast, the sieving coefficients of glucose and insulin quickly reached 1 (Fig. 2.7).

These data collectively demonstrate that SNM provide about 80% rejection of cytokine passage, while allowing complete transport of small molecules. Because concentration polarization and transmembrane diffusion were negligible in this experimental system, the observed sieving coefficient should be equal to the product of the solution partition coefficient (Φ) and the convective hindrance factor (K_c). Previously, Dechadilok and Deen derived an analytic expression for the product of ΦK_c which describes a rigid sphere passing in a slit-shaped pore:

$$\Phi K_c = 1 - 3.02\lambda^2 + 5.776\lambda^3 - 12.3675\lambda^4 + 18.9775\lambda^5 - 15.2185\lambda^6 + 4.8525\lambda^7$$ (Eq. 1)
where $\lambda$ is the relative solute size indicating the ratio between the diameter of the molecule and the width of slit-pore channel. Based on the observed sieving coefficients of cytokines (Fig. 2.7), we can calculate the corresponding relative solute sizes $\lambda$ from Deen’s model (Eq. 2) for TNF-$\alpha$, IFN-$\gamma$, and IL-1$\beta$ as 0.83, 0.74, and 0.74, respectively. The experimental relative solute sizes of these cytokines are larger than the theoretic values, as indicated by Stokes-Einstein’s radius\textsuperscript{10} (Fig. 2.8). This difference in relative solute sizes between the experimental and theoretical values could be explained by the fact that cytokines are not strictly spherical: TNF-$\alpha$ is a packed cubic shape consisting of trimers formed with $\beta$-sheet structure\textsuperscript{36}, IFN-$\gamma$ is a globular

Figure 2.7 Transport of various molecules through slit-pore of SNM under a pressure difference of ~2psi. Sieving coefficients (S) were expressed as the ratio of the concentration of the filtrate over the concentration of the feed (means ± SE). BSA was used as a negative control. Results showed that the sieving coefficients of TNF-$\alpha$, IFN-$\gamma$, and IL-1$\beta$ were 0.16, 0.27, and 0.27 after 6 hours, respectively. The sieving coefficients of glucose and insulin quickly reached 1. These data indicated that small molecules such as glucose and insulin completely passed the SNM whereas the entry of cytokines was greatly hindered under convective transport.
dimer with flattened elliptical shaped subunits\textsuperscript{362}, and IL-1\(\beta\) has \(\beta\)-strands wrapped around in a tetrahedron-like fashion\textsuperscript{363}. Furthermore, the electrostatic interactions associated with diffuse electrical double layer (EDL) around charged proteins could also increase the overall molecule size\textsuperscript{364,365}, thereby overestimating the experiment relative solute sizes.

In summary, the SNM enables higher levels of ultrafiltrate production and demonstrate selective rejection against middle molecules like cytokines. Therefore, by encapsulating islets in SNM, we postulate that the increased convective mass transport of nutrients and glucose can support islet viability and insulin production, while the selective rejection of immune components enables exceptional immunoisolation.

**Assessment of SNM-encapsulated islets cultured under mock-loop circuit**

The feasibility of developing an implantable SNM-encapsulated bioartificial pancreas device using convective transport was demonstrated using a mock-loop setup. The middle cell chamber is sandwiched between two membranes to closely mimic the \textit{in vivo} conditions where SNM-encapsulated islets will be mounted as an arterio-venous (AV) graft (Fig. 2.2). The
pressure difference between the artery and vein will generate the ultrafiltrate and drive transport of water, salts, glucose, insulin, and other small molecules through the SNM, while passage of immune components such as cytokines will be blocked.

After passing the cytokine-contained media from the reservoir through the mock-loop circuit for 6 hr under applied physiological pressure ~2psi, samples that were collected from the top, middle, and bottom chambers of the flow cell device were compared against the reservoir concentration. The level of cytokines TNF-α, IFN-γ, and IL-1β were significantly reduced to 30%, 35%, and 34% in the middle chamber, whereas small molecules insulin and glucose passed completely (~100%) through both membranes (Fig. 2.9). To further examine the SNM-encapsulated islets under convective transport in the proposed mock-loop circuit, mouse islets were loaded into the middle chamber with or without cytokine circulation for 6 hr. The static culture incubated with cytokines showed a more than 2.2-fold increase in cell death compared to the static culture without cytokines, mock-loop device without cytokines, and mock-loop flow cell device with cytokines (Fig. 2.10). Moreover, no significant change in islet viability was observed among the static culture without cytokines, mock-loop device without cytokines, and mock-loop flow cell device with cytokines (Fig. 2.10). This demonstrated the effectiveness of SNM to protect islets from pro-inflammatory cytokine attack maintaining islet viability.

Additionally, the static culture without cytokines, mock-loop device without cytokines, and mock-loop flow cell device with cytokines demonstrated a 3.0-fold, 2.6-fold, and 4.1-fold changes, respectively, in the amount of insulin secreted during high glucose challenge compared with those secreted during low glucose challenge, respectively (Fig. 2.11). However, the static culture incubated with cytokines exhibited little variation in insulin secretion upon changes in
Figure 2.9 Assessment of solute distribution in the mock-loop system. The mock-loop circuit was composed of two membranes dividing the flow cell into the top, middle, and the bottom compartments. Concentration of solutes from each chamber was assessed at the end of the 6 hr experiment and was expressed as a percentage (mean ± SE) relative to that of the feed solution. Silicon micropore membrane (SµM) consisted of 1000 nm diameter slit pores were used as control. The data showed that the amount of TNF-α, IFN-γ, and IL-1β were significantly reduced to 30%, 35%, and 34% in the middle chamber, whereas small molecules insulin and glucose passed completely (~100%) through SNM under convective flow. However, all molecules including cytokines passed into the middle chamber that were sandwiched between SµM. (n>3, *p < 0.05).

glucose level (Fig. 2.11) due to loss in islet viability (Fig. 2.10). The glucose challenge demonstrated that the SNM-encapsulated mouse islets responded properly to changes in glucose level, whereas cytokine-infiltrating mouse islets lost their insulin-secreting ability to sense glucose stimuli. These data confirmed the usefulness of SNM to provide desired immunoisolation to support the viability and functional performance of the encapsulated islets.
Figure 2.10 In vitro viability of mouse islets under cytokine exposure. A) Viability of SNM-encapsulated mouse islets was measured following the 6 hr experiment in which islets were subjected to culture solution circulating the mock-loop circuit at 5ml/min with a pressure difference of 2 psi. B) Viable (green) and dead (red) cells were stained for control static culture (a,b) and SNM-encapsulated mouse islets (c,d). Experiments with cytokine exposure (indicated by +Ck) consisted of media containing TNF-α, IFN-γ, and IL-1β. The viability of islets was calculated based on the ratio of dead cells (in red) over the islet area. Viabilities of islets in static cultures were evaluated as control for comparison. SNM protected encapsulated mouse islets from pro-inflammatory cytokines (SNM, +Ck), which showed similar viability to SNM-encapsulated mouse islets without cytokine exposure (SNM, -Ck) and control static culture without cytokine exposure (Control, -Ck). Control static culture with cytokine exposure (Control, +Ck) showed significantly more cell death compared with other groups. (n>3, *p < 0.05).
In this study, we have developed and characterized an improved silicon nanopore membrane, SNM, for the encapsulation of pancreatic islets under convective flow. The SNM structure was specifically designed to obtain a well-defined slit pore in the nanometer range with a remarkably high hydraulic permeability. Furthermore, we have showed for the first time that SNM achieved high molecule selectivity against middle molecules such as cytokines under convective flow. Islets were subjected to media containing low-glucose, high-glucose, and low-glucose for 15 min each. Experiments with cytokine exposure (indicated by +Ck) consisted of culture solution containing TNF-α, IFN-γ, and IL-1β. The static culture without cytokines (Control, -Ck), mock-loop device without cytokines (SNM, -Ck), and mock-loop flow cell device exposed with cytokines (SNM, +Ck) had a 3.0-fold, 2.6-fold, and 4.1-fold increase in the amount of insulin secreted during high glucose challenge over those secreted during low glucose phase, respectively. However, the control static culture with cytokine exposure (Control, +Ck) secreted limited amount of insulin upon high glucose challenge due to the dead cells damaged by cytokine infiltration. (n>3, *p < 0.05).

2.4 CONCLUSIONS

In this study, we have developed and characterized an improved silicon nanopore membrane, SNM, for the encapsulation of pancreatic islets under convective flow. The SNM structure was specifically designed to obtain a well-defined slit pore in the nanometer range with a remarkably high hydraulic permeability. Furthermore, we have showed for the first time that SNM achieved high molecule selectivity against middle molecules such as cytokines under convective flow.
convective transport and provided adequate immune-protection to the encapsulated islets while generating sufficient filtrate to support viability and functionality of the encapsulated islets. Successful islet encapsulation with SNM could potentially reduce the immunosuppressive drugs and their side effects resulted from current therapies and lead to the possibility of encasing xenogeneic and stem-cell derived cell sources to overcome donor shortage for T1D treatment in the future. Further work is needed to optimize the SNM, including configuration of the slit-pores and extra reduction of membrane thickness, and study SNM-encapsulated graft performance and its immune-barrier function *ex vivo*. 
CHAPTER III

GLUCOSE-STIMULATED INSULIN RESPONSE OF SILICON NANOPORE IMMUNOPROTECTED ISLETS UNDER CONVECTIVE TRANSPORT AND CYTOKINE EXPOSURE

3.1 INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease that results in destruction of the insulin-producing β-cells within the pancreatic islets of Langerhans. Islet transplantation offers a promising way to treat T1D by directly infusing cadaveric islets into the portal vein of the recipient’s liver. The transplanted islets engraft and produce insulin in response to serum glucose levels. However, primary challenges affecting islet graft survival are donor availability, poor engraftment, and compounded immune response to the transplant in addition to adverse side effects from life-long immunosuppression. Cell encapsulation with a selective, semi-permeable membrane provides an attractive means to protect islets from immune damage while allowing the exchange of nutrients and small molecules. Previous studies have showed that large antibodies (IgM: 950,000 Da) and complement (C1q: 410,000 Da) were blocked by a membrane with a maximum pore diameter of 30 nm. However, pro-inflammatory cytokines including Tumor Necrosis Factor-alpha (TNF-α) (17,300 Da; Stokes diameter: 3.80 nm), Interferon-gamma (IFN-γ) (15,600 Da; Stokes diameter: 3.67 nm), and Interleukin-1 beta (IL-1β) (17,500 Da; Stokes diameter: 3.81 nm) that are known to synergistically induce cytotoxic effects on islets are much smaller in size and are more challenging to exclude. Although a nominal
pore size on the scale of nanometers is advantageous for creating an immunoprotective
environment\textsuperscript{368-370}, solutes diffuse slower through the pores as their sizes approach the critical
geometric dimension of the pores\textsuperscript{371}. The slow diffusion and resulting impact on mass transport
have been widely investigated experimentally and computationally for porous materials with
nanometer-sized pores\textsuperscript{372-374}. For example, the diffusion of 45 nm nanoparticles in polyethylene
glycol (PEG)-passivated 300 nm cylindrical pores decreased by a factor of 2 due to
hydrodynamic friction\textsuperscript{375}. The large diffusion gradients imposed by nano-scale pores can result
in: (1) cell necrosis and hypoxia if insufficient nutrients and oxygen are delivered to the encased
islets, and (2) compromised insulin secretion kinetics due to delayed glucose diffusion. The
reduced nutrients and oxygen availability due to the slow diffusion can directly impact islet
viability in as little as six hours and cause \textasciitilde50% decrease in glucose-stimulated insulin
secretion\textsuperscript{376}.

This slow concentration-dependent diffusion through size-restricted nanoporous
membranes can potentially be circumvented by using convection-dominated transport since this
would create faster solvent movement under a transmembrane pressure gradient, which in turn
would efficiently drag small molecules such as glucose and insulin across membranes to the
encapsulated cells. We previously reported that silicon nanopore membrane (SNM) with 7 nm
wide slit-shaped pores, used under convective transport, allowed undisturbed passage of glucose
and insulin through the membrane, and also reduced the passage of pro-inflammatory cytokines,
namely TNF-\textalpha, IFN-\textgamma, and IL-1\textbeta, by 80\%\textsuperscript{370}. Before this approach can be used in the clinical
setting, it will be important to characterize the glucose-insulin kinetics of the encapsulated islets
in reflecting glucose-insulin kinetics on a physiologically relevant time-scale.
In the present study, SNM and silicon micropore membrane (SµM) with 7 nm and 1000 nm-wide slit-shaped pores respectively, were used to encapsulate mouse islets under diffusive and convective conditions with and without cytokine exposure (Fig. 3.1). The islets were then exposed to varying concentration of glucose inside the reservoir culture medium, and glucose-stimulated insulin responses and islet viability were evaluated. In addition, to determine the immunoprotective effect of the membranes, a highly concentrated cocktail of pro-inflammatory cytokines was added to the circulating system to challenge the encapsulated islets.

**Figure 3.1** Schematic diagram of the perfusion system for *in vitro* assessment of glucose-stimulated insulin secretion from SNM- and SµM-encapsulated mouse islets. A peristaltic pump circulated media through the upper compartment of a two-layered flow cell separated by a SµM/SNM. Islets were encapsulated in the bottom chamber. A 3-way valve was incorporated into the system to establish transmembrane pressure. A set of 75 µL perfusate samples were collected from the bottom chamber at 10-minute intervals for up to 1.5 hours. The bottom chamber was replenished with media following each collection and correction for dilution was made in calculation of the insulin concentration.
3.2 MATERIALS AND METHODS

SNM were designed to have an active membrane area (6 X 6 mm) consisting of ~10^6 rectangular slit pores with an average pore size of 7 nm in width, 2 μm in length, and 300 nm in depth (Fig. 3.2). The surface of SNM was coated with polyethylene glycol (PEG) to minimize protein fouling. All SNM used in this study exhibited a measured average pore size of ~7 nm post pegylation. The control silicon micropore membrane (SμM) had the same design, but with an average pore size of 1000 nm. In this study, we observed how encapsulated islets responded to changes in glucose concentration across a single silicon membrane under convective transport (~2 psi transmembrane pressure) or diffusive transport (0 psi transmembrane pressure) using a

![Diagram of silicon nanopore membranes](image_url)

**Figure 3.2** (a) Schematic for fabrication of silicon nanopore membranes (SNM). (A) Piranha clean of double side polished Si wafer. (B) Thermal oxidation growth of SiO2 and low pressure chemical vapor deposition (LPCVD) of polysilicon. (C) Dry-etch patterning of polysilicon. (D) Thermal oxidation growth of SiO2 for use as sacrificial layer defining nanopores. (E) Patterning of anchor layer by wet etch. (F) LPCVD of polysilicon. (G) Blanket-etch of polysilicon until exposure of vertical SiO2 nanopores. (H) Deposition of low temperature oxide (LTO) for membrane protection and backside etch of membrane with deep reactive ion etching. (I) Dry etch removal of LTO and wet etch release of SiO2.
pressure-driven filtration circuit. We further tested this glucose-insulin response by using highly concentrated cytokine solution in the circuit. We subsequently analyzed the respective stimulation index (SI) and shut-down index (SDI) of encapsulated islets under convective and diffusive conditions. We also studied the rate of change in insulin production based on the slopes of curves that were fitted on glucose-insulin kinetics graphs to describe the quickness of insulin being secreted as glucose concentration changes. Finally, we characterized the viability of encapsulated islets in the pressure-driven filtration assembly under various mass transfer and cytokine exposure conditions.

3.2.1 SUBSTRATE PREPARATION

Silicon Nanopore Membranes (SNM) and Silicon Micropore Membrane (SµM):
architecture and fabrication

Silicon nanopore membranes (SNM) have been prototyped from silicon substrates by MEMS technology as previously reported with some modifications (Fig. 3.2). Briefly, the process used the growth of a thin SiO₂ (oxide) layer on 400 µm-thick double side polished (DSP) silicon wafers followed by a low pressure chemical vapor deposition (LPCVD) of polysilicon (~500 nm). The wafers were then specifically patterned, dry oxidized, wet etched, deposited with a second polysilicon layer, and finally blanket-etched until 400 nm of polysilicon remained and the underlying vertical oxide layer was exposed. The vertical sacrificial oxide layer defined the critical nanoscale pore size of the membranes. The low temperature oxide (LTO) (~1 µm) was deposited onto polysilicon of the wafers to serve as the hard mask for membrane protection. Deep reactive ion etching (DRIE) removed the backside of each window until membranes were disclosed. Eventually, the sacrificial oxide was etched away in 49% hydrofluoric acid (HF) during the final step of the fabrication process to leave behind open
nanoscale slit pores. The wafers were subsequently cut into 1×1 cm chips with an effective area of 6×6 mm² containing 1500 windows each, with a total of 10⁶ pores per membrane. Each rectangular pore was 7 nm in width, 300 nm in depth, and 2 µm in length. Silicon Micropore membrane (SµM) were fabricated to produce wafer-scale arrays of 500 nm by 4 µm rectangular slit pores with 1000 nm-wide slit width using similar process. The wafers were diced to form 1X1 cm chips with an effective area of 6×6 mm² containing 1500 windows each, with a total of 3.12⁶ pores per membrane. All membranes were cleaned using a conventional “piranha” clean procedure, which involved a 20 min-immersion in 3:1 sulfuric acid (H₂SO₄)/hydrogen peroxide (H₂O₂) mixture, followed by thorough rinses in deionized (DI) water. Images of SNM were obtained using scanning electron microscope (SEM) (Leo 1550) (Fig. 3.2).

Surface modification of SNM with poly(ethylene glycol) (PEG)

SNM were covalently modified with PEG using a previously reported protocol with some modifications to prevent protein fouling on the membrane surface. The technique used for PEG attachment involved a single reaction step which covalently couples silicon surface silanol group (Si-OH) to a chain of PEG polymer through a trimethoxysilane group forming a Si-O-Si-PEG sequence. Briefly, SNM were immersed in a solution of 3 mM 2-[methoxy(polyethyleneoxy)propyl]trimethoxysilane (PEG-silane) (Gelest: SIM6492.7) in toluene for 2 hr at 70 °C. A series of extensive washing steps involving toluene, ethanol, and DI water were used to rinse away unbounded PEG residue.

Hydraulic permeability for SNM pore size characterization

An automated mass and pressure measurement system was utilized for characterizing liquid flow through the SNM under a tangential-flow filtration operation. The pore size of the SNM can be related to filtration flow parameters using

\[ h = \sqrt[3]{\frac{12 \mu Q}{nwAP}} \]  

(Equation 1), where h is pore...
width, μ is the viscosity, l is the membrane thickness, Q is the volumetric flow rate, n is the number of pores per membrane, w is the pore length, and ΔP is the transmembrane pressure. To assemble the overall system for SNM pore size characterization (Fig. 3.3), air was applied through a syringe pump (Sigma: Z675709) into a water reservoir. Water was circulated by a peristaltic pump (Masterflex: 07551-00) through a differential pressure transducer (Omega: PX429 015GI), a flow cell with enclosed membrane, and returned to the original water reservoir. The flow cell was assembled with the SNM submerged under water to remove air bubbles from all compartments. Specifically, a membrane was positioned with the polysilicon interface facing down with a customized silicone gasket positioned on top of the membrane, followed by the final

**Figure 3.3** Schematic diagram of the hydraulic permeability testing system. Air was applied through a pressure regulator into the liquid reservoir. A peristaltic pump circulated this liquid through the flow cell with enclosed membrane. The flow cell connected to a differential pressure transducer that was automatically controlled by a data acquisition laptop to adjust the transmembrane pressure. The permeated ultrafiltrate was collected into a liquid container on top of a precision mass balance. Data from the differential pressure transducer and the mass balance were automatically collected and stored in a data acquisition laptop.
placement of a filtrate chamber on top of the gasket. All sections were fastened together and secured to the base with hand-tightened hex bolts until the gasket was visibly compressed. The ultrafiltrate permeated through the membrane was routed to a liquid collection container that rested on a precision mass balance (Mettler Toledo: XS205). Measurements from the differential pressure transducer and the mass balance were automatically collected with a data acquisition laptop. A typical membrane hydraulic permeability test consisted of 5 ml/min flow rate and 4 pressure cycles (5, 1, 5, and 1 psi) for durations of 150 s each. Using the specifications for pore length, membrane thickness, and total number of pores provided based on individual wafer designs, the average pore size of SNM was calculated using Equation 1. All SNM membranes in this study were surface-modified with PEG and exhibited an average pore size of ~7 nm.

3.2.2 CULTURE OF MEMBRANE-ENCAPSULATED ISLETS IN THE PRESSURE-DRIVEN FILTRATION ASSEMBLY

All procedures described involving isolation of mouse islets were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, San Francisco (UCSF). Mouse islets were isolated from 8 to 10-week-old male B6 mice (Jackson Laboratories) based on previously described protocols370. Harvested islets were maintained in suspension culture with RPMI 1640 with L-glutamine and 11.1 mM glucose (Gibco: 11875-093), 10% fetal bovine serum (FBS) (Gibco: 16000), and 1% penicillin-streptomycin (P/S) (UCSF Cell Culture Facility: CCFGK003).

We assembled a mock-loop circuit with two flow cell components370. Briefly, one SNM with customized silicone gasket frames were sandwiched in between two flow cell components. A group of 40-50 mouse islets were introduced into the bottom chamber separated by the SNM
from the circulating fluid (5 ml/min) in the top chamber. A peristaltic pump drove the fluid through the top of the flow cell component, and finally back to the original medium reservoir. For convective experiments, a three-way valve was used to create flow resistance for a physiological pressure difference ~2 psi between the top and the bottom compartments of the flow cell. The membrane Peclet number (Pe) for the pressure-driven ultrafiltration system was significantly greater than 1, suggesting that convective transport dominates. For diffusive experiments, no transmembrane pressure was induced and fluid still circulated throughout the system. To study the effects of cytokines on SNM-encapsulated islets, solution consisting of mouse cytokines TNF-α (2,000 U/ml), IFN-γ (1,000 U/ml), and IL-1β (10,000 U/ml) was added to the original reservoir. Silicon membranes with 1 μm-wide slit pores (SμM) were used as the control with adjusted pressure (~0.127 psi) and flow rate (~20 µl/min) to produce similar amount of ultrafiltrate as the SNM in this mock-loop system. Naked mouse islets cultured under static conditions were also used as controls.

**Glucose challenge in the pressure-driven filtration mock-loop system**

The membrane-encapsulated mouse islets in the mock-loop systems were exposed to a series of low (1.6 mM), high (16.6 mM), and low (1.6 mM) glucose (Gibco: 11879) stimulation for 30 min each. Supernatant was sampled every 10 min from the bottom islet chamber during this series of glucose challenge. For convective experiments, an ultrafiltrate rate of ~3.5 ul/min was observed for the SNM with ~7 nm pore size and the same ultrafiltrate rate was obtained for the SμM with lowered transmembrane membrane pressure and system flow rate. For diffusive experiments, islet chambers were re-filled after individual sampling to ensure that the volume of islet chamber was kept constant at all time. This step minimized the any bubbles that might potentially be formed during the process which could hinder mass transfer within the system.
Insulin content was measured with mouse insulin enzyme-linked immunosorbent assay (ELISA) kits (Mercodia: 10-1247-01) with accounted dilutions and normalized by extracted total protein concentration (Thermo: 78505; 23225). Naked mouse islets were also challenged under static culture condition as controls. About 7-10 μl chamber fluid per islet were used in all cases.

