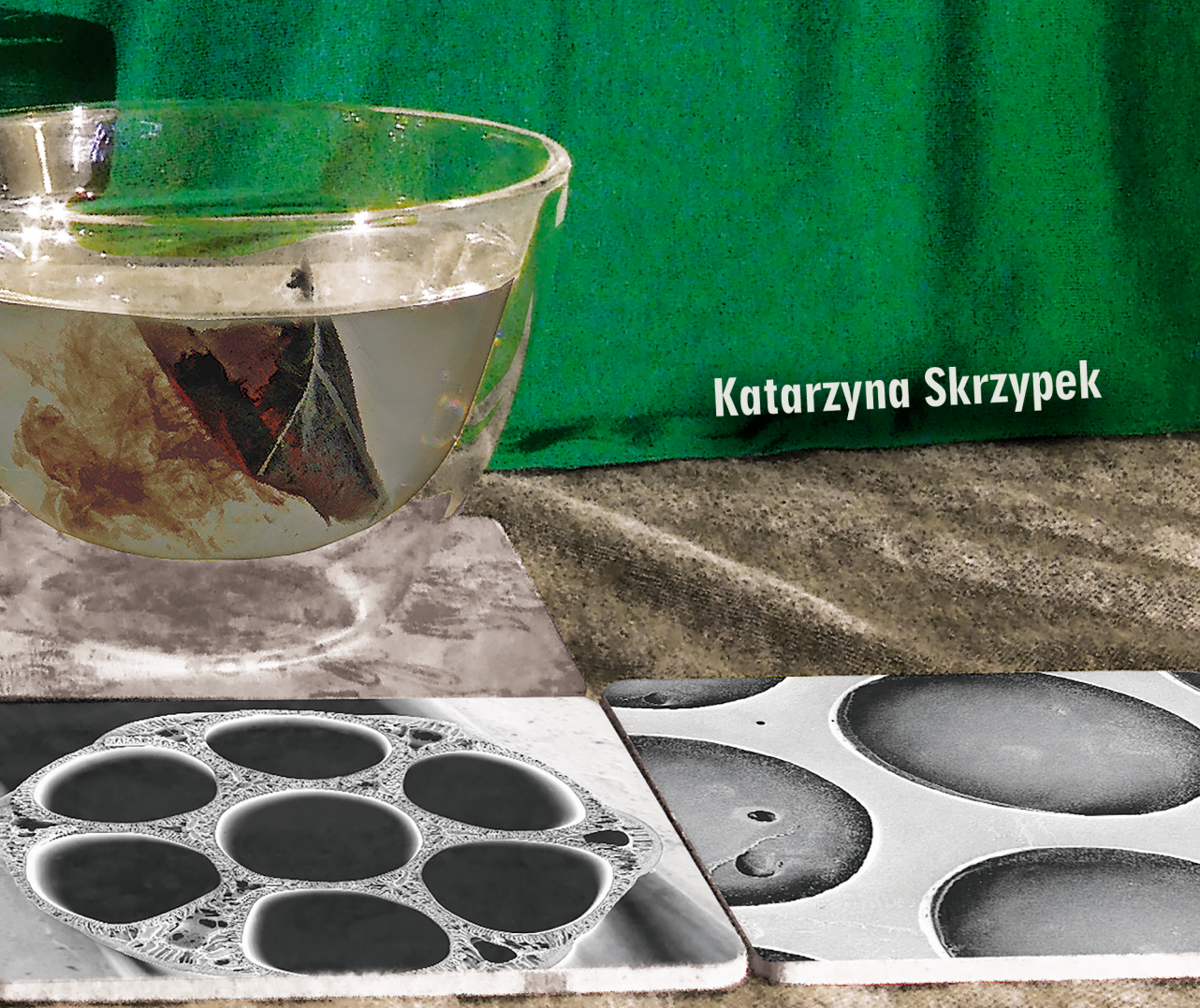
Membrane based macroencapsulation devices for improved pancreatic islet survival and function



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PhD Thesis, University of Twente, Enschede, The Netherlands

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MEMBRANE BASED MACROENCAPSULATION DEVICES FOR IMPROVED PANCREATIC ISLET SURVIVAL AND FUNCTION

DISSERTATION

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The dissertation has been approved by the supervisor:

Prof. Dr. D. Stamatialis

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Chapter 1

General introduction

1. General introduction

1.1. Pancreatic islets

The pancreas is a 12-15 cm-long organ located behind the lower part of the stomach and its main functions are production of exocrine enzymes aiding to digestion and production of endocrine hormones to regulate blood glucose [1]. Around 98% of the pancreatic mass performs the exocrine function and it is comprised of acinar cells responsible for synthesis, storage and secretion of digestive enzymes, while about 1-2% of pancreas' total mass, represented by pancreatic islets, is responsible for the endocrine function (Figure 1) [2].

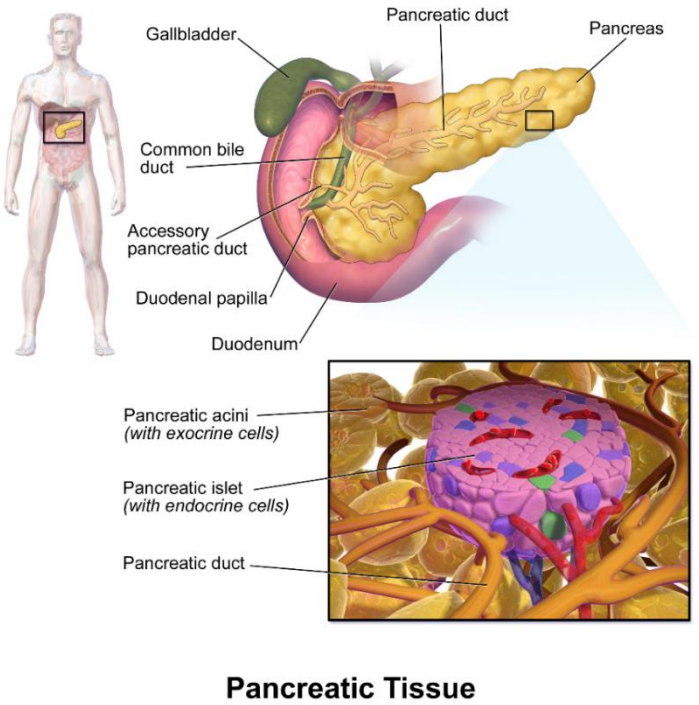

Figure 1. Schematic representation of pancreas and pancreatic islet. Reproduced from [9] with credits for Bruce Blaus.

Pancreatic islets, also called islets of Langerhans, are irregularly shaped clusters comprised of 5 distinct cell types: α-, β-, δ-, ε- and γ (PP) cells. The main population, β-cells, secrete amylin, C-peptide and insulin, while α-cells secrete glucagon. The small proportion of δ-cells and ε-cells secrete somatostatin and ghrelin, respectively. Finally, the PP cells produce pancreatic polypeptide that acts locally within the pancreas to auto regulate endocrine function and regulate gastrointestinal secretion [3].

Insulin and glucagon are the predominant hormones secreted by the pancreas and their interaction plays a key role in the regulation of glucose homeostasis. The release of insulin from the β-cells can be triggered by the growth hormone (somatotropin) or by glucagon, but the most important stimulator of insulin release is glucose [4]. When the blood glucose level increases, which occurs after the ingestion of a meal, insulin is released to counter it. The inability of the islet cells to secrete insulin or the failure to produce amounts sufficient to control blood glucose level are the causes of Diabetes mellitus.

1.2. Diabetes mellitus Type 1

Diabetes mellitus is a highly widespread disease with approximately 415 million individuals affected worldwide, where 5-10% of the patients suffer from Type 1 Diabetes [5]. Type 1 Diabetes, once known as juvenile diabetes or insulin-dependent diabetes, is a chronic disease characterized by destruction of insulin-producing β-cells due to an autoimmune reaction [6]. It mainly affects children and young adults, but it can occur at any age. The β-cells are the principal glucose sensors of the pancreas and their presence and function are absolutely required for proper glucose balance within the whole body. The damage of these cells results in a disorder of glucose homeostasis, insulin deficiency and hyperglycemia [7]. The first signs and symptoms of the disorder, caused by high blood sugar, may include frequent urination, excessive thirst, fatigue, blurred vision, tingling or loss of feeling in the

hands and feet, and weight loss. Long term symptoms include retinopathy, neuropathy and nephropathy [7, 8].

1.3. Treatment

Type 1 Diabetes patients need life-long insulin therapy via daily injections or using subcutaneous pumps combined with tight blood glucose level monitoring. However, this therapy is unable to reproduce a physiological insulin profile and patients with severe glycemic lability, recurrent hypoglycemia or hypoglycemia unawareness are in need for alternative therapies [1, 10]. Currently offered treatments include whole pancreas or islet transplantation.

The pancreas transplantation represents an effective solution to restore normoglycemia, however, the shortage of donors, the complexity of the transplantation surgical procedure and the need for immunosuppressive drug therapy to avoid organ rejection, make pancreas replacement a controversial solution for type 1 Diabetes treatment. It is often considered a viable option when kidney replacement is necessary too [1].

A compromise for reproducing a natural insulin release profile while avoiding some of the drawbacks of whole pancreas transplantation, is represented by pancreatic islet or β-cell transplantation via infusion in the liver portal vein. Although this transplantation has been shown as an effective treatment for type 1 Diabetes, with lower surgical risk and fewer complications compared to the whole pancreas transplantation, more than 60% of the islets are lost in the first days after the procedure [11]. This loss is mainly caused by complex immune responses and hypoxic conditions due to lack of adequate vascularization. As in the case of pancreas transplantation, irrespective of the source of the implanted islets, immunosuppressive drug administration is required to avoid rejection of the foreign tissue. Moreover, the intrahepatic system is suboptimal as the concentration of drugs and nutrients there is higher compared to pancreas, which negatively affects islet

function [10]. Thus, methods for islet or β-cell transplantation which do not require the use of immunosuppressive drugs and allow for the transplantation in other locations than the liver are highly desirable.

1.4. Bioartificial pancreas

In order to provide immunoisolation for the transplanted cells, research has focused on the development of a device consisting of semi-permeable membranes for β-cell or pancreatic islet encapsulation, called bioartificial pancreas [1]. The membrane, typically polymeric, has to be designed to allow the selective permeation of oxygen, glucose, nutrients, waste products and insulin, and should also be able to inhibit the immune rejection of the encapsulated cells (Figure 2A). In addition, the encapsulation device can be transplanted outside the suboptimal portal system, in other transplantation sites, such as subcutaneous site, omental pouch or peritoneal cavity (Figure 2B) [12].

Various strategies have been proposed for islet encapsulation [13]. Based on the amounts of cells encapsulated within the semipermeable membrane and implicitly the dimensions of the implantable capsules, we can distinguish nanocapsules (size<100μm), microcapsules (size: 250–1000μm) and macrocapsules (size: few cm), (Figure 2C).

Nanocapsules refer to single pancreatic β-cells with conformal coating directly bound to the cell membrane with a thickness varying between a few nanometers to micrometers. Due to their small dimensions, the distance for nutrients diffusion and insulin release is reduced to nanometers, which is beneficial for proper cell function. However, it is very challenging to achieve there stable and uniform coatings to provide sufficient cell immune protection [1, 14].

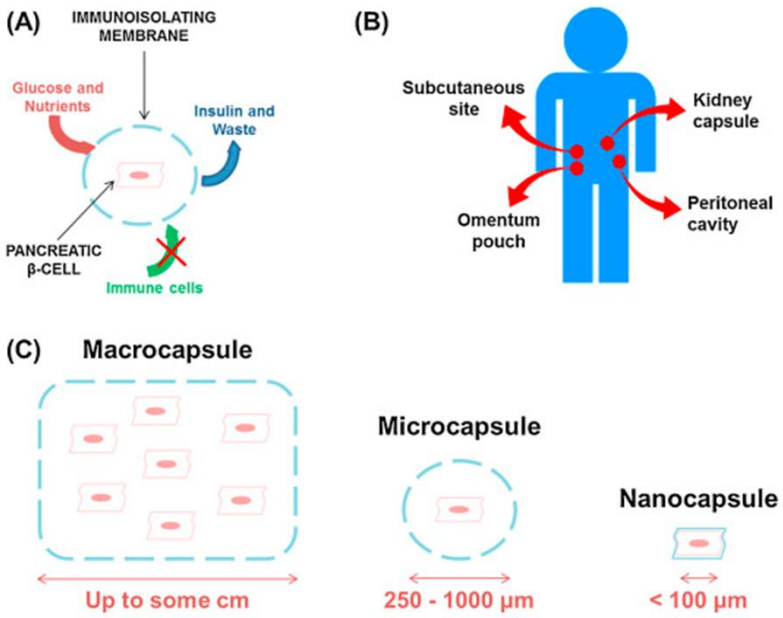

Figure 2. The bioartificial pancreas approach. A) Schematic representation of the diffusion mechanism across a semipermeable membrane; B) Typical bioartificial pancreas implant sites; C) Classification and average dimensions of extravascular encapsulation devices. Reproduced from [1] with permission from Elsevier.

Microcapsules contain mostly a small number of islets, which are fully embedded in a polymer gel matrix (hydrogel) providing sufficient immune protection [15]. They can be implanted using minimally invasive procedures, however they are very difficult to retrieve in case of graft failure.

In contrast to nano- and microcapsules, where a small number of islets/β-cells is used for encapsulation and therefore a high number of capsules is needed to implant sufficient amount insulin-producing cells, the macrocapsules comprise of a large number of pancreatic cells in a single, defined device, which can be fixed in one location in the body. Their dimensions also favor the formation of cell clusters, enabling intercellular communication, however the creation of large cells aggregates

may negatively affect islet function, leading to limited diffusion of nutrients and oxygen, and apoptosis [16]. Macrocapsules can rely on diffusion mechanisms or ultrafiltration, therefore islet encapsulation remains a difficult challenge because, by providing islet immune isolation, the mass transport of necessary nutrients, glucose and insulin is often compromised [17]. Nonetheless, they can be produced in a relatively simple way and can be easily implanted. Importantly, their great advantage is the possibility of their retrieval and/or reload if necessary. Depending on the implantation site, macroencapsulation systems can be distinguished into: extravascular, typically placed in the peritoneal cavity or subcutaneously and intravascular, which are connected to the patient's cardiovascular system [13]. A review of the materials and configurations used for macroencapsulation devices is presented in Chapter 2 of this thesis.

2. Aim and outline of the thesis

The aim of this thesis is the development of novel membrane based macroencapsulation devices for improved pancreatic islet survival and function. More specifically, poly(ethersulfone) (PES) / polyvinyl pyrrolidone (PVP) polymer blend was used to fabricate porous membranes in various configurations, with dimensions and transport properties optimized specifically for human islet encapsulation.

After presenting here (**Chapter 1)** a general introduction of the Diabetes Type 1 problem and the application of a bioartificial pancreas as a potential solution, **Chapter 2** gives a detailed literature overview of various aspects considering encapsulation devices and describes materials and strategies used for the development of macroencapsulation devices.

Chapter 3 presents the development of a flat macroencapsulation device in which islets are confined between two PES/PVP semipermeable membranes: one membrane contains microwells in which the islets are seeded and the other

membrane acts as a lid. For both membranes, the porosity is tailored to permit nutrient inflow and metabolite outflow. The microwell array is designed to provide islet separation and prevent both spreading and aggregation, maintaining the islet's rounded morphology. The encapsulated islet function is studied in vitro.

Chapter 4 presents the fabrication of porous, micropatterned PES/PVP membranes, which can be applied as a lid of the macroencapsulation device, developed in Chapter 3. We investigate the effect of surface pattern on human umbilical vein endothelial cell (HUVEC) alignment and interconnection in a co-culture with fibroblasts, as a first step towards the development of a stable prevascularized layer in vitro. The presence of a highly interconnected prevascularized layer, closely mimicking native tissue, could reduce the time of construct reconnection to host vasculature and improve islet survival within our macroencapsulation device.

In **Chapter 5**, we create stable composite aggregates consisting of mouse insulinoma MIN6 cells co-cultured with HUVECs to mimic the interaction between β- and endothelial cells in pancreatic islets. The composite aggregates are used for encapsulation within the flat device developed in Chapter 3 and the effect of HUVECs on MIN6 cell function is studied.

Chapter 6 presents the development of new multibore hollow fiber membranes with small bore diameter for islet encapsulation. The fiber consists of seven bores suitable for seeding a high number of islets. The membrane characteristics are tailored to achieve efficient nutrient delivery to the cells and insulin delivery by the cells. The insulin secretion of various number of human islets encapsulated within the new multibore fiber is studied.

Finally **Chapter 7** reflects back to the results of this thesis and presents an outlook on a number of topics for further optimization of the macroencapsulation devices proposed in this thesis.

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Chapter 2

Membranes for bioartificial pancreas ±macroencapsulation strategies

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1. Primary challenge: Diabetes type 1

Type 1 Diabetes mellitus is a chronic disease that mostly manifests in children and young people (usually <30 years) and is caused by destruction of the insulin producing β-cells due to an autoimmune reaction [1-4]. It is characterized by hyperglycemia as well as relative insulin deficiency. It is known for its severe acute and long-term complications due to micro- and macroangiopathic lesions and has a significant social and economic impact. Long term symptoms are retinopathy, neuropathy and nephropathy [5-12].

Since blood glucose levels are inadequately maintained due to the lack of insulin, type 1 Diabetes mellitus patients need proper diet management in combination with life-long insulin administration, either by multiple daily injections or more recently through pump delivery. Glycemic control has also been improved by development of the artificial pancreas , an integrated closed control system, which combines glucose monitoring with subcutaneous insulin infusion [13]. However, this system still shows several limitations mainly related to delayed glucose sensing and insulin absorption into the bloodstream. Moreover, glucose sensors have a relatively short lifetime. In order to enhance the artificial pancreas performance, new glucose sensing technologies, with higher reliability and longer lifetime are under development. Additionally, new algorithms, better predicting real time blood glucose levels and smart procedures (miniaturized tools, functionalized materials) are needed to make the implantable artificial pancreas a realistic treatment option for type 1 Diabetes patients [13, 14].

Patients with severe glycemic lability, recurrent hypoglycemia, hypoglycemia unawareness, or an insufficient response to the insulin therapy are in need for alternative therapies. One of these is total pancreas transplantation. Despite the increasing rate of successful pancreas transplantations, the intervention involves a complicated abdominal surgery with increased risk of comorbidity [15]. In addition,

the treatment is accompanied with long-term immunosuppressive therapy to avoid rejection of the donor tissue. Due to shortage of donors a whole pancreas transplantation is restricted to certain group of patients, often when kidney replacement is necessary too [15, 16]. An alternative for type 1 Diabetes treatment is clinical islet transplantation (CIT). Compared to total pancreas transplantation this treatment is associated with lower surgical risk. Additionally, often when whole pancreas is considered not suitable for transplantation, the islets from donor pancreas still can be utilized and used for CIT [17].

2. Clinical Islet Transplantation (CIT)

The CIT consists of the isolation of islets from a donor pancreas and transplantation in the patient via infusion in the portal vein (Figure 1). After infusion, the islets will embolize in the microvasculature of the liver and perform their endocrine function.

Over the years the success rate of CIT has increased from only 24% after 2 years and 15% after 5 years to 50-60% after 2-5 years [18-21]. However, many islets (60%) are lost in the first days after transplantation [22, 23].

The process of islet loss starts already during isolation, where islets are exposed to a variety of cellular stresses such as mechanical, enzymatic, osmotic, and ischemic stresses and disruption of cell matrix and vasculature [24]. This has a great impact on islet survival, as islets are particularly well-perfused in the pancreas. In fact, although islets consist of only 1% of the entire pancreas, they receive 5-15% of the total blood supply of the pancreas [25-27]. Due to disruption of their vasculature and the exposure to only venous blood in the first weeks after transplantation, the islets are exposed to relative hypoxic conditions. When this situation persists for more than 7 days, hypoxia results in cell death and endocrine dysfunction [6, 7, 19, 28, 29]. Besides, in the liver, islets are immediately exposed to relatively high concentrations of immunosuppressive drugs glucose and lipids, which can all negatively affect β-cell function and survival [11, 19, 29-34].