**Analysis of stimulation index (SI) and shut-down index (SDI)**

A stimulation index was calculated as the ratio of stimulated to basal insulin secretion. In our study, the stimulation index (SI) was the ratio of (1) the first insulin collection point in the high glucose phase to the last insulin collection point of the previous low glucose phase (stimulation/pre-stimulation), and (2) the highest insulin secretion in the high glucose phase to the last insulin collection point of the previous low glucose phase (highest stimulation/pre-stimulation). The shut-down index (SDI) was calculated as the ratio of (1) the first insulin collection point in the subsequent low glucose phase to the last insulin collection point in the high glucose phase (post-stimulation/stimulation), and (2) the lowest insulin secretion in the subsequent low glucose phase to the last insulin collection point in the high glucose phase (lowest post-stimulation/stimulation). The stimulation index indicates the magnitude of insulin released as stimulated by a higher concentration of glucose, whereas the shut-down index reflects the magnitude of cessation in insulin production once glucose concentration returns to normal.

**Analysis of rate of change in insulin secretion**

The rate of change in insulin secretion was calculated for the stimulation and shut-down phases. For the stimulation phase, a curve was fitted on the glucose-insulin kinetic graph with the last point of insulin produced during low glucose exposure to the highest point of insulin produced during high glucose exposure. For the shut-down phase, a curve was fitted on the glucose-insulin kinetic graph with the last point of insulin produced during high glucose
exposure to the first point of insulin produced during low glucose exposure. The rate of change was obtained by taking derivatives of those curves to study the quickness of insulin being secreted as changes in glucose concentration.

**Islet viability**

Islet viability was assessed by double staining with live green and dead red solutions (Invitrogen: R37601). Briefly, mouse islets were incubated in live green and dead red solutions for 15 min at room temperature followed by extensively washes in PBS to remove excess staining. Images of mouse islets were obtained using laser scanning Nikon Spectral C1si confocal microscope (Nikon Instruments). Viability of islets was calculated based on the percentage of live cells in the islets as described by protocol on assessment of islet viability by fluorescent dyes from Department of Surgery Division of Transplantation at University of Wisconsin-Madison.

**Statistical Analysis**

Sample pairs were analyzed using Student’s t-test. Multiple samples were evaluated with one-way or two-way analysis of variance (ANOVA) followed by Bonferroni and multiple comparison using Graphpad Prism software (San Diego, CA). A p value of <0.05 was accepted as statistically significant for all analyses.

**3.3 RESULTS AND DISCUSSION**

We constructed a benchtop flow loop circuit consisting of a single membrane that separated islets from the circulating fluid (Fig. 3.1). Using this system, we characterized the glucose-insulin kinetics of SNM- and SµM-encapsulated mouse islets under both convective and diffusive transport modalities. We further analyzed the effect of cytokine exposure to the function of SNM- and ability of SNM-encapsulated islets by adding a highly concentrated
cocktail of pro-inflammatory cytokines including TNF-α, IL-1 β, and IFN-γ to the circuit. The ability of membrane-encapsulated islets to secrete insulin upon changes in glucose concentration was characterized by: (1) computing the stimulation index (SI) and shut-down index (SDI) (see section 3.2.2), which reflect the magnitude of stimulatory and shut-down insulin response as a function of changes in glucose concentration, respectively; and (2) characterizing the rate of change in insulin secretion as the ambient fluid changed from low-to-high and high-to-low glucose concentrations. We also assessed the viability of encapsulated-islets in the mock-loop circuit at the end of the experimental conditions.

**Membrane fabrication and characteristics**

Sufficient amounts of antibodies, complement, and cytokines could transfer through the membrane to cause immune-mediated cell death if even 1% of the membrane pores exceed the desired pore size cut-off\(^{368}\). Conventional polymeric membranes with 30% nominal pore size distribution are clearly unsuitable for providing the necessary protection from these inflammatory substances\(^{339}\). We have developed new encapsulating membranes based on microelectromechanical systems (MEMS) technology initially pioneered by Ferrari and colleagues\(^{340,341}\) to create more uniform pore sizes at the nanometer scale. These semipermeable filtration membranes, silicon nanopore membranes (SNM), exhibit a pore size distribution of ~1% and a consistent controllable pore size in the range of 5-15 nm\(^{370,377}\). The slit pore microarchitecture of SNM is produced by dry oxidation of polysilicon for the growth of silicon dioxide (SiO\(_2\)) followed by backside patterning with deep ion-reactive etching (DRIE) that produces vertical walls in each membrane window (Fig. 3.2,a). The utilization of a sacrificial layer to define the nanopores results in a membrane with a straight pore path that exhibits a shorter travel path compared to the “L” pore design reported for the Nanogland device\(^{12}\). The
SNM wafer is diced into 1 cm x 1 cm chips, each with an active membrane area (6 x 6 mm) consisting of ~10^6 rectangular slit pores with ~7 nm width, 300 nm depth, and 2 μm thickness (Fig. 3.2, (b&c)). Using similar fabrication techniques, silicon micropore membranes (SµM) chips were produced, each with an active membrane area (6 x 6 mm) consisting of 3.12 X10^6 rectangular slit pores with ~1000 nm in width, 500 nm in depth, and 4 μm in length (Fig. 3.2, (d&e)). Previously, we demonstrated that SNM with ~7 nm pore size resulted in a 3.25-fold

Figure 3.2 (b) An SEM image of the tilted membrane surface which depicts nanopores with 2 μm in length. (c) An SEM image of the cross-section of the membrane which depicts nanopores with 7 nm in width and 300 nm in depth. (d) An SEM image of the membrane surface which depicts micropores with 4 μm in length. (e) An SEM image of the cross-section of the membrane which depicts micropores with 1 μm in width.
increase in hydraulic permeability compared with conventional polymer membranes used in other bioartificial pancreas devices\textsuperscript{370}. Whereas the S\textsubscript{µ}M allowed complete passage of all molecules, SNM demonstrated size selectivity with an \textasciitilde80\% rejection of cytokine passage, while allowing complete transport of glucose and insulin\textsuperscript{370}.

**Kinetics of glucose-stimulated insulin secretion of encapsulated islets**

Although the SNM has shown promise in immunoisolation while preserving islet viability and function under convective transport\textsuperscript{370}, it is important to investigate how SNM-encapsulated islets respond to glucose changes in surrounding fluid. Long-term glycemic control is perhaps the most important factor in the management of T1D and its related risks of microvascular complications and cardiovascular diseases\textsuperscript{367}. Chronic hyperglycemia leads to serious microvascular complications\textsuperscript{28} while hypoglycemia can result in cognitive impairment, unconsciousness, seizures, and even death\textsuperscript{28}. About 30-40\% patients with T1D have impaired awareness of hypoglycemia and this confers a 3 to 6-fold increased risk of severe hypoglycemic events\textsuperscript{379,380}. Therefore, understanding the real-time kinetics of SNM-encapsulated islets could offer us further insights on the responsiveness of our capsules, avoiding potential episodes of hypo- and hyperglycemia once they are implanted in the body.

**No cytokine exposure**

Convection-dominated transport can be used to improve solvent transport by efficiently dragging small molecules across size-restricted pores to encapsulated cells based on transmembrane pressure gradient, thus preventing the delays associated with concentration-dependent diffusion in nanoporous membranes\textsuperscript{359}. Based on this principle, we used a benchtop flow loop circuit incorporating membrane-encapsulated islets under applied physiological transmembrane pressure\textsuperscript{352} (Fig. 3.1). We observed how encapsulated islets responded to
changes in glucose concentration across a single silicon membrane under convective transport (~2 psi transmembrane pressure) or diffusive transport (0 psi transmembrane pressure) using this flow circuit. Unencapsulated islets cultured under static conditions were used as controls. Islets under all conditions reacted quickly to the high glucose concentration (16.6 mM) within the first 10 minutes by producing more insulin (40 minute time point; Fig. 3.4,a).
Figure 3.4 (a) Insulin release kinetics of SNM-encapsulated mouse islets during 90-minute low-high-low (1.6 mM, 16.6 mM, 1.6 mM) glucose stimulation under convective (2 psi) (Conv) and diffusive transport (Diff) without subjection to cytokines (-Ck). The naked islets cultured under static conditions were served as controls (Control, -Ck). The SNM-encapsulated islets under convective transport (SNM, Conv, -Ck) exhibited higher insulin secretion following stimulation at high glucose concentration and faster insulin release kinetics in response compared to those under diffusive transport (SNM, Diff, -Ck). (Mean±SEM, n≥3) (b) The stimulation index (SI) was calculated as the ratio of (1) the first insulin collection in the high glucose phase at 40 minutes to the last insulin collection point of the previous low glucose phase at 30 minutes (Stimulation/Pre-stimulation), and (2) the highest insulin secretion in the high glucose phase to the last insulin collection point of the previous low glucose phase at 30 minutes (Highest stimulation/Pre-stimulation). The SI indicates the magnitude of insulin released as stimulated by a higher concentration of glucose. Without cytokine exposure (-Ck), SNM-encapsulated islets under convection (SNM, Conv) and diffusion (SNM, Diff) in addition to the naked islets cultured under static conditions (Control) all exhibited similar magnitude of glucose-induced insulin secretion when transitioning from low glucose to high glucose (Stimulation/Pre-stimulation).
However, the SI of SNM-encapsulated islets under convection (SNM, Conv) was the highest compared to that under diffusion (SNM, Diff) and the naked islets cultured under static conditions (Control) when the highest insulin secretion in the high glucose phase was used (Highest Stimulation/Pre-stimulation). (Mean±SEM, n≥3) (c) The shut-down index (SDI) was the ratio of (1) the first insulin collection point in the subsequent low glucose phase at 70 minutes to the last insulin collection point in the high glucose phase at 60 minutes (Post-stimulation/Stimulation), and (2) the lowest insulin secretion in the subsequent low glucose phase to the last insulin collection point in the high glucose phase at 60 minutes (Lowest Post-stimulation/Stimulation). The SDI reflects the magnitude of cessation in insulin production once glucose concentration returns to normal. Without cytokine exposure (-Ck), SNM-encapsulated islets under convection (SNM, Conv) exhibited the highest magnitude of insulin reduction compared to the diffusive condition (SNM, Diff) and the naked islet culture (Control) as glucose dropped low (Post-stimulation/Stimulation & Lowest Post-stimulation/Stimulation). (Mean±SEM, n≥3, *p<0.05)

The unencapsulated islets under static culture and SNM-encapsulated islets under diffusion reached the peak of the response 20 minutes after high glucose exposure, whereas insulin secretion of the SNM-encapsulated islets under convection continued to increase during the entire 30-minute duration of high glucose challenge (Fig. 3.4,a). Importantly, the naked islets under static culture and SNM-encapsulated islets under convection secreted more insulin compared to SNM-encapsulated islets under diffusion within the first 10 minutes of high glucose exposure. The SNM-encapsulated islets under convection continued with higher insulin production during this high glucose challenge, whereas the diffusion and static control cases reached a plateau in insulin secretion after 50 to 60 minutes. The quick insulin response within 5-10 minutes of high glucose exposure was consistent with normal functioning islets releasing insulin in a biphasic manner\(^{381,382}\) (e.g. the first insulin phase appeared within 5-10 minutes followed by a second sustained phase). Furthermore, the stimulation index (SI), calculated as the
ratio of the first insulin collection in the high glucose phase to the last insulin collection in the previous low glucose phase (Stimulation/Pre-stimulation), were generally comparable among naked islets under static conditions and the SNM-encapsulated islets under convection and diffusion cases, which were \(3.92 \pm 1.07\), \(6.38 \pm 0.44\), and \(5.62 \pm 1.51\), respectively (Fig. 3.4,b). However, when the highest level of insulin secretion from high glucose phase was used to calculate the magnitude of stimulation (Highest Stimulation/Pre-stimulation), the naked islets under static conditions and SNM-encapsulated islets under convection and diffusion cases showed SI of \(5.29 \pm 0.69\), \(8.92 \pm 1.35\), \(5.97 \pm 1.16\), respectively (Fig. 3.4,b). The SI of SNM-encapsulated islets under convection showed a 1.49-fold increase than that under diffusion.

Once the circuit was switched back to low glucose concentration (1.6 mM) from 60 to 90 minutes, the SNM-encapsulated islets under convection exhibited a rapid shutdown in insulin production whereas a gradual decrease in insulin production occurred for the capsule under the diffusive mode. The shut-down index (SDI), calculated as the ratio of the first insulin collection in the subsequent low glucose phase to the last insulin collection in the previous high glucose phase (Post-stimulation/Stimulation), showed that the amount of insulin that was secreted significantly decreased for SNM-encapsulated islets under convection \(0.20 \pm 0.03\) compared with the naked islets under static culture \(0.59 \pm 0.17\) and SNM-encapsulated islets under diffusion \(0.93 \pm 0.19\) (Fig 4.4,c). When the lowest level of insulin secretion from the subsequent glucose phase was used to calculate the magnitude of shut down (Lowest Post-stimulation/Stimulation), the SDI showed that the amount of secreted insulin significantly decreased for SNM-encapsulated islets under convection \(0.11 \pm 0.02\) compared with the naked islets under static culture \(0.40 \pm 0.09\) and SNM-encapsulated islets under diffusion \(0.42 \pm 0.11\) (Fig. 4.4,c). The SDI of SNM-encapsulated islets under convection showed a 3.86-fold
decrease compared to that under diffusion. The slow insulin activation and delayed shut-down response associated with diffusive transport is consistent with previous studies on islet encapsulating in hydrogels\textsuperscript{383,384} and restricted pore size in the sub-10 nm range\textsuperscript{12}. For example, the islet-encapsulating Nanogland device with lateral positioned 3.6 and 5.7 nm nanochannels reported a significant reduction in glucose diffusivity by 40% and 25% compared to the diffusivity in bulk medium, respectively\textsuperscript{12}. This delay of insulin shut-down that occurred in the diffusive scenario could potentially lead to problems in the clinical setting, since insulin production would be high in the face of falling blood glucose levels\textsuperscript{28}. In contrast, the SNM-encapsulated islets under convection showed the ability to quickly activate and cease insulin production, which is extremely important to simulating the physiological glucose-insulin response and avoiding dangerous episodes of hypoglycemia in the body.

As illustrated in Table 3.1, the rate of change in insulin production was monitored when conditions transitioned from low-high to high-low glucose phases. The rates of change in insulin activation and cessation were on the same scale in the naked islets under static culture as in the SNM-encapsulated islets under diffusion (0.86 and 0.84 for the stimulation and -0.71 and -0.42 for deactivation, respectively; Table 3.1). The SNM-encapsulated islets under convection showed 1.16- and 1.19-fold increase in the rate of glucose-stimulated insulin response and 3.82- and 6.45-fold decrease in the rate of insulin shut-down compared with the naked islets under static culture and SNM-encapsulated islets under diffusion, respectively. In short, the magnitude of glucose-stimulated insulin secretion was higher for SNM-encapsulated islets under convection compared to the naked islets under static culture and SNM-encapsulated islets under diffusion as indicated by the SI (Fig. 3.4,b). The SNM-encapsulated islets under convection showed the fastest rate of insulin production (~1 normalized insulin content min\textsuperscript{-1} (X 10\textsuperscript{-2})) and cessation (~-
2.7 normalized insulin content min\(^{-1}\) (X 10\(^{-2}\)) compared to the other two conditions (Table 3.1). These data demonstrated that the responsiveness of convection-based SNM encapsulation was significantly better compared to the islet culture under static conditions and SNM encapsulation under diffusion.

Table 3.1 The rate of change in insulin secretion without cytokine exposure. The rate of change in insulin production was calculated based on the slopes of curves that were fitted on glucose-insulin kinetics graphs to describe the quickness of insulin being secreted as glucose concentration changes. Without subjection to cytokines (-Ck), S\(\mu\)M-encapsulated mouse islets under convection (S\(\mu\)M, Conv) showed the fastest response following high glucose exposure while SNM- and S\(\mu\)M-encapsulated mouse islets under convection (SNM, Conv & S\(\mu\)M, Conv) exhibited similar rate of insulin cessation when glucose concentration returned to normal.

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>Low-High Glucose Stimulation (10(^{-2})) (normalized insulin content * min(^{-1}))</th>
<th>High-Low Glucose Shut-Down (10(^{-2})) (normalized insulin content * min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, -Ck</td>
<td>0.86</td>
<td>-0.71</td>
</tr>
<tr>
<td>SNM, Conv, -Ck</td>
<td>1</td>
<td>-2.71</td>
</tr>
<tr>
<td>SNM, Diff, -Ck</td>
<td>0.84</td>
<td>-0.42</td>
</tr>
<tr>
<td>S(\mu)M, Conv, -Ck</td>
<td>3.15</td>
<td>-3.36</td>
</tr>
</tbody>
</table>

Further comparison with the silicon micropore membrane (S\(\mu\)M)-encapsulated islets under convection showed that pressure-driven convection yields faster mass transport as the pore size becomes larger (1 \(\mu\)m). The naked islets under static culture, SNM-encapsulated islets under convection, and S\(\mu\)M-encapsulated islets under convection all quickly released more insulin during high glucose exposure from 40 to 60 minutes (Fig. 3.5,a). Whereas the level of insulin plateaued in the naked islets, the amount of secreted insulin increased in the SNM-encapsulated islets under convection from 50 to 60 minutes. However, the S\(\mu\)M-encapsulated islets under convection showed a maximum level of secreted insulin at 50 minutes followed by an immediate concentration drop at 60 minutes. The difference in the glucose-insulin kinetics between SNM-
and SμM-encapsulation under convection during high glucose challenge can be explained by: (1) the variation in the ultrafiltration rate produced by two different types of membranes despite efforts to adjust both membranes to obtain the same amount of ultrafiltrate (section 3.2); and (2) possible protein adsorption on the SNM\textsuperscript{377,385} that resulted in the lack of negative feedback inhibition of insulin secretion\textsuperscript{386} due to additional fouling resistance (Fig. 3.5,a). Furthermore, the SI indicating the magnitude of insulin secretion during pre-stimulation and stimulation (Stimulation/Pre-Stimulation) were higher for SNM- and SμM-encapsulation under convection compared to naked islets under static conditions, which were 6.38 ± 0.44, 6.44 ± 1.41, and 3.92 ±

![Graph a.](image1)

![Graph b.](image2)
Figure 3.5 (a) Insulin release kinetics of SNM- and SµM-encapsulated mouse islets during 90-minute low-high-low (1.6 mM, 16.6 mM, 1.6 mM) glucose stimulation under convective (2 psi) (Conv) without subjection to cytokines (-Ck). The naked islets cultured under static conditions were served as controls (Control, -Ck). Without cytokine exposure (-Ck), the SµM-encapsulated islets under convective transport (SµM, Conv) exhibited higher insulin secretion following stimulation at high glucose concentration and faster insulin release kinetics in response to glucose compared to the SNM-encapsulated islets under convective transport (SNM, Conv). (Mean±SEM, n≥3) (b) The stimulation index (SI) was calculated as the ratio of (1) the first insulin collection in the high glucose phase at 40 minutes to the last insulin collection point of the previous low glucose phase at 30 minutes (Stimulation/Pre-stimulation), and (2) the highest insulin secretion in the high glucose phase to the last insulin collection point of the previous low glucose phase at 30 minutes (Highest stimulation/Pre-stimulation). The SI indicates the magnitude of insulin released as stimulated by a higher concentration of glucose. Without cytokine exposure (-Ck), the SNM- and SµM-encapsulated islets under convection (SNM, Conv & SµM, Conv) all showed a higher magnitude of secreted insulin compared to the naked islets cultured under static conditions (Control). Furthermore, the SI of SµM-encapsulated islets under convection (SµM, Conv) was the greatest compared to that for the SNM (SNM, Conv) and naked islets cultured under static conditions (Control) when the highest insulin secretion in the high glucose phase was used (Highest Stimulation/Pre-stimulation). (Mean±SEM, n≥3) (c) The shut-down index (SDI)
was the ratio of (1) the first insulin collection point in the subsequent low glucose phase at 70 minutes to the last insulin collection point in the high glucose phase at 60 minutes (Post-stimulation/Stimulation), and (2) the lowest insulin secretion in the subsequent low glucose phase to the last insulin collection point in the high glucose phase at 60 minutes (Lowest Post-stimulation/Stimulation). The SDI reflects the magnitude of cessation in insulin production once glucose concentration returns to normal. Without cytokine exposure (-Ck), both SNM- and SµM-encapsulated islets under convection (SNM, Conv & SµM, Conv) exhibited significant magnitude of insulin reduction compared to the islets cultured under static conditions (Control) once glucose dropped back low (Post-stimulation/Stimulation & Lowest Post-stimulation/Stimulation). (Mean±SEM, n≥3, *p<0.05)

1.07, respectively (Fig. 3.5,b). When the highest amount of insulin secretion in the high glucose phase was used to calculate SI (Highest Stimulation/Pre-stimulation), SµM-encapsulation under convection (8.92 ± 1.34) and SµM-encapsulation under convection (11.8 ± 1.64) showed significantly higher SI compared to naked islets under static conditions (5.29 ± 0.69) (Fig. 3.5,b). The SDI calculated from the ratio of insulin secretion from post-stimulation and stimulation (Post-stimulation/Stimulation) for SNM- and SµM-encapsulation under convection were 0.20 ± 0.03 and 0.25 ± 0.09, which showed a significant decrease in the magnitude of insulin secreted during low glucose exposure compared to the naked islets (0.59 ± 0.17) (Fig. 3.5,c). This trend was also observed for SNM-encapsulation under convection (0.11 ± 0.02), SµM-encapsulation under convection (0.11 ± 0.01), and the naked islets (0.40 ± 0.09) when the SDI was calculated based on the ratio of lowest insulin secretion from post-stimulation and stimulation (Lowest post-stimulation/Stimulation) (Fig. 3.5,c).