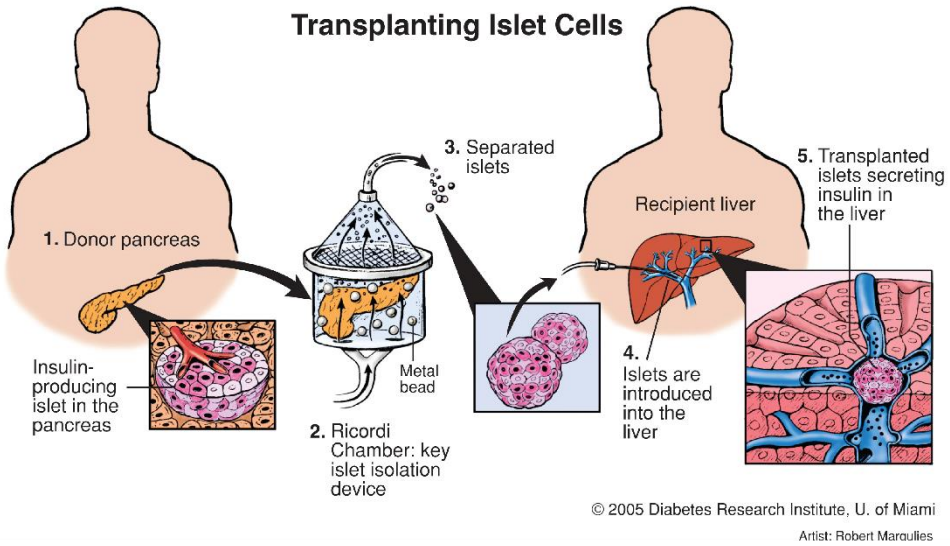

Figure 1. Schematic representation of the CIT procedure (reproduced with permission from Diabetes Research Institute, University of Miami).

The isolation procedure also brings islets in a pro-inflammatory state as a consequence of cellular stresses [35]. This induces expression of pro-inflammatory cytokines which trigger different immune responses involved in the loss of islets after transplantation. There is in fact one immune response directly related to the transplantation site; Instant Blood Mediated Immune Response (IBMIR), which is triggered by direct contact of islets with ABO blood components in the hepatic portal system [11, 12].

Isolated islets express at least 50 inflammation-associated genes, most likely up-regulated due to stress started before organ procurement till their transplantation [18, 36]. This results in a cascade of reactions with many components, such as, tissue factor (TF, 47 kDa), complement activation, and the deposition of immunoglobulins [1, 19-21, 37]. Consequently, islets are infiltrated by several immune cells finally resulting in cell lysis and apoptosis [12, 24, 25]. As IBMIR triggers many different

components of the immune system, it plays an important role in the activation/enhancement of other immune responses involved in islet loss [18].

To prevent islet loss due to the attack of the immune system, immunosuppressive drugs are administered to the patients. Although these drugs are necessary to enhance islet survival, their life-long use is associated with numerous complications such as; infections, neoplasms, and failure to control rejection [1]. Additionally, the hepatic location for CIT increases the effect of toxic drugs, as their concentration is high in the liver as the liver metabolizes drugs [2]. Some drugs have negative effects on islet function by causing metabolic alterations or by direct drug toxicities. For example, calcineurin inhibitors and steroids have shown to interfere with β-cell function [1-8]. Consequently, multiple donor pancreata are necessary to obtain complete insulin dependency, which is again a problem with limited donor availability [3, 9].

Based on the above it is obvious that methods for islet transplantation which do not require the use of immunosuppressive drugs are highly desirable. To increase CIT efficacy, research has focused on developing devices for either immune protection or improved islet survival. In addition, due to the disadvantages of the portal system as a transplantation site, other transplantation sites are investigated too, such as subcutaneous site, omental pouch, peritoneal cavity, bone marrow or muscle [14, 38, 39].

3. Encapsulation devices

3.1. Introduction - requirements

Combining islet immune protection while maintaining islet viability might be the ultimate solution to overcome issues related to intrahepatic islet transplantation. It is thought that an encapsulation device could both maintain islet viability and act as a barrier for the immune system. Therefore, the encapsulation of islets within a semipermeable membrane, called a bioartificial pancreas (BAP), has been widely

investigated for type 1 Diabetes treatment (Figure 2) [14, 40]. The most essential requirements of this device are [6-8, 19, 41-43]:

- optimal mass transfer properties, namely fast nutrient and oxygen transport to the cells and fast and efficient response to high glucose concentration in the blood - encapsulated cells should be able to rapidly detect an increase in blood glucose levels and release insulin to prevent delays in blood glucose regulation.
- immune protection - islets need to be separated from the blood stream to avoid contact with immune cells, antibodies, etc., immune protection should not compromise transport of glucose, insulin, oxygen, and nutrients as this will lead to islet necrosis and dysfunction of the bioartificial pancreas.
- biocompatibility – the material used for encapsulation should not activate inflammatory responses and should not stimulate fibrosis.
- the device should be sterilizable and long lasting, preferably non degradable to sustain its functions over a long period of time.
- easily implantable – using minimally invasive surgical procedures.
- retrievable – it should be possible to remove the device in an easy and safe manner in case of failure.

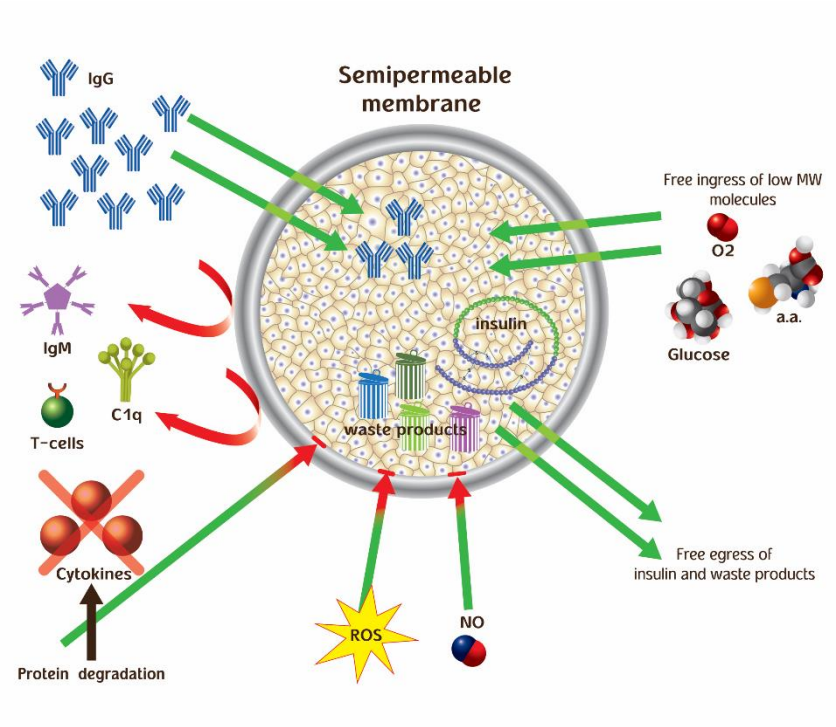

Figure 2. Bioartificial pancreas ± schematic representation of mechanisms by which semipermeable membrane protects grafted cells from host immune system. Reproduced with permission from [44].

3.2. Encapsulation

The need for immune protective device

When designing an immune protective device for transplantation of islets, a balance needs to be found between optimal survival of islets and their shielding from the immune system [45]. The challenge then becomes finding the optimal pore size or the molecular weight cut-off (MWCO) for the membrane. MWCO is the molecular weight of the solute that can be retained at least 90% by the membrane.

The overall strategy in the membrane fabrication process is to obtain a highly selective membrane, with high diffusivity of the low molecular weight nutrients and low diffusivity of the high molecular weight immunoglobulins. Usually membranes used for encapsulation have MWCO of 50-150 kDa [46]. The selection of 150 kDa MWCO is often based on the retention of the smallest immunoglobulin - IgG. However, it is important to note that even if the IgG passes through the membrane, it is not an effective cell killer on its own [44]. The immune rejection is mediated by cytotoxic T-cells and therefore, the immune protective encapsulation device needs to prevent cell-to-cell contact. In fact, it has been reported that membranes with 0.4 m pore size were successful in shielding allogeneic cells from the immune system (e.g., immunoisolation TeraCyte™ device) [31, 47, 48].

Decreasing the membrane pore size would improve retention of potentially harmful molecules (immunoglobulins and some of cytokines), however it might negatively affect the device s mass transport properties.

Encapsulation strategies

Based on the amount of islet cells encapsulated within the semipermeable membrane, there are three strategies investigated: nano-, micro- and macroencapsulation (see Table 1).

Nanoencapsulation devices are less than 100 m in diameter and can envelop single pancreatic β-cells in a semipermeable membrane. The membrane is directly bound to the cell as conformal coating with thickness between nanometers to some micrometers [14]. Several methods of conformal coating have been proposed, including covalent surface attachment of polyethylene glycol (PEG) and layer-by-layer encapsulation [49-51]. The advantage of these coatings is that islets are less prone to hypoxia and nutrient deprivation since diffusion distances are reduced to nanometers [49]. The challenge, however, remains to achieve a uniform and stable coating in order to provide sufficient islet immune protection [49, 50].

Microencapsulation refers to individual or small clusters of islets enclosed in a polymer gel matrix [16]. Most common materials used there are hydrogels like; alginate, chitosan, agarose, HEMA-MMA, copolymers of acrylonitrile (AN69), and polyethylene glycol [22]. Alginate has shown advantages over other materials, as it does not interfere with cell function, is mechanically stable, and capsules are easily manufactured at physiological conditions [22, 52, 53]. Additionally, various materials such as PEG and different polycations have been incorporated into alginate to reduce plasma adsorption. Some coatings (e.g. poly-D-lysine (PDL) and poly-L-ornithine (PLO)) may act as pro-inflammatory agents. The poly-L-lysine (PLL) coating was found to be the most optimal since it is the least reactive to the host [7, 22, 52, 54, 55]. As islets are fully embedded in the hydrogel with adequate MWCO, the device can provide sufficient immune protection. Problems there could be large diffusion distances due to thickness of the capsule, fragility, limited islet viability, no connection to the vascular network, fibrosis, lot-to-lot variability of alginates. In addition microcapsules are difficult to retrieve in case of implant failure [56, 57].

Macroencapsulation systems often comprise of the total transplanted cell volume in a single, defined container with centimeter range dimensions. Macrocapsules can be produced in relatively simple way and easily implanted with minimally invasive surgical procedure. Their greater advantage is the possibility of their retrieval and/or reload if necessary but this may come at the expense of mass transfer limitations [58]. Further in this chapter we will focus on the macroencapsulation strategies using polymeric membranes.

Macroencapsulation with membranes

Depending on the implantation site, macroencapsulation devices can be distinguished into: extravascular, mostly placed subcutaneously or in the peritoneal cavity and intravascular devices connected to the patient s cardiovascular system [58].

Table 1. Overview of encapsulation strategies advantages and disadvantages

Encapsulation type	**Advantages**	**Disadvantages**
Nanoencapsulation	Very small diffusion distances	Low coating stability Non-retrievable
Microencapsulation	Easy manufacturing procedure	Large diffusion distances No connection to the vasculature Fibrosis Difficult to retrieve
Macroencapsulation (intravascular)	Close proximity to blood Direct access to the nutrients and oxygen Can be reseeded and retrieved Flexible in size	Complex surgical procedure High risk of thrombosis, clot formation Necessity of anticoagulation therapy
Macroencapsulation (extravascular)	Easy to implant retrieve and reload Flexible in size	Large diffusion distances

The intravascular devices contain islets encapsulated within hollow biocompatible tubes or fibers directly attached to the patient s cardiovascular system allowing for rapid diffusion of the oxygen and nutrients to encapsulated islets [59, 60]. Consequently, the islets are able to react fast and efficient to changes in glucose concentration. However, these systems can have several disadvantages including: required complex surgical intervention, high risk of thrombosis and clot formation, and live-long, systemic administration of anticoagulation therapy. In case of material failure, there is a risk of damaging blood vessels and retrieval of the device is associated with a complex surgical procedure [49, 52]. Some of the intravascular devices are listed in Table 2.

Table 2. Examples of intravascular devices.

Material	**Islet source**	**Animal model**	**Outcome**	**Reference**
Polyacrylonitrile-polivinyl chlorine (PAN-PVC)	Rat islets Monkey islets	Rat Monkey	Restored normoglycemia	[59]
Polyethylene-vinyl alcohol (EVAL) fibers and poly-amino-urethane-coated, non-woven polytetrafluoroethylene (PTFE) fabric	Porcine isles	Pig	Restored normoglycemia	[67]
Polycarbonate membrane	Rat islets	Dog	Restored normoglycemia	[64]
Nylon microporous membrane	Rabbit fetuses	Human	Restored normoglycemia	[66]

The first intravascular systems with membranes were made using the copolymer polyacrylonitrile-polivinyl chlorine (PAN-PVC) and were reported to reverse diabetes in a rodent model after transplantation [59, 61-63]. Similar results were obtained by Scharp et al. using tubular polycarbonate membrane [64]. While modified versions of intravascular devices have been tested in allogeneic and xenogeneic transplantation models, coagulation and further complications occurred, thus making development of intravascular devices very challenging [60, 65]. In 2008, Prochorov et al. reported the use of a nylon microporous membrane as intravascular macrocapsule transplanted into diabetic human patients [66]. Even though, in this approach, islets from fetal rabbits were used and no immunosuppressive therapy was applied, positive results in several patients were observed even after two years of transplantation. The device was immune protective

and thrombosis did not occur. Still, approximately 40% of the islets were lost during the first weeks after transplantation due to poor vascularization of the device. Despite this success, intravascular devices have not been implemented to the clinic so far.

The extravascular devices are mostly placed subcutaneously or in the peritoneal cavity and can be implanted without need of anastomosis which is advantageous in terms of clinical implementation. Additionally, extravascular devices are relatively easy to implant and retrieve. They can be re-seeded and they have flexibility in size. However, the design of such device needs to overcome the lack of direct vascular access and the fact that the islets experience diffusional limitations, due to the relatively large size of these devices. Glucose and nutrients transport to the cells can be slower and as a consequence the release of insulin is delayed. Additionally decreased oxygen delivery towards islets may cause necrosis and cell death. Therefore the membranes used there have to be thin but at the same time mechanically and chemically stable [40].

Examples of the extravascular devices are listed in Table 3. The materials used for extravascular device fabrication can be organic (polymeric) or inorganic. The inorganic materials such as silicon [76-78], aluminum/aluminum oxide [79, 80] or titanium/titanium oxide [81, 82] have been used for the fabrication of nanoporous membranes with controlled pore size and geometry. Their advantages over polymeric membranes are tighter pore distribution and better diffusivity due to reduced membrane thickness [83]. Since inorganic membrane development is rather new, they have not been extensively tested for cell encapsulation.

Table 3. Examples of extravascular devices.

Material	Transplantation side	Islet source	Model	Configuration	Ref.
Modified polyacrylonitrile-polivinyl chlorine (PAN-PVC)	Subcutaneous	Human islets	Patients with Diabetes type1 and 2	Tubular device	[68]
Cellulose acetate	Intraperitoneally	Human islets	Rat	Tubular device	[69]
Acrylic copolymer (XM-50 Amicon)	Intraperitoneally	Rat	Mice	Tubular device	[70]
Polysulphone	-	Rat	In vitro	Tubular device	[71]
Nitro-cellulose acetate (Milipore)	Intraperitoneally	Mice	Mice	Flat device	[72]
2-hydroxyethyl methacrylate	Sutured to parietal peritoneum	Rat or rabbit pancreatic tissue	Rat	Flat device	[73]
Acrylonitrile (AN62)	Intraperitoneally	Rat	Rat	Flat device	[74]
Alginate	Omentum	Dog	Dog	Flat device	[75]

Membrane configuration

Hollow fiber membranes

Following the development of hollow fiber technology for renal dialysis, hollow fibers have been used for cell encapsulation [84-86]. Hollow fiber extravascular devices were produced from different materials such as; modified PAN-PVC [68, 87], regenerated cellulose and polyamide [86], acrylic copolymer (XM-50 Amicon)

[58, 69, 70] and polysulphone [71]. Using these systems, in vitro and in vivo studies were performed with encapsulated islets. Proper insulin release in response to glucose level changes was obtained and the transplanted islets were viable and functional after several weeks of implantation [88, 89]. However, usually graft survival was shorter compared to intravascular devices, due to limited oxygenation and nutrient transport. In fact, the large volume of islets encapsulated in hollow fiber devices can lead to aggregation and creation of big islet clumps. Consequently necrosis occurs in the central core of islets resulting in graft failure [70]. This problem can be overcome by using hydrogels (alginate, collagen, chitosan) for islet separation [70, 90, 91]. Additionally surface modifications of hollow fiber devices can allow better immunoisolation, biocompatibility and minimal fibrotic response [70, 92, 93].

Hollow fiber devices are adaptable and relatively easy to transplant. However, due to their shape they tend to bent and break and require a large volume of islets to achieve insulin independence, an important issue in case of clinical trials [90].

Flat devices

Flat devices consist of flat, circular or rectangular membranes encapsulating islets. It is believed that this configuration can provide better stability than hollow fiber membranes and can improve oxygen supply to the entire graft. Algire et al. used a flat device by gluing two thin microporous membranes made of nitro-cellulose acetate and investigated immune rejection mechanisms with non pancreatic tissue [94, 95]. The Millipore company later adapted the design of Algire and produced an e travascular device with 0.45 m pore size [64]. Many other researchers used the same device to confirm improved immune protection [72, 96]. Besides nitro-cellulose, other materials have been applied for development of flat devices such as 2-hydroxyethyl methacrylate [73], acrylonitrile and sodium-methallysulfone [74], poly(vinyl alcohol) hydrogel in combination with a mesh [97, 98] and alginate [75,

99]. Despite initial successes there, the problem of poor oxygenation and lack of vascularization remained.

Flat devices are mostly implanted subcutaneously or in the peritoneal cavity due to their size and shape. They stay intact and remain in original configuration after transplantation. Nonetheless, if the material is not selected properly, the formation of thick layer of fibrotic tissue around the device can occur, limiting transport of nutrients and oxygen and contributing to graft failure [40, 100]. Recently, researchers also focused on addition of oxygen supply or possibility of device vascularization [101]. There are several companies active in this field, developing the products for macroencapsulation. Some of them are described in detail in the next section.

3.3. Upscaling of the devices

Encapsulation devices need to fit a certain number of islets to be able to restore normoglycemia. Shapiro et al. have shown that about 9,000 islets per kilogram of bodyweight are needed to obtain insulin independency in patients, however the final number of viable islets is not known [102]. Therefore, optimal macroencapsulation devices, designed for in vitro testing, as well as, in vivo using smaller animals, should be able to be upscaled in order to be applied to humans. The modeling study of Dulong et al. has shown that, by increasing islet density in the device, the surface of planar devices or the length of tubular devices often needs to be increased in order to encapsulate an optimal number of viable islets, resulting in very large devices not suitable for implantation [103]. Additionally, any compromise of the space required for islet encapsulation may result in mass transport limitation causing cell necrosis and resulting in a less functional device. Decreasing membrane thickness could improve diffusion of nutrients and oxygen, however it may compromise the membrane mechanical properties. Different approaches can be considered in order to solve these issues. The most obvious is implantation of more than one device in

order to obtain the required number of functional islets. In case of tubular devices, the fibers can be coiled, decreasing the space needed for implantation. To achieve this the device needs to be mechanically stable to avoid damages and deformations. Besides, for planar devices, multi-layer stacking of the membranes is possible [104]. Here, optimal transport properties need to be maintained for each layer of encapsulated islets.

4. Macroencapsulation devices under development

TheraCyte™ system

In the late 1990 s, Ba ter Healthcare developed an islet macroencapsulation, planar device that is still in use today in several laboratories [48, 105]. The device is composed of two thin, polytetrafluoroethylene membranes sealed at all sides complemented with a loading port. The device is teabag shaped, 4 cm in length and has at one end a polyethylene port for islet seeding. The outside, 5 m pore membrane improves strength of the device and allows for infiltration of vasculature, whereas the inner 0.4 m pore membrane provides immunoisolation. The device is suitable for subcutaneous implantation but since it is designed to be incorporated into the host vasculature, it is difficult to remove and replace. Animal studies have shown promising results related to biocompatibility and functionality of the device [105-110]. There is also possibility to improve the system performance by inducing neovascularization before transplantation [111], addition of vascular endothelial growth factor [27], or exploiting encapsulation of human embryonic stem cell (ESC's) derived from islet tissue [112].