In addition, the SµM-encapsulated islets under convection showed the fastest rate of response when switching from low to high glucose condition (3.15 normalized insulin content min⁻¹ (X 10⁻²)) to the high to low glucose situation (3.36 normalized insulin content min⁻¹ (X 10⁻
The SμM-encapsulated islets under convection demonstrated 3.66- and 3.15-fold increase in the rate of glucose-stimulated insulin response, and 4.73- and 1.24-fold decrease in rate of insulin shut-down compared with the naked islets under static culture and SNM-encapsulated islets under convection, respectively (Table 3.1). All rates of change in insulin production and cessation were comparable among the naked islets under static culture, SNM-encapsulated islets under diffusion, and SμM-encapsulated islets under diffusion (Table 3.1). Noticeably, membrane-encapsulation under diffusive scenarios showed a slower insulin response when stimulated with high concentration of glucose (Fig. 3.6 & 3.7). This could be due to the potential formation of boundary layer by adsorption of molecules in the nanoscale pores. Depending on the choice of membranes and methods to stimulate the islets (diffusion vs. convection), all experimental conditions had a SI ranging from 2.89 to 6.44 (including Fig. 3.6 & 3.7), which is consistent of typical values (2 to 20) for healthy mouse islets. Convective conditions with SNM- and SμM-encapsulation outperformed the pure diffusive scenarios during the glucose-insulin activation and shut-down phases. In particular, convective transport with SμM encapsulation demonstrated superior response in insulin activation while the insulin shut-down was observed to be similar for both SNM and SμM encapsulation under convection.
a. Normalized Insulin Content

b. Stimulation/Pre-stimulation


c. Post-stimulation/stimulation

Legend:
- Control, -Ck
- SuM, Conv, -Ck
- SuM, Diff, -Ck

Graphs showing various experimental conditions and measurements.
Figure 3.6 (a) Insulin release kinetics of SµM -encapsulated mouse islets during 90-minute low-high-low (1.6 mM, 16.6 mM, 1.6 mM) glucose stimulation under convective (2 psi) (Conv) and diffusive transport (Diff) without subjection to cytokines (-Ck). The naked islets cultured under static conditions served as controls (Control, -Ck). The SµM-encapsulated islets under convective transport (SµM, Conv, -Ck) exhibited higher insulin secretion following stimulation at high glucose concentration and faster insulin release kinetics in response compared to those under diffusive transport (SµM, Diff, -Ck). (Mean±SEM, n≥3) (b) The stimulation index (SI) was calculated as the ratio of (1) the first insulin collection in the high glucose phase at 40 minutes to the last insulin collection point of the previous low glucose phase at 30 minutes (Stimulation/Pre-stimulation), and (2) the highest insulin secretion in the high glucose phase to the last insulin collection point of the previous low glucose phase at 30 minutes (Highest stimulation/Pre-stimulation). The SI indicates the magnitude of insulin released as stimulated by a higher concentration of glucose. Without cytokine exposure (-Ck), SµM-encapsulated islets under convection (SµM, Conv) and diffusion (SµM, Diff) in addition to the naked islets cultured under static conditions (Control) all exhibited similar magnitude of glucose-induced insulin secretion (Stimulation/Pre-stimulation). However, the SµM-encapsulated islets under convection (SµM, Conv) showed the highest magnitude of insulin secreted when the highest insulin secretion in the high glucose phase was used (Highest stimulation/Pre-stimulation). (Mean±SEM, n≥3) (c) The shut-down index (SDI) was the ratio of (1) the first insulin collection point in the subsequent low glucose phase at 70 minutes to the last insulin collection point in the high glucose phase at 60 minutes (Post-stimulation/Stimulation), and (2) the lowest insulin secretion in the subsequent low glucose phase to the last insulin collection point in the high glucose phase at 60 minutes (Lowest Post-stimulation/Stimulation). The SDI reflects the magnitude of cessation in insulin production once glucose concentration returns to normal. Without cytokine exposure (-Ck), SµM-encapsulated islets under convection (SµM, Conv) exhibited the highest magnitude of insulin reduction compared to the diffusive condition (SµM, Diff) and the naked islet culture (Control) as glucose dropped low (Post-stimulation/Stimulation). When the lowest insulin secretion in the low glucose phase was used, SµM-encapsulated islets under convection (SµM, Conv) also showed the largest magnitude of insulin reduction (Lowest Post-stimulation/Stimulation). (Mean±SEM, n≥3, *p<0.05)
Figure 3.7 Insulin release kinetics of SNM- and SμM-encapsulated mouse islets during 90-
minute low-high-low (1.6 mM, 16.6 mM, 1.6 mM) glucose stimulation under diffusion (2 psi) (Diff) without subjection to cytokines (-Ck). The naked islets cultured under static conditions served as controls (Control, -Ck). Without cytokine exposure (-Ck), SµM-encapsulated islets under diffusive transport (SµM, Diff) exhibited higher insulin secretion that slowly plateaued following stimulation at high glucose concentration compared to the SNM-encapsulated islets under diffusive transport (SNM, Diff). (Mean±SEM, n≥3) (b) The stimulation index (SI) was calculated as the ratio of (1) the first insulin collection in the high glucose phase at 40 minutes to the last insulin collection point of the previous low glucose phase at 30 minutes (Stimulation/Pre-stimulation), and (2) the highest insulin secretion in the high glucose phase to the last insulin collection point of the previous low glucose phase at 30 minutes (Highest stimulation/Pre-stimulation). The SI indicates the magnitude of insulin released as stimulated by a higher concentration of glucose. Without cytokine exposure (-Ck), the SNM- and SµM-encapsulated islets under diffusion (SNM, Diff & SµM, Diff) all showed a similar magnitude of insulin secretion compared with the naked islets cultured under static conditions (Control) (Stimulation/Pre-stimulation). Moreover, the SNM-encapsulated islets under diffusion (SNM, Diff) and naked islets cultured under static conditions showed an increase in SI compared to the SµM-encapsulated islets under diffusion (SµM, Diff) when the highest insulin secretion in the high glucose phase was used (Highest stimulation/Pre-stimulation). (Mean±SEM, n≥3) (c) The shut-down index (SDI) was the ratio of (1) the first insulin collection point in the subsequent low glucose phase at 70 minutes to the last insulin collection point in the high glucose phase at 60 minutes (Post-stimulation/Stimulation), and (2) the lowest insulin secretion in the subsequent low glucose phase to the last insulin collection point in the high glucose phase at 60 minutes (Lowest Post-stimulation/Stimulation). The SDI reflects the magnitude of cessation in insulin production once glucose concentration returns to normal. Without cytokine exposure (-Ck), SNM- and SµM-encapsulated islets under diffusion (SNM, Diff & SµM, Diff) exhibited similar magnitude of insulin reduction compared to the islets cultured under static conditions (Control) once glucose dropped back low (Post-stimulation/Stimulation). However, the level of shut down was more significant in SµM-encapsulated islets under diffusion (SµM, Diff) than in the other two conditions (SNM, Diff & Control) when the lowest insulin secretion was used (Lowest Post-stimulation/Stimulation). (Mean±SEM, n≥3, *p<0.05)
**Cytokine exposure**

A highly concentrated solution of pro-inflammatory cytokines consisting of TNF-α, IFN-γ, and IL-1β was used to investigate how the glucose-insulin kinetics of SNM-encapsulated islets are influenced by cytokine exposure. When challenged with high glucose concentration, SNM-encapsulated islets under convection immediately secreted insulin to the maximum level within first 10 minutes followed by a slight decrease in insulin secretion in the next 20 minutes (Fig. 3.8,a). However, SNM-encapsulated islets under diffusion showed an incremental increase in insulin secretion during high glucose exposure. Although we also observed an increase in the insulin secretion level for the naked islets under static culture during the high glucose challenge, the maximum level of insulin secreted was not as amplified as the other two conditions. Furthermore, the magnitude of insulin secretion during pre-stimulation and stimulation (Stimulation/Pre-Stimulation) was significantly different among the naked islets under static conditions, and SNM encapsulation under convection and diffusion as indicated by the SI, which were 2.98 ± 0.06, 6.22 ± 0.69, and 4.29 ± 0.34, respectively (Fig. 3.8,b). When the highest amount of insulin secretion in the high glucose phase was used to calculate SI (Highest Stimulation/Pre-stimulation), SNM-encapsulation under convection (6.50 ± 0.42) showed an increase in SI compared to SNM-encapsulation under convection (4.99 ± 0.51) and naked islets under static conditions (3.85 ± 1.51) (Fig. 3.8,b). As the circuit was switched back to low glucose concentration, SNM-encapsulated islets under convection showed the most significant drop in insulin secretion compared to the naked islets and SNM encapsulation under diffusion (Fig. 3.8,a). The SDI calculated based on the ratio of insulin secretion from post-stimulation and stimulation (Post-stimulation/Stimulation) for naked islets, and SNM encapsulation under convection and diffusion were 1.1 ± 0.36, 0.42 ± 0.19, and 0.8 ± 0.12, respectively (Fig. 3.8,c).
The similar trend was observed for SNM-encapsulation under convection (0.26 ± 0.02), SNM-encapsulation under diffusion (0.57 ± 0.15), and the naked islets (0.70 ± 0.12) when the SDI was calculated based on the ratio of lowest insulin secretion from post-stimulation and stimulation (Lowest post-stimulation/Stimulation) (Fig. 3.8, c) Further analysis of the rate of change in insulin production from low to high glucose stimulation showed that SNM-encapsulated islets under convection produced 2.89 normalized insulin content min⁻¹ (X 10⁻²), whereas naked islets under static conditions and SNM-encapsulated islets under diffusion produced 0.22 and 0.73 normalized insulin content min⁻¹ (X 10⁻²) (Table 3.2).
Figure 3.8 (a) Insulin release kinetics of SNM-encapsulated mouse islets during 90-minute low-high-low (1.6 mM, 16.6 mM, 1.6 mM) glucose stimulation under convective (2 psi) (Conv) and diffusive transport (Diff) with subjection to cytokines (+Ck). Experiments with cytokine exposure (+Ck) consisted of media containing TNF-α (2,000 U/mL), IFN-γ (1,000 U/mL), and IL-1β (10,000 U/mL). The naked islets cultured under static conditions served as controls (Control, +Ck). The SNM-encapsulated islets under convective transport (SNM, Conv, +Ck) exhibited higher insulin secretion following stimulation at high glucose concentration and faster insulin release kinetics in response compared to those under diffusive transport (SNM, Diff, +Ck) and naked islets cultured under static conditions (Control, +Ck). (Mean±SEM, n≥3) (b) The stimulation index (SI) was calculated as the ratio of (1) the first insulin collection in the high glucose phase at 40 minutes to the last insulin collection point of the previous low glucose phase at 30 minutes (Stimulation/Pre-stimulation), and (2) the highest insulin secretion in the high glucose phase to the last insulin collection point of the previous low glucose phase at 30 minutes (Highest stimulation/Pre-stimulation). The SI indicates the magnitude of insulin released as stimulated by a higher concentration of glucose. With cytokine exposure (+Ck), all conditions including SNM-encapsulated islets under convection (SNM, Conv) and diffusion (SNM, Diff), and the naked islets cultured under static conditions (Control) all exhibited varying level of magnitude in glucose-induced insulin secretion (Stimulation/Pre-stimulation). However, when using the highest insulin secretion in the high glucose phase (Highest Stimulation/Pre-stimulation),
The rate of change in insulin production from high to low glucose cessation of SNM-encapsulated islets under convection (SNM, Conv) compared to that under diffusion (SNM, Diff) and naked islets cultured under static conditions (Control). \( \text{(Mean±SEM, n≥3, *p<0.05)} \) (c) The shut-down index (SDI) was calculated as the ratio of (1) the first insulin collection point in the subsequent low glucose phase at 70 minutes to the last insulin collection point in the high glucose phase at 60 minutes (Post-stimulation/Stimulation), and (2) the lowest insulin secretion in the subsequent low glucose phase to the last insulin collection point in the high glucose phase at 60 minutes (Lowest Post-stimulation/Stimulation). The SDI reflects the magnitude of cessation in insulin production once glucose concentration returns to normal. With cytokine exposure (+Ck), the SNM-encapsulated islets under convection (SNM, Conv) exhibited the highest magnitude of insulin reduction compared to the diffusive condition (SNM, Diff) and the naked islet culture (Control) as glucose dropped low (Post-stimulation/Stimulation & Lowest Post-stimulation/Stimulation). \( \text{(Mean±SEM, n≥3, *p<0.05)} \)

The rate of change in insulin production from high to low glucose cessation of SNM-encapsulated islets under convection was -1.76 normalized insulin content min\(^{-1}\) \( \times 10^{-2} \), whereas that of the naked islets under static culture and SNM-encapsulated islets under diffusion were -0.092 and -0.32 normalized insulin content min\(^{-1}\) \( \times 10^{-2} \). The SNM-encapsulated islets under convection exhibited a 13.1- and 3.96-fold increase in the rate of insulin production compared with naked islets and SNM encapsulation under diffusion respectively, when conditions were changed from low to high glucose exposure with cytokines. The SNM-encapsulated islets under convection also demonstrated a 19.1- and 5.5-fold increase in the rate of shutting down insulin secretion from high to low glucose conditions compared with naked islets and SNM encapsulation under diffusion. In summary, the SNM-encapsulated islets under convection exceeded both naked islets and SNM encapsulation under diffusion in terms of the magnitude of insulin produced when stimulated with high level of glucose (Fig. 3.8, (b&c)) and
the rate at which insulin was produced and ceased due to changes in glucose concentration (Table 3.2).

**Table 3.2** The rate of change in insulin secretion with cytokine exposure. The rate of change in insulin production was calculated based on the slopes of curves that were fitted on glucose-insulin kinetics graphs to describe the quickness of insulin being secreted as glucose concentration changes. With subjection to cytokines (+Ck), SNM-encapsulated mouse islets under convection (SNM, Conv) showed the fastest response following high glucose exposure while SNM- and SµM-encapsulated mouse islets under convection (SNM, Conv & SµM, Conv) exhibited similar rate of insulin cessation when glucose concentration returned to normal.

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>Low-High Glucose Stimulation (10²) (normalized insulin content * min⁻¹)</th>
<th>High-Low Glucose Shut-Down (10²) (normalized insulin content * min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, +Ck</td>
<td>0.22</td>
<td>-0.092</td>
</tr>
<tr>
<td>SNM, Conv, +Ck</td>
<td>2.89</td>
<td>-1.76</td>
</tr>
<tr>
<td>SNM, Diff, +Ck</td>
<td>0.73</td>
<td>-0.32</td>
</tr>
<tr>
<td>SµM, Conv, +Ck</td>
<td>1.57</td>
<td>-2.84</td>
</tr>
</tbody>
</table>

Unlike the SNM-encapsulated islets under convection in which the maximum level of insulin secreted within 10 minutes of high glucose challenge, SµM-encapsulation under convection showed a continuous rise in insulin secretion and reached the highest peak within 30 minutes of high glucose exposure (Fig. 3.9,a). Moreover, SNM-encapsulated islets under convection exhibited the largest magnitude of glucose-stimulated insulin secretion possessing a SI value of 6.22 ± 0.69, which was significantly higher than that for the SµM-encapsulation case with a SI value of 4.66 ± 0.07 (Fig. 3.9,b). When the highest amount of insulin secretion in the high glucose phase was used to calculate SI (Highest Stimulation/Pre-stimulation), SNM- and SµM-encapsulation under convection (6.50 ± 0.42 & 6.37 ± 0.11) showed an increase in SI compared to naked islets under static conditions (3.85 ± 1.51) (Fig. 3.9,b). However, the SDI of SNM- and SµM-encapsulated islets under convection were similar in which the SDI were 0.42 ±
0.19 and 0.40 ± 0.04, respectively (Fig. 3.9,c). The same trend was observed when examining the SDI of SNM- and SμM-encapsulation under convection (0.26 ± 0.02 & 0.28 ± 0.04) and the naked islets (0.70 ± 0.12) where the SDI was calculated based on the ratio of lowest insulin secretion from post-stimulation and stimulation (Lowest post-stimulation/Stimulation) (Fig. 3.9,c). Further analysis of the rate of changes in insulin production was calculated for SμM-encapsulated islets under convection which showed a 1.46-fold decrease and 1.61-fold increase compared with SNM-encapsulated islets under convection in transitioning from low to high glucose stimulation and from high to low glucose shut-down, respectively (Table 3.2). Noticeably, all diffusive conditions with both SNM- and SμM-encapsulation showed reduction in the magnitude of insulin produced as well as decline in the rate of insulin production compared to all convective scenarios (Fig. 3.10 & 3.11, Table 3.3). In summary, convective transport with SNM encapsulation demonstrated better performance than SμM-encapsulation in terms of the magnitude of insulin produced and ceased during high and low glucose phases as indicated by SI and SDI factors under cytokine exposure (Fig. 3.8 & 3.9), while the rate of changes in insulin secretion was similar between the two (Table 3.2).
a. 

Stimulation/Pre-stimulation

b. 

Highest Stimulation/Pre-stimulation

Post-stimulation/stimulation

Lowest Post-stimulation/stimulation
Figure 3.9 (a) Insulin release kinetics of SNM- and SµM-encapsulated mouse islets during 90-minute low-high-low (1.6 mM, 16.6 mM, 1.6 mM) glucose stimulation under convective (2 psi) (Conv) with subjection to cytokines (+Ck). The naked islets cultured under static conditions served as controls (Control, +Ck). Experiments with cytokine exposure (+Ck) consisted of media containing TNF-α (2,000 U/mL), IFN-γ (1,000 U/mL), and IL-1β (10,000 U/mL). With cytokine exposure (+Ck), the SµM-encapsulated islets under convective transport (SµM, Conv) exhibited a continuous insulin secretion following stimulation at high glucose concentration from 40 minutes to 60 minutes, while the SNM-encapsulated islets under convection (SNM, Conv) showed a plateau in insulin production during this period of challenge. (Mean±SEM, n≥3) (b) The stimulation index (SI) was calculated as the ratio of (1) the first insulin collection in the high glucose phase at 40 minutes to the last insulin collection point of the previous low glucose phase at 30 minutes (Stimulation/Pre-stimulation), and (2) the highest insulin secretion in the high glucose phase to the last insulin collection point of the previous low glucose phase at 30 minutes (Highest stimulation/Pre-stimulation). The SI indicates the magnitude of insulin released as stimulated by a higher concentration of glucose. With cytokine exposure (+Ck), the SNM- and SµM-encapsulated islets under convection (SNM, Conv & SµM, Conv) and the naked islet culture under static conditions (Control) all showed a significant difference in the magnitude of insulin secreted upon high glucose challenge (Stimulation/Pre-stimulation). However, the SNM- and SµM-encapsulated islets under convection (SNM, Conv & SµM, Conv) showed greater difference in the magnitude of insulin secreted upon high glucose challenge when the highest insulin secretion was used (Highest stimulation/Pre-stimulation). (Mean±SEM, n≥3, *p<0.05) (c) The shut-down index (SDI) was the ratio of (1) the first insulin collection point in the subsequent low glucose phase at 70 minutes to the last insulin collection point in the high glucose phase at 60 minutes (Post-stimulation/Stimulation), and (2) the lowest insulin secretion in the subsequent low glucose phase to the last insulin collection point in the high glucose phase at 60 minutes (Lowest Post-stimulation/Stimulation). The SDI reflects the magnitude of cessation in insulin production once glucose concentration returns to normal. The SNM- and SµM-encapsulated islets under convection (SNM & SµM, Conv) exhibited the highest magnitude of insulin reduction compared to the naked islet culture (Control) as glucose dropped low (Post-stimulation/Stimulation & Lowest Post-stimulation/Stimulation). (Mean±SEM, n≥3, *p<0.05)
Figure 3.10 (a) Insulin release kinetics of SμM-encapsulated mouse islets during 90-minute
low-high-low (1.6 mM, 16.6 mM, 1.6 mM) glucose stimulation under convective (2 psi) (Conv) and diffusive transport (Diff) with subjection to cytokines (+Ck). Experiments with cytokine exposure (+Ck) consisted of media containing TNF-α (2,000 U/mL), IFN-γ (1,000 U/mL), and IL-1β (10,000 U/mL). The naked islets cultured under static conditions served as controls (Control, +Ck). The SµM-encapsulated islets under convective transport (SµM, Conv, +Ck) exhibited higher insulin secretion and faster insulin release kinetics in response to stimulation at high glucose concentration compared to those under diffusive transport (SµM, Diff, +Ck) and naked islets cultured under static conditions (Control, +Ck). (Mean±SEM, n≥3) 

(b) The stimulation index (SI) was calculated as the ratio of (1) the first insulin collection in the high glucose phase at 40 minutes to the last insulin collection point of the previous low glucose phase at 30 minutes (Stimulation/Pre-stimulation), and (2) the highest insulin secretion in the high glucose phase to the last insulin collection point of the previous low glucose phase at 30 minutes (Highest stimulation/Pre-stimulation). The SI indicates the magnitude of insulin released as stimulated by a higher concentration of glucose. With cytokine exposure (+Ck), all conditions including SµM-encapsulated islets under convection (SµM, Conv) and diffusion (SµM, Diff), and the naked islets cultured under static conditions (Control) all exhibited varying level of magnitude in glucose-induced insulin secretion (Stimulation/Pre-stimulation). The SµM-encapsulated islets under convection (SµM, Conv) and naked islets cultured under static conditions (Control) showed an increase in the magnitude of insulin secretion when the highest insulin secretion in the high glucose phase was used (Highest stimulation/Pre-stimulation). (Mean±SEM, n≥3, *p<0.05) 

(c) The shut-down index (SDI) was the ratio of (1) the first insulin collection point in the subsequent low glucose phase at 70 minutes to the last insulin collection point in the high glucose phase at 60 minutes (Post-stimulation/Stimulation), and (2) the lowest insulin secretion in the subsequent low glucose phase to the last insulin collection point in the high glucose phase at 60 minutes (Lowest Post-stimulation/Stimulation). The SDI reflects the magnitude of cessation in insulin production once glucose concentration returns to normal. The SµM-encapsulated islets under convection (SµM, Conv) and under diffusion (SµM, Diff) both exhibited the highest magnitude of insulin reduction compared to the naked islet culture (Control) as glucose dropped low (Post-stimulation/Stimulation & Lowest Post-stimulation/Stimulation). (Mean±SEM, n≥3, *p<0.05)
Figure 3.11 (a) Insulin release kinetics of SNM- and SµM-encapsulated mouse islets during
90-minute low-high-low (1.6 mM, 16.6 mM, 1.6 mM) glucose stimulation under diffusion (Diff) with subjection to cytokines (+Ck). The naked islets cultured under static conditions served as controls (Control, +Ck). Experiments with cytokine exposure (+Ck) consisted of media containing TNF-α (2,000 U/mL), IFN-γ (1,000 U/mL), and IL-1β (10,000 U/mL). With cytokine exposure (+Ck), the SµM-encapsulated islets under diffusive transport (SµM, Diff) exhibited the fastest insulin secretion at high glucose concentration from 40 minutes to 60 minutes followed by the SNM-encapsulated islets under diffusion (SNM, Diff). The level of glucose-induced insulin secretion from the naked islets cultured under static conditions (Control) was not as significant as the other two groups. (Mean±SEM, n≥3) (b) The stimulation index (SI) was calculated as the ratio of (1) the first insulin collection in the high glucose phase at 40 minutes to the last insulin collection point of the previous low glucose phase at 30 minutes (Stimulation/Pre-stimulation), and (2) the highest insulin secretion in the high glucose phase to the last insulin collection point of the previous low glucose phase at 30 minutes (Highest stimulation/Pre-stimulation). The SI indicates the magnitude of insulin released as stimulated by a higher concentration of glucose. The SNM- and SµM-encapsulated islets under diffusion (SNM,Diff & SµM, Diff) and the naked islet culture under static conditions (Control) all showed a significant difference in the magnitude of insulin secreted upon high glucose challenge (Stimulation/Pre-stimulation). The SNM-encapsulated islets under diffusion (SNM,Diff) and naked islets cultured under static conditions (Control) showed an increase in the magnitude of insulin secretion when the highest insulin secretion in the high glucose phase was used (Highest stimulation/Pre-stimulation). (Mean±SEM, n≥3, *p<0.05) (c) The shut-down index (SDI) was the ratio of (1) the first insulin collection point in the subsequent low glucose phase at 70 minutes to the last insulin collection point in the high glucose phase at 60 minutes (Post-stimulation/Stimulation), and (2) the lowest insulin secretion in the subsequent low glucose phase to the last insulin collection point in the high glucose phase at 60 minutes (Lowest Post-stimulation/Stimulation). The SDI reflects the magnitude of cessation in insulin production once glucose concentration returns to normal. The SµM-encapsulated islets under diffusion (SµM, Diff) exhibited the highest magnitude of insulin reduction compared to the SNM-encapsulated islets under diffusion (SNM, Diff) and naked islet culture (Control) as glucose dropped low (Post-stimulation/Stimulation & Lowest Post-stimulation/Stimulation). (Mean±SEM, n≥3, *p<0.05)
Table 3.3. The rate of change in insulin secretion. The rate of change in insulin production was calculated based on the slopes of curves that were fitted on glucose-insulin kinetics graphs to describe the quickness of insulin being secreted as glucose concentration changes. The SµM-encapsulated mouse islets under diffusion without cytokine exposure (SµM, Diff, -Ck) showed similar rate of insulin secretion in glucose-induced stimulation and a slightly faster insulin cessation compared with the SµM-encapsulated mouse islets under diffusion with cytokine exposure (SµM, Diff, +Ck).

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>Low-High Glucose Stimulation (10^-2)</th>
<th>High-Low Glucose Shut-Down (10^-2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SµM, Diff, -Ck</td>
<td>1.13</td>
<td>-0.73</td>
</tr>
<tr>
<td>SµM, Diff, +Ck</td>
<td>1.15</td>
<td>-1.46</td>
</tr>
</tbody>
</table>

Comparing previous conditions that were not subjected to cytokines, we observed that conditions with cytokine exposure had a slight decrease in SI values ranging (including Fig. 3.10 & 3.11). We observed no significant difference in the magnitude of insulin secreted before and after cytokine exposure for SNM-encapsulation under convection (SI (Stimulation/Pre-stimulation): 6.38 ± 0.44 and 6.23 ± 0.69, respectively) (Fig. 3.4,b & 3.8,b), while the SµM-encapsulation under convection and naked islets under static culture all declined slightly in their SI values (Stimulation/Pre-stimulation): SµM-encapsulation under convection dropped from 6.44 ± 1.41 to 4.66 ± 0.07 (Fig. 3.5,b & 3.9,b), and naked islets decreased from 3.92 ± 1.06 to 2.98 ± 0.06 (Fig. 3.4,b & 3.8,b). The naked islets under static culture showed a higher SDI value (Post-stimulation/Stimulation) with cytokine exposure (0.59 ± 0.17) (Fig. 3.4,c) than the no-cytokine condition (1.1 ± 0.36) (Fig. 3.8,c), whereas SNM- and SµM-encapsulation under convection showed consistent SDI values (Post-stimulation/Stimulation) before and after cytokines were added (Fig. 3.4,c; Fig. 3.5,c; Fig. 3.8,c; Fig. 3.9,c). When switching from high to low glucose conditions, the naked islets showed a large variation in the SDI value (Post-stimulation/Stimulation), indicating partial loss of islet regulatory function with insulin (Fig. 3.10 & 3.11).
In contrast, both membrane-encapsulated conditions showed sharp drop in insulin production once they were switched back to low glucose environment (Fig. 3.8 & 3.9). Cytokines namely TNF-α, IFN-γ, and IL-1β are known to be synergistically cytotoxic through a cascade of inflammatory events such as production of nitric oxide and chemokines, and trigger of endoplasmic reticulum stress to cause loss of islet viability and functionality. We speculated that cytokines damaged the naked islets as shown by their changes in SI and SDI values mentioned above, whereas the selectivity of the SNM and SμM membranes hindered cytokine infiltration and preserved islet function.

**Islet viability**

In addition to the glucose-insulin kinetics of SNM- and SμM-encapsulation described above, we investigated the islet viability to understand if cytokines caused excessive islet dysfunction (Fig. 3.12). The naked islets with cytokine exposure showed significantly more cell death compared to all other groups including SNM- and SμM-encapsulation under convection (Fig. 3.12,a). All membrane-associated diffusive conditions showed normal health comparable to the untreated naked islets under static culture (Fig. 3.13). Some level of cytokine-induced death damage was observed in the SμM-encapsulation under convection as a result of their inability to completely exclude cytokines likely due to the large membrane pore size (Fig. 3.12,b). However, the islet death in the SμM-encapsulation under convection was not as significant as in the control scenario with naked islets. The SNM-encapsulated islets under convection with cytokine exposure showed similar viability compared to SNM-encapsulating and healthy control conditions without cytokines. These observations confirm that membrane protection afforded by SNM provides sufficient immunoisolation to support viability and functional performance of the encapsulated islets, which is indicated by the glucose-kinetics data.
Figure 3.12 (a) Viability of mouse islets was measured following the 90-minute low-high-low (1.6 mM, 16.6 mM, 1.6 mM) glucose stimulation in which islets were subjected to the mock-loop circuit with or without cytokine exposure (+Ck vs -Ck) for SNM- and SµM-encapsulation under convection (SNM, C & SµM, C). The naked islet culture under static culture with cytokine exposure (Control, +Ck) showed significantly less viability compared to all other conditions. (Mean±SEM, n≥3, *p<0.05) (b) Viable (green) and dead (red) cells were stained for control static culture without cytokines (A: Control, -Ck), control static culture with cytokines (B: Control, +Ck), SNM-encapsulated mouse islets under convection without cytokines (C: SNM, C, -Ck), SNM-encapsulated mouse islets under convection with
cytokines (D: SNM, C, +Ck), SµM-encapsulated mouse islets under convection without cytokines (E: SµM, C, -Ck), and SµM-encapsulated mouse islets under convection with cytokines (F: SµM, C, +Ck). Experiments with cytokine exposure (indicated by +Ck) consisted of media containing TNF-α, IFN-γ, and IL-1β. Both control static culture with cytokines (B: Control, +Ck) and SµM-encapsulated mouse islets under convection with cytokines (F: SµM, C, +Ck) showed a higher level of islet damage compared to other groups, however, the viability of SµM-encapsulated mouse islets under convection with cytokines (F: SµM, C, +Ck) was not statistically significant (Fig. 3.12,a).

Figure 3.13 (a) Viability of mouse islets was measured following the 90-minute low-high-
CONCLUSIONS

In this study, we characterized the glucose-insulin kinetics of an improved silicon nanopore membrane, SNM, for the encapsulation of pancreatic islets under convective flow. The glucose-insulin responsiveness of membrane-encapsulated islets was analyzed under a series of low, high, and low glucose challenge by: (1) SI and SDI values, which show the magnitude of insulin secreted when transitioning from low to high glucose condition or vice versa; and (2) rate of change in insulin secretion, which indicates how quickly the system responds from low to high glucose condition or vice versa. Based on these parameters, we found that convective mode performed better than diffusive mode in both SNM and SµM encapsulations. In addition, once exposed under cytokines, convective transport with SNM encapsulation demonstrated superior performance over SµM encapsulation in terms of the magnitude of insulin produced and ceased during high and low glucose phases with healthy islet viability, while the rate of changes in

low (1.6 mM, 16.6 mM, 1.6 mM) glucose stimulation in which islets were subjected to the mock-loop circuit with or without cytokine exposure (+Ck vs -Ck) for SNM- and SµM-encapsulation under diffusion (SNM, D & SµM, D). The naked islet culture under static culture with cytokine exposure (Control, +Ck) showed significantly less viability compared to all other conditions. (Mean±SEM, n≥3, *p<0.05) (b) Viable (green) and dead (red) cells were stained for control static culture without cytokines (A: Control, -Ck), control static culture with cytokines (B: Control, +Ck), SNM-encapsulated mouse islets under diffusion without cytokines (C: SNM, D, -Ck), SNM-encapsulated mouse islets under diffusion with cytokines (D: SNM, D, +Ck), SµM-encapsulated mouse islets under diffusion without cytokines (E: SµM, D, -Ck), and SµM-encapsulated mouse islets under diffusion with cytokines (F: SµM, D, +Ck). Experiments with cytokine exposure (indicated by +Ck) consisted of media containing TNF-α, IFN-γ, and IL-1β. The control static culture with cytokines (B: Control, +Ck) showed significant level of islet damage compared to all other conditions.
insulin secretion was on the same scale as that for the SμM encapsulation. In summary, SNM encapsulation under convective transport enables rapid glucose-insulin sensing to activate and cease insulin production based on the surrounding glucose concentration while retaining healthy islet viability even under cytokine exposure. Our data demonstrates the importance of using convective transport to obtain a faster insulin activation and shut-down, which is a critical issue to address in many islet-encapsulating devices\textsuperscript{367,389} with undesired delay in glucose-insulin response. Successful islet encapsulation with selective SNM under convective transport could potentially lower the immunosuppressive drugs and their side effects resulted from current therapies, lead to the possibility of using xenogeneic or stem-cell derived cell sources to overcome donor shortage, and reduce dangerous episodes of hypoglycemia for T1D patients in the future.
CHAPTER IV

AN INTRAVASCULAR BIOARTIFICIAL PANCREAS DEVICE (IBAP) WITH SILICON NANOPORE MEMBRANE (SNM) FOR ISLET ENCAPSULATION UNDER CONVECTION

4.1 INTRODUCTION

Islet encapsulation has shown great promise in treating unstable Type 1 Diabetes (T1D); however, this treatment is severely limited by the shortage of donor tissue and the need for lifelong systemic immunosuppression that can be detrimental to islet cell function and result in immunosuppression-associated adverse events\textsuperscript{390}. Immunoisolation via cell encapsulation may circumvent the need of immunosuppression by preventing the passage of the host’s immune factors, while allowing the exchange of glucose, insulin, nutrients and small molecules to sustain the survival of islet graft\textsuperscript{58}. However, the clinical success with islet encapsulation, thus, far, has been limited\textsuperscript{79,318,367,391,392}. Reasons for poor performance with islet micro- and macro-encapsulation include the large capsule sizes that lead to considerable diffusional gradients and implant volumes, which result in decreased islet cell viability and delayed glucose-insulin kinetics. Solutes that are critical for islet survival and function, including oxygen, nutrients, and insulin, are exchanged by diffusion across the semipermeable encapsulating membrane\textsuperscript{368}. Cell necrosis occurs when islets are placed beyond the diffusion limit of tissue, which is \(\sim 150-200\) \(\mu\)m away from the nearest blood vessels\textsuperscript{133}. This distance is significantly smaller than the half-length/radial distance of most encapsulating devices\textsuperscript{393,394}. In T1D patients, the delayed glucose-insulin response due to large diffusion gradients could also endanger patients by exposing them
to potential episodes of hypoglycemia and hyperglycemia\textsuperscript{367}. Furthermore, transplant sites able to accommodate such large volumes of encapsulated-islets at a clinically relevant dose are restricted to the intraperitoneal space, which is poorly vascularized and therefore less favorable for cell engraftment\textsuperscript{76,166,395}.

Our lab has developed the silicon nanopore membrane (SNM) based intravascular bioartificial pancreas (iBAP) to overcome the oxygenation and glucose-insulin kinetics challenges of diffusion-based BAP devices\textsuperscript{7,396-400} by convective mass transfer of nutrients and small molecules within the encased islet chamber (IC) (Fig. 4.1). We designed a new generation of encapsulating membranes for immunoisolation of transplanted islets based on microelectromechanical systems (MEMS) technology initially pioneered by Ferrari and colleagues\textsuperscript{340,341} to create more uniform pore sizes at nanometer scale. These semipermeable filtration membranes can be engineered with precise slit-shaped pore sizes with critical widths down to 5 nm (Fig. 4.1,b) and an uniform pore size distribution (~1\%) for superior selectivity\textsuperscript{342}. The pore geometry influences the trade-off between selectivity and permeability of the membranes. The permeability – selectivity analysis for ultrafiltration showed that SNM with monodispersed slit-shaped pores exhibited greater selectivity at a given value of permeability, than membranes with cylindrical pores for pore size below 100 nm\textsuperscript{401}. Due to the thickness of BAP devices, convection-dominated mass transport is advantageous\textsuperscript{359}, because it efficiently transports solutes such as glucose, oxygen, and insulin between the device and adjacent vascular system. In a previous study, we showed 80\% reduction in cytokine passage using SNM with an
average pore size of ~7 nm, providing immunoisolation and preserving the islet viability under
While previous attempts with ultrafiltration-based devices showed some promise, they provided insufficient ultrafiltrate flow rates. In contrast, the SNM produces high levels of ultrafiltrate (Table 4.1), which is ten times greater than the polymer membranes used in previous ultrafiltrate-based iBAP devices at physiological blood pressures. Given advantages in the selectivity and permeability of the SNM, our long-term objective is to develop the silicon nanopore membrane (SNM) based intravascular bioartificial pancreas (iBAP), where the iBAP will be connected to arterio-venous grafts and a pressure drop between the artery and ultrafiltrate vein produces ultrafiltrate to flow through the SNM-encapsulated islet chamber (IC), carrying nutrients to the islets and insulin to the ultrafiltrate vein (Fig. 4.2).

**Table 4.1** Silicon nanopore membrane (SNM) hydraulic permeability as a function of pore size.

<table>
<thead>
<tr>
<th>SNM Pore Size (nm)</th>
<th>Hydraulic Permeability (ml/hr/m²/mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>$1.3 \times 10^2$</td>
</tr>
<tr>
<td>15</td>
<td>$3.0 \times 10^2$</td>
</tr>
<tr>
<td>30</td>
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<td>40</td>
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In this study, we tested an iBAP prototype containing SNM-encapsulated mouse islets under diffusion and convection (Fig. 4.1). For in vitro experiments, we investigated the SNM-encapsulated islet viability and glucose-insulin kinetics of the iBAP with varying islet densities by volume (10% vs 20%) and membrane pore sizes (10 nm vs 40 nm) under both diffusion and convection. Subsequently, the SNM-encapsulated islets iBAP were intravascularly implanted into pigs for three days. The SNM hemocompatibility, device patency, and islet viability and functionality from porcine experiments were investigated to determine the feasibility of developing a refined SNM-based iBAP with convective transport to support a clinically relevant islet dosage to treat a T1D patient in the future.

Figure 4.2 (a) Illustration of the full-scale iBAP connected to arterial-venous grafts and an Ultrafiltrate Outlet catheter delivering insulin rich ultrafiltrate to the ultrafiltrate vein. Blood flows into the iBAP and a looped blood channel transports blood to a vein. The SNM encapsulated IC is placed directly above and below the blood channel. (b) A cross-sectional view perpendicular to blood flow illustrating the blood channel surrounded by the SNM (green) encapsulated IC (blue). Ultrafiltrate (black arrows) crosses the SNM encapsulated IC into ultrafiltrate channels (side) and exits the Ultrafiltrate Outlet catheter into the ultrafiltrate vein.
4.2 MATERIALS AND METHODS

4.2.1 SUBSTRATE PREPARATION

Silicon Nanopore Membranes (SNM) architecture and fabrication

Silicon nanopore membranes (SNM) have been prototyped from silicon substrates by MEMS technology as previously reported\(^{370}\). Briefly, the process used the growth of a thin SiO\(_2\) (oxide) layer on 400 µm-thick double side polished (DSP) silicon wafers followed by a low pressure chemical vapor deposition (LPCVD) of polysilicon (~500 nm). The wafers were then specifically patterned, dry oxidized, wet etched, deposited with a second polysilicon layer, and finally blanket-etched until 400 nm of polysilicon remained and the underlying vertical oxide layer was exposed. The vertical sacrificial oxide layer defined the critical nanoscale pore size of the membranes. The low temperature oxide (LTO) (~1 µm) was deposited onto polysilicon of the wafers to serve as the hard mask for membrane protection. Deep reactive ion etching (DRIE) removed the backside of each window until membranes were disclosed. Eventually, the sacrificial oxide was etched away in 49% hydrofluoric acid (HF) during the final step of the fabrication process to leave behind open nanoscale slit pores. The wafers were subsequently cut into 1×1 cm chips with an effective area of 6x6 mm\(^2\) containing 1500 windows each, with a total of 10\(^6\) pores per membrane. Each rectangular pore was 300 nm in depth and 2 µm in length. The SNM with an average pore size width of 10 nm and 40 nm were used in this study. All membranes were cleaned using a conventional “piranha” clean procedure, which involved a 20 min-immersion in 3:1 sulfuric acid (H\(_2\)SO\(_4\))/hydrogen peroxide (H\(_2\)O\(_2\)) mixture, followed by thorough rinses in deionized (DI) water. Images of SNM were obtained using scanning electron microscope (SEM) (Leo 1550) (Fig. 4.1,b).
**Surface modification of SNM with poly(ethylene glycol) (PEG)**

SNM were covalently modified with PEG using a previously reported protocol to prevent protein fouling on the membrane surface\(^3\). The technique used for PEG attachment involved a single reaction step which covalently couples silicon surface silanol group (Si-OH) to a chain of PEG polymer through a trimethoxysilane group forming a Si-O-Si-PEG sequence. Briefly, SNM were immersed in a solution of 3 mM 2-[methoxy(polyethyleneoxy)propyl]trimethoxysilane (PEG-silane) (Gelest: SIM6492.7) in toluene for 2 hr at 70 °C. A series of extensive washing steps involving toluene, ethanol, and DI water was used to remove unbounded PEG residue.

**Hydraulic permeability for SNM pore size characterization**

An automated mass and pressure measurement system was utilized for characterizing liquid flow through the SNM under a tangential-flow filtration operation\(^3\). The pore size of the SNM can be related to filtration flow parameters using \( h = \frac{\sqrt{12 \mu Q}}{nw\Delta P} \) (Eq. 1), where \( h \) is pore width, \( \mu \) is the viscosity, \( l \) is the membrane thickness, \( Q \) is the volumetric flow rate, \( n \) is the number of pores per membrane, \( w \) is the pore length, and \( \Delta P \) is the transmembrane pressure. To assemble the overall system for SNM pore size characterization, air was applied through a syringe pump (Sigma: Z675709) into a water reservoir. Water was circulated by a peristaltic pump (Masterflex: 07551-00) through a differential pressure transducer (Omega: PX429 015Gi), a flow cell with enclosed membrane, and returned to the original water reservoir. The flow cell was assembled with the SNM submerged under water to remove air bubbles from all compartments. Specifically, a membrane was positioned with the polysilicon interface facing down with a customized silicone gasket positioned on top of the membrane, followed by the final placement of a filtrate chamber on top of the gasket. All sections were fastened together and secured to the base with hand-tightened hex bolts until the gasket was visibly compressed. The
ultrafiltrate permeated through the membrane and was routed to a liquid collection container that rested on a precision mass balance (Mettler Toledo: XS205). Measurements from the differential pressure transducer and the mass balance were automatically collected with a data acquisition laptop. A typical membrane hydraulic permeability test consisted of 5 ml/min flow rate and 4 pressure cycles (5, 1, 5, and 1 psi) for durations of 150 s each. Using the specifications for pore length, membrane thickness, and total number of pores provided based on individual wafer designs, the average pore size of SNM was calculated using Equation 1. All SNM membranes in this study were surface-modified with PEG and exhibited an average pore size of 10 nm and 40 nm.

4.2.2 ASSEMBLY OF THE INTRAVASCULAR DEVICE

Development of the islet chamber (IC)

In this study, IC possessed a thickness of 1000 μm and high islet densities of 10% (5,700 IE/cm²) and 20% (11,400 IE/cm²). Islet density by percentage was calculated as the ratio of total islet volume expressed in islet equivalents and the IC volume. Islet density by surface area was calculated by dividing the total number of islet equivalents (IE) by the device membrane surface area. A biocompatible acrylic sheet (McMaster: 8589K11) was first laser-cut to create ~2.4 mm x ~2.4 mm x ~1 mm thick void region which was inserted with eight 100 μm diameter polytetrafluoroethylene (PTFE) coated wires (McMaster: 1749T11). A 2% agarose-islet mixture was then poured into this void region of acrylic sheets. After the agarose-islet mixture was cured, all wires were removed (Fig. 4.1,c,i). Using this process, a hexagonal arrangement of eight 800-μm cylindrical agarose-islet regions (dotted red circle) with a central 100 μm cylindrical channel (solid red circles) was obtained for the IC (Fig. 4.1,c,ii). This configuration created a diffusion
distance ≤ 400 μm between the islets and ultrafiltrate. After IC construction, it was assembled in the iBAP as described in Figure 5.1,a with gaskets between the various iBAP components.

**Assembly of an intravascular bioartificial pancreas device (iBAP) for islet encapsulation**

The intravascular bioartificial pancreas device (iBAP) is shown in an exploded view in Figure 1,a: the polycarbonate flow path component containing the blood flow path, two SNM sandwiching the islet chamber (IC) containing the agarose (Sigma: A2576)-seeded mouse islets, the polycarbonate backside (PC Backside), and the ultrafiltrate port (Ultrafiltrate Outlet). The parallel-plate blood flow path was modeled with SolidWorks and computational fluid dynamics (CFD) to create ideal flow characteristics to minimize thrombosis\(^{327}\). The iBAP was symmetrical on both sides and could be assembled with one IC on each side. The iBAP can possess up to 0.72 cm\(^2\) of SNM area. In operation, fluid flows through the Flow Path component at an elevated pressure creating a transmembrane pressure (TMP) of ~80 mmHg between the blood flow path and the Ultrafiltrate Outlet resulting in ultrafiltrate flow through the SNM, IC, PC Backside, and Ultrafiltrate Outlet, which was collected in a tube *in vitro* or drained into interstitial tissue space *in vivo*. Under the diffusive condition, the PC Backside was capped-off resulting in no ultrafiltrate flow through the system. All device components were individually sterilized either by autoclave or Nolvasan for both *in vitro* and *in vivo* experiments.

**4.2.3 TESTING AND IMPLANTATION OF THE INTRAVASCULAR DEVICE IN VITRO AND IN VIVO**

**Testing of the intravascular bioartificial pancreas device (iBAP) in vitro**

All procedures involving isolation of mouse islets were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, San Francisco (UCSF). Mouse islets were isolated from 8 to 10-week-
old male B6 mice (Jackson Laboratories) based on previously described protocols. Harvested islets were maintained in suspension culture with RPMI 1640 with L-glutamine and 11.1 mM glucose (Gibco: 11875-093), 10% fetal bovine serum (FBS) (Gibco: 16000), and 1% penicillin-streptomycin (P/S) (UCSF Cell Culture Facility: CCFGK003). A 2% agarose gel mixed with appropriate amount of mouse islets was dispensed into the previously described IC to create high islet densities of 10% or 20% by volume, respectively. The SNM with 10 nm and 40 nm pore sizes were chosen to encapsulate the IC with 10% and 20% islet densities. For in vitro viability tests, a mock-loop circuit was set up with a peristaltic pump flowing culture medium through the iBAP at TMP of ~80 mmHg to generate ultrafiltrate for convective condition (Fig. 4.1,a,ii), whereas no ultrafiltrate was produced for the diffusive condition. The viability experiments studied both 10 nm and 40 nm SNM encapsulating both 10% and 20% islet densities. After 3 days of culture, the devices were disassembled and the islets were assessed for viability. Using this same mock-loop circuit, glucose-insulin kinetics was explored in iBAPs containing either 10% or 20% islet density and either 10 nm or 40 nm pore sized SNM. SNM-encapsulated mouse islets in the iBAP were exposed to a low, high, and low glucose (Gibco: 11879) challenge on day 0. Ultrafiltrate directly produced from the IC under convection was collected for insulin measurements. Insulin content was analyzed with mouse insulin enzyme-linked immunosorbent assay (ELISA) kits (Mercodia: 10-1247-01) with accounted dilutions.