Cell Pouch system

Serenova Corporate commercializes a biocompatible macrodevice for subcutaneous implantation which can create a natural environment in the body for long term survival and function of therapeutic pancreatic cells. The device is made of a non-

biodegradable knitted polymer mesh with large pores to allow there the development of fibrous tissue rich in vessels. Inside the device, a rod-like polymer plug is positioned to guide the growth of the microvessels and connective tissue in order to create lumen for the future transplantation of islets [113]. When the lumen is created, the rod-like plug is removed and islets can be transplanted. Addition of sertolin, a testicular protein, provides immune protection and reduces the need for immunosuppressive therapy [114].

Islet sheet

Islet sheet is another planar device. Its development began in the late 1990s. Firstly islets were seeded on a collagen matrix in order to create a cell monolayer that allows better diffusion of nutrients and oxygen in comparison to islet clusters. This monolayer was then embedded in 3% wt./vol. alginate gel creating an islet sheet [115]. Alginate layers formed a uniform immune protective barrier for the encapsulated cell and the sheet thickness was maintained as small as possible (~ 250 m). The main advantage of this device is the easy implantation (in peritoneal cavity or subcutaneous site) and removal or replacement. Studies in rodent models have demonstrated satisfactory insulin secretion, however the implantation into larger animals has limited success due to short graft survival [75].

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Beta-O2 Technologies Ltd. has focused on an important problem of extravascular macrodevices: their insufficient oxygen supply after implantation resulting in large islet loss at the early stage. Therefore, they developed an oxygen-refueled macrochamber composed of islets immobilized in two flat alginate, immune protective sheet with oxygen supply chamber placed between the membranes (Figure 3) [116]. A subcutaneous port connected to the implanted device permits daily replacement of oxygen, which slowly diffuses to the islet compartment. The chamber is housed in a plastic case, providing mechanical protection, wrapped in a porous

PTFE membrane impregnated with hydrophobically modified alginate to avoid immune responses [14, 117]. Studies in animals and patients with type 1 Diabetes have shown proper device functionality and regulated insulin secretion without need for immunosuppressive agents [118, 119].

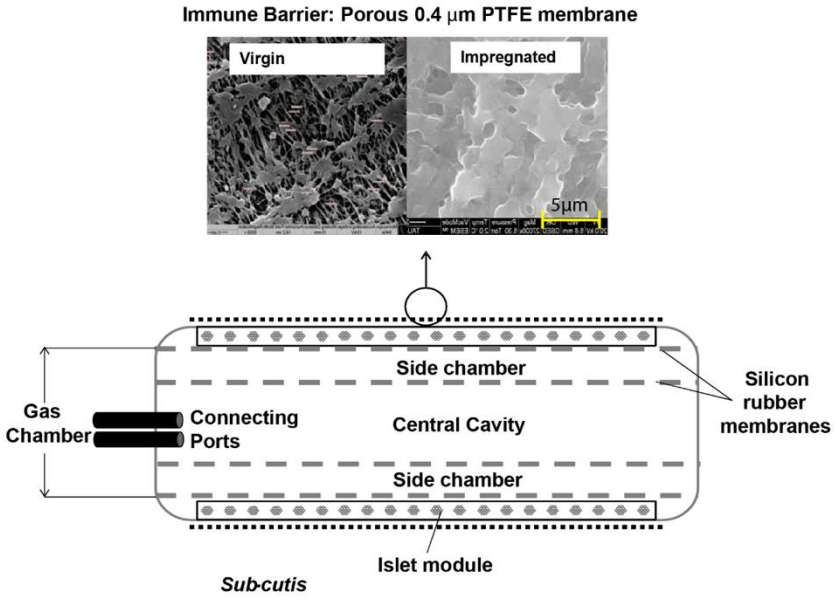

Figure 3. Schematic view of the VIVO device (adapted with permission from [118]).

VC-01 system

ViaCyte developed a subcutaneous macrocapsule based on embryonic stem cell–derived precursors of insulin-producing β-cells and a semipermeable membrane made of undisclosed polymers. The cells are expected to further differentiate to produce mature pancreatic cells, which will synthesize and secrete insulin and other factors. Promising results in mice were obtained in terms of neovascularization and blood glucose regulation [117].

5. Cells used for encapsulation

Encapsulation of pancreatic islets may allow for their transplantation without need for immunosuppressive therapy by shielding the graft from immune system via a semipermeable membrane. This protection can even allow for the transplantation of animal tissue or novel insulin-producing cells as an alternative to solve the critical problem of the shortage of human islet donors [101]. Several possibilities have been considered in the literature as an alternative cell source for islet transplantation [14, 120-122].

Porcine islets

Porcine islets contain physiologically compatible insulin-producing cells [123]. Pigs are readily available species and there is close homology between porcine and human insulin. Additionally porcine islets are morphological similar to human islets, however, less sensitive to destruction by autoimmunity in comparison to human islets and there are no ethical issues [124, 125]. Since porcine islets have less insulin-secreting capacity than humans, new protocols to isolate large quantities of porcine islets have recently been developed [126, 127]. Furthermore, effective methods have been established for genetic modification of pigs to improve xenotransplantation outcomes [123-125, 128, 129]. Despite the above, issues in using porcine cells are the risk of retroviral disease transmission and there is absolute need for immune protective encapsulation strategies.

Exocrine cells

Recently a new approach has been proposed based on reprograming of exocrine acinar cells to insulin-producing β-cells [130-132]. Normally the exocrine pancreatic tissue is discarded after the islet isolation procedure, but it appears to be still useful and valuable. However, in order to guarantee high efficacy and safety, the techniques of reprogramming still need improvement.

Stem cells

Stem cells represent a novel approach for the production of transplantable β-cells, thanks to their regenerative and proliferative abilities [133-135]. Theoretically they can be used to generate insulin-producing β-cells, multicellular islets or even the whole pancreas [136, 137]. Viable insulin-producing β-cells can be derived from various kinds of stem cells, such as, human embryonic (hESC) or induced pluripotent stem cells (iPSC) [134, 135, 138-141]. However, there are still concerns regarding the limited ability to retain insulin independence using β-cells generated from stem cells in preclinical models [14]. Additionally, the use of immune protective encapsulation devices is of high importance there since the non-differentiated cells can form tumors [142]. Therefore, the risk of cells escaping from the device should be prevented.

6. Conclusions and perspectives

Clinical islet transplantation, although successful, is associated with several complications hindering the transplantation outcomes. The development of a bioartificial pancreas seems to be a promising strategy to overcome the issues related to intrahepatic transplantation. The use of porous membranes for macroencapsulation devices gives the opportunity to optimize the device properties regarding the transport of nutrients and the exchange of glucose and insulin. Here, the great advantage of macroencapsulation devices is that they can be easily retrieved, replaced or reloaded. The promising results obtained with macroencapsulation devices have led to first clinical studies. However, there is still room for improvement in order to develop a life-long, fully functional islet encapsulation device for type 1 Diabetes treatment.

Macroencapsulation systems are advantageous because they contain a high density of islets in a single device. Therefore, cells are in close proximity to each other (similar to healthy pancreas), improving communication and synchronization

regarding insulin secretion [66]. At the same time, a high number of islets placed in one location may lead to their fusion, which negatively affects islet native structure. As a result, large cell aggregates form and islets suffer from limited nutrient diffusion [143]. In order to avoid islet aggregation, islets can be immobilized using gels [8, 41]. However, the gel brings an additional barrier for diffusing nutrients and oxygen, impeding proper insulin secretion and device function. In order to improve islet separation without the use of gels, a microwell array has been proposed [144, 145]. Buitinga et al. described an open, microwell scaffold for vessel ingrowth, where individual islets are captured in separate microwell pockets [144].

Lower aggregation allows for better transport of nutrients, increasing islet viability and survival. After improving islet performance and therefore providing more functional islets, one could consider using a lower density of islets without conceding the function of the macroencapsulation device.

Since the islets are highly metabolic active, they require large amounts of oxygen and access to nutrients to function properly within the encapsulation devices. Different strategies to improve islet performance, such as, additional supply of oxygen to the construct, the use of growth factors and induction of prevascularization is required. In fact, administration of growth factors has been shown to improve graft functionality by increasing angiogenesis. However it can also result in abnormal and unsustainable vasculature formation [146]. Therefore, the addition of a prevascularized layer as part of the encapsulation device seems to be a promising strategy, which would overcome the need for an external oxygen supply. The use of prevascular networks leads to a long term solution for vasculature reconnection to the host [147].

List of abbreviations

BAP	Bioartificial pancreas
CIT	Clinical islet transplantation
EVAL	Polyethylene-vinyl alcohol
hESC	Human embryonic stem cells
IMBIR	Instant Blood Mediated Immune Response
iPSC	Induced pluripotent stem cells
MWCO	Molecular weight cut off
PAN-PVC	Polyacrylonitrile-polivinyl chlorine
PDL	Poly-D-lysine
PEG	Polyethylene glycol
PLL	Poly-L-lysine
PLO	Poly-L-ornithine
PTFE	Polytetrafluoroethylene
TF	Tissue factor

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Chapter 3

Pancreatic islet macroencapsulation using microwell porous membranes

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Abstract

Allogeneic islet transplantation into the liver in combination with immune suppressive drug therapy is widely regarded as a potential cure for type 1 Diabetes. However, the side effects of immunosuppressive agents are an important barrier for wider application of this therapy. Islet encapsulation that obviates the needs for immunosuppressive therapy is a promising strategy. In this study, we develop a macroencapsulation device based on thin microwell membranes. The islets are seeded in separate microwells to avoid aggregation, whereas the membrane porosity is tailored to achieve shielding of the islets from the host immune cells without compromising their secretory responses. The non-degradable, microwell membranes are composed of poly (ether sulfone)/polyvinylpyrrolidone and manufactured via phase separation micromolding. Our results show that the device prevents DJJUHJDWLRQDQGSUHVHUYHVWKHLVOHW¶VQDWLYHPRUSKRORJ\RUHRYHUWL islets maintain their glucose responsiveness, comparable to free-floating non-encapsulated controls and function very well after 7 days of culture, demonstrating the potential of this novel device for islet transplantation.

1. Introduction

Type 1 Diabetes is an autoimmune disorder, characterized by the specific destruction of insulin-SURGXFLQJ-cells within the islets of Langerhans, resulting in an absolute insulin deficiency. Currently, type 1 Diabetes accounts for 5-10% of the total cases of diabetes worldwide, occurring mainly in children and young adults [1]. In fact, more than 500 000 children under 15 years of age were diagnosed with type 1 Diabetes in 2015 [2]. Although insulin therapy is e¡ ective in regulating the blood glucose levels, it still lacks the precise glycemic control that the normal physiological system has. Therefore, it results often in hypoglycemic events, while in the long term micro/macrovascular complications affect many patients [3].

7KHUHSODFHPHQWRI-cells by intrahepatic islet transplantation in combination with immunosuppressive drugs can restore insulin independence. However, while often successful, intrahepatic islet transplantation is associated with a high degree of islet loss, due to a multifactorial response involving an immediate blood mediated inflammatory response, auto and alloimmunity, and loss of innervation and vascularization [4]. In addition, life-long immune suppressive therapy is necessary resulting in increased risks of attacking infections or certain cancers, while the supply of high quality donor pancreas available for islet isolation and transplantation is very limited. Encapsulation using biomaterials, to provide a physical barrier between transplanted -cells and their recipients, has emerged as a promising approach to improve transplantation outcomes eliminating the need for immunosuppression [4, 5]. Moreover, encapsulation could allow for using of not only human donor islets, but also the use of *de novo* -cells derived from stem cells, or even xenogeneic islets and help overcome the islet donor shortage limitations.

Commonly applied strategies often focus on encapsulation of single islets using either hydrogels, or nanometer-scale coatings, such as, alginate, polyethylene glycol, polylactide-derived, or cation-anion layer by layer systems [6-8]. Although, initial

results suggest that islets can maintain their function, long-term survival cannot be guaranteed. Hydrogels are, in most cases, not stable enough to support islets transplantation over long time. In recent years, the creation of alternative transplantation sites using three-dimensional scaffolds has been explored too. Highly porous scaffolds such as poly(lactide-glycolide) sponge, vicryl or, poly(glycolic-acid) fibers meshes, with high interconnectivity have been proposed as suitable islet encapsulation devices [9-11]. Islets seeded into the macropores of these constructs can easily and quickly be provided with oxygen and the necessary nutrients. However, in most cases these constructs have large pores that permit tissue ingrowth and cell penetration. Therefore, this approach still requires the use of immunosuppressive drugs.

The main advantage of macroencapsulation technique is the control of confining the islets to one location in the body as well as the ability to retrieve the device and the possibility of islet replenishment, if necessary. However, islet encapsulation remains a difficult challenge because, by preventing cytotoxic T-lymphocyte interaction with the allogeneic -cells, the mass transport of necessary nutrients, glucose and insulin is often compromised.

A variety of different macroencapsulation designs have been studied such as tubular chambers, sealed hollow fibers and planar devices [12]. However, poor oxygen and nutrient diffusion across the membranes was the main reason for eventual graft failure, leading to compromised islet viability. Additionally, the lack of physical separation of the islets in these macroencapsulation devices causes aggregation of the islets. This negatively affects islet structure, leading to limited diffusion of nutrients and oxygen, loss of function and apoptosis. Design of an immune protective macroencapsulation device for islets, should strike a balance between optimal survival of islets and shielding the same islets from the immune system.

In this study, we propose a novel concept for a macroencapsulation device in which islets are confined between two porous membranes. One membrane consists of microwells in which the islets are seeded and the other membrane acts as a lid, see Figure 1. For both, the microwell and the lid membranes, the porosity is tailored to permit nutrient inflow and metabolite outflow, but protects the islets from immune cells. The microwell array allows good islet separation and prevents both spreading DQGDJJUHJDWLRQDPLQWDLQLQJWKHLVOHW¶URXQGHGPRUSKRORJ\

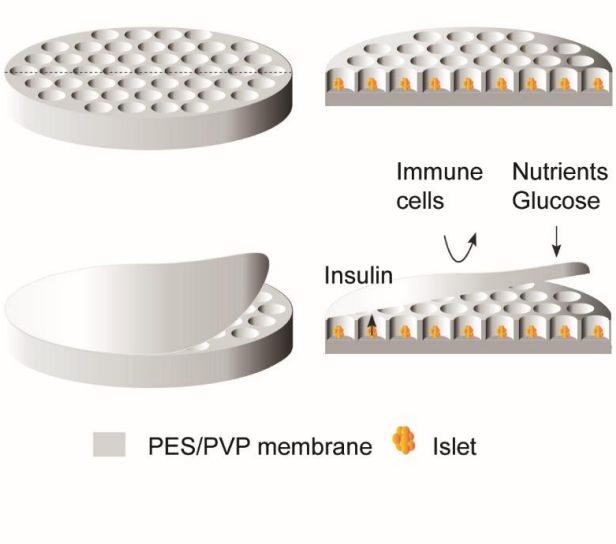

Figure 1. A schematic overview of the PES/PVP flat membrane encapsulation device.

In earlier studies, we developed open microwell scaffolds for vessel ingrowth using poly(ethylene oxide terephthalate)-poly(butylene terephthalate) (PEOT/PBT) biodegradable polymers [13]. Our aim here is to achieve a non-degradable functional closed encapsulation device. Therefore a closed porous system is developed using poly(ethersulfone) (PES) / polyvinyl pyrrolidone (PVP) polymer blend via phase separation micromolding, which is a unique method for preparation of porous

microstructured membranes in one step [14-16]. PES is a non-degradable material that has high stability and good mechanical properties, and is widely used as a membrane material for blood purification and other biomedical membrane applications. Blending PES with PVP results in more hydrophilic membranes that have low fouling and, importantly for islet encapsulation, low cell adhesion properties. The porosity of the PES/PVP microwell membranes is tailored to allow insulin and glucose transport and the device performance is evaluated by analyzing the glucose responsiveness of encapsulated MIN6 mouse insulinoma cell aggregates and of human islets. Our results indicate that the PES/PVP microwell membrane, as a crucial part of macroencapsulation device, is a potential carrier for extrahepatic islet transplantation.

2. Materials and methods

2.1. Microwell membrane fabrication

Microwell membranes were fabricated using phase separation micromolding 360 [14-16]. For this, we used a polymer blend of 15 wt.% poly(ethersulfone) (PES) (Ultrason, E6020P) and 5 wt.% polyvinylpyrrolidone (PVP) (MW= 40000, Sigma Aldrich) in N-Methylpyrrolidone (NMP) (Acros organic). This polymer solution was stirred on a roller bank overnight, at room temperature. The membranes were prepared by casting the solution on a custom made, silicon, micropatterned mold with spatially organized dome-OLNHVWUXFWXUHVRIPKHLJKWDQGHL diameter presented on Figure 2. A custom-made casting machine, with micrometric screws to regulate the casting WKLFNQHVV ZDV XVHG WR REWDLQ PDQG P thick membranes. Casting was followed by immersion into a coagulation bath, containing demineralized water. After the polymer solution became turbid and precipitated, the membranes were removed from the mold, rinsed with demineralized water in order to remove remaining solvent traces and stored in demineralized water till further use. In order to increase the membrane porosity, the membranes were treated with 4000 ppm sodium hypochlorite aqueous solution

(NaClO, Fluka) for either 2 h or 24 h. Subsequently, the membranes were washed and stored in demineralized water.

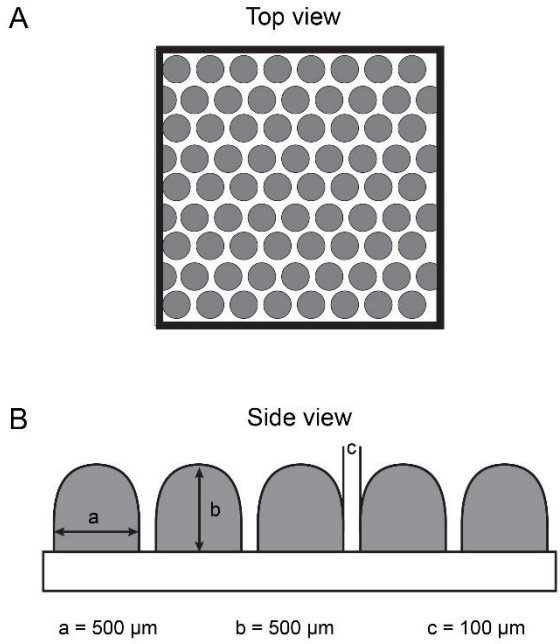

Figure 2. Silicon micropatterned mold. A) Top view; B) Side view.

2.2. Scanning electron microscopy

For scanning electron microscopy (SEM), the membranes were dried overnight in air at room temperature and cryogenically broken in liquid nitrogen when needed for cross section images.

Microwell membranes with cells were fixed in 4% paraformaldehyde for 1 h at room temperature, dehydrated in water-ethanol solutions following a gradient (volume ratio water: ethanol of 100:0, 50:50, 25:75, 10:90, 5:95 and 0:100) and dried after dipping in hexamethyldisilizane overnight. Dried membranes were placed on the SEM holders and sputter-coated with nm-thick gold layer prior to imaging.

2.3. Cell culture and controlled cell aggregate formation

MIN6-B1 mouse insulinoma cells (kindly provided by Dr. P. Halban, University 0HGLFDO &HQWHU *HQHYD 6ZLW]HUODQG ZHUH FXOWXUHG LQ 'XOEHFFR¶V 0R (DJOH¶V 0HGLXP'0(0*LEFR) supplemented with 10% (v/v) FBS (Lonza), 100 8P/ SHQLFLOOLQ DQG PJP/ VWUHSWRP\FLQ *LEFR DQG 0 +UHVKO\DGGHG beta-PHUFDSWRHWKDQRO*LEFRDWÛ& and 5% CO_2.

Sterile agarose microwells were fabricated as described previously [17]. In short, SRO\GLPHWK\OVLOR[DQH 3'06 QHJDWLYH PROGV FDUU\LQJ P SLOODUV ZHUH sterilized with 70% ethanol. 3% UltraPure™ agarose (Gibco) was dissolved in PBS. 7KHVROXWLRQZDVKHDWHGWRÛ&QDPLFURZDYHRYHQ0ROGVZHUHSODFHGLQ a 6-well plate and filled with 8 mL of 3% agarose solution. The plates were centrifuged at 300 G IRUPLQWRUHPRYHDLUEXEEOHVDQGVWRUHGDWÛ&IRUD 30 min. After the gel was formed, the molds were gently removed from the agarose using a sterile spatula. Using a sterile punching device, chips were punched out leaving a thin agarose wall on all sides to fit into a 12-well plate. Stable pseudo-islets were then prepared based on the work of Hilderink et al [18]: MIN6 cells were then seeded onto the agarose chips (250 cells per pseudo-islet). The plates were centrifuged at 150 G for 1 min and 2 mL of medium was carefully added to the chips. 0HGLXPZDVUHIUHVKHGKDIWHUVHHGLQJ$IWHUKDWÛ&DJJUHJDWHV0 PLQGLDPHWHUZHUHflushed out of the chips and used for seeding on the microwell structured membranes.

+XPDQLVOHWVRI/DQJHUKDQVLVRODWHGIURPGRQRUSDQFUHDWDSXULW\DQG UHVSHFWLYHO\ZHUH SURYLGHG E\WKH +XPDQ ,VOHW ,VRODWLRQ /DERUDWRU\ /HLGHQ 8QLYHUVLW\0HGLFDO&HQWHU /HLGHQ 7KH 1HWKHUODQGV 6WXGLHV ZH SHUIRUPHGRQLVOHWVWKDWFRXOGQRWEHXVHGIRUFOLQLFDOWUDQVSODQ UHVHDUFKFRQVHQWZDVDYDLODEOHDFFRUGLQJWRQDWLRQDOODZV7KHLVO LQ &5/ PHGLX P PPRO/ JOXFRVH FRQWDLQLQJ)%6 P0

6XWD0$P8P/SHQLFLOOLQDQGPJP/VWUHSWRPFLQ*EFRPPRO/
(63 (6DQGPJP/QLFRWLQDPLGH

2.4. Membrane transport properties: water permeability and insulin, glucose diffusion

Microwell membranes with an effective surface area of 0.9 cm^2 were used for clean water flux measurements. The experiments were performed at room temperature using nitrogen pressurized dead-HQG SLFRQ WSH XOWUDILOWUDWLRQ FHOO DQG 0L water. Firstly, the membranes were pre-pressurized for 30 minutes at 0.7 bar. Afterwards, the clean water flux through the membranes at various transmembrane pressures was measured for at least 20 minutes. The membrane hydraulic permeability was calculated from the slope of the linear part of the flux versus the transmembrane pressure relation.

In order to determine the diffusion coefficient of insulin and glucose through the microwell membranes, a two-compartment transwell system was used. The commercial membrane was removed from the Transwell insert (Corning) and a VHDOLQJ\ polyether ether ketone (PEEK) ring was fabricated to seal the microwell membrane to the insert (Figure 3A). The assembled Transwell insert was placed in the well plate, creating top and bottom compartments separated by the microwell membrane. The membranes were sterilized in 70% ethanol, washed with PBS and pre-incubated in culture medium. Modified Krebs buffer (115 mM NaCl, 5 mM KCl, 24 mM $NaHCO_3$, Sigma) supplemented with 2.2 mM $CaCl_2$, 20 mM HEPES (Gibco), 30% bovine serum albumin, 1 mM $MgCl_2$, and 0.1 mM Theophylline (Sigma) was prepared at pH 7.4 [19]. From this Krebs buffer, a high (16.7 mM) glucose solution was prepared. For the glucose diffusion experiment, the top compartment of the transwell system contained the prepared buffer, whereas the bottom compartment contained glucose solution. In the case of insulin diffusion H[SHULPHQWDJ/L nsulin in glucose solution was prepared and put in the top

compartment, while the bottom compartment contained the earlier prepared buffer. After 1 hour, samples were collected separately from both compartments (n=6).

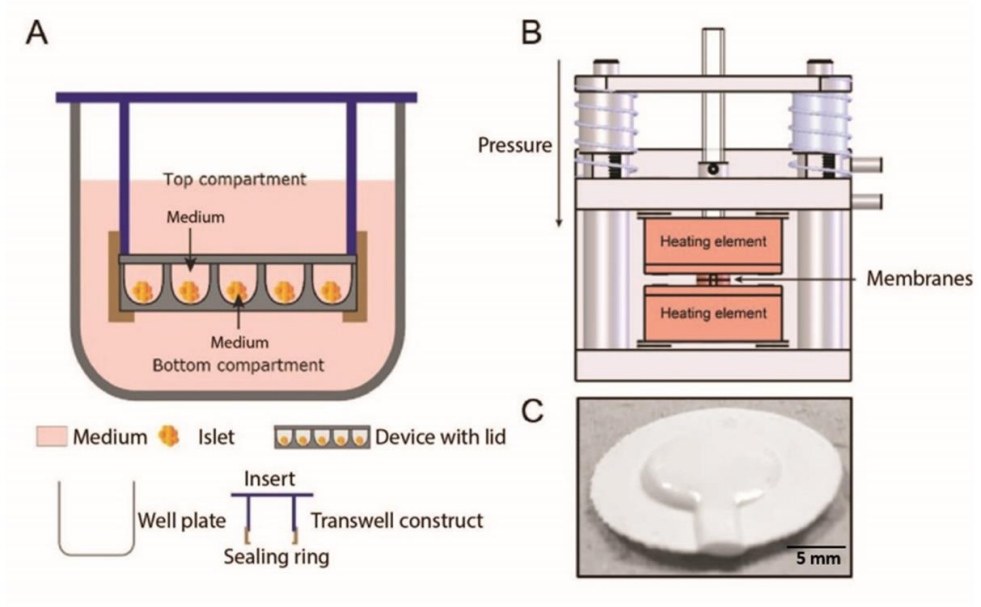

Figure 3. A) Schematic representation of two-compartment transwell system; B) Schematic overview of the custom-made sealing machine. Two Teflon coated heating elements with a circle with a small opening were used to obtain a seal between the microwell membrane and the PES lid; C) The sealed device.

In order to evaluate whether the membrane limits the response to glucose concentration, 150 MIN6 pseudo islets were seeded on the top of the microwell membrane. Subsequently, the medium was replaced by the premade buffer on the top compartment and glucose solution on the bottom compartment. In response to glucose diffusing through the membrane to the top compartment, the MIN6 aggregates there secreted insulin. During a period of 1 hour, samples were taken separately from the top and bottom compartment every 10 minutes. The samples

were analyzed for glucose concentration using a Vitros DT60 chemistry system and insulin concentration using a Mouse ELISA immunoassay (Mercodia). The amount of glucose and insulin that passes through the membrane, from the donor compartment to the acceptor compartment, in time, indicates whether the porosity of the membrane is optimal for transport of these molecules. The permeability of glucose and insulin through the membranes was calculated using the equation:

$$\text{LANIA=EHEP:J}\ ^{6}\text{Ơ}^{5};\ \text{L}\ \frac{\text{ê}\ \text{:Úà}^{7}\cdot\text{æ}^{7-};}{\text{¿¼:Úà}^{7/};}\ \text{H H:I}\ ; \qquad (1)$$

:KHUH &V WKH FRQFHQWUDWLRQ GLIIHUHQFH EHWZHHQ WKH GRQRU DQG WH compartment and l is the geometrical thickness of the membrane. Flux was calculated using the equation:

$$\text{BHQ:C I}\ ^{?6}\text{Ơ}^{5};\ \text{L}\ \frac{\text{c¼ê}\quad \text{kÚà}^{7/}\text{oëÏê}\quad \text{:à}^{/};\ ^{o}\text{:à}^{\cdot};\text{g}}{\text{ê}\quad \text{:æ;}} \qquad (2)$$

Where, $C_{acceptor}$ is the glucose or insulin concentration in the acceptor compartment, $V_{acceptor}$ is the volume of the acceptor compartment and A is the surface area of the membrane. From the permeability coefficient, we calculated the diffusion coefficient using equation 3:

$$\text{@BBQOEKKABBEE:AJ?I}\ ^{6}\text{Ơ}^{5};\ \text{L}\ \frac{\text{ê}\quad \text{kà}\cdot\text{æ}^{7-}\text{o}}{\text{Ä}} \qquad (3)$$

Where K is the partition coefficient of glucose and insulin in the membrane, which was considered to be equal to one.

2.5. MIN6 aggregates functionality in vitro using open system

The membranes for cell seeding were placed in the transwell system (figure 3A), sterilized with 70% ethanol for 30 min and washed 3 times in PBS. 150 MIN6 DJJUHJDWHVLQRIIPHGLXPZHUHVHHGHGRQWKHWRSRIWKHPLFURZHOOPHPEUI DQGRIIPHGLXPZDVDGGHGWRWKHERWWRPFRPSDUWPHQWSWHUGRIXOWXU

a glucose induced insulin secretion test (GIIST) was performed, with free-floating pseudo-islets in a commercial transwell system (MilliPore), as a control. The modified Krebs buffer was prepared as previously described and was used to prepare low (1.67 mM) and high (16.7 mM) glucose concentration solutions. Both the free-floating pseudo-islets and the microwell membranes with MIN6 aggregates were washed three times (5 min) in the Krebs buffer, followed by a pre-incubation of 90 min in the low glucose concentration buffer. All samples were then incubated for 60 min in subsequent low, high and low glucose concentration buffer with three times 5 min washing in the Krebs buffer, between each high and low. The final function test was performed with additional high and low glucose concentration buffer incubation. During the test, both top and bottom compartments of the transwell system contained the same buffer for each step respectively. Samples were taken after each incubation time, spun down (300 G, 3 min) and the supernatant was stored at - ̂&. Samples were analyzed using insulin Mouse ELISA immunoassay 0HUFRGLDDFFRUGLQJWRWKHPDQXIDFWXUHU¶VLQVWUXFWLRQV7KHIXQFWLR aggregates was determined by the stimulation index that is defined as the insulin secretion when stimulated with the first low glucose buffer.

2.6. Human islets viability in closed system

The closed microwell system (see details about its preparation in the next section), containing 150 human islets, was opened after 1 day of culture and live/dead analysis was performed in order to examine cell viability. Membranes with islets inside the ZHOOVZHUHSODFHGLQDVROXWLRQFRQWDLQLQJ OP/FDOFHLQJUHHQDQG OP/ HWKLGLXP KRPRGLPHU UHG LQ 3%6 DQG LQFXEDWHG IRU PLQ DW ̂& in the dark. Green-fluorescent (ex 494 nm / em 517 nm) live cells and red florescent (ex 517 nm / em 617 nm) dead cells were imaged using an EVOS digital inverted fluorescence microscope and photomicrographs were taken.

2.7. Human islets functionality in vitro

Closed system: a closed system containing a microwell membrane covered with a flat PES membrane lid was developed. For the lid, we investigated membranes with WZR GLIIHUHQW SRURVLWLHV PDQG P6WHUOLWHFK 7KHVH PHPEUDQHV ZHU sterilized with 70% ethanol for 30 min, washed 3 times in PBS and pre-incubated in culture medium overnight. Afterwards, the microwell membrane was assembled ZLWKLQ WKH WUDQVZHOO VVWHP DQG KXPDQ LVOHWV LQ ORI PHGLXP ZKHU seeded on the top. Medium was carefully aspirated, leaving the islets inside the wells of the membrane. The insert was removed while the microwell membrane with islets remained within the sealing ring. The lid was placed on the top of the microwell membrane and the system was sealed with the insert fitted in the sealing ring.

Sealed device: A sealed device was designed with the aim of implantation. The microwell membrane and the flat lid membrane were placed between the two shaped molds of a custom-made sealing machine (Figure 3B, C). A temperaWXUH RI & was applied for 10 seconds and the membranes were sealed on the edges, leaving open the middle part and a small inlet for cell seeding. The sealed device was sterilized with 70% ethanol, washed in PBS and pre-incubated in culture medium overniJKW7KHLVOHWVLQORIPHGLXPZHUHVHHGHGLQVLGHWKHGHYLFHYLDWK inlet, which was closed after seeding using sterile, surgical staples (Teleflex Medical, HORIZON, Ligating clips).

7RDVVHVVWKHHQFDSVXODWHGLVOHWV¶IXQFWLRQDJOXFRVHLQGXFHGLQV (GIIST) was performed after culturing them statically for 1 day and 7 days. Free-floating islets in transwell system (MilliPore) (n=3) were used as a control. Islets were first pre-incubated in modified Krebs buffer (115 mM NaCl, 5 mM KCl, 24 mM $NaHCO_3$, 2.2 mM $CaCl_2$, 20 mM HEPES, 1 mM $MgCl_2$, 2 mg/mL bovine serum albumin, pH IRUPLQDWRQG& 2. All samples were then stimulated for 1 hour in subsequent low (1.67 mM), high (16.7 mM), low, and again high and

low glucose buffer with three times 5 min washing in the Krebs buffer between the high and low glucose incubation step. Samples were taken after each incubation, spun down (300 G, 3 min) and the supernatant was stored at -8[illegible] DPSOHVZHUH analyzed using an insulin ELISA (Mercodia). The functionality of human islets was determined by the stimulation index, which is defined as the insulin secretion after stimulation with high glucose buffer relative to the insulin secretion when stimulated with the first low glucose buffer. Islets with stimulation index higher than two were regarded functional.

3. Results

3.1. Microwell structured membranes fabrication and characterization

We used phase separation micromolding to fabricate uniform, porous, microstructured flat membranes. A custom designed mold allowed the formation of structures of defined shape and size. Figure 4 shows flat PES/PVP membranes with microwells of excellent quality. The microwells are homogenously distributed over the membrane, reflecting the features of the mold used for casting. Each well has [illegible] The cross-section of the membrane presents asymmetric pore morphology. A dense selective layer containing small pores (1-[illegible] is present on the bottom of the microwell membrane and provides protection from cell infiltration towards the microwells. A finger-like, porous sub-layer [illegible] formed between the microwell structures and the top of the selective layer (Fig. 4B). This microwell array allows for good separation of the islets, preventing aggregation and spreading which could cause further transport limitation [13]. The islets do not adhere to the PES-based membranes due to its tailored hydrophilicity by addition of PVP. Therefore, their rounded morphology is preserved after prolonged culture (Fig. 4C and 4D). The number of the microwells determine the number of the islets possible for encapsulation in order to maintain their viability and function. Higher number of islets than the number of available wells used for encapsulation leads to

transport limitation, thereby decreasing the functionality of the islets. Here, considering the number of wells, we could encapsulate 150 islets using 8 mm microwell membrane.

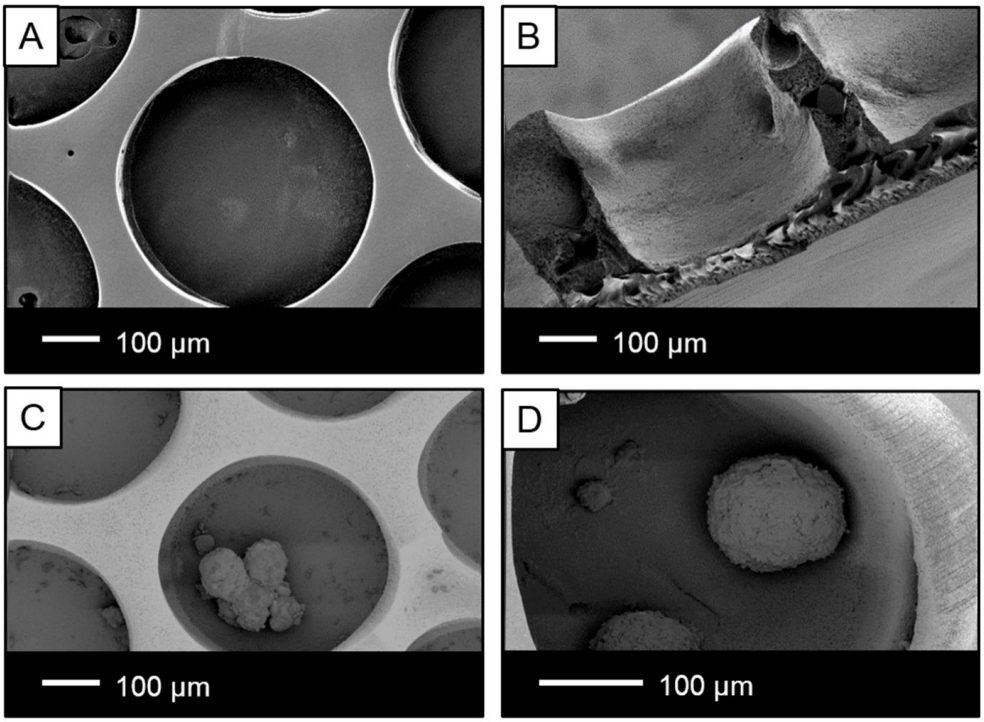

Figure 4. Scanning electron microscopy images of microwell membranes: A) top view, B) cross section, C) Human islets inside the well, D) MIN6 aggregates inside the well

Figures 5 A and B present the clean water fluxes (CWF) of the membranes at various transmembrane pressures. In all cases, the graph is linear indicating high stability of the membranes in this pressure range. Table 1 presents the water hydraulic permeability of all prepared membranes. When reducing the membrane thickness IURP WR ~~P~~ WKH KGUDXOLF SHUPHDELOLW\LQFUHDVHV IURP WR L/m^2/h/bar. Additionally, treatment with NaClO solution removes part of PVP and

the membranes become more porous. In fact, the hydraulic permeability of the membranes treated with NaClO for two hours is more than double compared to the untreated membrane. Longer treatment, namely for 24 hours, results in even more porous membranes with a hydraulic permeability of 3845 L/m^2/h/bar.

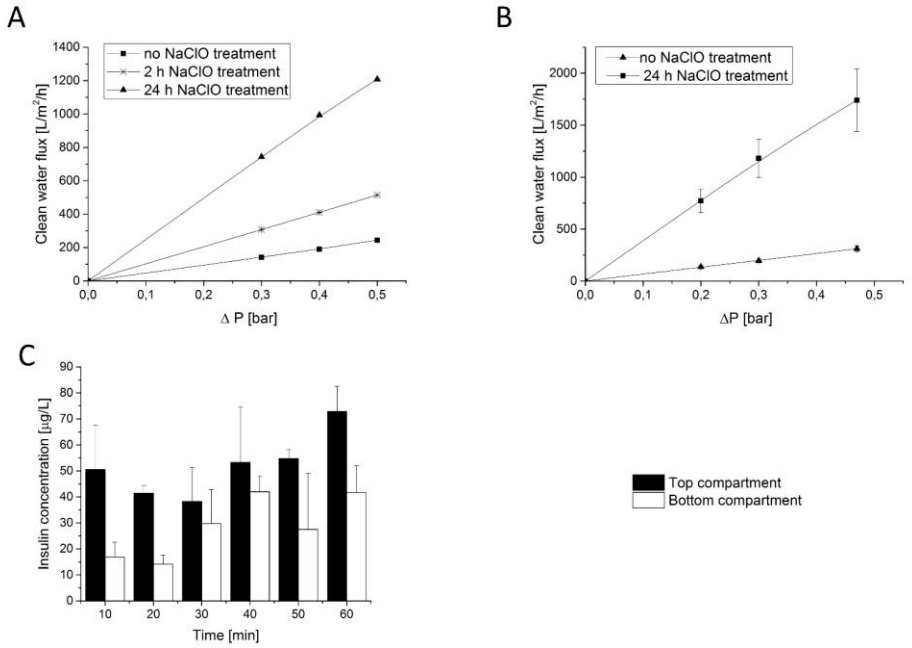

Figure 5. Transport characteristics. A) Clean water flux vs. pressure for membranes; B) Clean water flux vs. pressure for 24 h NaClO treatment; C) MIN6 aggregates insulin secretion through the membrane in response to a high glucose concentration. Error bars indicate standard deviation (n=3).

The important requirement for the microwell membrane to be suitable for islet encapsulation is having high insulin and glucose permeability. To test this, MIN6 aggregates consisting of 250 cells per aggregate were seeded in the microwell membranes assembled in the transwell system and exposed to 16.7 mM glucose

solution in the bottom compartment. Over time, glucose diffusing through the membrane to the top compartment induces insulin secretion from the aggregates.

Table 1. Membrane water permeability.