**Implantation of the intravascular bioartificial pancreas device (iBAP) in pigs**

A preliminary proof-of-concept study was designed with a swine model because of the comparably sized vasculature and hematologic similarities with humans. The study was approved by the IACUC review committee at PMI Preclinical CRO, San Carlos, CA.
For pig #1, the device was assembled as previously described with each chamber containing a 5% islet equivalents (IE) density by volume of mouse islets suspended in agarose gel. Oral aspirin (81mg) and clopidogrel (75mg) were given to a 75kg female Yorkshire pig for 3 days preoperatively and then daily thereafter. After general anesthesia was induced, a vertical incision was made to the left of midline to expose the left external jugular vein. A 15Fr double-lumen tunneled catheter (NextStep®, Teleflex, Morrisville, NC) was placed in the left external jugular vein for blood sampling. The right carotid artery and right external jugular vein were then exposed via a similar vertical incision on the right side of the neck. Once the vessels were exposed a subcutaneous pocket was created caudally for eventual device placement. Heparin was given intraoperatively targeting an activated clotting time (ACT) of greater than 200 seconds. The 6mm externally-supported polytetrafluoroethylene (PTFE) grafts were then anastomosed end-to-side to the internal carotid artery for inflow and the external jugular vein for outflow. The device was then placed in the subcutaneous pocket and anchored to surrounding soft tissue. The inflow and outflow grafts were then connected to the device and clamps were removed to allow blood flow through the device, which was visually confirmed. The overlying soft tissue and skin were then closed in layers and then animal was extubated and allowed to recover. Meloxicam and buprenorphine were administered as needed for post-operative pain.

For pig #2, the device was assembled as previously described with 10% density by volume for each chamber. One chamber had a channel in communication with the islets that was open to atmosphere, allowing for ultrafiltrate flow through the chamber (Fig. 4.1,a,ii). The ultrafiltrate then passed through the channel and was freely deposited into the surrounding tissue and subcutaneous pocket. The other chamber’s ultrafiltrate outlet was capped resulting in a diffusive chamber. The technical aspects of the implant procedure were identical to Pig #1.
Blood flow through the device and ultrafiltrate deposition into the surrounding tissue was visually confirmed prior to closure of the incision.

**Assessment of islet function in vivo**

Islet function was assessed using standard intravenous glucose tolerance tests (IVGTT) with administration of glucose (0.5g/kg in 40% solution) via the tunneled venous catheter. Blood was drawn to measure serum glucose using a standard glucometer (Accu-Chek Compact Plus: 1002-5021) at time 0, 5, 10, 15, 30, 60 and 90 minutes. The IVGTT was administered on post-operative day (POD) 1 and 2 prior to the animals eating their morning meals. On POD 3 the test was performed intra-operatively prior to planned explant of the device and islet retrieval. For pig #1, blood sampling for the intra-operative IVGTT was performed via direct cannulation of the external jugular venous outflow tract immediately distal to the anastomosis. All samples from systematic circulation and directly collected from ultrafiltration port were stored on ice prior to testing for mouse insulin enzyme-linked immunosorbent assay (ELISA) (Mercodia: 10-1247-01) and c-peptide ELISA kits (EMD Millipore: EZRMCP2-21K).

**Patency assessment and device explant with islet retrieval**

On POD 3 both animals were taken back to the operating room for assessment of patency and device retrieval. Once the animal was intubated and sedated the incision was re-opened and the device was delivered into the superficial tissue for visual assessment and confirmation of maintained patency. A final IVGTT was administered. As mentioned, for pig #1, blood was sampled directly from the outflow vein via direct cannulation of the external jugular vein distal to the anastomosis. For pig #2, blood was sampled from the tunneled catheter. Once the IVGTT was completed, the carotid was cannulated proximal to the anastomosis with a 5Fr catheter. Radiopaque contrast media was then injected (Visipaque™, GE Healthcare, Little Chalfont,
United Kingdom) to fluoroscopically confirm flow through the device. The inflow and outflow grafts were then clamped and the device was then explanted and subsequently flushed with culture media prior to disassembly and retrieval of the islets from the chamber.

**Islet viability**

Islet viability was assessed by double staining with the Live/Dead Cell Imaging Kit (488/570) (Life Technology: R37601). Live cells are distinguished by the presence of intensely fluorescent calcein (green) which is well-retained within live cells, whereas dead cells are stained with red. Briefly, agarose-encapsulated mouse islets were incubated in the mixture of live (green) and dead (red) kit components for 15 min and extensively washed in phosphate buffered saline (PBS) to remove excess staining. Images of mouse islets were obtained using laser scanning Nikon Spectral C1si confocal microscope (Nikon Instruments). The percentage of viability was calculated based on the ratio of non-dead or the green area over the entire area of that islet.

**Explanted membrane analysis**

For observation, SNM were fixed in a solution containing 3% glutaraldehyde (Sigma: G7651), 1 M sodium cacodylate (Polysciences) and 0.1 M sucrose (Sigma). After 2 days, the substrates were washed with distilled water. Dehydration was achieved by placing these scaffolds in an increasing concentration of ethanol (50–100%). Dehydrated samples were then mounted on aluminum stubs, sputter-coated with gold–palladium, and examined with scanning electron microscopy (SEM) (Ultra 55, Carl Zeiss).

**Blood platelet adhesion and activation**

The SNM were fixed with 4% paraformaldehyde followed by PBS washes and incubated in blocking solution (PBS, 1% bovine serum albumin (BSA)) for 30 min. Samples were then
incubated with CD41 antibody (Biorbyt: orb181793) for platelet adhesion (green) and CD62p antibody (Bioss: bs-0561R-Cy3) for platelet activation (red) at a dilution of 1:300 for 4 h and repeatedly washed with PBS to remove residues. Images were obtained using 6D High Throughput Perfect Focus System (Nikon Instruments).

**Statistical analysis**

Sample pairs were analyzed using Student’s t-test. Multiple samples were evaluated with one-way or two-way analysis of variance (ANOVA) followed by Bonferroni and multiple comparison using Graphpad Prism software (San Diego, CA). A p value of <0.05 was accepted as statistically significant for all analyses.

**4.3 RESULTS**

**The iBAP testing in vitro**

The iBAP comprising a 10 nm pore sized SNM with 10% (5,700 IE/cm²) or 20% (11,400 IE/cm²) mouse islet densities was investigated for glucose-stimulated insulin response, and viability and functionality of the encapsulated islets after three days. Under convection, the iBAP with 10% mouse islet density and 10 nm pore sized SNM showed an increase in insulin secretion within 10 minutes of high glucose exposure (Fig. 4.3,a,i), which was consistent with normal islet function of biphasic insulin release (i.e. the first insulin phase appeared within 5-10 minutes followed by a second sustained phase that is slower and delayed as times goes longer). Furthermore, during the period when the glucose concentration decreased, the corresponding stimulated-insulin secretion also dropped. However, when the cell density increased from 10% to 20% in the iBAP with 10 nm pore sized SNM under convection, no significant change in glucose-stimulated insulin level was observed within the first few minutes of high glucose
exposure (Fig. 4.3,a,ii). The stimulation index (SI), the ratio of stimulated to basal insulin secretion normalized by the insulin content, was calculated as \(4.4 \pm 0.6\) and \(1.1 \pm 0.1\) for the iBAP of 10 nm pore sized SNM with 10\% and 20\% mouse islet densities, respectively. It is well-recognized that delay of insulin secretion in response to glucose (>20 min) has been a common problem encountered in the early extravascular hollow-fiber systems\(^{142}\). Our iBAP with 10 nm pore size under convection supported the normal insulin function at 10\% islet density with no significant delay in glucose-stimulated insulin response. However, the glucose stimulated-insulin response at 20\% islet density under convection with 10 nm pore sized SNM showed abnormal insulin-functioning behavior, indicating that encased cells were likely not in optimal health in that environment.

The viability study of 10 nm pore sized SNM in the iBAP demonstrated that 10\% mouse islet density under convection (40 ± 11\%) showed a higher viability compared to that under diffusion (4.0 ± 1.3\%) (Fig. 4.3, (b&c)). Furthermore, as the islet density increased to 20\% within the islet chamber, the viability significantly decreased regardless the model of diffusion (11 ± 5.8\%) or convection (17 ± 11\%) used in the system (Fig. 4.3, (b&c)). In summary, although sufficient nutrients were delivered to support the viability and glucose-insulin response of 10\% mouse islet density using the 10 nm pore sized SNM under convection, the use of convection with 10 nm pore sized SNM could not deliver the necessary oxygen and nutrients keep the cells viable and functional at a 20\% cell density.
Figure 4.3 *In vitro* testing of the intravascular bioartificial pancreas device (iBAP) with 10%
To verify whether the pore size was the limiting factor in causing cell death at the higher density, the 40 nm pore size SNM with 10% or 20% mouse islet densities was studied in the iBAP under diffusion and convection. The glucose-stimulated insulin study showed that both 10% (Fig. 4.4,a,i) and 20% (Fig. 4.4,a,ii) demonstrated the characteristic insulin biphasic release curves. Particularly, the iBAP at 10% and 20% mouse islet densities with 40 nm pore size SNM indicated that the first spike in glucose-stimulated insulin production occurred within 10 minutes of high glucose exposure. Both conditions showed insulin shut down as glucose concentration slowly dropped off. The SI at 10% and 20% mouse islet densities with 40 nm pore sized SNM were 3.2 ± 1.3 and 9.1 ± 1.2, respectively. Although the absolute amount of insulin secreted did not double when cell density increased from 10% to 20%, the latter showed a 1.9-fold increase in SI factor, indicating the magnitude of insulin stimulated from basal to high glucose level almost
doubled. The viability study demonstrated that 10% mouse islet density under convection (66 ± 4.8%) showed a higher viability compared to that under diffusion (24 ± 6.8%) (Fig. 4.4, (b&c)). Furthermore, as the islet density increased to 20% within the islet chamber, the viability of islets under convection (61 ± 3.0%) exhibited a significant increase in viability compared with that
under diffusion (5.2 ± 1.3%) (Fig. 4.4, (b&c)). Overall, the iBAP using 40 nm pore sized SNM under convection supported the viability and glucose-insulin response at both 10% and 20% mouse islet densities.

Compared to the previous *in vitro* experiments using 10 nm pore sized SNM, we observed that islet viability correlates positively with an increase in pore size dimension under convection. Specifically, the 40 nm pore sized SNM enhanced the viability at 10% and 20% mouse islet densities to 66 ± 4.8% and 61 ± 3.0% as compared to 40 ± 11% and 17 ± 11% for the
10 nm pore sized SNM under convection, respectively (Fig. 4.3,b & 4.4,b). The greater amount of ultrafiltrate produced by 40 nm pore size under convection enhanced the viability and functionality of encapsulated islets. In contrast, diffusion provides inadequate mass transfer to support a greater islet density. Although an increase in pore size improved islet viability at a lower cell density (10%) under diffusion from 10 nm (4.0 ± 1.3%) to 40 nm pore size (24 ± 6.8%), the islet viability at a higher cell density (20%) showed no significant difference under diffusion ((11 ± 5.8%) vs. (5.2 ± 1.3%)). These data show that nutrients and oxygen remained severely depleted under diffusion even when the pore size was increased to 40 nm. The slow diffusion and its impact on mass transport have been widely reported for porous materials with nanometer-sized pores\textsuperscript{373,374}, as one study showed that the diffusion of 45 nm nanoparticles was slowed down by a factor of 2 in 300 nm cylindrical pores due to hydrodynamic friction\textsuperscript{375}. Therefore, given the greater cell density, the large diffusion distance, and the restriction of nanoscaled pores under diffusion, insufficient transfer of nutrients and oxygen would likely result in cell necrosis and hypoxia. In summary, our in vitro testing of the iBAP demonstrated that convection is the key to supporting 10% or 20% mouse islet density with either 10 nm or 40 nm pore sized SNM with higher islet viability and providing appropriate glucose-stimulated insulin response.

**The iBAP implantation in pigs**

As a first step to study the device and membrane patency, the diffusion-based iBAP with 10 nm pore sized SNM and a 5% mouse islet density (2,850 IE/cm\(^2\)) was intravascularly grafted in the porcine model for three days. The angiogram showed no thrombosis formation and obstruction in the blood flow path of the device during explant (Fig. 4.5,a, (i&ii)). This data matched with previous study in which the iBAP device was intravascularly implanted into Class
A dogs where device was patent throughout the experiment, possessed no thrombus formation, and generated 27.5 ml of ultrafiltrate based on a SNM pore size of 5.6 nm after explanted at 8 days\textsuperscript{403}. The SEM images of blood-contacting SNM displayed some attachment and aggregations of cells and adhesive proteins (Fig. 4.5,a,iii).
Figure 4.5 In vivo testing of the intravascular bioartificial pancreas device (iBAP) with 5% islet density encapsulated with 10 nm-pore size SNM for 3 days. (a) An image of the explanted diffusion-based iBAP (i). The angiogram showed no obstruction of flow after injecting the contrast agent into the device (ii). An SEM image of the implanted membrane showing attachment of red blood cells and platelets (iii) (scale bar = 10 µm). (b) Immunofluorescence staining of platelet adhesion CD41 marker (green) and platelet activation CD62p marker (red). The rectangular pore-containing regions surrounded by solid silicon regions were shown in the bright field image (i). The platelet adhesion (green) mostly occurred in the window regions where pores reside, whereas minimal platelet activation (red) was detected (ii) (scale bar = 20 µm). (c) The SNM-encapsulated iBAP with 5% islet density under diffusion both in vitro (in vitro 5% diffusion) and in vivo (in vivo 5% diffusion)
In particular, we observed red blood cells, white blood cells, and platelets deposit on the membrane surface. Subsequent immunohistochemical analysis showed that while platelet adhesion mostly in the porous regions of SNM (which contain the nanopores) as indicated by the green CD41 marker, there was minimal platelet activation observed as stained by the red CD62p marker (Fig. 4.5,b). The viability study demonstrated that the diffusion-based iBAP in the pig supported the viability at 5% mouse islet density with 10 nm pore size (88 ± 4.9%) which was comparable with the in vitro conditions (89 ± 2.1%) (Fig. 4.5,c). To avoid hypoxia and necrosis of cells located at the center of diffusion-based devices, islet density of the macrocapsules has been suggested to be 5-10% of the volume fraction in order to ensure the proper exchange of nutrients and waste of islets\textsuperscript{367}. Our iBAP with 10 nm pore sized SNM demonstrated sufficient mass transfer to support the viability of 5% islet density under diffusion (Fig. 4.5,d).

Unfortunately, the concentration of mouse insulin and c-peptide in the porcine systemic circulation was below the detection limit. Cytokine panel indicated an expected increase in the pro-inflammatory response from pig immediately after the surgery (Fig. 4.6).
Next, we evaluated the performance of the iBAP with SNM-encapsulated mouse islets under diffusion and convection at a higher islet density to demonstrate the effectiveness of mass transfer for supporting islet viability and functionality. Specifically, the iBAP with 10 nm pore sized SNM and a 10% mouse islet density (5,700 IE/cm²) with convective and diffusive mechanism was grafted to the carotid artery and vein of a pig for three days. No ultrafiltration was generated for the diffusive side, whereas ultrafiltrate production was observed on the convective side. The ultrafiltrate was directly drained into the interstitial space of the animal. After three days, we observed no significant change in device blood flow rate and the ultrafiltration appeared to be clear, indicating the membranes were intact during the *in vivo*
experiment (Fig. 4.7,a,i). The angiogram also showed no thrombus formation and obstruction in the blood flow path of the device during explant (Fig. 4.7,a,iii). Gross inspection of the blood-contacting membrane surfaces showed minimal cellular adhesion for both diffusive and convective conditions; however, the back side of the SNM under convection exhibited a white layer of proteinaceous materials (Fig. 4.7,b,iii). Our previous study showed that a pore size of ~7 nm SNM can prevent the passage of large molecules such as bovine serum albumin (66.5 kDa)\textsuperscript{370}. Given the pore size (~10 nm) used in this convective transport study, we speculated that some blood-rich proteins such as albumin or fibrinogen passed through and deposited on the back side of the membrane. The SEM images of blood-contacting SNM displayed minimal cellular attachment for the diffusive case, whereas cellular and protein deposition were present for the convective condition (Fig. 4.7,a,ii). Subsequent immunohistochemical analysis showed that the convection resulted in more platelet adhesion and activation on the blood-exposed SNM surface compared with diffusion (Fig. 4.7,b). More importantly, the viability of 10% mouse islet density with 10 nm pore size was higher in the convective condition (85 ± 4.4%) compared to the diffusive scenario (73 ± 4.1%). Interestingly, the \textit{in vivo} viability at 10% mouse islet density with 10 nm pore size under diffusion (73 ± 4.1%) was greater than the \textit{in vitro} viability of those under diffusion (2.0 ± 1.3%) and convection (40 ± 11%). The difference in viability between \textit{in vivo} and \textit{in vitro} testing under diffusion could potentially be due to that blood serves as a better culture medium with essential nutrients for the islets. Encapsulating hemoglobin with islets showed improved islet viability and function as compared to islet encapsulation without
hemoglobin$^{279,406}$. This may result from the ability of hemoglobin from the blood flow path to scavenge nitric oxide (NO), which could be produced by islets under hypoxia$^{407}$.

a.

i. 

ii. 

iii. 

b.

i. 

ii. 

iii. 

iv.
Figure 4.7 In vivo testing of the intravascular bioartificial pancreas device (iBAP) with 10% islet density encapsulated with 10 nm-pore size SNM under either diffusion or convection for
Furthermore, the ultrafiltrate generated directly from the islet chamber on the convective side indicated a mouse c-peptide concentration of 144 pM (or 12 pg/min/IE insulin), exhibiting the functionality of the encapsulated islets. These data demonstrate that SNM encapsulation under convection preserved islet viability and functionality of the encapsulated cells at a higher cell density for macroencapsulation. A pro-inflammatory response was also observed for this pig immediately after the surgery (Fig. 4.8). To summarize, the in vitro testing of the iBAP
demonstrated that SNM with 10 nm pore size showed an improved viability at 10% mouse islet density (5,700 IE/cm²) under convection, and SNM with 40 nm pore size demonstrated an increase in viability at 10% (5,700 IE/cm²) and 20% (11,400 IE/cm²) mouse islet densities compared to those tested under diffusion. Furthermore, the glucose-insulin kinetics experiments showed physiological glucose-insulin response and a clinically relevant absolute insulin production rate. Furthermore, porcine studies demonstrated both device and membrane patency under convection and diffusion, a higher islet viability at 10% mouse islet density with convection, and a clinically relevant mouse insulin production rate. Overall, these studies suggest the feasibility of designing a full-scale SNM-based iBAP to achieve long-term blood flow.

**Figure 4.8** Daily measurement of the systematic cytokine concentration in the pig. The intravascular bioartificial pancreas (iBAP) with 10% islet density encapsulated with 10 nm-pore size SNM. Cytokines namely Interferon gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), interleukin 1-alpha (IL-1α), interleukin 1-beta (IL-1β), interleukin 2 (IL-2), interleukin 6 (IL-6), interleukin 12 (IL-12), interleukin 18 (IL-18), interleukin-1 receptor antagonist (IL-1Ra), interleukin 4 (IL-4), and interleukin 10 (IL-10) were analyzed. Granulocyte-macrophage colony-stimulating factor (GM-CSF) was not detected. About 25.44 pg/ml of interleukin 8 (IL-8) was detected on Day 2 only.
patency, improved islet viability with convection in comparison to diffusion at clinically relevant densities, and sustained clinically relevant insulin secretion.

4.4 DISCUSSION

A biocompatible, retrievable, and immunoisolating bioartificial pancreas (BAP) is a promising approach to expand islet transplantation to additional T1D patients. Native islets are highly perfused with arterial blood at high pO$_2$ (~100 mmHg) levels and possess short diffusion distances between capillaries and cells (<50 μm) resulting in high mass transfer rates of nutrients, wastes, and insulin$^{397,408}$. Cell necrosis occurs when islets are placed beyond the diffusion limit of tissue, which is ~150-200 μm away from the nearest blood vessels$^{133}$. The high mass transfer rates meet the islets’ high metabolic demands and ensure rapid glycemic control$^7$. Consequently, a successful BAP must possess sufficient mass transfer to achieve physiologic glycemic control. While diffusion-based macrocapsule BAP devices have demonstrated successful alloimmunoisolation and efficacy in small animal models$^{154,259,409}$, previous BAP in large animal models have demonstrated poor islet viability and insufficient glycemic control due to inadequate mass transfer. More specifically, inadequate mass transfer resulted from diffusive mass transport over large distances (>500 μm), which created large diffusion gradients of glucose, oxygen, and insulin. The unfavorable gradient reduced islet viability and resulted in poor glucose-insulin kinetics$^{397}$. In addition, many of these devices were implanted extravascularly resulting in low pO$_2$ (10-50 mmHg) and fibrous tissue formation at the device surface severely limiting oxygen delivery$^{410}$. Recently, the Viacyte VC-01 device demonstrated successful alloimmunoisolation, efficacy in small animal models, and promising early clinical data on cell viability and differentiation, and device biocompatibility and vascularization$^{154}$. 
However, the low oxygen concentration and fibrous tissue at the device surface as well as the large diffusion distances could significantly reduce oxygen, glucose, and insulin mass transfer ultimately resulting in a prohibitively large device for implantation and poor clinical outcomes.

Recognizing the serious oxygenation barrier to BAP development, recent BAP devices have attempted to enhance islet oxygenation. Beta-O2 Technologies (Rosh-Haayin, Israel) has implemented exogenous oxygen supplementation by daily oxygen injection through transcutaneous ports, while Giner (Newton, MA) and their collaborators have been developing an implantable electrochemical oxygen generator. Both devices enable continuous and long-term supply of oxygen to implanted islets. While these devices have been designed and optimized for enhanced BAP oxygenation and demonstrated superior islet viability and pre-clinical functionality, early clinical translation of the Beta-O2 device yielded poor graft function with a clinically relevant islet dose (160,000 IE). While clinical testing of the Beta-O2 device may have achieved poor glycemic control for various reasons, the large diffusion distance (>600 um) (>10x normal islet physiology) for glucose into the device and especially insulin out of the device is likely a significant factor. The large diffusion distance requires a significantly higher insulin production rate within the device to achieve the same systemic insulin delivery rate. The large diffusion distance may also cause high insulin concentrations within the islet local microenvironment, consequently inhibiting insulin production. In addition to the Giner device being susceptible to the Beta-O2 device limitations, the Giner device’s small surface area (3-9 cm²) may also significantly reduce insulin release, since the rate of insulin release is directly dependent on device surface area. Consequently both the Beta-O2 and Giner devices may yield a significant delay in insulin release and consequently poor glucose-insulin kinetics. Proper glucose-insulin kinetics is essential to create a functional BAP and even
small delays of insulin release may result in hyperinsulinemia and hypoglycemia. In summary, while these devices supply adequate oxygen, they are not optimized for glucose-insulin kinetics, and may ultimately yield poor glycemic control in the patient.

The use of vascular perfusion devices have been studied to avoid the primary limitations, namely hypoxia and necrosis of cells and the delay of insulin secretion in response to glucose, associated with diffusive transport in extravascular macroencapsulation. Chick and co-workers developed an intravascular hollow fiber device consisting of neonatal islets where the unidirectional blood flow causes the pressure of first part of the fiber lumen to be greater than the pressure in the periphery of the islet compartment, resulting in ultrafiltrate crosses from the bloodstream to the islet graft. Because the hydrostatic pressure drops as a function of distance within the fiber lumen, the low hydrostatic pressure in the second half of the fiber creates an equal, reverse flux where ultrafiltrate moves from the islet compartment back to the bloodstream. This approach ensures encapsulated islets in close contact with the blood circulation, allowing fast exchange of glucose and insulin to correct blood glucose levels in almost real time. Other blood-contacting hollow-fiber shaped devices with islets at the outside of the artificial capillaries also failed due to excess blood clotting in the lumen of those small-diameter capillaries.