Microwell membrane	**Membrane** *W K L F N Q H V V*	**Sodium hypochlorite treatment [h]**	**Water hydraulic permeability (n=3) [L/m^2/h/bar]**
M1	250	-	“
M2	250	2	“
M3	250	24	“
M4	100	-	“
M5	100	24	“

Figure 5C shows that within 10 min the aggregates release insulin, which diffuses through the membrane to the bottom compartment. After one hour, the insulin concentration in the bottom compartment increases further. Based on these data, the estimated diffusion coefficients of insulin and glucose through the membranes are 0.3 x 10^{-10} and 3.6 x 10^{-10} m^2/s respectively and they are in the same order of magnitude as the free diffusion coefficient of the molecules in solution, namely 1.5 x 10^{-10} m^2/s for insulin and 9.59 x 10^{-10} m^2/s for glucose. These results indicate that the porosity of microwell membranes is sufficient to achieve high transport of insulin and glucose without transport limitations.

In summary, we successfully fabricated microwell membranes suitable for our encapsulation device. The membrane performance was assessed further regarding the fuQFWLRQDOLW\RIWKHSDQFUHDWLF16 aggregates in an open system.

3.2. MIN6 aggregates function using open system

A glucose induced insulin secretion test (GIIST) was performed for the MIN6 aggregates seeded on the microwell membranes placed in the transwell system. Figure 6 compares the stimulation index of the aggregates within the microwell membranes to the free-floating aggregates, our positive control. For the estimation of the stimulation index, the insulin secretion of all samples was normalized to the insulin secretion of the first experiment of low glucose stimulation. Therefore, in all results presented here, the stimulation index of the first low is always equal to one.

In all cases, free-floating aggregates function well (stimulation index more than 2 for high glucose concentration) and show a clear response to glucose concentration changes. The insulin concentration was analyzed separately on the top and bottom compartments of the open microwell system with M1 and M2 membranes (Figure 6A). The MIN6 aggregates on the top compartment respond to glucose concentration changes. Additionally, when more open, M2 membrane is used, the insulin concentration in the bottom compartment reaches a similar value to the one detected in the top compartment, where cells were in direct contact with the high glucose concentration solution. Finally, a function test of MIN6 aggregates over 5h was performed in an open system, uVLQJWKHPRVWSHUPHDEOHPWKLFNPHPEUDQHV ± M3 (Figure 6B). An increase in insulin release following stimulation, compared to basal insulin release levels, was observed there similar to the free-floating aggregates.

In summary, we showed here the development of an optimized functional microwell membrane open system where the MIN6 aggregates within the wells respond to glucose concentration changes. The M3 membrane will be investigated in the next section as part of a closed, immune protective system for human islet encapsulation.

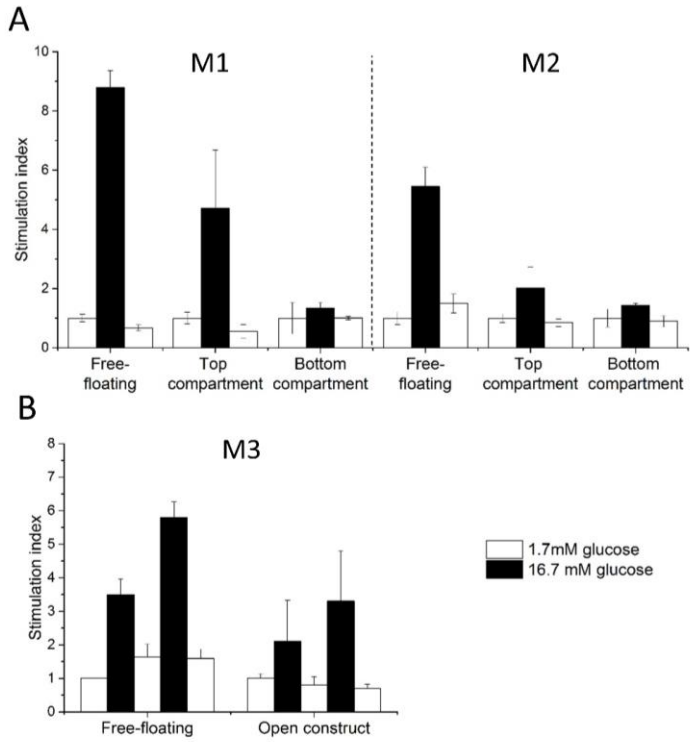

Figure 6. MIN6 aggregates functionality. A) A comparison of MIN6 aggregates insulin secretion detected on the top and bottom compartment separately, in an open transwell system using untreated microwell membranes - M1 and microwell membranes after 2h treatment with NaClO solution - M2; B) Total insulin secretion of MIN6 aggregates over 5h using final open construct with microwell membranes after 24h of treatment with NaClO solution - M3. Insulin secretion is normalized to the first low glucose incubation and presented as a stimulation index. Error bars indicate standard deviation (n=3).

3.3. Human islet viability and functionality using closed system

Human islets were seeded in the closed system with the M3 microwell membrane and cell survival was studied after one day of culture. Figure 7A shows that the islets are viable in the closed system, as represented by the green viable cells.

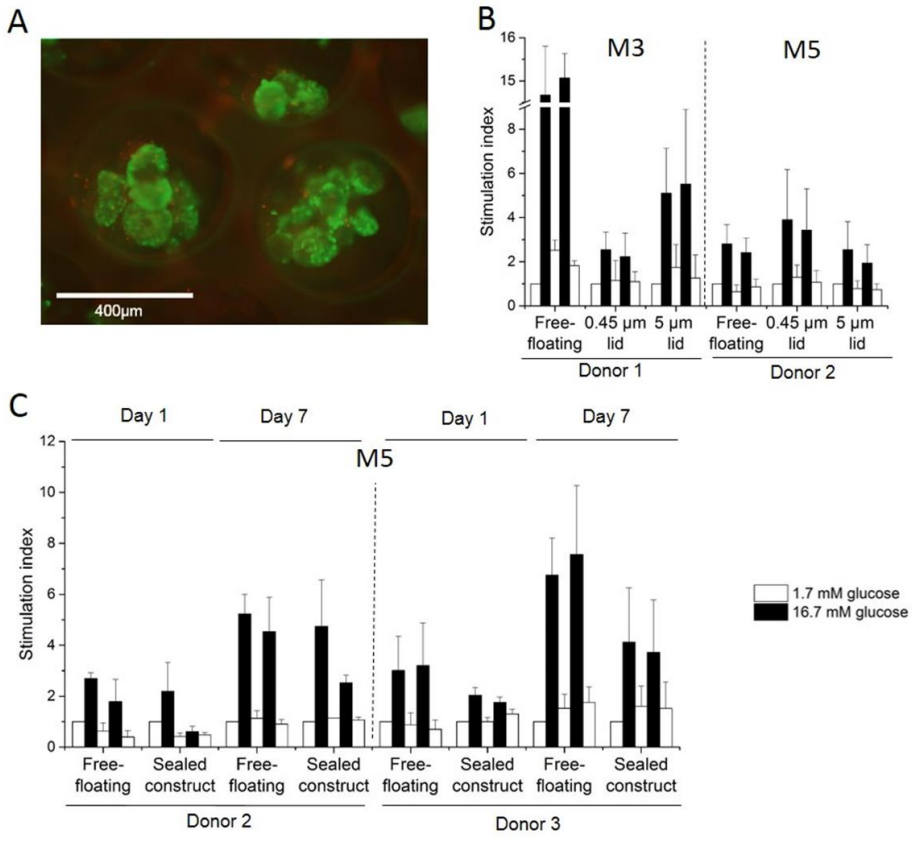

Figure 7. A) Islets viability after 1 day of culture in closed system with M3 microwell membrane, green-live cells, red-dead cells; B) Islets functionality using a closed system with various porosities of the lid and various microwell membrane after 24h treatment –M3 and 100 µm thick micro 24 h treatment –M5; C) Functionality of islets from two donors over 7 days using a sealed device. Insulin secretion is normalized to the first low glucose stimulation and presented as a stimulation index. Error bars indicate standard deviation (n=3).

We also investigated the islet function in our closed system consisting of microwell membrane covered with a flat PES membrane as a lid. We compared flat membranes with YDULRXV SRUH VL]HV XVHG RQ WKH WRS RI WKH PRVW SHUPHDEOH P0 DQG

100 P 0 WKLFN PLFURZHOO PHPEUDQHV)LJXUH % FRPSDUHV WKH VWLPXODWL
index obtained for free-floating islets and islets encapsulated in the closed system, for two different donors of islets. Both free-floating and encapsulated islets function well and respond fast to glucose concentration changes. Islets encapsulated in closed system with the thin M5 microwell membrane respond over 5 h in similar manner to free-floating islets regardless of the porosity of the lid. However, for the thicker, M3, PLFURZHOOPHPEUDQHDOLGZLWKSRUHVL]HRI PL VUHTXLUHGWRDFKLHYHVX glucose transport and obtain better insulin secretion. Since a membrane lid with 0.4 - 0.PSSRUHVL]HLVH[SHFWHGWRSURYLGHVXIILFLHQWEDUULHUEHWZHHQWKH UHFLSLHQW¶VWLVVXHIURPWKHWRSRI[24, 25], we have selected this lid in FRPELQDWLRQZLWKWKH PWKLFNPLFURZHOOPHPEUDQH0IRUWKHSUHSDUDW of the sealed device. Figure 7C shows the stimulation index for the islets encapsulated there in comparison to free-floating islets. The sealed islets produce and release insulin upon stimulation after one day of culture and they remain functional, indicating also their viability after 7 days of culture. Interestingly, during the 7 days culture period, they show an increase in performance comparable to the increase observed for the free-floating islets. As it is clearly shown in Figure 5, all these results are independent on the donor variability showing the great potential of our device for encapsulation of islets and the treatment of diabetes.

4. Discussion

In this study, we propose a novel membrane-based islet macroencapsulation device. We developed a sealed device that consists of a microwell membrane for hosting the islets and a flat membrane as a lid. Our device was developed using non-degradable polymer blend of PES/PVP [14, 23]. PES is an excellent membrane forming material with very good chemical and mechanical properties, therefore, it has been widely used in medical devices, artificial organs and blood purification processes, such as hemodialysis membranes [24-27]. By blending PES with PVP, we can obtain a more hydrophilic material with better biocompatibility [28, 29], low fouling and non-cell

adhesive properties [30, 31]. All these properties are essential for preventing islet clustering and attachment, and increase islet survival after transplantation [20, 32, 33].

7KHPLFURZHOOPHPEUDQHVZHUHIDEULFDWHGXVLQJ[36,25] through immersion precipitation of the polymer on a micropatterned mold, we obtained in one step a highly porous material with controllable micrometer-scale pores having excellent quality microwells (see Figure 4). Recently, Buitinga et al [13] developed an open poly(ethylene oxide terephthalate)-poly(butylene terephthalate) (PEOT/PBT) microwell scaffold fabricated by microthermoforming, where heated polymer porous film was stretched into a negative mold in order to obtain similar microwells. The microthermoforming method, however, affects pore morphology causing stretching and collapsing thus can have a negative effect on transport properties. Important adYDQWDJHV RI 360 ÐSSOLHG KHUH DUH the fabrication of a porous microstructured membrane in one step and the easy upscaling. The fabrication parameters can be tuned to control material shrinkage and obtain the suitable pore size, adequate porosity and interconnectivity of the microwell membranes, all very important for sufficient transport of nutrients to the encapsulated islets. In our device, the membrane porosity is carefully tailored to allow glucose diffusion to the islet and corresponding insulin release in response to blood glucose levels. The selective layer of the microwell membrane is microporous (1-2ÐQGWKHUHIRUHLVH[SHFWHGWREORFNWKHLPPXQHFHOOV [12] HRIaP and can provide sufficient immune isolation for encapsulated islets. Additionally, the device avoids aggregation of the islets, since they are seeded on separated microwells. According to the modeling studies of Dulong and Legallais [34], in order to increase the number of functional islets, a higher islet density needs to be used. However, high islet density with no cell aggregation would require the application of a gel system to keep the islets separated leading to the need of large devices, almost impossible to apply in humans. Our microwell device prevents islet

aggregation, thereby, improving their chances of survival and function, since separation allows for a proper supply of nutrients and oxygen to all islets. The dimensions of the microwells (500 x 400 –m) are suitable for fitting the broad range of pancreatic islets obtained after islet isolation. Lehmann et al [20] describe the superiority of small islets over large ones due to higher survival in both normoxic and hypoxic conditions and better insulin secretion indicating that optimal mass WUDQVSRUW SODV DQ LPSRUWDQW UROH ,VOHWV ZLWK D GLDPHWHU DUR recommended for encapsulation in order to avoid necrosis, which usually occurs in larger islets [35]. Using our fabrication method, it is possible to create membranes containing microwells with smaller dimensions to accommodate a population of smaller diameter islets, or pseudoislets created from stem cell derived *de novo* - FHOOVWKHUHEIXUWKHULQFUHDVLQJ cell survival and function. This is one of the main goals of a follow up study.

Clark *et al*. [36] proposed heat-sealing as an effective method to close polysulfone hollow fiber membranes in order to prevent cell infiltration. Here, we applied heat sealing only on the edges of the membranes to prevent surface damage, and preserve pore morphology and porosity, which are important for adequate transport properties. Our device also features a practical small inlet, which makes islet seeding quite simple. After seeding, the islets settle on the bottom of the wells and remain stable during further handling procedures.

7KHSRUHVL]HRIWKHOLGPHPEUDQHLV–PLQDJUHH with other studies, which have shown that this pore size does not allow host cells to permeate to the device providing protection to allogeneic and xenogeneic transplants [21, 22, 37, 38]. For comparison, the TheraCyte™ VVWHPLV FRPSRVHG RI D FHOO LPSHUPHDEOH P– pore membrane laminated to a P–RXWHU PHPEUDQH IRU VXSSRUW DQG WLVVXH integration [12]. Besides, Cell Pouch System™ - a biocompatible polymeric macrocapsule, mimicking natural environment in the host for encapsulated pancreatic cells contains large pores to allow the development of fibrous tissue rich

LQYHVVHOVZLWKRXWLPPXQRSURWHFWLRQZKHUHDVWKH VOHWVKHHWPDF system consists of flat, thin alginate sheets in which islets are entrapped without efficient islet separation [39, 40].

In order to evaluate the function of our device, we used MIN6 insulin secreting cells, which are able to create stable aggregates and mimic pancreatic islet function [41]. These aggregates function well when seeded in thHRSHQ3VWKLFNPLFURZHOO membranes (M1). By tuning the membrane porosity (via partly washing PVP with NaClO) we obtained membranes with optimal diffusion of insulin (M2). In fact, the concentration of insulin, which passes through the membrane equalizes after 30 minutes to the one above the membrane, where cells in the microwells are in direct FRQWDFWZLWKWKHJOXFRVHVROXWLRQVKRZLQJWKDWLQVXOLQVHFUHWHG transported relatively unhindered across the membrane. When using microwell membranes with 5 times higher water permeability (M3) than the original membrane (M1), pseudo-islets seeded in the membrane functioned very well, comparable to free-floating aggregates. Wienk *et al.* [42] first reported an increase in water permeability of PES/PVP membranes attributed to PVP degradation and leaching during membrane treatment with a NaClO solution. This observation was also later confirmed by other researchers [30, 43, 44]. Here, the membrane treatment with NaClO allowed increase of membrane porosity and optimization of the membrane transport properties, however, at the same time not all PVP was removed so the membranes have still low fouling and low adhesive properties (see Figure 4).

Islets encapsulated in our closed system usLQJ3VWKLFNPLFURZHOOPHPEUDQH (hydraulic permeability 3845 [L/m^2/h/bar]) function very well and comparable to free-floating islets, regardless of the porosity of the membrane lid. This fits well with the results of literature studies which indicate that when the diffusion distance of FHOOVWRQXWULHQWVLVORZHUWKDQFHOOVXUYLYDO[12, 45] Besides, our results confirm that decreasing membrane thickness, thereby reducing the distance that molecules such as glucose and insulin would need to travel through

the membrane, has also a positive effect on islet functionality. In comparison to other devices reported in the literature, our device combines two important characteristics: it avoids aggregation of the islets, since they are seeded on separated microwells, and can protect them from the host immune cells via the tailored membrane porosity. It is finally important to note here that the developed membranes are mechanically stable and all the above steps can be performed without problems. Preliminary implantation studies in mice (results not shown) indicated that the device can be easily implanted and retrieved.

5. Conclusions

In this study, we have developed a novel PES/PVP device for macroencapsulation, in which islets are physically separated in microwells and closed by a membrane lid, without compromising their function. Non-degradable PES/PVP membranes are mechanically stable and can offer long-term protection of encapsulated islets. Moreover, low adhesive material properties combined with our specific microwell design prevent islet spreading and aggregation. Additionally, the tailored membrane porosity allows for sufficient glucose and insulin transport, crucial for maintaining islet viability and function.

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Grant title: New islet encapsulation method for ideal mass transport and immune-protection

Grant key: 17-2013-303

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%LNHO 0 3QW ,0 /DPPHUWLQN 5*HVVOLQJ 0 6KULQNDJH HIIHFWV GXULQJ SRO\PHU SKDVH separation on microfabricated molds. Journal of Membrane Science 2010;347:141-9.

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Chapter 4

Effect of micropatterned PES/PVP membranes on cell organization ±a step towards prevascularized islet macroencapsulation devices

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Abstract

Immune protective devices for islet encapsulation allow for transplantation in the absence of immunosuppression. However, the hypoxic environment that occurs due to lack of adequate vascularization negatively affects islet viability. Islets are highly vascularized in the pancreas and their optimal function requires an oxygen supply to be metabolically active. However, the islet¶ own vasculature is disrupted due to the isolation procedure. Prevascularization of the devices in vitro can improve the connection between the device and the host vasculature after implantation, providing encapsulated islets with sufficient blood supply and oxygenation. However, during in vitro generation of microvascular networks, it is important to guide their formation, since native vasculature is a highly organized tissue.

In this work, we fabricate porous, micropatterned poly(ether sulfone)/polyvinylpyrrolidone (PES/PVP) membranes, which can be applied as a lid used for the flat islet encapsulation device, and we investigate the effect of patterns (bricks and channels) on human umbilical vein endothelial cell (HUVEC) alignment and interconnection as a first step towards the development of a stable prevascularized layer in vitro. In contrast to non patterned membranes where HUVECs form typical randomly spread HUVEC branch-like structures, in the case of micropatterned membranes we achieved a clear alignment of these structures in the direction of the patterns. Additionally, the presence of intermittent bricks allows for communication between cells and the connection of HUVEC branch-like structures creating a network over the membrane surface. We obtained this by co-culture of HUVECs on the monolayer of fibroblasts grown on the fibronectin coated membrane surface. This was achieved without the addition of hydrogels, often used in angiogenesis assays, as gels could block the pores of the membrane and limit the transport properties of the islet encapsulation device.

1. Introduction

Over the last decade, improvements in islet isolation techniques have made islet transplantation an option for treatment of certain groups of patients with type 1 Diabetes. Although islet transplants have shown improved graft function, the patients still require immunosuppression to prevent rejection [1-4]. Islet encapsulation using semi-permeable membranes could offer a solution to avoid the need for toxic immunosuppression while increasing the chances of graft survival and function [5, 6]. The primary role of encapsulation is to create a barrier against immune cells and cytotoxic molecules, thus avoid rejection while still allowing for the diffusion of oxygen, nutrients and hormones [7].

Islets are highly metabolic and require high amounts of oxygen and glucose to function properly [8]. In fact, they are highly vascularized and receive up to 10-20% of pancreatic blood flow while they account for only 1-2% of the entire pancreas mass [9, 10]. Unfortunately, the isolation procedure disrupts their own vasculature, thus, immediately following transplantation, islets face hypoxic stress that contributes to a loss of 60% of transplanted islets during the first 48 hours post-transplantation [11, 12]. Therefore, a very important consideration in the development of a bioartificial pancreas is the transplantation site where the encapsulated islets are in close proximity to the blood stream [13]. Due to the significantly greater graft volume, the geometry and the material, encapsulated islets cannot be safely transplanted into the liver through the portal system [11]. Therefore, other sites like subcutaneously, the omental pouch, bone marrow or the peritoneal cavity have been proposed as well [5, 14-16] 'HVSLWH HDFK VLWH¶V distinctive advantages, the significant graft failure is attributed to lack of adequate oxygenation.

Currently, various strategies are under investigation to improve islet vascularization, including the addition of growth factors to induce a faster

vascularization rate and prevascularization of the device [1, 17, 18]. The application of angiogenic growth factors such as vascular endothelial growth factor, nerve growth factor and basic fibroblast growth factor has shown to improve graft functionality by increasing angiogenesis locally [19]. However, the complete vascularization of large implants by angiogenesis still needs a prolonged time period while hypoxic conditions contribute to a large tissue loss [20]. Moreover, inappropriate administration of growth factors can also result in abnormal and unsuitable vasculature formation [8]. Prevascularization of the encapsulation device can be obtained directly in vivo, where a non-vascularized construct is implanted a few days/weeks before the islets are seeded in a highly vascularized area [1]. In fact, Serenova Corporate proposed a biocompatible macrodevice where non-biodegradable knitted polymer mesh with large pores allows for the development of fibrous tissue rich in vessels [5]. Inside the device, a rod-like polymer plug is positioned to guide the growth of the microvessels and connective tissue in order to create lumen for the future transplantation of islets. When the lumen is created, the rod-like plug is removed and islets are transplanted. However, this approach requires advanced imaging technics in order to optimize the time period required for the development of vasculature after which islets can be implanted [21].

Another islet macroencapsulation device designed to be incorporated into the host vasculature is the TheraCyte^{TM} system [22]. The device is composed of two thin, polytetrafluoroethylene membranes. The outer membrane with pores of 5 m improves the strength of the device and allows for infiltration of vasculature, whereas the additional inner 0.4 m pore membrane provides islet immunoisolation. Although, the device is well vascularized, it requires several months to provide optimal blood perfusion in the surrounding microcirculation and therefore glucose and insulin diffusion is impaired during the first period after transplantation [23-25]. Nonetheless, it has been shown that the preimplantation

can improve vascularization of immune protective devices before islet transplantation [26]. Although this seems to be a promising strategy, it requires several surgical steps that could be reduced by induced prevascularization in vitro. Here, after prevascularized structures are formed, islets can be encapsulated and the final construct implanted to the patient. This method is time saving as the blood vessels from the host do not have to infiltrate through the construct but only connect to the pre-existing network [27].

The most widely applied in vitro prevascularization approach is the seeding of vessel-forming cells onto scaffolds [20]. The main cell type in the native vasculature is the endothelial cell [28]. However, years of research on angiogenesis has shown that co-culture systems of multiple cell types are required to have the advantage of cell-cell contact and cross-talk between different cell types [29]. In fact, co-cultures with fibroblasts, mesenchymal stem cells and smooth muscle cells have shown promising results in the development of capillary like structures in vitro [30-32]. However, during in vitro generation of microvascular networks it is important to achieve close representation of native tissues, which are highly organized at the microscale level [33]. Here, the construct microarchitecture has a potential to support and guide prevascular network formation. Often, physical guides (e.g. wells, channels) have been applied to create cell patterns [34]. This approach can be used to enhance 2 dimensional (2D) endothelial cells alignment and organization to promote 3 dimensional (3D) vasculature formation in tissue engineering constructs.

In our previous study, we developed a functional flat macroencapsulation device consisting of two polyethersulfone/polyvinylpyrrolidone (PES/PVP) membranes, where a bottom microwell membrane provides good separation of encapsulated islets and the top flat membrane acts as a lid. In order to reduce the time required for the device to be incorporated within host vasculature and therefore provide proper islet oxygenation after transplantation, the outer membrane surface of our

device could be used for the creation of a prevascularized layer in vitro (see Figure 1).

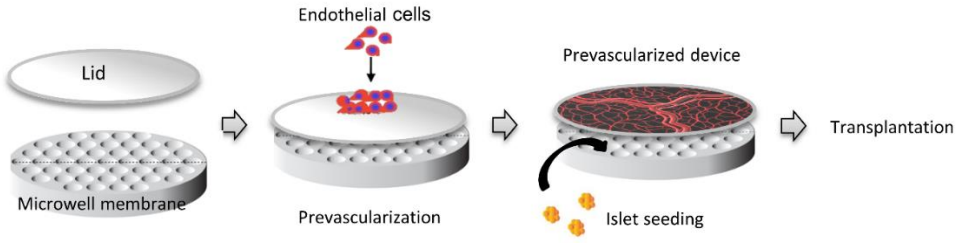

Figure 1. Schematic representation of the final flat macroencapsulation device consisting of two PES/PVP membranes: microwell membrane for islets separation inside the device and covering membrane designed for prevascularization of the device.

In this work, we investigate the effect of the membrane micropatterns on cell alignment and orientation which are important parameters for the development of a prevascularized network [35]. We aim to obtain this without the addition of hydrogels (e.g. matrigel) often used as angiogenesis assays [36-38], as these gels can result in the blocking of membrane pores causing transport limitations which negatively affect islet function. Various surface topographies have been shown to affect cell morphology, differentiation and proliferation [39-41], which could be used to control cell growth and their orientation. The formation of capillary like structures was obtained using grooves, stripes or adjacent fibers [42-44]. However, these methods did not result in interconnected capillary-like networks but in the formation of separated aligned endothelial cell structures and often required the addition of gels. Here, we prepare flat membranes with two patterns: one of intermittent bricks and another with a combination of bricks and channels (Figure 2) with a GLVWDQFH RI ~~P~~EHWZHHQ WKH SDWWHUQV DV VWXGLHV KDYH VKRZ alignment occurs LQ FKDQQHOV DV VPDOO DV ~~P~~XS WR ~~P~~– [45]. We selected

these patterns (channels and bricks) in order to provide not only alignment but also interconnectivity of the cellular network, better mimicking the situation in the native tissues. Papenburg et al. have shown that such cell organization can be controlled using poly(L-lactic acid) membranes with continuous and interconnected microchannels [46].

In this study, we fabricated micropatterned PES/PVP membranes via phase separation micromolding method 360– , which allowed us to obtain porous membranes with desired surface topography in one step [46]. Our membranes have low cell adhesion, which is favorable for islet encapsulation, therefore we apply a thin fibronectin coating on one side of the membrane to improve cell attachment properties. We study the attachment and alignment of normal human dermal fibroblasts (NHDFs) and human umbilical endothelial cells (HUVECs) on our micropatterned membranes in comparison to non patterned PES/PVP membranes. It has widely been demonstrated that fibroblasts promote endothelial cells proliferation, migration, and angiogenesis, both in vivo and in vitro [47, 48]. Therefore, we further use NHDFs as support cells in the co-culture with HUVECs and investigate whether these micropatterns have an effect on HUVEC structure formation and organization in comparison to a non patterned membranes.

2. Materials and methods

2.1. Membrane preparation

A 15wt% poly-(ether sulfone) (PES, ULTRASON, the Netherlands), 5wt% poly-(vinylpyrrolidon) (PVP, 40.000kDa, Sigma-Aldrich) polymer blend in N-methylpyrrolidone (NMP, Sigma-Aldrich) was used to fabricate porous membranes via 360– [46, 49, 50]. The polymer blend was cast on a glass plate (for non patterned membranes) or on a custom made, micropatterned silicon wafer for the creation of the different micropatterns (Figure 2). A casting thickness of 300 P– was used for all membrane fabrication. Directly after casting, the polymer was

submerged in a coagulation bath containing demineralized water (dH_2O). After the polymer solution became turbid and precipitated, the membranes were removed from the glass plate or micropatterned silicon wafer, and rinsed with dH_2O to remove remaining solvent traces. Membranes were plated in a 24-well plate and fixed with o-rings, after which they were sterilized (70% ethanol, 30 minutes) and washed with phosphate buffered saline (PBS, 3x). Membranes were washed 3x with dH_2O before use.

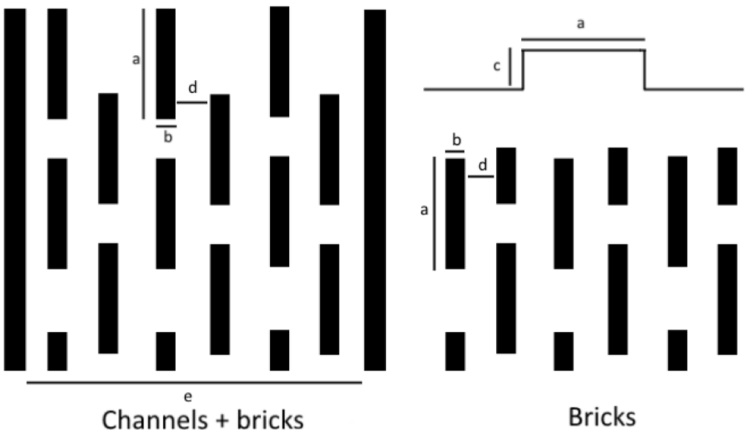

Figure 2. Micropatterns and their dimensions: a = 100µm, b = 20µm, c = 40µm d = 100µm, e = 540µm.

2.2. Cell culture

Human umbilical vein endothelial cells (HUVEC, Lonza CC2519A) and normal human dermal fibroblasts (NHDF, ThermoFisher SCIENTIFIC C-013-5C) were purchased and upon arrival directly transferred to liquid nitrogen. Upon the start of a new culture, cells were grown to 80% confluence using Endothelial Growth Medium-2 (EGM-2, Lonza) and Fibroblast Growth Medium (FGM, Lonza). When 80% confluence was reached, cells were trypsinized. When the cells were

completely detached from the cell culture flask, trypsin neutralizing medium (EGM-2 with 10% fetal bovine serum (FBS) or FGM respectively) was added and the cells were seeded. A seeding density of 2500 cells/cm^2 (NHDF) or 3500 cells/cm^2 (HUVEC) was used for the start of a new culture. After the seeding, the cells were cultured in an incubator (37| C, 5% CO_2) and medium was changed every other day. All cells used had passage numbers less than 6.

2.3. Cell attachment

To improve cell adhesion, the membranes were coated with fibronectin solution. Fibronectin solutions of 1mg/ml JPO DQG JPO were obtained by dissolving fibronectin (Sanquin, Amsterdam) in PBS and then poured on the membranes and incubated for 30 minutes at 37| C. After coating, the membranes were incubated with culture medium for 1 hour. The cells were seeded (10000 cells/cm^2) and cultured for 1, 4 and 7 days. The experiment consisted of 3 groups: a non-coated coverslip as positive control, a non-coated porous PES/PVP membrane as negative control, and a fibronectin coated membrane.

To examine the effect of microstructured topography on the cell attachment, membranes with 2 different micropatterns (bricks and channels with bricks) were coated with the optimal concentration of fibronectin and the cells were seeded on top. Non-coated coverslip served as a positive control and fibronectin coated membrane without topography served as a negative control.

For the microscopy images, cells were fixed in 10% formalin (10 minutes, room temperature) and washed with PBS (2x). Following fixation, all samples were washed 2x with dH_2O and stained for 10 seconds with methylene blue (Sigma-Aldrich), after which, they were washed 3x with dH_2O. Directly after staining, images were taken using a Nikon SMZ800 microscope. Quantification of the amount of cells on the fibronectin coated membranes was done by taking 3 pictures of each membrane and counting the amount of cells.

2.4. Statistical analysis

To determine the effect of fibronectin concentration on cell attachment, a statistical analysis was performed. From all samples, three photos were taken. Since all used cell types have the tendency to grow in clusters after initial attachment, the photos were taken of more danse, least dense and average covered areas. The average cell numbers on 1mm^2 were determined for each sample. Statistical differences in cell QXPEHUV EHWZHHQ WKH FRQGLWLRQV DQG FRQWURO ZHUH GHWHUPLQHG XVLQJ D W-test (P<0.05 = *, P<0.01 = ** and P<0.001 = ***).

2.5. Cell alignment

To examine the effect of surface topography on cell alignment, the cells were seeded on the top of the micropatterned membranes previously coated with ~~JP~~OILEURQHFWLQ Cells were seeded (10000 cells/cm^2) and cultured for 7 days, while medium was changed every other day. After culture, the cells were fixed in 10% formalin (10 minutes, room temperature). After fixation, samples were washed 2x with PBS, permeabilized with 0.05% triton X-100 for 15 minutes and blocked with 10% goat serum in PBS for 1 hour. DAPI was diluted 1:10 in MiliQ water and then 1:10 in PBS and added for 10 minutes to the samples. Images were taken using a BDpathway 435 microscope and analyzed using CellProfiler (v 2.1.1). Using this method the nucleus alignment was analyzed. The orientation of the nucleus is defined as the angle between the x-axis of the picture and the major axis of the nucleus. All images with cells on micropatterned membranes were aligned horizontally with the x-axis, resulting in a nucleus orientation relative to the patterns.

All experiments were performed in triplicate for both cell types. Membranes without surface topography served as a negative controls.

2.6. Co-culture

Co-culture experiments of HUVECs together with NHDFs was performed following the protocol provided by Friis et al. [51]. NHDFs were first seeded on the membrane with a concentration of 10000 cells/cm^2 and cultured in FGM. After a confluent layer of NHDFs was obtained, three times as many HUVECs as the starting concentration of NHDFs were seeded on top (30000 cells/cm^2). The co-culture was kept for 3 days in reduced medium (500ml EBM-2 supplemented with 0.5ml ascorbic acid, 2% FBS, 0.5ml hEGF, 0.5ml gentamicin sulfate, 0.5ml heparin, all from the HUVEC media-kit), 1 ng/ml human basic fibroblast growth factor and 10 ng/ml human vascular endothelial growth factor (Preprotech). The experiments were performed in triplicate and medium was changed every other day. After 3 days of culture, the cells were fixed in 4% paraformaldehyde (10 min, at room temperature (RT)) and washed with PBS (2x). After fixation, the cells were stained for CD31 with Alexa 488 and DAPI. Cells cultured on non-coated coverslips served as positive controls and HUVECs cultured alone on membranes served as negative controls.

2.7. Co-culture alignment

The effect of surface topography on the formation of HUVEC structures was studied. HUVECs were seeded in a ratio of 1:3 on top of a confluent layer of NHDFs. The co-culture was kept in reduced medium with the same composition as previous experiments. Medium was changed every other day. After 3 days of co-culture, the cells were fixed with 4% paraformaldehyde (10 min, RT) and washed with PBS (2x). After fixation, the cells were stained for CD31 with Alexa 488 and DAPI. All experiments were performed in triplicate. Cell cultured on non patterned membranes served as negative controls.

2.8. Immunostaining

Samples were washed 2x with PBS and permeabilized with 0.1% triton X-100 (SigmaAldrich) for 15 minutes. A 0.1% Tween-20 (Sigma-Aldrich) solution in PBS was made (PBST). The samples were blocked/permeabilized with 10% BSA (SigmaAldrich) and 22.52 mg/ml glycerin (Sigma-Aldrich) in PBST. Primary and secondary antibody were both diluted in 10% BSA in PBST, 1:200 and 1:400 respectively. Cells were incubated in the diluted primary CD31 antibody (Ab32457, Abcam) for 1 hour at RT. After 3x washing in PBS, the cells were incubated in secondary antibody Alexa 488 (Invitrogen) for 1 hour at RT in the dark. After 3x washing in PBS, cells were counterstained with DAPI (Invitrogen, 1:100 in PBS) for 10 minutes. Membranes were mounted on coverslides using mounting medium (Hard-set mounting medium, Vectashield). Images were taken using a BDpathway 435 microscope. Autofluorescence from the membrane was manually subtracted from the images by decreasing the range of grey values from the pictures from 0-4095 to 800/1000/1200-4095 (depending on the strength of the autofluorescence and signal) using Fiji software.

Images of HUVECs stained with CD31 were analyzed using the skeletonization plugin in ImageJ (providing region-based shape of the structures). All images with HUVEC structures on micropatterned membranes were aligned horizontally to the x-axis. The orientation of skeletonized HUVEC structures relative to surface topography (x-axis) was quantified using the OrientationJ plugin.

3. Results

3.1. Cell attachment on the membranes

In order to enhance cell attachment to porous PES/PVP membranes, which have low adhesive properties, we applied a fibronectin coating. Figure 3 compares cell attachment on PES/PVP membranes coated with various fibronectin solutions.

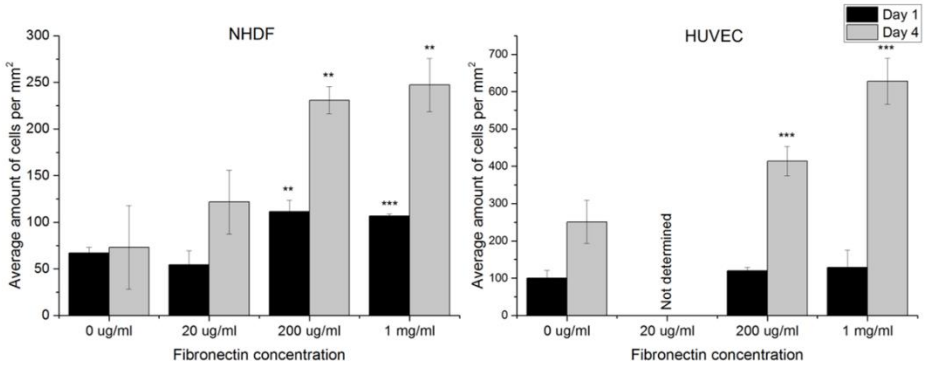

*Figure 3. Average number of NHDF and HUVEC attached per mm² on PES/PVP membranes coated with various fibronectin concentrations. Significance levels: P<0.05= *, P<0.01 =**, P<0.001= ***).*

After 1 day of culture, a significant increase in NHDF attachment was observed on WKH PHPEUDQHV FRDWHG ZLWK JPO 3 DQG PJPO 3 RI fibronectin, in comparison to non-coated membranes. Longer culture, for 4 days, confirmed the positive effect of the coating on NHDF attachment.

Besides the increase in initial NHDF attachment to the membranes, the fibronectin coating also improved cell proliferation. In fact, all coated membranes performed better after prolonged culture in terms of amount of cells per mm², in comparison to non-FRDWHGPHPEUDQHV7KHFRDWLQJZLWKILEURQHFWLQFRQFHQWUDWLR and 1 mg/ml showed positive results in case of NHDF attachment, therefore, these two concentrations were used for the HUVEC attachment study. Here, although there was no difference in initial HUVEC attachment, we observed improved cell proliferation on coated membranes after 4 days of culture, similar to NHDFs.

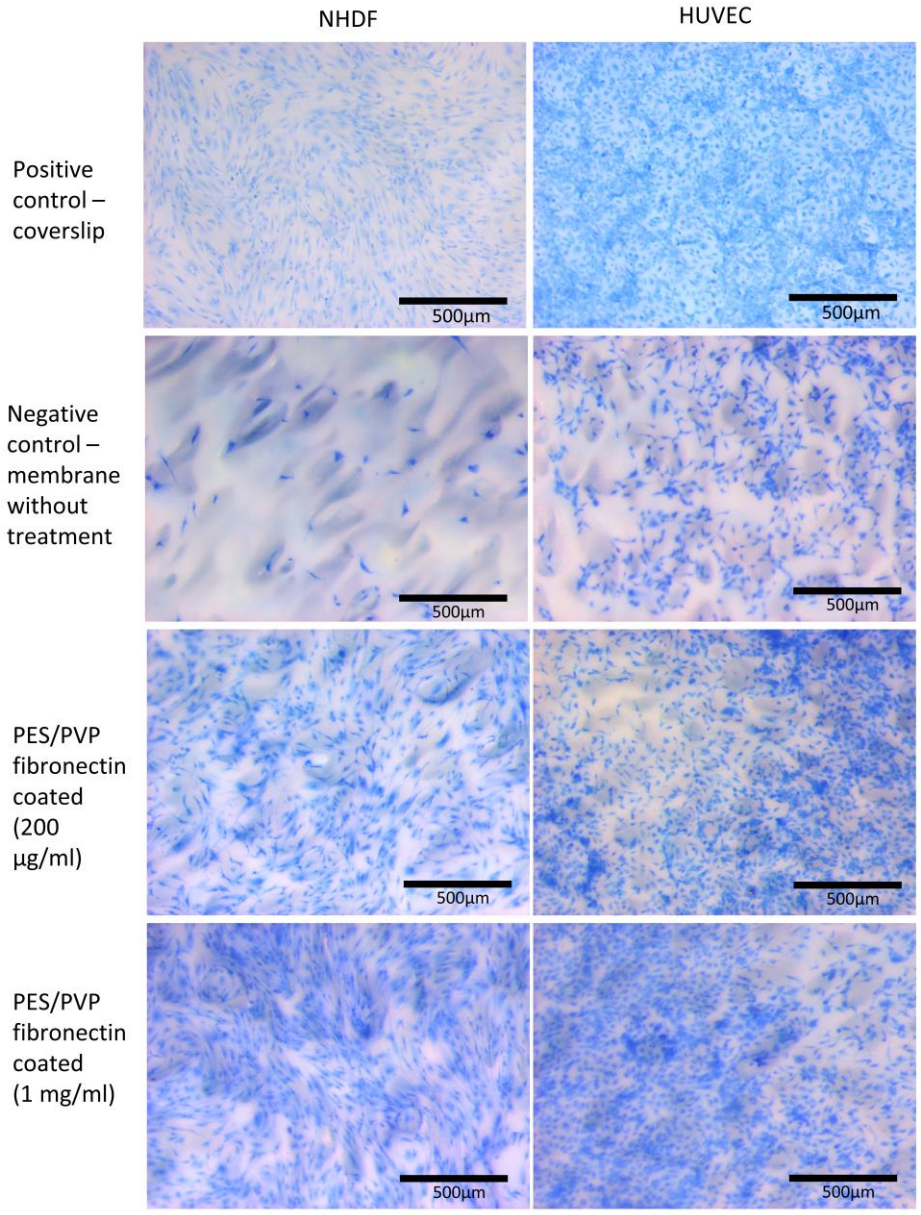

Figure 4. Images of methylene blue stained NHDFs and HUVECs cultured for 4 days on coverslip-positive control, non-coated PES/PVP membranes and membranes coated with 200 µg/ml and 1mg/ml fibronectin.