Whereas the early extravascular hollow-fiber systems shows a common delay of insulin secretion in response to glucose (>20 min). Maki and co-workers first demonstrated the long-term use of vascular perfusing BAP in large animals. The hybrid pancreas device contained an acrylic housing with islets separated from the common iliac artery and vein in dogs through the semipermeable membrane (80 kDa). They reported that allogeneic islets controlled pancreatectomy-induced diabetes for up to 1 year with zero or minimal exogenous insulin in dogs. Devices retrieved from two recipient dogs showed a 50-70% islet viability after one
year. A 50% reduction in insulin requirements was observed in the allogeneic and xenogeneic recipients after 284 days and 106 days, respectively. Although no gross fibrosis detected throughout the membrane, clotting occurred at either the anastomosis sites or the junction of the graft. Layers of fibrin-like materials were adhered to the luminal surface of the membrane. Implanted hybrid BAP devices in three dogs failed immediately due to excessive clotting and thrombosis, collapse of membrane and vascular connection, and loss of islet function related to device patency. Although these studies demonstrated the feasibility and clinical applicability of the intravascular hybrid BAP devices, it did not move to the clinical stage due to potential risks associated with thrombosis and hemorrhage. Vascular perfusion devices are advantageous in creating high oxygen tension of the arterial blood exposed to islets, however, risks associated with the surgery required for creating arterio-venous (AV) or arterio-arterio (AA) shunts, vascular thrombosis, and potentials risks in diversion of large volume of blood from the distal extremity limited the development and application of this approach.

There are three major areas to be considered for making the successful design of next generation of intravascular macroencapsulation devices: (1) sustaining cell viability by minimizing hypoxia and necrosis, (2) demonstrating proper glucose-insulin kinetics, and (3) exhibiting device patency with no blood clotting and thrombosis with minimal systematic anticoagulants. As mentioned above, although intravascular ultrafiltration devices performed better than diffusion-based devices, the low hydraulic permeability of the polymer membranes used in vascular perfusion devices severely limited their performance. The polymer membranes produced inadequate levels of ultrafiltrate resulting in insufficient convective mass transport; the hydraulic permeability of the polymer membranes was about an order of magnitude too low to achieve ideal glucose-insulin kinetics (~10x too low). Additionally, the low hydraulic permeability also
severely restricted convective oxygen transport, which adversely affected islet viability and function. Consequently, a membrane with a significantly greater hydraulic permeability is needed to provide the convective mass transport rates necessary to ensure proper long-term performance of the promising ultrafiltrate-based intravascular bioartificial pancreas device. Conventional polymer membranes used in previous ultrafiltration-based intravascular BAP devices exhibited hydraulic permeabilities of \( \sim 0.40 \times 10^2 \text{ ml/hr/m}^2/\text{mmHg} \). Our SNM provides an extremely high hydraulic permeability (6 and 120-fold greater for 10 and 40 nm, respectively; Table 4.1) and molecular selectivity with \( \sim 1\% \) pore size variation at nanometer scale\(^{370}\) to enhance immunoisolation in comparison to polymer membranes which possess large pore size distributions \( \sim (30\%)^{339} \).

With the decades of profound technological advances and improved clinical experience with prosthetic vascular grafts used in arterial bypass and dialysis access to develop a safe and effective intravascular device, the development of vascular perfusion devices is now more achievable. Our current \textit{in vitro} and \textit{in vivo} porcine studies of the SNM-encapsulated iBAP demonstrated the advantages of using convective transport to support a high islet viability at a density by volume of 10\% and 20\% by volume, providing fast glucose-insulin kinetics, and maintaining device and membrane patency with only aspirin and Plavix. Specifically, the SNM-based iBAP feasibility was demonstrated by: (1) \textit{in vitro} experiments in which 10 nm and 40 nm pore sized SNM enabled high islet viability (\( >60\% \)) and functionality (\(<15 \text{ minute insulin response to glucose stimulation} \)) at clinically relevant islet densities (5,700 and 11,400 IE/cm\(^2\)) under convection; and (2) porcine studies in which 10 nm pore sized SNM enabled high islet viability (\( >85\% \)) at clinically relevant islet density (5,700 IE/cm\(^2\)), c-peptide concentration of 144 pM (or 12 pg/min/IE insulin) and hemocompatibility under convection.
4.5 CONCLUSIONS

The iBAP with SNM-encapsulated mouse islets under convection provides ultrafiltrate rates at physiologic blood pressure supporting functional islets at clinically relevant islet densities (5,700 and 11,400 IE/cm$^2$). A full-scale SNM-encapsulated iBAP must next be developed to accommodate a clinically relevant number of islets (50,000-300,000 IE) and investigated for the long-term hemocompatibility, ultrafiltrate production, and cell viability and functionality in large diabetic animals and then T1D patients in the future.
CHAPTER V

AN OXYGEN TRANSPORT MODEL AND A DEVICE FOR LONG-TERM EVALUATION OF IMMUNOISOLATION IN VIVO

5.1 INTRODUCTION

Transplantation of pancreatic islet cells can normalize metabolic control in a manner that is not achievable with exogenous insulin. Islet encapsulation with the large capsule sizes and implant volumes can greatly reduce cell viability and poor functional engraftment with the host. Key solutes that are critical for encased islet survival and function include oxygen, glucose, and insulin. Within this context, a quantitative model that will offer insights into the SNM encapsulated-islet environment is of considerable relevance to the optimization of future designs. Furthermore, islet health and engraftment could be impacted by the compounded immune and autoimmune response. An understanding of the effects of the humoral and cellular part of the host immune system on membrane-encapsulated islets is of great importance.

A modified SNM design previously developed by Desai et al. with 18, 66, and 78 nm pore sizes were used to encapsulate rat islets$^{341,356}$. In the investigation, the 18 nm pore sized membranes significantly hindered the passage of IgG as compared to the larger pores, although with incomplete blockage. They also incubated islet-filled capsules in a serum complement and antibody solution for two weeks. At day 14, the insulin secretion following glucose stimulation was approximately five times higher for the encapsulated islets (18 and 78 nm pores) as
compared to unencapsulated islets, demonstrating the potential of immunoisolation by SNM. Additional testing with 7 nm pore sized SNM showed that the diffusion of IgG was greatly hindered and albumin was unable to pass through\textsuperscript{181}. They also encapsulated mouse insulinoma cells that were then implanted intraperitoneally in mice for 8 days. The insulinoma cells encapsulated by 18 nm membranes exhibited higher insulin secretion upon glucose stimulation than the cells in capsules with 66 nm pores. With the data, they concluded that SNM with pores just below 20 nm should be able to immunoprotect cells while allowing sufficient diffusion of glucose and insulin. In contrast to the nanoporous biocapsule (Fig. 1.8) developed by Desai et al, we designed a much thinner extravascular SNM-capsule that can be used to study the long-term immunoisolation in various animal models. In this chapter, we present an analytical modeling of the membrane-encapsulated islet chamber (section 5.2) and describe the extravascular macrocapsule that can be used to explore the complex immune response in mice (section 5.3).

### 5.2 ANALYTICAL MODELING OF THE ISLET CHAMBER

Our objective is to develop an implantable bioartificial pancreas where transplanted islets are encapsulated between two SNM sheets in a device that will be mounted similarly to an arterio-venous (AV) graft (Fig. 5.1). The concept involves using the pressure difference between the artery and vein to generate ultrafiltrate and drive transport of glucose, insulin, and other small molecules through the SNM to support function of encased islets while preventing passage of immune components. Here we report the development of an analytical solution to model the thickness of islet chamber, cell density, and transport of oxygen.
Based on assumptions that (1) the device reaches a quasi-steady state; (2) one-dimensional modelling that variables (e.g. concentration, velocity) change predominantly in one-dimensional illustration of the arterial (red) and venous (blue) blood sweeping the SNM surfaces (black) (adapted from Fig. 5.1). The entire thickness of the chamber is denoted as “L” with x-direction indicating the distance into the islet chamber. At the arterial blood and arterial SNM interface (x = 0), the solute concentration is the same as the initial arterial concentration (C = C₀). At the venous blood and venous SNM interface (x = L), the solute concentration is the same as the initial venous concentration (C = C₀).

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defined direction; and (3) a consumption factor is added to account for encased cells inside the islet chamber (Fig. 5.2), the Navier-Stokes can be written as \( \frac{dc}{dt} + V \cdot \nabla c = D \nabla^2 c - R \) (Eq. 1), where \( c \) is solute concentration (mol/cm\(^3\)), \( t \) is time (min), \( v \) is velocity (cm/min), \( D \) is diffusivity (cm\(^2\)/min), and \( R \) is consumption rate (mol/cm\(^3\)/min). Assuming the steady state is reached and solute concentration does not change with time \( \frac{dc}{dt} = 0 \), then Eq. 1 can be simplified as

\[
V \frac{\partial c}{\partial x} = D \frac{\partial^2 c}{\partial x^2} - R \quad \text{(Eq. 2)}
\]

\[
\frac{\partial^2 c}{\partial x^2} - \frac{V}{D} \frac{\partial c}{\partial x} + \frac{R}{D} = 0 \quad \text{(Eq. 3)}
\]

Solving Eq. 3

\[
c(x) = -\frac{a}{b} x + C_1 + C_2 e^{bx} \quad \text{(Eq. 4), where } a = -\frac{R}{D} \text{ and } b = \frac{v}{D}
\]

Based on Figure 5.2, boundary conditions (BC) are defined as

BC 1: \( x = 0, c(0) = C_0; \ c(0) = C_0 = C_1 + C_2, C_1 = C_0 - C_2 \)

BC 2: \( x = L, c(L) = C_0; \ c(L) = C_0 = -\frac{aL}{b} + (C_0 - C_2) + C_2 e^{bL} \)

\[
C_1 = C_0 - \frac{aL}{b(e^{bL} - 1)} \quad \text{(Eq. 5)}
\]

\[
C_2 = \frac{aL}{b(e^{bL} - 1)} \quad \text{(Eq. 6)}
\]

Now Eq. 4 can be substituted with Eq. 5 and 6,

\[
c(x) = -\frac{a}{b} x + C_0 - \frac{aL}{b(e^{bL} - 1)} + \frac{aL}{b(e^{bL} - 1)} e^{bx}
\]
The change in concentration as a function of thickness of the islet chamber can be written as

\[ c(x) = C_0 + \frac{a}{b} x \left( e^{\frac{L}{e^b x - 1}} - x - \frac{L}{e^b x - 1} \right) \] (Eq. 7)

where \( a = -\frac{R}{D} \), \( b = \frac{V}{D} \), and \( C_0 \) is the initial concentration in the blood.

If we assume the worst scenario occurs where 100% cell density (a fully-packed islet chamber) consume at a maximum rate of oxygen, now Eq. 7 can be plotted to illustrate the relationship between oxygen concentration and thickness of the islet chamber (Fig. 5.3). To prevent hypoxia within the islet chamber, the largest thickness for this islet chamber is 550 µm. Oxygen parameters used in this plot: \( C_0 \) (37 °C) in water = \( 3.73 \times 10^{-7} \) mol/cm³; \( D \) (37 °C) in water \( = 1.61 \times 10^{-7} \) mol/cm³; \( V = 0.2 \) cm/min based on properties of a 15 nm pore sized SNM

**Fig. 5.3** The concentration of oxygen as a function of distance inside the islet chamber with 100% cell density (c.d.) (blue). When the oxygen concentration drops under 50 mmHg, hypoxia can occur leading to massive cell death inside the islet chamber (orange). The oxygen concentration inside the islet chamber was above the hypoxia limit with a maximum thickness of 550 µm.
at physiological pressure difference of 2 psi and a total area of 40 cm$^2$; R (maximum O$_2$

consumption rate)$^{393} = 2.04 \times 10^{-6}$ mol/cm$^2$/min.

Furthermore, we can non-dimensionalize Eq. 1 to obtain
\[
\frac{\partial^2 c^*}{\partial x^*^2} - P_e \frac{\partial c^*}{\partial x^*} + D_{ii} C^* = N_F \frac{\partial c^*}{\partial t^*} \quad \text{(Eq. 7)}
\]
where $P_e$ is the Péclet number, $D_{ii}$ is the Damkohler number ii, and $N_F$ is the Fourier number. The Péclet number signifies the relative magnitude of convection over diffusion as $P_e = \frac{V L}{D}$, where $V$ is the velocity (cm/min), $L$ is the distance (cm), and $D$ is the diffusivity (cm$^2$/min). When the Péclet number is greater than one, the effects of convection exceed those of diffusion in determining the overall mass flux. It is known that time for molecules to travel under pure diffusion is $t = \frac{L^2}{D}$, where $L$ is the distance (cm), and $D$ is the diffusivity (cm$^2$/min).

By combining the information provided by the Péclet number and pure diffusion case, we can calculate the time for molecules such as oxygen, glucose, and insulin to travel through a given distance with respect to diffusion and convection (Table 5.1). The convection significantly enhances solute transport regardless of the molecular weight of the solute.

**Table 5.1** The time required for molecules to travel through the islet chamber with a thickness of 550 μm under diffusion and convection based on a physiological pressure difference of 2 psi and a 15 nm pore sized SNM.

<table>
<thead>
<tr>
<th>Thickness, L=550 μm</th>
<th>Oxygen (16 Da)</th>
<th>Glucose (180 Da)</th>
<th>Insulin (5808 Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diffusion</td>
<td>1.87 min</td>
<td>5.25 min</td>
<td>25min</td>
</tr>
<tr>
<td>Convection</td>
<td>16s</td>
<td>16.5s</td>
<td>16.5s</td>
</tr>
</tbody>
</table>

In this section, we explored the quantitative relationship among solute concentration, chamber thickness, and cell density inside the islet chamber. Although oxygen transport was
given as the only example here, other solutes like glucose and insulin with various pore sizes of SNM can be similarly evaluated and analyzed for different mass transfer conditions.

5.3 DESIGN OF EXTRAVASCULAR SNM CAPSULES FOR IMMUNOISOLATION TESTING IN VIVO

Membrane encapsulation should support the function of encased cells and prevent immune rejection against them. In particular, T cells are the primary drivers of alloimmune responses. They can be activated “directly” by a major histocompatibility complex (MHC) expressed on the transplanted cells or “indirectly” by peptides from allogeneic donor cells which are acquired and presented by recipient antigen-presenting cells. Although the direct pathway can be prevented by establishing a physical barrier between host immune cells and intact graft cells, the indirect pathway is potentially more difficult to block using physical separation due to antigenic fragments shed from the graft. In addition, recipients may become sensitized to alloantigens because of prior or concurrent transplants, and thus, present pre-formed effector T cells with lower threshold of activation. Therefore, an effective encapsulation device should prevent not only activation of both the direct and indirect T cell responses, but also pre-formed alloimmune and autoimmune effector cells. The in vitro model described in previous chapters is ideal in assessing toxicity of defined mediators, but cannot accurately reproduce the complexity of an immune response to allogeneic or xenogeneic graft encapsulated in an engineered or inorganic device. Thus, it is necessary to augment the above in vitro approach with in vivo models. We propose the use of a sealed SNM chamber loaded with various allo-auto, and xeno-antigens for subcutaneous implantation into recipient mice as listed in Table 5.2.
**Table 5.2** Experiments for assessing SNM barrier function for auto-, allo- and xeno-immune responses.

<table>
<thead>
<tr>
<th>Response monitored</th>
<th>Assay</th>
<th>Encapsulated graft</th>
<th>Recipient</th>
<th>Readout</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute autoimmune</td>
<td>Autoreactive T cell proliferation</td>
<td>NOD.Rag2KO islets</td>
<td>NOD</td>
<td>CFSE dilution of BDC2.5 and 8.3 cells on day 7, 14, and 30</td>
</tr>
<tr>
<td>Chronic autoimmune</td>
<td>Prevention of diabetes induced by diabetogenic T cells</td>
<td>Newborn NOD.Rag2KO islets</td>
<td>NOD.Rag2KO reconstituted with diabetic NOD splenocytes or BDC2.5 and 8.3 T cells 100 days after islet transplant</td>
<td>Blood glucose for 100 days after T cell transfer</td>
</tr>
<tr>
<td>Acute alloimmune</td>
<td>Alloreactive T cell proliferation</td>
<td>BALB/c spleen and islets</td>
<td>B6</td>
<td>CFSE dilution of alloreactive T cells on day 7, 14, and 30</td>
</tr>
<tr>
<td>Chronic alloimmune (mouse)</td>
<td>Newborn islet and MIN6 graft survival</td>
<td>Newborn islets from B6.MIP-luc mice; MIN6-luc cells</td>
<td>BALB/c</td>
<td>Luciferase activity for 100 days</td>
</tr>
<tr>
<td>Chronic alloimmune (human)</td>
<td>Human immunogenic tumor cell and insulinoma survival</td>
<td>Luciferase and HLA-A2 transduced K562 myeloleukemia cells; EndoC-bH1 cells</td>
<td>NOD.Rag2KO reconstituted with human PBMCs from HLA-A2 negative donors</td>
<td>Luciferase activity for 30 days; Glucose stimulated serum C-peptide</td>
</tr>
<tr>
<td>Chronic xenoimmune</td>
<td>Human immunogenic tumor cell survival</td>
<td>Luciferase and HLA-A2 transduced K562 myeloleukemia cells</td>
<td>B6</td>
<td>Luciferase activity for 100 days</td>
</tr>
<tr>
<td>Chronic xenoimmune</td>
<td>Human insulinoma cell survival</td>
<td>EndoC-bH1 cells</td>
<td>B6</td>
<td>Glucose stimulated serum C-peptide</td>
</tr>
</tbody>
</table>
In this study, we designed implantable extravascular silicon nanopore membrane (SNM) macrocapsules for investigating allogeneic mouse pancreatic progenitor cells against alloimmune- and autoimmune-mediated rejection in mouse models.

### 5.3.1 SNM Capsule Preparation and Implantation

**Silicon Nanopore Membranes (SNM) fabrication**

Silicon nanopore membranes (SNM) have been prototyped from silicon substrates by MEMS technology as previously reported\(^{347,348,377}\) with some modifications. Briefly, the process used the growth of a thin SiO\(_2\) (oxide) layer on 400 \(\mu\)m-thick double side polished (DSP) silicon wafers followed by a low pressure chemical vapor deposition (LPCVD) of polysilicon (~500 nm). The wafers were then specifically patterned, dry oxidized, wet etched, deposited with a second polysilicon layer, and finally blanket-etched until 400 nm of polysilicon remained and the underlying vertical oxide layer was exposed. The vertical sacrificial oxide layer defined the critical nanoscale pore size of the membranes. The low temperature oxide (LTO) (~1 \(\mu\)m) was deposited onto polysilicon of the wafers to serve as the hard mask for membrane protection. Deep reactive ion etching (DRIE) removed the backside of each window until membranes were disclosed. Eventually, the sacrificial oxide was etched away in 49% hydrofluoric acid (HF) during the final step of the fabrication process to leave behind open nanoscale slit pores. The wafers were subsequently cut into 1\(\times\)1 cm chips with an effective area of 6X6 mm\(^2\) containing 1500 windows each, with a total of \(10^6\) pores per membrane. Each rectangular pore was 7 nm in width, 300 nm in depth, and 2 \(\mu\)m in length. All membranes were cleaned using a conventional “piranha” clean procedure, which involved a 20 min-immersion in 3:1 sulfuric acid (H\(_2\)SO\(_4\))/hydrogen peroxide (H\(_2\)O\(_2\)) mixture, followed by thorough rinses in deionized (DI) water.
Surface modification of SNM with poly(ethylene glycol) (PEG)

SNM were covalently modified with PEG using a previously reported protocol with some modifications to prevent protein fouling on the membrane surface. The technique used for PEG attachment involved a single reaction step which covalently couples silicon surface silanol group (Si-OH) to a chain of PEG polymer through a trimethoxysilane group forming a Si-O-Si-PEG sequence. Briefly, SNM were immersed in a solution of 3 mM 2-[methoxy(polyethyleneoxy)propyl]trimethoxysilane (PEG-silane) (Gelest: SIM6492.7) in toluene for 2 hr at 70 °C. A series of extensive washing steps involving toluene, ethanol, and DI water were used to rinse away unbounded PEG residue.

Assembly of SNM macrocapsules

To prepare the polydimethylsiloxane (PDMS) thin film, liquid PDMS base and curing agent (Sylgard 184; Dow Corning) were mixed at a ratio of 10:1, degassed, and poured uniformly on top of a silanized silicon wafer on a spin coater. The SDS 6800 spin coater was programmed with the following command: (a) 5 s ramp to 500 rpm; dwell 10 s; (b) 22 s ramp to 6000 rpm; dwell 300 s; and (c) 24 s ramp to 0 rpm; dwell 0 s. After spin coating was complete, the PDMS-silicon wafer was cured at 85 °C for 2 h. The thickness of the PDMS thin film was ~300 µm. The PDMS thin film was then cut into hollow ring with an inner diameter of $\frac{3}{8}$" and an outer diameter of 1” (Mayhew Pro 66002). Next, SNM chips and PDMS rings were placed on plasma bonder tray and inserted into plasma oven (M4L O₂ Plasma System) with oxygen plasma exposure at 100 W and 200 sccm for 10 s. About 5 µl isopropyl alcohol was deposited on the spot where the PDMS rings would contact the SNM chips. The PDMS rings were immediately placed on top of the SNM chips with applied pressure to ensure the bonding. After 24 hr, the PDMS-SNM pieces were complete. Using the same process described above, two pieces of the
PDMS-SNM rings were made into one SNM capsule with 10-15 µl volume without leakage (Fig. 5.4).

**Figure 5.4** The SNM macrocapsule with 10-15 µl volume. (a) A top view of SNM macrocapsule consisted of two SNM chips and PDMS rings (scale bar = 1 cm) (b) A side view of SNM macrocapsule consisted of two SNM chips and PDMS rings (scale bar = 600 µm).

**Implantation of SNM macrocapsules in mice**

The empty SNM macrocapsules were implanted subcutaneously into the flank sides of mice for 45 days (Fig. 5.5). The round geometry showed good biocompatibility with no tissue and skin tearing. Although no fibrotic pockets were observed for the first two weeks, large fibrotic tissues were presented on day 45. All SNM macrocapsules remained clean and no bacterial infection detected at the end of explant.
To study the allogeneic immune response in the mouse model, we will implant SNM macropodculus-encapsulated mouse allogenic islets or cell lines into B6 mice. One day after transplantation, carboxyfluorescein succinimidyl ester (CFSE)-labeled lymph node (LN) cells from 4C, TCR75, and 2C TCR mice can be injected into recipient B6 mice. Briefly, Tg 4C T cells are derived from H-2b B6 background and directly recognize the MHC class II molecule I-A^d (CD4+ direct pathway)\textsuperscript{421}. Transgenic 2C T cells are derived from H-2b B6 background and directly recognize the class I MHC H-2L^d (CD8 direct pathway)\textsuperscript{422}. Transgenic TCR75 T cells indirectly recognize a peptide derived from H-2K^d MHC class I molecule presented by MHC class II molecule I-A^b (CD4+ indirect pathway)\textsuperscript{423}. After 7 and 12 days, axillary LN from recipient mice can be analyzed for individual expansion of T cell subsets using flow cytometry. By understanding the expansion of T cell subsets and their corresponding activation pathway, we
can assess the efficacy of SNM macrocapsule in protecting allogeneic mouse cells against alloimmune-mediated rejection in mice.
The work (Ch. 1 – Ch. 5) presented in this research dissertation will provide future investigators utilizing convective silicon nanopore membrane (SNM) encapsulated transplantation technology with a basis from which to potentially improve the current Type 1 Diabetes (T1D) treatment. Specifically, the SNM encapsulation demonstrated middle molecule selectivity against pro-inflammatory cytokines and provided sufficient nutrients and oxygen with convective transport to overcome the mass transfer limitations associated with diffusion through nanometer-scale pores (Ch. 2). The glucose-stimulated insulin response demonstrated that membrane-encapsulation of islets with convection outperformed the diffusive conditions in terms of the magnitude of insulin secreted (1.49-fold increase in stimulation index & 3.86-fold decrease in shut-down index) and the rate of insulin production during high (1.19-fold increase) and low glucose (6.45-fold decrease) challenges (Ch. 3). In addition, the SNM-based intravascular bioartificial pancreas (iBAP) device was shown to support high cell viability and function at clinically relevant islet densities utilizing convective mass transfer both in vitro and in vivo (Ch. 4). The hemocompatibility of the iBAP blood flow path after intravascular implantation was also demonstrated in the porcine model (Ch. 4).