Figure 4 compares images of cells cultured for 4 days on coverslip-positive control, non-coated membranes and membranes coated with ILEURQHFWLQ JPO DQG 1mg/ml). A similar amount of cells is present on the coated membranes and coverslip controls, while the non-coated membranes clearly have low cell attachment properties. Moreover, on the fibronectin coated membranes, cells were spread over the surface and their morphology was preserved comparably to the SRVLWLYHFRQWUROV7KHILEURQHFWLQFRDWLQJVRI PJPO DQG JPO KDG VLPL UHVXOWV LQWHUPV RI FHOO DWWDFKPHQW:HWKHUHIRUH VHOHFWHG JPO IL concentration for coating of PES/PVP membranes.

3.2. Cell alignment on micropatterned membranes

Figure 5 shows scanning electron microscopy images of micropatterned porous PES/PVP membranes. We obtained high quality micropatterns which closely replicate the designed topography of the silicon wafer used for the membrane fabrication. First micropatterned design consists of equally spaced bricks distributed over the membrane surface, while in the second design, every four rows of bricks are separated by a continuous channel. The width of channels and bricks is the same as well as the space between each row.

We observed typical shrinkage during phase separation process, which helped the release of the micropatterned membrane from the silicon wafer. As a result, we obtained patterns without sharp edges and the distance between the rows of patterns as well as the length of the bricks was 20% smaller compared to the designed features on the silicon wafer. The fabricated membranes were porous with the pore size uSWR PRQWKHSDWWHUQHGVXUIDFHDQG VRQWKHERWWRP flat surface (obtained from SEM images, see example Figure 5). The water permeability of the uncoated and of membranes coated with fibronectin is identical (supplemental figure 1) proving that the very thin coating does not alter the membrane transport properties. Besides, the graph of the clean water fluxes (CWF)

of the membranes at various transmembrane pressures is linear indicating good mechanical stability of the membranes in applied pressure range.

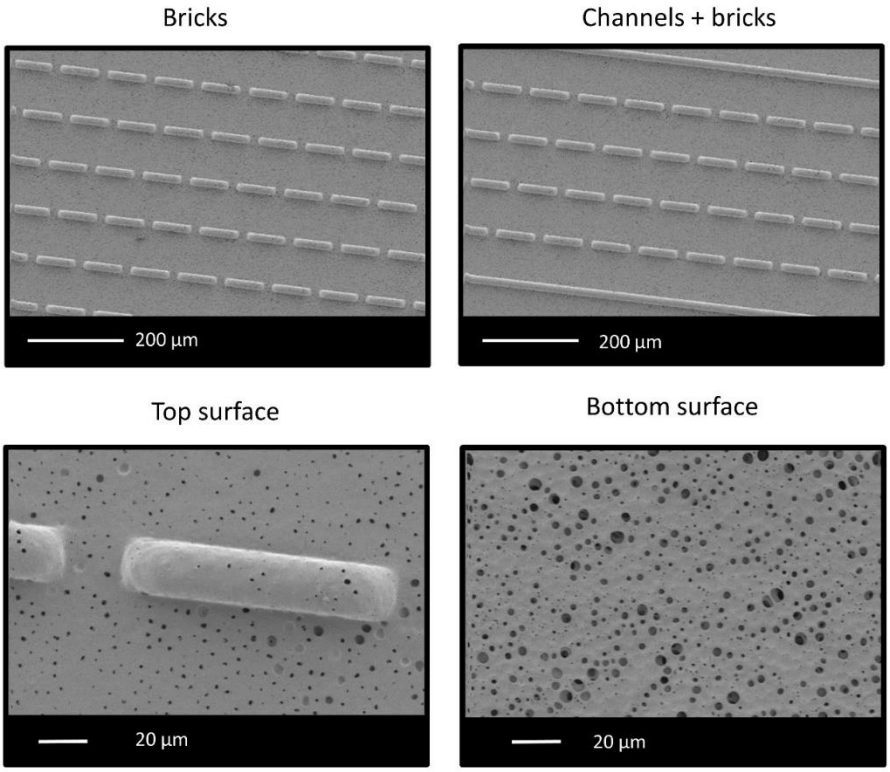

Figure 5. Scanning electron microscopy images of micropatterned membranes with bricks and channels and bricks.

Both NHDFs and HUVECs were cultured for 1 and 4 days on micropatterned PHPEUDQHVFRDWHGZLWKJPOILEURQHFWLQVROXWLRQ)LJXUH VKRZVLPDJHV methylene blue stained NHDFs and HUVECs after 1 day of culture. Initial cell attachment to the micropatterned membranes was similar to the results obtained for non patterned membranes. Although there are minor differences in cell distribution between membranes with bricks and membranes with bricks and channels (Figure

6), after 4 days of culture, all membranes were equally covered with cells (data not shown).

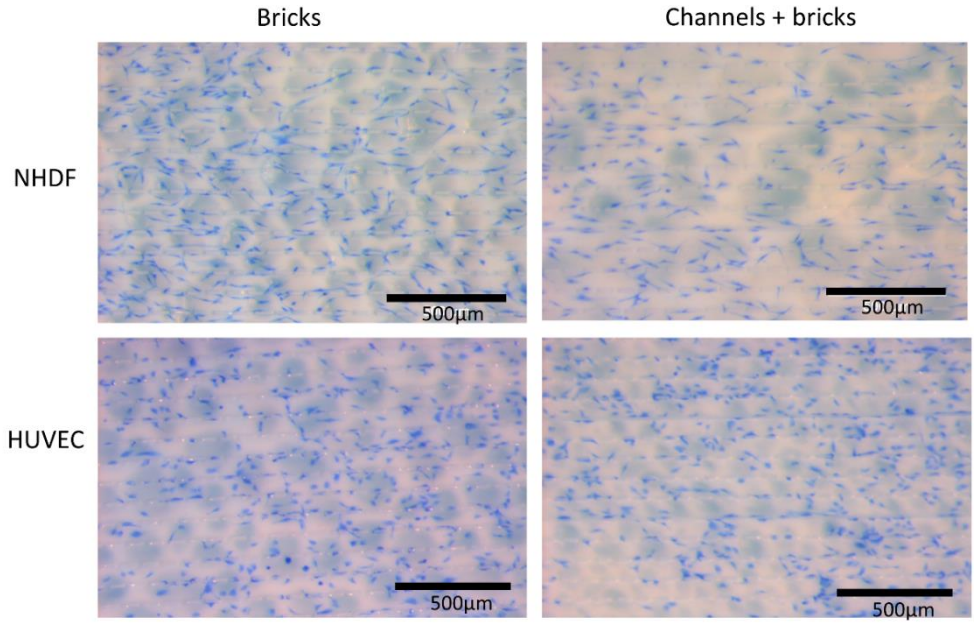

Figure 6. Images of methylene blue stained NHDF and HUVEC after 1 day of culture on micropatterned PES/PVP membranes.

After 1 day, we also observed cell alignment in both NHDF and HUVEC cultures. Figure 7 compares the orientation of cell nuclei on flat and micropatterned PES/PVP membranes, relative to the x-axis of the images with methylene blue stained cells. Both NHDFs and HUVECs cultured on the non patterned membranes have no particular orientation (similar percent of nuclei for all angles relative to the x-axis of the picture). In contrast, when NHDFs are cultured on a patterned membrane, they show a strong tendency to align to the patterns (angle of 0| -20| relative to the microstructures, Figure 7A), while there are almost no cells growing

perpendicular to them. The HUVECs also align to the micropatterns, although the orientation is lower than the NHDFs (Figure 7B).

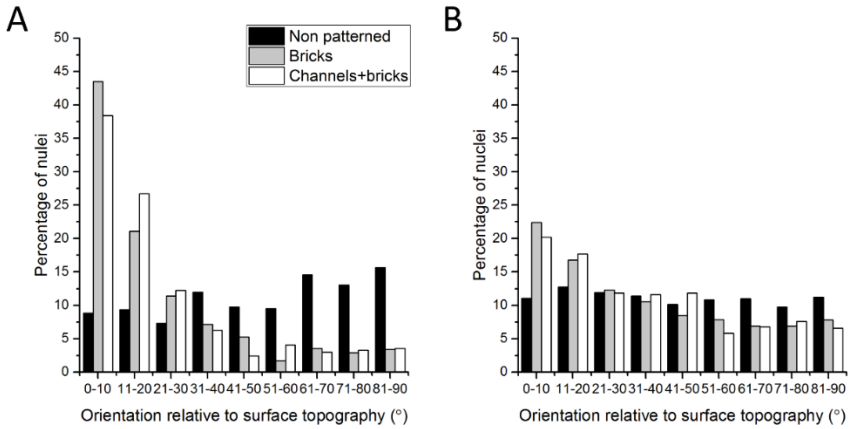

Figure 7. Nucleus alignment in relation to the surface topography for A) NHDFs and B) HUVECs.

3.3. Co-culture of HUVECs and NHDF on micropatterned membranes - effect of surface topography

We investigated whether micropatterned membranes have an effect on the HUVEC migration and organization in the subsequent co-culture with NHDFs. HUVECs were seeded on the top of a confluent layer of NHDFs cultured on non patterned and micropatterned membranes coated with fibronectin. Figure 8 A-C shows CD31 positive HUVECs which form a network on the PES/PVP membranes after 3 days of culture. As expected, HUVECs cultured on the non patterned membranes have no specific orientation and they migrate and connect, creating elongated branch-like structures within the network in all directions (Figure 8A,D). The micropatterned membranes, however, showed clear cell orientation following the membrane topography (Figure 8B,C).

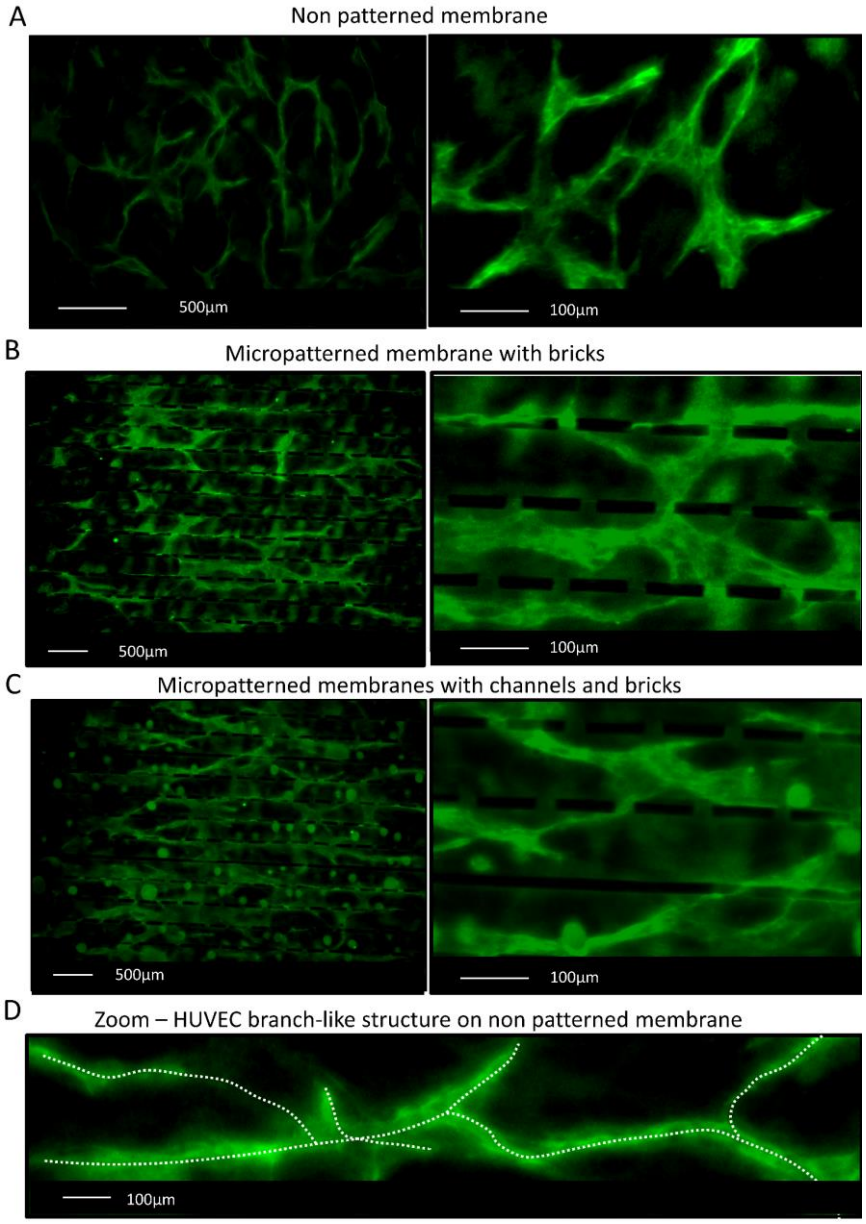

Figure 8. Co-culture of NHDFs and HUVECs resulting in HUVEC network formation. In green the immunostaining for CD31 of HUVEC cells on A) non patterned membranes, B) membrane with bricks, C) membrane with bricks and channels, D) example of elongated HUVEC branch-like structure of the network. The dotted line is drawn to guide the eye of the reader.

The membrane patterns guided and assisted cell growth during HUVEC network formation. The bricks present on both micropatterned membranes allowed for cell interconnection within this network. We also observed that HUVECs sometimes fill the space between the rows of patterns and even grow over the bricks (Figure 8B ±high magnification). As the height of the patterns was the same, cells could also connect over the continuous channels, indicating that the addition of this pattern to the bricks still allowed for the interconnection between HUVEC branch-like structures within the network (Figure 8C ±high magnification).

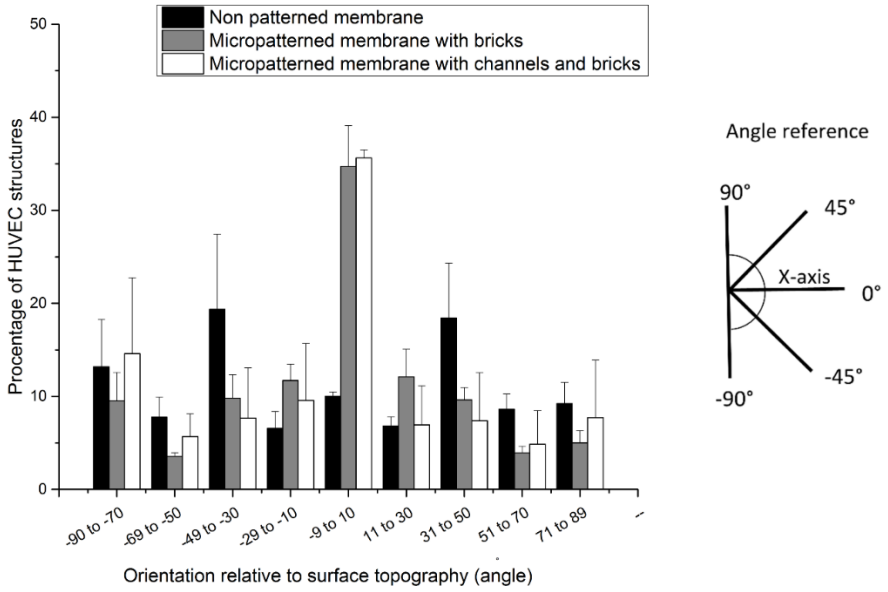

Figure 9. Quantification of HUVEC branch-like structure alignment relative to the x-axis of the immunostaining images (n=3). Membrane patterns were aligned parallel to the x-axis.

Figure 9 compares the percentage of HUVEC branch-like structures within the network on the non patterned and patterned membranes, for various orientations relative to the x-axis of the immunostaining images. It is clear that cells grew

following the direction of bricks and channels forming HUVEC branch-like structures parallel to membrane micropatternes (angle -9| -10| relative to the surface topography), while cells on non patterned membranes formed unorganized HUVEC branch-like structures over the membrane surface. Both micropattern designs led to the creation of highly interconnected and aligned branch-like structures within the HUVEC network without significant differences between them (Supplemental figure 2).

4. Discussion

Islet survival in encapsulation devices is often hindered by lack of adequate vascularization and therefore limited oxygen supply. Vascularization of encapsulation devices often occurs after implantation in an uncontrolled way, triggered by the use of highly porous membranes or addition of stimulating growth factors [1, 7, 17, 18]. Nevertheless, often a period of weeks or months is needed to provide the encapsulated islets with close proximity to functional blood vessels, while hypoxic conditions negatively affect their viability [20]. One of the possible solutions is prevascularization of the device in vitro in order to minimize the time required for the device connection to the host vasculature therefore improving islet survival during the first weeks after implantation [52]. Moreover, it is important to control and guide cell¶ organization in order to enhance the microvasculature formation [35].

We developed, in our earlier study, a flat macro-encapsulation device consisting of a microwell membrane for islet separation and a flat lid membrane. In order for islets to be in close proximity to the blood vessels and avoid harmful hypoxic conditions, we would like to tailor the outer membrane surface topography of our device to induce prevascularization in vitro, allowing for a faster connection with the host vasculature after transplantation. Therefore, here we developed PES/PVP micropatterned membranes with bricks alone and combination of bricks and

channels, which could be used as a part of our flat device and we investigated the effect of the patterns on cell alignment and organization, as a first step towards prevascularization. We hypnotized that these micropatterns would assist and guide cell orientation and therefore achieve highly organized endothelial cell structures similar to the native tissues. The presence of bricks would allow for the interconnection of the cell network. Extensive research has been performed to examine the effect of nano/micro surface topography on cell behavior [39-41]. It has been found that cells respond better to the topographies at the cell scale than to URXJKVXUIDFHVDQGWRSRJUDSKLHVDERYHWKHFHOOVFDOH[53]. Therefore our micropatterns were PLQKHLJKWDQG PZLGH

We fabricated PES/PVP micropatterned membranes using phase separation micromolding, which is a unique method for the preparation of porous membranes in one step [46]. The material of choice used for membrane fabrication is non-degradable and has low cell attachments properties, which are important factors in the development of devices for islets transplantation [54-56]. Therefore, in order to induce cell adhesion on one side of the PES/PVP membrane, we applied a thin fibronectin coating. Fibronectin is a protein of the extracellular matrix (ECM) that, when used as a coating, improves the attachment of the cells by providing more attachment points for the cell focal adhesion points [57]. Indeed, with the fibronectin coating, we achieved an increased attachment of NHDFs and HUVECs on coated membranes in comparison to no-coated ones, similar to coverslip positive controls. The positive effect of fibronectin coating on cell attachment to various materials (e.g. poli(tetrafluoroetylen), polyethersulfone) was also observed by other researchers [57-59]. Cells are able to attach to ECM molecules such as fibronectin through integrins, transmembrane receptors activating intracellular signaling pathways directing cell viability, proliferation and differentiation [60-62]. Therefore, integrin mediated adherence plays an important role in improving cell survival and proliferation [63]. Usually the fibronectin concentration used to

HQKDQFHFHOODWWDFKPHQWYDULHVIURPJPOWR[64-66]. In order to find the optimal coating for PES/PVP membranes, we tested various concentrations of fibronectin. We selected JPO ILEURQHFWLQ FRQFHQWUDWLRQ IRU FRDWLQJ IRU RX membranes as the coating of 1mg/ml had similar results in terms of cell attachment. Moreover, the higher coating concentration could result in blocking of membrane pores and transport limitations. Besides an increase in initial cell attachment, the fibronectin coating also improved the proliferation of both NHDFs and HUVECs. Our findings are consistent with the results obtained by Lotz et al. and Pendegrass et.al., who used fibronectin to improve attachment of HUVECs and NHDFs on hydroxyapatite discs and poli(tetrafluoroetylen) films respectively [57, 59].