However, significant work remains before successful clinical translation and the potential benefits of using convective SNM encapsulation are realized. As described in the previous chapters, the SNM could be further optimized by changing the configuration of the slit-pores, increasing pore density on the membrane, and extra reduction of membrane thickness. These changes would potentially enhance mass transport across the membrane barrier and provide a greater hydraulic permeability to sustain the viability and functionality of encased cells, while
rejecting undesired immunocellular components with the inherent membrane selectivity. In addition, SNM-encapsulated graft performance and its immune-barrier function could be further evaluated in a diffusion-based extravascular mouse model or in a convective intravascular pig model for an extended period of time based on strategies mentioned in studying alloimmune- and autoimmune-mediated rejections (Ch. 5). It is also important to consider other biocompatibile materials and encapsulation methods that uniformly coat islets before SNM encapsulation to reduce cell aggregation and improve cell viability. Alternative microfabrication-based strategies to enhance the microenvironment within islet chamber are worth exploring. For example, the appendix chapter illustrates some fabrication techniques to create microtextured substrates to enhance cell growth, and might be adapted to control and organize islet aggregation. Based on the promising results from iBAP that supported functional islets at clinically relevant islet densities (5,700 and 11,400 IE/cm²) for 3 days in pigs, a full-scale SNM-encapsulated iBAP needs to be developed to accommodate a clinically relevant number of islets (50,000-300,000 IE) and investigated for the long-term hemocompatibility, ultrafiltrate production, and cell viability and functionality in large animals in the future.

In summary, this dissertation suggests the design of a bioartificial pancreas device consisted of SNM-encapsulated insulin-producing pancreatic islets under convective transport that could effectively treat T1D in the future.
APPENDICES

EFFECT OF SURFACE TOPOGRAPHY AND BIOCHEMICAL CUES ON OSTEOREGENESIS AND ANGIOGENESIS OF HUMAN MESENCHYMAL STEM CELLS

Substantial portions of this Chapter have been published in Acta Biomaterialia 2015;18:100-111.

A.1 INTRODUCTION

Bone is the second most common transplanted tissue\(^\text{424,425}\) with an estimated 2.2 million grafting procedures performed annually worldwide\(^\text{426}\). The gold standard for bone regeneration is to use autologous bone grafts for traumatic injuries, fracture non-unions, spinal fusion, and hip joint replacements, and other bone-related diseases\(^\text{427}\). However, autologous grafting is associated with limited availability of donor tissue for clinical applications\(^\text{428-430}\) and significant patient morbidity and complication rates of 8.6\%-20.6\%\(^\text{424,431,432}\). Allograft technologies have been developed to address the limited availability of autograft bone. However, the process of decellularization, sterilization, and allograft storage disrupts the osteoinductive nature of the tissue and results in clinical failure rates of 16-35\% predominantly due to the inability of these grafts to adequately re-vascularize\(^\text{433,434}\). As such, there is a significant and growing need for strategies to promote vascularized bone graft substitutes for this unmet clinical need. Several strategies have been used to stimulate musculoskeletal healing, such as controlled release of growth factors to promote vascularization\(^\text{435-438}\) or regulation of osteogenesis with mechanical properties\(^\text{439-444}\). Nonetheless, the lack of host vasculature penetration and integration between engineered bone scaffold constructs and host tissue still remains a fundamental challenge during
bone fracture healing\textsuperscript{445-447}. An understanding of the scaffold’s underlying physical properties and its biochemical environment is crucial to elicit the desired biological responses for bone regeneration\textsuperscript{448,449}.

Surface topographical characteristics are important aspects in designing biological implants, as they have great implications in cell guidance and behavior\textsuperscript{450-458}. The development of microfabrication and related microelectromechanical systems (MEMS) technologies such as soft lithography offers unprecedented reproducibility and precision to create surface topography that can interact with cells and tissues in a systematic manner\textsuperscript{459-462}. For instance, Dalby \textit{et al.} demonstrated that different arrangements of topographical disorder could modulate osteogenic differentiation of human mesenchymal stem cells (hMSCs)\textsuperscript{463}. The Chen group demonstrated that the inherent rigidity of different sized micropatterns could shift the balance of hMSC fates, alternating between osteogenesis and adipogenesis\textsuperscript{464,465}. Our laboratory also reported that 10 µm diameter post microtextures on polydimethylsiloxane (PDMS) significantly increased proliferation and osteogenesis of human bone marrow-derived connective tissue progenitor cells (CTPs) compared with those cultured on smooth surface\textsuperscript{444,466}. These results suggest that varying geometry and arrangement of the surface topography can result in the modification of cell proliferation and morphology as well as the potential to enhance lineage specificity. However, there has been little investigation on the synergistic effects of microscale surface topography and biochemical attributes for tuning hMSC fate \textit{in vivo}. Particularly, \textit{in vivo} studies concerning microtextured materials have demonstrated mixed results in which microgrooved surfaces produced the same cellular and tissue response as smooth surfaces\textsuperscript{467,468}. It has been suggested that an amorphous layer was formed between the surface microtexture and the connective tissue.
due to the influence of inflammatory cells present at the site of implant surface. Hence, cells from the connective tissue were unable to sense and respond to the micro-topography\textsuperscript{467,469}.

The goal of this study is to evaluate the long-term combined effect of microtextured topography and biochemical cues on hMSC proliferation and osteogenic differentiation, and re-vascularization in mice for potential bone tissue engineering applications. Specifically, PDMS substrates with 10 µm cylindrical post (diameter, height, and interspace) were created to culture hMSCs for 6 weeks under two different conditions: (1) one group in the proliferative basal medium (BM) during the entire study; and (2) another group in the BM for the first five weeks and in the differentiative osteogenic medium (OM) for the last week. The \textit{in vitro} pre-culture maximized material-cell interaction for lineage specificity on microtextured scaffolds, ensuring adequate hMSCs sensing and response to topographical signals for \textit{in vivo} development. The \textit{in vivo} study investigated the potential osteogenic differentiation and vascular integration of hMSCs with the host environment by subcutaneous implantation of hMSCs on microtextured substrates in BM and OM for 6 weeks.

\section*{A.2 MATERIALS AND METHODS}

Bone marrow derived hMSCs were cultured for 6 weeks on 10 µm micropost PDMS substrates under two conditions: (1) in the proliferative basal medium (BM) during the entire study, and (2) in the BM for the first five weeks and in the differentiative osteogenic medium (OM) for the last week. Cell seeded scaffolds were analyzed \textit{in vitro} using fluorescent microscopy, scanning electron microscopy (SEM), histological stains, and real time polymerase chain reaction (RT-PCR). Cell seeded scaffolds were also subcutaneously implanted into mice for 6 weeks.
A.2.1 SUBSTRATE PREPARATION

The microfabricated-patterned mold was prepared using soft lithography techniques as previously described (Fig. A.1). To prepare the PDMS substrate, liquid PDMS base and curing agent (Sylgard 184; Dow Corning) were mixed at a ratio of 10:1, degassed, and poured uniformly on top of the patterned mold. After curing the patterned mold at 85 °C for 2 h, solidified PDMS casts with 10 μm cylindrical posts were released from the mold. Patterned

Figure A.1 Fabrication of PDMS post microtextures by soft lithography. Briefly, 10 μm thick SU-8 2010 photoresist was spin coated on top of silicon wafers. The post microtexture pattern with 10 μm inter-space was transferred from a photomask onto the photoresist under UV exposure. The liquid PDMS and curing photocatalyst were mixed at a ratio of 10:1, degassed for 20 min, and then poured uniformly on top of the patterned mold. The PDMS substrates were cured at 85 °C for 2 h.
PDMS substrates were cut into 4 mm diameter disks, sterilized for 30 min with 70% ethanol, and washed 3 times with phosphate buffered saline (PBS) for subsequent cell culture.

A.2.2 CELL CULTURE

Human MSCs (PT-2501, Lonza, Allendale, NJ) were cultured on microtextured 4 mm disks under standard culture conditions. For the BM condition, hMSCs seeded on microtextured PDMS scaffolds were grown in Dulbecco's modified Eagle's medium (DMEM) low glucose (CCFAA001, UCSF Cell Culture Facility) supplemented with 10% fetal bovine serum (FBS) (Life Technologies), 1% penicillin-streptomycin (P/S) (CCFGK003, UCSF Cell Culture Facility), and 10 ng/mL recombinant human FGF-2 (R&D Systems, Minneapolis, MN) for 6 weeks. For the OM condition, hMSCs seeded on PDMS substrates were first grown under BM for 5 weeks before exposed to osteogenic media (OM) in DMEM high glucose (CCFAA005, UCSF Cell Culture Facility) supplemented with dexamethasone (10⁻⁷ M) (D4902, Sigma), L-Ascorbic acid 2-phosphate (100 µM) (A8960, Sigma), β-glycerol phosphate (10 mM) (50020, Sigma), sodium pyruvate (100X) (CCFGE001, UCSF Cell Culture Facility), ITS+ (BD Biosciences, NJ), and 1% P/S for another week. Seeding density for both conditions was 2X10⁶ cells/cm².

Mouse preosteoblast cell lines MC3T3 (obtained from R.Franceschi, University of Michigan, Ann Arbor, MI) and caiB 2T3 (obtained from S.E.Harris, University of Texas, San Antonio, TX) were cultured on microtextured substrates as positive controls. For the BM condition, they were grown on microtextured PDMS scaffolds in α- Minimum Essential Medium w/o nucleosides (α-MEM) (CCFAC006, UCSF Cell Culture Facility), 10% FBS, and 1% P/S. For the OM condition, they were moved to OM comprising α-MEM, 2% FBS, L-Ascorbic acid
2-phosphate (10 µg/mL), β-glycerol phosphate (10 mM), and 1% P/S. Seeding density for both conditions was 2X10^6 cells/cm^2.

For all data shown, hMSCs were used between passages 5-7. MC3T3 and calB2T3 were used between passages 12-16 and passages 15-25, respectively. Individual experiments were repeated at least 3 times with different cell preparations.

A.2.3 CELL CULTURE ANALYSIS

Cell proliferation and morphology

After cells were cultivated for 6 weeks on microtextured substrates, the media was removed and washed with PBS. For the proliferation study, they were stained with 0.4% trypan blue and counted. For morphology observation, they were fixed in a solution containing 3% glutaraldehyde (G7651, Sigma), 1M sodium cacodylate (Polysicences) and 0.1M sucrose (Sigma). After 2 days, the substrates were washed with distilled water. Dehydration was achieved by placing these scaffolds in an increasing concentration of ethanol (50-100%). Dehydrated samples were then mounted on aluminum stubs, sputter-coated with gold-palladium, and examined with SEM (Ultra 55, Carl Zeiss).

Quantitative gene expression

Total RNA of hMSCs from different conditions was extracted using the RNeasy® Mini Kit (Qiagen). The quantity of the RNA was determined using NanoDrop™ ND-1000 spectrophotometer (NanoDrop Technologies, DE, US). cDNA was obtained using 100 ng of total RNA plus master mix from Fast SYBR® Green Cells-to-CT™ Kit (Life Technologies) with BioRad iCycler (BioRad, CA). Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed using 7900HT PCR system (Applied Biosystems) and Fast SYBR Green PCR kit (Life Technologies). For in vitro cell-based scaffolds, relative
quantification of human Collagen X (COL X), Collagen I (COL I), Alkaline Phosphatase (ALP), Bone Sialoprotein (BSP), Osteocalcin (OC), Collagen II (COL II), and Peroxisome Proliferators-Activator Receptor γ (PPARγ) was performed using the comparative $C_T$ (crossing of threshold) method ($\Delta \Delta C_T$ method) with GAPDH from hMSCs cultured on smooth surface and human osteosarcoma cell line Saos-2 cultured on smooth surface as negative and positive controls, respectively. Primers (IDT Technologies) used in this study were previously published sequences (Table A.1). *In vivo* scaffolds were also analyzed with previous mentioned markers. Relative quantification of mouse platelet endothelial cell adhesion molecule (PECAM) and mouse vascular endothelial growth factor (VEGF) (Table A.2) were analyzed with mouse GAPDH used as an internal control and no cDNA used as a negative control. Each experiment was performed at least in triplicate.

**Table A.1** A list of primers used in RT-PCR analysis of *in vitro* and *in vivo* microtextured scaffolds.

<table>
<thead>
<tr>
<th>Species</th>
<th>Gene Makers</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>COL I</td>
<td>CAGCCGCTTCACCTACAGC</td>
<td>TTTTGTTATTCAATCAGCTGCTC</td>
</tr>
<tr>
<td></td>
<td>COL X</td>
<td>CAAGGCACCACCATCTCAGGAA</td>
<td>AAAGGTTATTTGTGCCCAGCA</td>
</tr>
<tr>
<td></td>
<td>ALP</td>
<td>ACGTGGCTAAGATGTCATC</td>
<td>CTGGTACGGAGATGCCCTCTTA</td>
</tr>
<tr>
<td></td>
<td>BSP</td>
<td>CATTCTGGGAATTGCCTGCT</td>
<td>ATTGTCTCCGCTGCT</td>
</tr>
<tr>
<td></td>
<td>OC</td>
<td>GAAAGCAGCCGGGTCAA</td>
<td>CACTACCCGGTCCCTC</td>
</tr>
<tr>
<td></td>
<td>COL II</td>
<td>TATGCCACCTCCCACACG</td>
<td>AAGGCCCTTTGTAAGAAGCTA</td>
</tr>
<tr>
<td></td>
<td>PPARγ</td>
<td>CCAGATGCCAACTCCCACACG</td>
<td>AAGGCACCTTTGTAAGACCCTA</td>
</tr>
<tr>
<td></td>
<td>GAPDH</td>
<td>ATGGGGAAAGGTGAAGGTC</td>
<td>TAAAAGGCAGCCCTGGTGAC</td>
</tr>
</tbody>
</table>

170
Table A.2. A list of primers used in RT-PCR analysis of in vivo cell-cultured microtextured scaffolds.

<table>
<thead>
<tr>
<th>Species</th>
<th>Gene Makers</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>PECAM</td>
<td>TGCTCTCGAAGCCCAGTATT</td>
<td>CGCTGAACACCGCGGGGTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GGAATGGGC</td>
</tr>
<tr>
<td></td>
<td>VEGF</td>
<td>GGAGATCCTTCGAGGAGCACTT</td>
<td>GGCGATTAGCAGCAGATAT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AAGAA</td>
</tr>
<tr>
<td></td>
<td>GAPDH</td>
<td>TGCCCCCATGTATTGTGATG</td>
<td>TGTGGTCATGAGCCCTTCC</td>
</tr>
</tbody>
</table>

**Immunofluorescence of bone-specific markers**

Cells on microtextured substrates were fixed with 4% formaldehyde followed by PBS washes, permeabilized with 0.1% Triton X-100 for 10 min, and incubated in blocking solution (PBS, 1% bovine serum albumin (BSA)) for 30 min. Samples were incubated with primary antibodies (COL IA: sc-8783, OC: sc-30044; Santa Cruz Biotechnology) at a dilution of 1:200 for 1 hr and washed twice for 5 min with PBS to remove residues. Another incubation with secondary antibodies (COL1A: anti-goat sc-2783, Santa Cruz Biotechnology; OC: anti-rabbit A-21206, Life Technologies) at a dilution of 1:500 for 1 hr was used followed by PBS washes for 5 min. DAPI (Vectashield) was added for nuclear staining. Images were obtained using 6D High Throughput Perfect Focus System (Nikon Instruments) for in vitro scaffolds. In vivo scaffolds were analyzed with laser scanning spectral C1si confocal microscope (Nikon Instruments).

**Mineral content**

Quantitative analysis of ALP activity was performed as previously described\(^{471}\), using Sigma Fast p-nitrophenyl phosphate tablet sets (Sigma). Cell seeded scaffolds were washed with PBS, then substrate buffer made from p-nitrophenyl phosphate tablet sets were added to each sample. After 10 min, NaOH was added to supernatants taken from each sample to stop
enzymatic activity. ALP activity was measured by Spectra Max M2 plate reader (Molecular Devices) at 405 nm using p-nitrophenyl as a standard and analyzed according to the manufacturer’s protocol (85L-3R, Sigma).

**Calcium deposition**

Samples were fixed with 70% ethanol at room temperature for 15 min. After washes with distilled water for 5 min, 1% alizarin red stain (Sigma) was added to samples for 20 min. Samples were washed with distilled water 4 times. Images were obtained using Leica DFC 295 (Leica Microsystems).

**Histology**

Hall Brunt Quadruple stain\(^4\)\(^7\)\(^2\) was used to distinguish bone (red) from cartilage (blue). Samples were fixed with 70% ethanol. They were stained with 0.5% celestine blue solution (51050, Fisher Scientific) consisted of 14% glycerin and 5% ammonium sulphate for 5 min, Mayer’s haematoxylin (75290, Fluka) for 5 min, 1% alcian blue in 1% acetic acid (74240, Merckmillipore) for 6 min, 1% phosphomolybdic acid for 1 min, and 0.5% direct red solution (28160, Polysicences) for 6 min. Samples were washed with distilled water for 2-5 min between each stain. Images were obtained using Leica DFC 295 (Leica Microsystems).

**A.2.4 Harvested Scaffolds Analysis**

All animal studies were approved by the UCSF IACUC. Immunocompromised nude mice (Homozygus Nu/J strain #002019; Jackson Laboratories, Sacramento CA) were used for transplantation in order to prevent any graft rejection. A total of 6 adult male mice (9-14 weeks old) were anesthetized with an intraperitoneal injection of 1.5 mg ketamine and 0.15 mg medetomidine. The skin was cleaned with povidone-iodine and chlorhexidine gluconate (4% w/v). Using a scalpel or surgical scissors, 1-2 mm incisions were created in the skin on the dorsal
side of the mice approximately 10-15 mm from the midline. Subcutaneous pockets were generated in the connective tissue beneath the incision using blunt dissection and scaffolds were placed at least 1 mm laterally from the incision site with the printed scaffold features facing the subcutis. Each mouse received 8 scaffolds. Scaffolds were placed approximately 20 mm from each other in a linear fashion, dorsal to caudal, such that scaffolds are sufficiently isolated from each other. The incisions were closed with two 6.0 silk sutures and covered with a triple antibiotic ointment. Anesthesia was reversed and animals were provided with post-surgical analgesics per approved protocol. Animals were socially housed, allowed to ambulate freely, and survived for 6 weeks prior to cellular and molecular analyses. We have previously found that this protocol is well tolerated by the animals.

Three types of scaffolds were prepared by methods described in previous section (A.2.3) for transplantation: (1) microtextured PDMS without cells, (2) hMSCs on microtextured PDMS in BM, and (3) hMSCs on microtextured PDMS in OM. Harvested scaffolds were analyzed using methods illustrated previously in this section (A.2.3).

**Immunofluorescence of angiogenic marker**

*In vivo* scaffolds were fixed using same procedure in section 1.2.3. Primary antibodies anti-human mitochondria (MAB1273, Millipore) with 1:10 dilution and anti-mouse PECAM-1 (B&D Biosciences) with 1:200 dilution were used. Secondary antibodies anti-rat (A-11006, Life Technologies) and anti-mouse (A-11014, Life Technologies) were used to incubate harvested scaffolds. DAPI was added for nuclear staining. Images were obtained using laser scanning spectral C1si confocal microscope (Nikon Instruments).

**Histology**
Harvested scaffolds were fixed in 4% paraformaldehyde for 15 min and processed to paraffin using standard protocols. Sections were cut at 5 µm and mounted on glass slides. Hematoxylin and Eosin (HE) was performed on the subsequent slides. Slides were deparafinized using 3 changes of xylene for 2 min, rehydrated through a graded ethanol series (100%, 95%, 70%) each for 1 min. Slides were washed in water followed by 7 min in Mayers Haematoxylin to stain nuclei (HXMMHGAL, American Mastertech). Excess haematoxylin was removed with water for 1 min followed by bluing (HXB00242E, American Mastertech) for 1 min. Excess bluing was removed with water for 1 min. Slides were then placed in 70% ethanol for 1 min and Eosin/Phloxine were used to stain cytoplasm and connective tissues (STE0457, American Mastertech). Eosin was differentiated for 1 min in 95% alcohol and the tissue sections are dehydrated in 2 changes of 100% alcohol followed by 3 changes of 2 min in xylene and mounted in a resinous based mounting media (361254D Depex, Fisher Scientific). Slides were dried and viewed under Leica DFC 295 (Leica Microsystems).

Statistical Analysis

Sample pairs were analyzed by the Student’s t-test. Multiple samples were evaluated with one way or two way analysis of variance (ANOVA) followed by Bonferroni and multiple comparison using Prism (San Diego, CA). A P value of <0.05 was accepted as statistically significant for all samples.

A.3 RESULTS

A.3.1 IN VITRO CULTURE ANALYSIS

Cell proliferation and morphology
The microtextured PDMS disks cultured with hMSCs exhibited a higher proliferative capacity in BM than OM after a 6-week culture period (Fig. A.2). Positive control mouse pre-osteoblast cell lines calB2T3 and MC3T3 demonstrated significantly more cell growth in both BM and OM compared with that of the hMSCs (Fig. A.2). The SEM images also revealed that hMSCs tended to attach onto cylindrical posts by spreading their processes to other posts and neighboring cells in BM (Fig. A.3,b), whereas hMSCs cultured in OM condition displayed layers of ECM that covered most of the microtextured surfaces (Fig. A.3,c).

Figure A.2 Number of cells on PDMS microtextured substrates in basal medium (BM) and osteogenic medium (OM) for 6 weeks. The positive control mouse pre-osteoblast cell lines calB2T3 and MC3T3 and hMSCs were cultured on microtextured substrates. Proliferation study showed that hMSCs have less proliferative capacity than the positive controls in both BM and OM conditions. The number of hMSCs on microtextured surfaces increased significantly in BM compared to OM. (n>3, *p<0.05, **p<0.005, ****p<0.0001)
Gene and extracellular matrix protein (ECM) expression

The expression of osteoblast-specific markers was assessed in hMSCs on microtextured substrates under BM and OM conditions in vitro using real time RT-PCR (Fig. A.4). Findings indicated that hMSCs on microtextured substrates differentiated toward osteogenic lineage even when cultured under proliferative BM condition as shown by the relative expression of osteoblast-specific markers (COL I, ALP, BSP, and OC) (Fig 1.4,a). The osteogenic capacity of hMSCs on microtextured substrates in BM was significantly elevated by switching to OM as indicated by a 5.5-to-6 fold increase in COL I, 2.7-fold increase in ALP, 1.3-to-1.7 fold increase in BSP, and 1.6-fold increase in OC (Fig. A.4). The chondrogenic and adipogenic markers (COL II and PPARγ) were not expressed (Fig. A.4,a).
a. hMSCs in BM on smooth surface as negative control

b. hMSCs in OM on smooth surface as negative control

c. Human Saos-2 on smooth surface as positive control
The osteogenic differentiation of hMSCs on microtextured substrates was also confirmed by immunohistological detection of osteoblast markers COL I and OC. Human MSCs on microtextured substrates in both BM and OM showed a greater intensity of osteoblast markers compared with hMSCs on smooth surfaces (Fig. A.5).
Histological analysis of hMSCs on microtextured substrates

Direct visualization of mineralization and calcium deposition verified bone cell differentiation and bone formation. The hMSCs on microtextured substrates in OM possessed a significant increase in ALP activity compared with cells in BM (Fig. A.6). Results from alizarin red data indicated that hMSCs on microtextured substrates stained more intensely in OM than

**Figure A.5** Immunofluorescence staining of osteogenic markers COL I (red, b,f,j) and OC (green, c,g,k) on microtextured scaffolds. Nuclei were stained with DAPI (d,h,l). *(Note: the original color images were converted to grayscale and reversed to provide visual clarity.)* (a-d) hMSCs on smooth PDMS substrates. (e-h) hMSCs on microtextured surfaces in BM. (i-l) hMSCs on microtextured surfaces in OM (i-l). hMSCs on microtextured surfaces in BM (f,g) and OM (j,k) revealed high intensity of COL I and OC compared to the negative control, undifferentiated hMSCs on smooth surface (b,c), indicating the differentiation of hMSCs toward osteogenic lineage.
BM (Fig. A.7). Overall, the spatial distribution and intensity of both alizarin red stain were found to be consistently greater for cells cultured in OM than BM.

**Figure A.6** Alkaline phosphatase levels were normalized for cells cultured on microtextured surfaces in basal medium (BM) and osteogenic medium (OM). The level of mineralization enhanced significantly for hMSCs on microtextured substrates in OM compared to BM. The positive control pre-osteoblast cell lines calB2T3 and MC3T3 showed a greater level of mineralization in both BM and OM. (n=3, **p<0.005)

**Figure A.7** Alizarin red staining of cells cultured on microtextured surfaces in basal medium (BM) (a-d) and osteogenic medium (OM) (e-h). (a,e) microtextured substrates. (b,f) calB2T3 on microtextured surfaces. (c,g) MC3T3 on microtextured surfaces. (d,h) hMSCs on microtextured surfaces. The OM condition (f-h) enhanced calcium deposition of cells on microtextured surfaces compared to the BM condition (b-d). hMSCs on microtextured substrates in both BM (d) and OM (h) showed significant amount of calcium deposition.
A.3.2 IN VIVO ANIMAL STUDY

Scaffold morphology

After a 6-week implantation period in the mice, harvested scaffolds displayed various levels of vascularization, which we observed during the explant process (Fig. A.8) (Table A.3). Generally, hMSCs on microtextured substrates in OM showed a more extensive vascular invasion throughout the entire scaffold (Fig. A.8(c,d)), while hMSCs on post microtexture in BM exhibited vascularization mostly on the edge of the scaffolds (Fig. A.8,b). Minimal vasculature was observed in the microtextured substrates without hMSCs (Fig. A.8,a). SEM images demonstrated that all implanted scaffolds developed ECM rich capsules with uniform shape and thickness around the entire microtextured substrates (Fig. A.9). However, the sprouting of new blood vessels\textsuperscript{15} in tissues surrounding the implants was significantly higher for hMSCs on microtextured substrates in OM (Fig. A.9(e,f)) compared with microtextured substrates without hMSCs (Fig. A.9(a,b)) and hMSCs on microtextured substrates in BM (Fig. A.9(c,d)).

**Table A.3** The number of harvested scaffolds with vascular formation based on a double-blind observation.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Overall Vascularization</th>
<th>Edge Vascularization</th>
<th>No Vascularization</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDMS</td>
<td>1</td>
<td>--</td>
<td>2</td>
</tr>
<tr>
<td>hMSCs + PDMS in BM</td>
<td>2</td>
<td>2</td>
<td>--</td>
</tr>
<tr>
<td>hMSCs + PDMS in OM</td>
<td>4</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>
Figure A.8 Vasculature observed from harvested scaffolds after a 6-week implantation in mice. (a) microtextured substrates without hMSCs. (b) hMSCs on microtextured surfaces in BM. (c,d) hMSCs on microtextured surfaces in OM. Microtextured substrates without hMSCs (a) that were implanted in mice lacked any microvasculature and integration with the surrounding host tissues. hMSCs on microtextured surfaces in BM (b) exhibited micro-vessel growth surrounding the edge of the substrates, whereas hMSCs on microtextured surfaces in OM (c,d) showed a more perfused and extensive network of blood vessels in all directions.
Gene and ECM expression

The expression of osteoblast-specific markers was assessed in harvested scaffolds using quantitative RT-PCR (Fig. A.10,a). Similar to the gene analysis results in vitro, hMSCs on microtextured substrates in BM differentiated toward osteogenic lineage with increased expression of osteoblast-specific markers (ALP, BSP, and OC) (Fig. A.10,a). Compared with hMSCs on microtextured substrates in BM, the osteogenic capacity of hMSCs on microtextured substrates in OM was expedited as shown by a 4-fold increase in COL I, 3.3-fold increase in ALP, 2.2-fold increase in BSP, and maintained a constant level of OC expression (Fig. A.10,a). The chondrogenic and adipogenic markers (COL II and PPARγ) were not expressed (Fig A.10,a). The synergistic effects of microtextured surfaces and the OM enhanced osteogenesis of hMSCs in vivo. The expression of angiogenic genes showed that PECAM and VEGF were 5.8- and 58.7-fold higher in hMSCs on microtextured substrates in OM compared with BM,

Figure A.9 SEM images of harvested scaffolds after a 6-week implantation in mice. (a,b) microtextured substrates without hMSCs. (c,d) hMSCs on microtextured surfaces in BM. (e,f) hMSCs on microtextured surfaces in OM. All implanted scaffolds developed ECM rich capsules with uniform shape and thickness around the entire microtextured substrates. However, the sprouting of new blood vessels in tissue surrounding the implants was significantly higher for hMSCs on microtextured surfaces in OM (e,f). Red blood cells (black arrows) were observed for implants of hMSCs on microtextured surfaces in OM (e,f).
suggesting that both the microtopology and growth-factor induction contribute to angiogenic

Figure A.10 RT-PCR analysis of harvested scaffolds after a 6-week implantation in mice. (a) The expression of osteogenic markers (COL I, COL X, ALP, BSP, and OC) were determined for blank microtextured substrates (PDMS) without hMSCs, hMSCs on microtextured surface in basal medium (hMSCs + PDMS in BM), and hMSCs on microtextured surface in osteogenic medium (hMSCs + PDMS in OM). (b) The expression of angiogenic markers (PECAM and VEGF) were measured for blank microtextured substrates (PDMS), hMSCs on microtextured surface in basal medium (hMSCs + PDMS in BM), and hMSCs on microtextured surface in osteogenic medium (hMSCs + PDMS in OM). Gene analysis of harvested implants demonstrated that hMSCs on microtextured surfaces in OM (hMSCs + PDMS in OM) provided the optimal condition to accelerate the early and late stage of hMSCs osteogenesis in vivo. hMSCs on microtextured surfaces in OM (hMSCs + PDMS in OM) also promoted vascularization based on the increased expression of angiogenic makers, indicating successful integration of vascularized osteogenic bone grafts. (n>3, *p<0.05, ***p<0.001)
The osteogenic differentiation of harvested scaffolds was further confirmed by the immunohistochemistry of COL I and OC. Both hMSCs on microtextured substrates in BM and OM exhibited high intensity of COL I and OC (Fig. A.11). The vascularization of harvested scaffolds was further confirmed by the immunofluorescence staining of osteogenic markers COL I (red, b,f,j) and OC (green, c,g,k) for harvested scaffolds after a 6-week implantation. Nuclei were stained with DAPI (d,h,l). (Note: the original color images were converted to grayscale and reversed to provide visual clarity.) (a-d) microtextured substrates without hMSCs. (e-h) hMSCs on microtextured surfaces in BM. (i-l) hMSCs on microtextured surfaces in OM. Blank microtextured substrates showed minimal cell attachment on the surface (d) and exhibited no COL I and OC stainings (b,c). hMSCs on microtextured surfaces in BM (f,g) and OM (j,k) revealed high intensity of COL I and OC compared to the negative control blank microtextured substrates (b,c), indicating the differentiation of hMSCs toward osteogenic lineage on microtextured surfaces in vivo.
scaffolds was observed using mouse PECAM (Fig. A.12). Although microtextured substrates showed minimal cell attachment and lacked any vasculature, hMSCs on microtextured substrates

![Image of immunofluorescence staining](image)

**Figure A.12** Immunofluorescence staining of human mitochondria (green, e,d,g) and mouse PECAM (red, b,e,h) for harvested scaffolds after a 6-week implantation. Nuclei were stained with DAPI (c,f,i). *(Note: the original color images were converted to grayscale and reversed to provide visual clarity.)* (a-c) microtextured substrates without hMSCs. (d-f) hMSCs on microtextured surfaces in BM. (g-i) hMSCs on microtextured surfaces in OM. Blank microtextured substrates without hMSCs showed minimal cell attachment on the surface (c) and exhibited neither human nor mouse markers (a,b). hMSCs on microtextured surfaces in BM (d,e) and OM (g,h) revealed elevated signals of human mitochondria and mouse PECAM compared to the negative control blank microtextured substrates without hMSCs (a,b), which confirmed the presence of hMSCs on the microtextured surfaces and identified mouse blood vessels attracted to the scaffolds.
in both BM and OM confirmed the presence of hMSCs and demonstrated vascularization to various degrees (Fig. A.12).

**Histological analysis**

The ALP activity of harvested scaffolds was comparable between hMSCs on microtextured substrates in BM (116 mU/L) and OM (120 mU/L) (Fig. A.13). Microtextured substrates alone exhibited ALP activity of 65 mU/L, which was significantly lower compared with PDMS substrates that were cultured with cells (BM and OM), probably due to non-specific cell attachment to the substrate surfaces over the period of *in vivo* implantation. Alizarin red results showed that hMSCs on microtextured substrates in both BM and OM were stained more intensely compared with microtextured substrates, indicating improved calcium deposition from bone matrix formation (Fig. A.14). HE stain showed densely nucleated epithelial layers surrounding the scaffolds for hMSCs on microtextured substrates in BM (Fig. A.15,b) (Fig. A.16,a), whereas loose epithelial layers with extensive blood vessels were identified for hMSCs

![Figure A.13](image)

**Figure A.13** Alkaline phosphatase levels were determined in harvested scaffolds after a 6-week implantation. hMSCs on microtextured surfaces in basal medium (BM) and osteogenic medium (OM) demonstrated bone matrix mineralization characterized by an increased level of alkaline phosphatase activity in vivo. (n=3, **p<0.005, *p<0.05)**
on microtextured substrates in OM (Fig. A.15,c-e) (Fig. A.16,b). Blood was also detected in the vascular lumen of hMSCs on microtextured substrates in OM (Fig. A.15,c-e).

**Figure A.14** Alizarin red staining of harvested scaffolds after a 6-week implantation. (a,d) microtextured substrates without hMSCs. (b,e) hMSCs on microtextured surfaces in BM. (c,f) hMSCs on microtextured surfaces in OM. The microtextured substrates showed minimal calcium deposition on top of the substrate surfaces. hMSCs on microtextured surfaces in BM (b,e) and OM (c,f) showed significant amount of calcium deposition characterized by the amount of red staining, indicating the successful differentiation of hMSCs toward osteogenic lineage.
**Figure A.15** Cross-sectional images of HE staining for harvested scaffolds after a 6-week implantation. (a) microtextured substrates without hMSCs. (b) hMSCs on microtextured substrates in BM. (c-e) hMSCs on microtextured substrates in OM. Microtextured substrates were shredded into PDMS pieces during HE sectioning (a). No stained tissue debris detected for microtextured substrates (a). hMSCs on microtextured substrates in BM (b) showed densely nucleated epithelial layers surrounding the substrates, whereas hMSCs on microtextured substrates in OM (c-e) exhibited very loose tissues accompanied by extensive blood vessels (black arrows). Blood (red arrows) was also observed in the vascular lumen for hMSCs on microtextured substrates in OM (c-e).

**Figure A.16** Cross-sectional HE staining of harvest scaffolds after a 6-week implantation. (a) hMSCs on microtextured substrates in BM. (b) hMSCs on microtextured substrates in OM. hMSCs on microtextured substrates in BM (a) exhibited dense epithelial-like tissue surrounding the scaffolds, while hMSCs on microtextured substrates in OM (b) showed loose tissue structure and blood vessels.
A.4 DISCUSSION

Bone regeneration and repair require the coordination of multiple cellular processes, the first of which is the migration, proliferation, and differentiation of osteoprogenitors. This process is regulated in part by soluble and adhesive factors from ECM that bind to cell surface receptors, but recent advances suggest that the mechanical properties of the ECM also play an important role in mediating cell signaling, migration, proliferation, and differentiation.

Therefore, to better mimic the natural environment of the cells, synergistic effects of mechanical, chemical, and topographical cues at the micro- and nanoscale is necessary to modulate specific cell function. We postulate that the osteogenic differentiation of hMSCs can be optimized both in vitro and in vivo with a combined effect from topographical cues based on post microtexture design and biochemical supplements. Our group previously demonstrated that microposts with 10 µm in diameter, height, and interspace provide the greatest cell-surface contact area, longer residence time to establish adhesive contacts and corresponding cell proliferation. In the present study, we assessed the ability of 10 µm microposts in two different conditioning medium (BM & OM), independently of surface chemistry, to influence hMSC morphology, proliferation, and osteogenic differentiation in vitro and in vivo.

The number of hMSCs that remained on the substrates after 6 weeks did not change significantly compared with the initial seeding density. This was probably due to the poor cell adhesion properties of PDMS which could reduce initial cell attachment and affect subsequent cell proliferation for long-term studies. The proliferation (Fig. A.2) analysis demonstrated that the cells on microtextured substrates were more proliferative in BM than OM. Cells on microtextured substrates in BM showed excellent attachment, spreading and even distribution on the substrate surface (Fig. A.3). Cells on microtextured substrates in OM exhibited dense layers
of matrix coverage on the substrate surface. Since the surface topography and surface chemistry of underlying substrates were identical, these results suggest that the BM condition helped the growth of hMSCs, whereas the OM condition deposited more ECM proteins for matrix support. Such effect is contributed by the different biomolecular components of the culture medium: serum and growth factor in BM support the proliferation of hMSCs, but the presence of dexamethasone, ascorbic acid and β-glycerol phosphate in OM induce the differentiation of hMSCs.

A group of osteoblast-specific markers based on the model of osteoblast differentiation demonstrated by Stein and Lian\textsuperscript{479} was used to assess different stages of osteogenic differentiation of hMSCs on microtextured substrates in BM and OM conditions (Fig. A.4). We found that hMSCs on microtextured substrates in OM showed an increased level of COL I compared with BM (Fig. A.4(a,b)), suggesting the early matrix formation for hMSCs stimulated by osteogenic factors\textsuperscript{480}. Despite culturing in BM, hMSCs on microtextured surface differentiated into osteogenic lineage, as evidenced by the increase in osteoblast-specific markers such as ALP, BSP, and OC (Fig. A.4(a,b)). The addition of OM further elevated the expression of these markers with negligible chondrogenic expression (COL II), indicating hMSCs on microtextured surfaces undergo a direct and specific osteogenesis. The substantial amount of ALP from hMSCs on microtextured substrates in BM and OM (Fig. A.4(a,b)) was observed which signified the ECM maturation post proliferative period\textsuperscript{481,482}. The increased levels of BSP and OC (Fig. A.4) indicated final stage of extracellular-matrix mineralization\textsuperscript{481,482}. Our data showed that the addition of OM significantly augmented the osteogenic capability of hMSCs on microtextured substrates during each stage of differentiation (Fig. A.4). More importantly, the expression level of late stage makers for matrix mineralization process (BSP & OC) were greatly
improved compared with those involved during matrix maturation (COL I) (Fig. A.4(a,b)). The combined osteoinductive effects from topography and biochemical cues far exceeded that of using either one alone (Fig. A.4). Additional verification from immunofluorescence staining of COL I and OC (Fig. A.5), quantitative measurement of ALP (Fig. A.6), and calcium deposition from alizarin red (Fig. A.7) all confirmed osteogenic differentiation of hMSCs with adequate matrix deposition and maturation. These results suggest that the combination of microtextured surfaces and differentiation factors provided by the OM significantly improve osteogenic differentiation and mineralization of hMSCs in vitro.

To further assess the osteogenic differentiation of hMSCs on microtextured surfaces, scaffolds consisting of microtextured substrates without cells, hMSCs on microtextured substrates in BM, and hMSCs on microtextured substrates in OM were implanted subcutaneously in mice for 6 weeks. Gene expression analysis showed that hMSCs on microtextured substrates in OM exhibited higher levels of COL I, ALP, and BSP compared with BM, suggesting that pre-culture in OM is more effective in promoting hMSCs osteogenic differentiation in vivo (Fig. A.10,a). The expression of OC, a marker of late-stage matrix mineralization was expressed equally in hMSCs on microtextured substrates in OM and BM (Fig. A.10,a). In vivo matrix maturation, mineralization, and bone formation was further analyzed with ALP (Fig. A.13) and alizarin red (Fig. A.14). Both hMSCs on microtextured substrates in BM and OM showed significant ALP activity, extensive calcium deposition, and substantial amount of bone matrix formation. The ALP activity was also observed in microtextured substrates without cells (Fig. A.13), which was probably a non-specific catalytic and enzymatic activity due to the invasion of subepithelial connective tissues of the animals into scaffolds. Clearly, the differentiative OM condition accelerates the number of hMSCs into
early osteoblast differentiation in vivo. However, the osteogenic phenotype of the hMSCs pre-cultured under BM suggests that the in vivo microenvironment was sufficient to enable these constructs to promote osteogenesis and mineralization.

We observed that hMSCs on microtextured substrates in OM showed a more expansive local microvasculature around the implants, whereas hMSCs on microtextured substrates in BM displayed some level of vascularization on the edge of the scaffolds, and microtextured substrates alone exhibited no vascularization at all (Fig. A.8, A.12, A.15) (Table A.3). We discovered abundant blood vessels in tissues surrounding the implants as well as regions with clusters of red blood cells for scaffolds consisted of hMSCs on microtextured substrates in OM (Fig. A.9(e,f)). This observation was further confirmed by the high level of expression in mouse vascular-specific markers PECAM and VEGF (Fig. A.10,b). Immunofluorescence staining showed that individual endothelial cells scattered around the scaffolds (Fig. A.12). Observation of mouse angiogenic-related markers implied that the endothelial cells were recruited from the host. Based on the evidence of invasion of host capillaries into the cell-based scaffolds, the appearance and differentiation of hMSCs into mature osteoblasts, and the lack of chondrogenic expression (COL II) in vivo (Fig. A.10,a), we speculated that the implanted hMSCs on microtextured substrates in OM underwent an intramembraneous ossification process. Cells on microtextured substrates in BM demonstrated dense nucleated epithelial-like tissue layers, whereas hMSCs on microtextured substrates in OM exhibited very loose tissue structure occupied with numerous blood vessels (Fig. A.15). The formation of blood vessels for scaffolds consisted of hMSCs on microtextured substrates in OM establishes mass transport between the body and the implanted microtextured scaffolds. Past reports have suggested that hMSCs, without differentiation, have the capacity to enhance angiogenesis through active synthesis and
release of paracrine and autocrine factors (e.g. VEGF)\textsuperscript{486,487}. Human MSC-derived progenies such as osteoblasts and chondrocytes are not as capable of elaborating angiogenesis when populated in microchannels of anastomized scaffolds\textsuperscript{488}. Although the \textit{in vivo} gene expression study (Fig. A.10) confirmed the osteogenic differentiation of harvested scaffolds, it is possible that undifferentiated hMSCs could contribute to the increased angiogenic response through paracrine and autocrine signaling from cell-based scaffolds. Overall our \textit{in vivo} data suggested that engineered scaffolds with controlled physical topography and proper biochemical environment could enable angiogenesis of cell-seeded scaffolds. Further investigation is needed to elucidate the mechanistic links through which the vascular endothelial cells and the hMSCs-derived osteoblasts on microtextured substrates interact.

A.5 CONCLUSIONS

We have showed for the first time that the combined effects of biochemical supplements and micro-topographical signals can synergistically generate highly functional microvascular networks for tissue engineered bone constructs in animals. Human MSCs on post microtextures cultured in the OM condition consistently expressed higher level of osteoblast-specific markers and induced greater amount of bone matrix and mineralization compared with the BM condition. The \textit{in vivo} study found increased osteogenic differentiation and identified the presence of substantial amounts of microvasculature for hMSCs on the post microtextures in OM, indicating well-vascularized grafts within the host. This study demonstrates the potential advancement toward engineering vascularized cell-based bone scaffolds through combining topographical and biochemical cues by leveraging the precision and reproducibility of microfabrication and related MEMS techniques for developing therapeutic orthopedic grafts in the future.
A.6 FUTURE WORK

Critical failures associated with current engineered bone grafts involve insufficient induction of osteogenesis of the implanted cells and lack of vascular integration between graft scaffold and host tissue. This study investigated the combined effects of surface microtextures and biochemical supplements to achieve osteogenic differentiation of hMSCs and revascularization of the implants in vivo. Cells were cultured on 10 µm micropost-textured polydimethylsiloxane (PDMS) substrates in either proliferative basal medium (BM) or osteogenic medium (OM). In vitro data revealed that cells on microtextured substrates in OM had more coverage of extracellular matrix and higher gene expression of osteoblast-specific markers, accompanied by substantial amount of bone matrix formation and mineralization. To further investigate the osteogenic capacity, hMSCs on microtextured substrates under different biochemical stimuli were implanted into subcutaneous pockets on the dorsal aspect of immunocompromised mice to study capacity for ectopic bone formation. In vivo data revealed greater expression of osteoblast-specific markers coupled with increased vascular invasion on microtextured substrates with hMSCs cultured in OM. Together, these data represent a novel regenerative strategy that incorporates defined surface microtextures and biochemical stimuli to direct combined osteogenesis and re-vascularization of engineered bone scaffolds for musculoskeletal repair and relevant bone tissue engineering applications.

However, the intra-cellular mechanism by which cell behavior changes in response to differential geometrical and biochemical stimuli remain unclear. Further investigation is needed to elucidate and establish definitive mechanism between cell-molecule-surface interactions and understand the process by which cells convert mechanical and biochemical stimuli into functional response. Furthermore, other biocompatible materials such as cellulose acetate (CA)
and poly(lactic-co-glycolic acid) (PLGA) could also be fabricated and studied using similar strategy. It is also important to realize that the material used here are lower in stiffness (~MPa) than those of normal bone tissues or metallic implants (~GPa). Therefore, there is a clinical need to combine different biodegradable materials with metallic implants to produce better implantable scaffolds that promote tissue integration and vascularization with the host body.
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258