We also applied a JPO ILEURQHFWLQ FRDWLQJ on our micropatterned PES/PVP membranes. As expected, the coating slightly decreased membrane water permeability (6.5%), however, the coating vas very thin and it was not visible on the coated membranes (SEM images similar to non-coated membranes, data not shown).

We further investigated the effect of surface topography on cell alignment. Our results indicate that both cell types, NHDFs and HUVECs, have a tendency to orient and grow along the surface topographies. Cells guided by both membrane topographies (bricks and combination of bricks and channels) align parallel to the micropatterns, while on the non patterned membranes they do not show a particular orientation. These findings are consistent with the study of Papenburg et al., where cell alignment was observed on the PLLA membranes with continuous and interconnected channels [46].

Besides the possibility to guide and control the alignment of the single cells, we also investigated whether our surface topographies could affect the formation of endothelial cell organized networks in a co-culture system. The co-culture has been

found to closely mimic the in vivo situation and to form stable endothelial cell networks [32]. Therefore, we adapted a protocol from Friis et al. for the co-culture of HUVECs and NHDFs, where the NHDF monolayer cultured on our membranes served as a support for HUVEC network formation [51]. By using this co-culture protocol, we successfully obtained HUVEC network formation on PES/PVP membranes after 3 days of culture, without additional application of hydrogels (e.g. matrigel), often used as angiogenesis assays [36-38]. The use of gels could result in blocking of membrane pores and severely hinder membrane transport properties. The endothelial cells, co-cultured on the monolayer of fibroblasts, grew and connected on the surface of non patterned PES/PVP membranes forming HUVEC networks without specific orientation, similar to the ones obtained on polystyrene surfaces by Friis et al. [51]. Fuchs et al. have also obtained similar HUVEC networks on polycaprolactone disks using co-culture of endothelial cells and primary osteoblasts [67]. Although our results present only preliminary endothelial cell network formation, it has been shown that prolonged co-culture of endothelial cells and fibroblasts can result in capillary lumen formation [51]. However, in this study, we focused on the possible guidance and interconnectivity of the HUVEC networks in order to mimic closely the highly organized native tissues, as a first step towards the prevascularization of our encapsulation device in vitro. Importantly, the brick pattern allowed cell communication and interconnection between the cells growing in parallel rows of patterns. In other studies, aligned and elongated HUVEC structures were obtained using grooves, stripes or adjacent fibers to affect HUVEC orientation [42-44]. However, these methods did not allow for the connection between the structures, which is important the formation of the microvascular network. The advantage of our micropattern design is that, besides assisting cell alignment, the bricks allow for the interconnection of HUVEC branch-like structures, forming a network. In case of both membranes (with bricks and channels with bricks) we observed a similar effect on HUVEC branch-like structures organization. The addition of continuous channels to the bricks did not

block the connections between forming HUVEC branch-like structures. We observed that HUVECs connected over the channels, indicating that the channel GLPHQVLRQV P LQ KHLJKW DQG P ZLGH DOORZHG IRU signaling and communication between the cells.

5. Conclusions

In this study, we established the co-culture of HUVECs and fibroblast grown without addition of hydrogels on micropatterned PES/PVP membranes, coated with a thin layer of fibronectin. By using these membranes with bricks as well as channels and bricks, we achieved interconnected HUVEC branch-like structures oriented in the direction of the patterns, which is an important step towards obtaining a stable endothelial cell network for the prevascularization of our flat encapsulation device.

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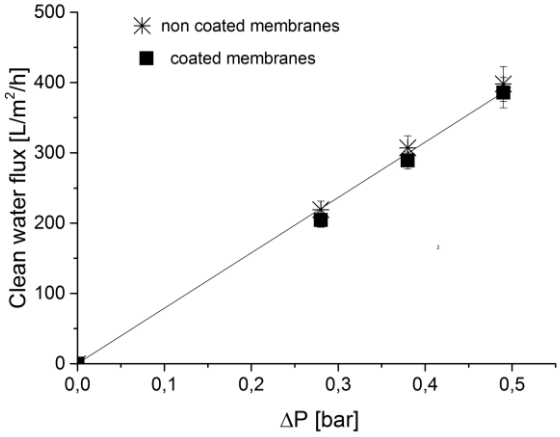
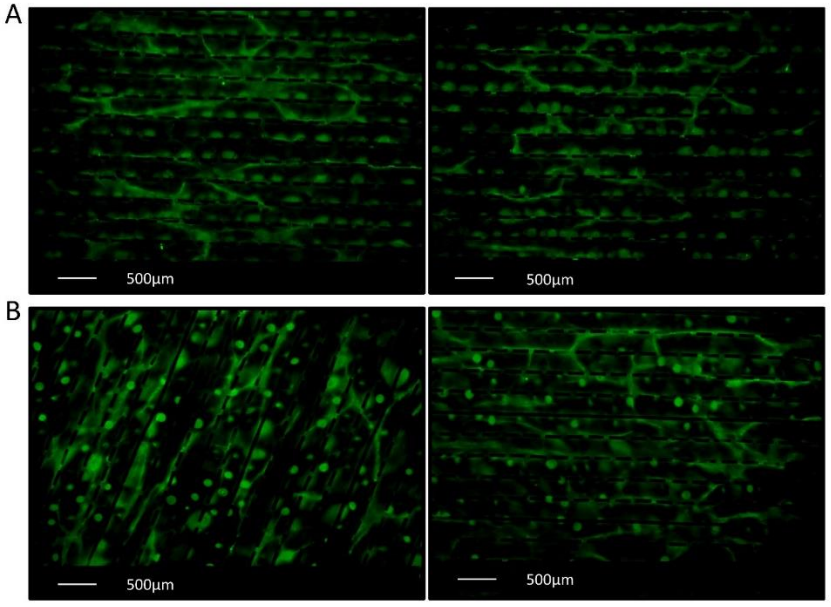
Grant title: New islet encapsulation method for ideal mass transport and immune-protection

Grant key: 17-2013-303

Supplemental material



Supplemental figure 1. Water permeability of the patterned PES/PVP membranes



Supplemental figure 2. Co-culture of NHDFs and HUVECs resulting in HUVEC network formation. In green the immunostaining for CD31 of HUVEC cells on A) membranes with bricks, B) membranes with bricks and channels.

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Chapter 5

Endothelial and -cell composite aggregates for improved function of a bioartificial pancreas encapsulation device

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The manuscript of this chapter has been submitted for a publication.

Abstract

Encapsulation of pancreatic islets or -cells is a promising strategy to improve transplantation outcomes in treatment of type 1 Diabetes by providing an immune isolated environment and allowing for transplantation in a different location than the liver. However, islets used for encapsulation often show lower functionality due to the damaging of islet endothelial cells during the isolation procedure. Factors produced by endothelial cells have been shown to have great impact on -cell insulin secretion. Therefore, mutual signaling between endothelial cells and -cells should be considered during the development of islet encapsulation systems in order to achieve high insulin secretion after encapsulation and maintain beta cell viability.

In this work, we created stable composite aggregates by co-culturing of mouse insulinoma MIN6 cells with human umbilical vein endothelial cells (HUVECs) on a non-adherent agarose microwell platform. The presence of HUVECs there results in improved insulin secretion upon glucose stimulation in comparison to aggregates consisting of only MIN6 cells. Moreover, the composite aggregates encapsulated within a flat poly (ether sulfone)/polyvinylpyrrolidone (PES/PVP) sealed device maintain their high performance and secrete more insulin than encapsulated aggregates consisting of only MIN6 cells. This shows that the interaction of -cell and endothelial cell is crucial for the development of a highly functional encapsulation system.

1. Introduction

The bioartificial pancreas represents a viable solution for the treatment of type 1 Diabetes. The encapsulation of pancreatic islets or -cells in a semipermeable membrane can allow for nutrient, glucose and insulin exchange and provide necessary immunoisolation to avoid the administration of immunosuppressive drugs [1]. However, reproducing a natural insulin release profile, while applying a membrane as an immune barrier, requires using of highly functional islets or -cells and maintaining their viability [2].

The native pancreatic islet is highly vascularized, with an extensive capillary network [3]. However, the isolation of islets by enzymatic digestion disrupts the islet vascular connection contributing to lower islet viability and loss of function [4]. In the pancreas, islet vasculature provides nutrients and oxygen to the endocrine cells and transports the hormones to the peripheral circulation [3]. Therefore, it is important to provide encapsulated islets with close proximity to blood vessels. In addition, islet endothelial cells, which form capillaries, are an important source of signals that enhance survival and function of the islet -cell [3]. In fact, each -cell in the native islet is surrounded by at least one endothelial cell, and therefore these FHOOVEQHFHVVLWDUHH[SRVHGWRHDFKRWKHU¶SURGXFWV@

After the isolation process, human islets suffer from hypoxia and express high levels of vascular endothelial growth factor (VEGF) [6]. In the islet, the cell is a major source of VEGF production which is required to maintain endothelial cell (EC) viability and promotes proliferation of EC. It has been shown that -cell-specific deletion of VEGF-A in mice results in islet capillary loss and decreased insulin release in vivo [7]. Insulin is also a major signal for endothelial cell function. It is, for example, required for phosphorylation (activation) of endothelial nitric oxide synthase (NOS3), which catalyzes production of the vasodilator nitric oxide (NO) [3]. Thus, signals produced by the -cell influence the islet endothelial cell,

contributing to the overall islet health. Importantly, endothelial cells signal back and contribute to the maintenance of -cell viability and function [8, 9]. Johansson et al examined the effects of multiple endothelial derived molecules on insulin release in vitro. This study was based on a model where exposure of whole islets to factors secreted from cultured islet endothelial cells resulted in no change in basal insulin release, but significantly enhanced glucose-stimulated insulin release and increased insulin content [10], similar to findings from a very recent study [11]. Besides, there are indications that -cells, in contrast to exocrine pancreatic cells, do not form a basement membrane. Instead, by using VEGF-A, they attract endothelial cells which form a vascular basement membrane containing laminins next to -cells [12]. Exposure of -cells to various laminin isoforms can increase insulin gene transcription and insulin release, enhancing -cell function [10, 11]. This effect is at least partly dependent on integrins, a family of heterodimeric cell-surface receptors with broad specificity for extracellular matrix (ECM) molecules (e.g. laminins, collagens, fibronectin), some of which are expressed by the -cells [13]. Sebara and Vermette have also studied the influence of separation distance between -cells and endothelial cells on insulin secretion. They have shown that the insulin release of rat insulinoma cells (INS-1) was significantly increased when the cells were co-cultured LQFORVHSUR[LPLWHPWRKXPDQXPELOLFDOYHLQHQGRWKHOLDOFHOOVLQFRPS to INS-1 cells cultured alone [14]. All the above show the importance of the presence of endothelial cells in close proximity to -cells. Hence, this should be considered during the development of islet encapsulation systems in order to achieve high insulin secretion after encapsulation and maintain -cell viability.

In this study, we hypothesize that the encapsulation of -cells co-cultured with endothelial cells can be beneficial for improved insulin secretion upon glucose stimulation due to possible signaling between these two cell types. We create stable composite aggregates consisting of MIN6 cells co-cultured with human umbilical vein endothelial cells (HUVECs) using a non-adherent agarose microwell platform.

These aggregates are used for encapsulation within our earlier developed PES/PVP sealed device (see Chapter 3) and their functionality, assessed by glucose induced insulin secretion test, is compared to encapsulated MIN6 aggregates without endothelial cells. Mouse insulinoma MIN6 cell line is often used as a model for primary -cells as it closely resembles native -cells and reflects physiological conditions, while HUVECs have been employed in many studies as an endothelial cell model for experiments attempting to achieve micro vessel formation and vascular remodeling [15, 16].

2. Materials and methods

2.1. Microwell membrane fabrication

Microwell membranes were fabricated using phase separation micromolding 360> -19] as described in Chapter 3. Shortly, a polymer blend of 15 wt.% poly(ethersulfone) (PES) (Ultrason, E6020P) and 5 wt.% polyvinylpyrrolidone (PVP) (MW= 40000, Sigma Aldrich) in N-Methylpyrrolidone (NMP) (Acros organic) was used for casting on a custom-made, micropatterned mold with spatially organized dome-OLNH VWUXFWXUHV RI PKHLJKW DQG PLQ diameter. The FDVWLQJ WKLFNQHVV ZDV P$WHU FDVWLQJ IROORZHG E\LPPHUVLRQ LQWR ZI coagulation bath, the polymer solution precipitated and the membranes were removed from the mold. In order to increase the membrane porosity, the membranes were treated with 4000 ppm sodium hypochlorite aqueous solution (NaClO, Fluka) for 24 hours. Subsequently, the membranes were washed and stored in demineralized water.

2.2. Cell culture and labeling

MIN6-B1 mouse insulinoma cells (kindly provided by Dr. P. Halban, University 0HGLFDO &HQWHU ¥HQHYD 6ZLW]HUODQG ZHUH FXOWXUHG LQ ;OEHFFR¶ 0I (DJOH¶V 0HGLXP0(0ŁEFRVXSSOHPHQWHGZLWKYY)%6/RQ]D 8P/ SHQLFLOOLQ DQG PJP/ VWUHSWRPŁFLQŁEFR DQG 01UHVKOVDGGHG

beta-mercaptoethanol (Gibco) DW & DQG &2 ;PDQ XPELOLFDO YHLQ endothelial cells (HUVECs, Lonza CC2519A) were cultured in Endothelial Growth Medium-2 (EGMTM-2 BulletKitTM, Lonza). HUVECs used for the experiments had passage numbers lower than 6. In order to distinguish HUVECs from MIN6 FHOOV89(&VZHUHODEHOHGZLWKL,UHGVRoXWLRQDFFRUGLQJWRWKHPDQXID protocol (Life Technologies).

2.3. Pseudo-islet formation

Non-adherent agarose microwell chips were fabricated, as described previously [20]. ,QVKRUWSRoGLPHWKoVLOR[DQH306QHJDWLYHPROGVFDUULQJPSLOODUVZHU sterilized with 70% ethanol. 3% UltraPureTM agarose (Gibco) was dissolved in PBS. The solXWLRQZDVKHDWHGWR&LQDPLFURZDYHRYHQ0ROGVZHUHSODFH inside a 6-well plate and filled with 8 mL of 3% agarose solution. The plates were centrifuged at 300 G IRUPLQWRUHPRYHDLUEXEEOHVDQGVWRUHGWR&IRUDWO 30 min. After the gel was formed, the molds were gently removed from the agarose using a sterile spatula. Using a sterile punching device, chips were punched out leaving a thin agarose wall on all sides to fit into a 12-well plate. Prior to cell seeding, the agarose chips were incubated in medium prepared for MIN6 cells (for MIN6 aggregates) or mixture of this medium and EGM-2 medium in ratio 1:1 (for composite aggregates consisting of MIN6 cells and HUVECs).

Stable cell aggregates were prepared based on the work of Hilderink et al [21]. MIN6 cells were seeded onto the agarose chips (250 cells per/aggregate). The plates were centrifuged at 150 G for 1 min and 2 mL of medium was carefully added to the chips. In order to prepare composite aggregates, the suspension of HUVECs (1500 cells/aggregate) was added to the MIN6 aggregates after 1 day of culture. The plates were centrifuged at 150 G for 30 seconds and then, every 10 min, plates were stirred on the shaker for 5 min during the first hour of co-culture. MIN6 aggregates were cultured in DMEM medium described earlier and composite aggregates were

cultured in mixture of DMEM medium and EGM-2 medium in ratio 1:1. The PHGLXP ZDV UHIUHVKHG K DIWHU VHHGLQJ $IWHU K DW & DJJUHJDWHV ZHUH flushed out of the chips and used for the experiments.

2.4. Free-floating aggregates functionality

After 1 day of culture, a glucose induced insulin secretion test (GIIST) was performed, with 150 free-floating MIN6 aggregates as a control, and 150 free-floating composite aggregates, using a commercial transwell system (MilliPore). Modified Krebs buffer (115 mM NaCl, 5 mM KCl, 24 mM $NaHCO_3$, Sigma) supplemented with 2.2 mM $CaCl_2$, 20 mM HEPES (Gibco), 30% bovine serum albumin, 1 mM $MgCl_2$, and 0.1 mM Theophylline (Sigma) was prepared at pH 7.4 [22] and was used to prepare low (1.67 mM) and high (16.7 mM) glucose concentration solutions. The free-floating aggregates were washed three times (5 min) in the Krebs buffer, followed by a pre-incubation of 90 min in the low glucose concentration buffer. All samples were then incubated for 60 min in subsequent low, high and low glucose concentration buffer with three times, 5 min, washing in the Krebs buffer between each high and low glucose incubation. Samples were taken after each incubation time, spun down (300 G, 3 min) and the supernatant was stored at - & 6DPSOHV ZHUH DQDO]HG XVLQJ LQVXOLQ 0RXVH (/,6$ LPPXQRDVVD\ 0HUFRGLD DFFRUGLQJ WR WKH PDQXIDFWXUHU¶V LQVWUXFWLRQV 7KH IXQFW aggregates was assessed by determining the amount of insulin secreted and displayed as the glucose induced insulin stimulation index. For the calculation of the stimulation index, the insulin secretion of all samples was normalized to the insulin secreted during the first low glucose incubation (1.67 mM glucose).

2.5. Functionality of encapsulated aggregates

The sealed device was prepared as previously described (Chapter 3 Materials and methods section). Briefly, the microwell membrane and the flat lid membrane (0.45 P SRUH VL]H 6WHUOLWHFK ZHUH VHDOHG & VHDOHG the edges using a

custom-made sealing device, leaving open the middle part and a small inlet for cell seeding. The sealed device was sterilized with 70% ethanol, washed in PBS and pre-incubated in culture medium overnight. 150 MIN6 aggregates or 150 composite aggregates were seeded inside the device via the small inlet, which was closed after seeding using sterile, surgical staples (Teleflex Medical, HORIZON, Ligating clips). After one day of culture, the functionality of encapsulated aggregates was determined following the GIIST procedure earlier described for free-floating aggregates (section 2.4.).

3. Results

3.1. Formation of multicellular aggregates

$JDURVH PLFURZHOO FKLSV ZLWK ZHOOV RI — aggregation. Figure 1A shows the images of MIN6 aggregates and composite aggregates, consisting of MIN6 cells co-cultured with HUVECs. MIN6 single cells cultured on non-adherent agarose chips clustered over time, resulting in stable in size, rounded aggregates of 80-PLQGL ameter (Figure 1, left). In order to prepare composite aggregates, the red-labeled HUVEC suspension was added to the MIN6 aggregates. Over time, HUVECs attached to the MIN6 aggregates and upon culturing become more uniformly distributed over the aggregates (Figure 1, right). After 24 hours, aggregates consisting of only MIN6 cells as well as composite aggregates were similar in size, however, not all of the composite aggregates were spheroidal shaped.

Figure 1B shows higher magnification images of the formed composite aggregates over time. Initially, HUVECs (red labeled) surrounded MIN6 aggregates and attached to them as well as to each other (Figure 2A). After 24 hours, we obtained the composite aggregates where HUVECs where either uniformly integrated with MIN6 aggregates (Figure 2B, right) or HUVECs formed clusters attached to the MIN6 aggregate (Figure 2B, left).

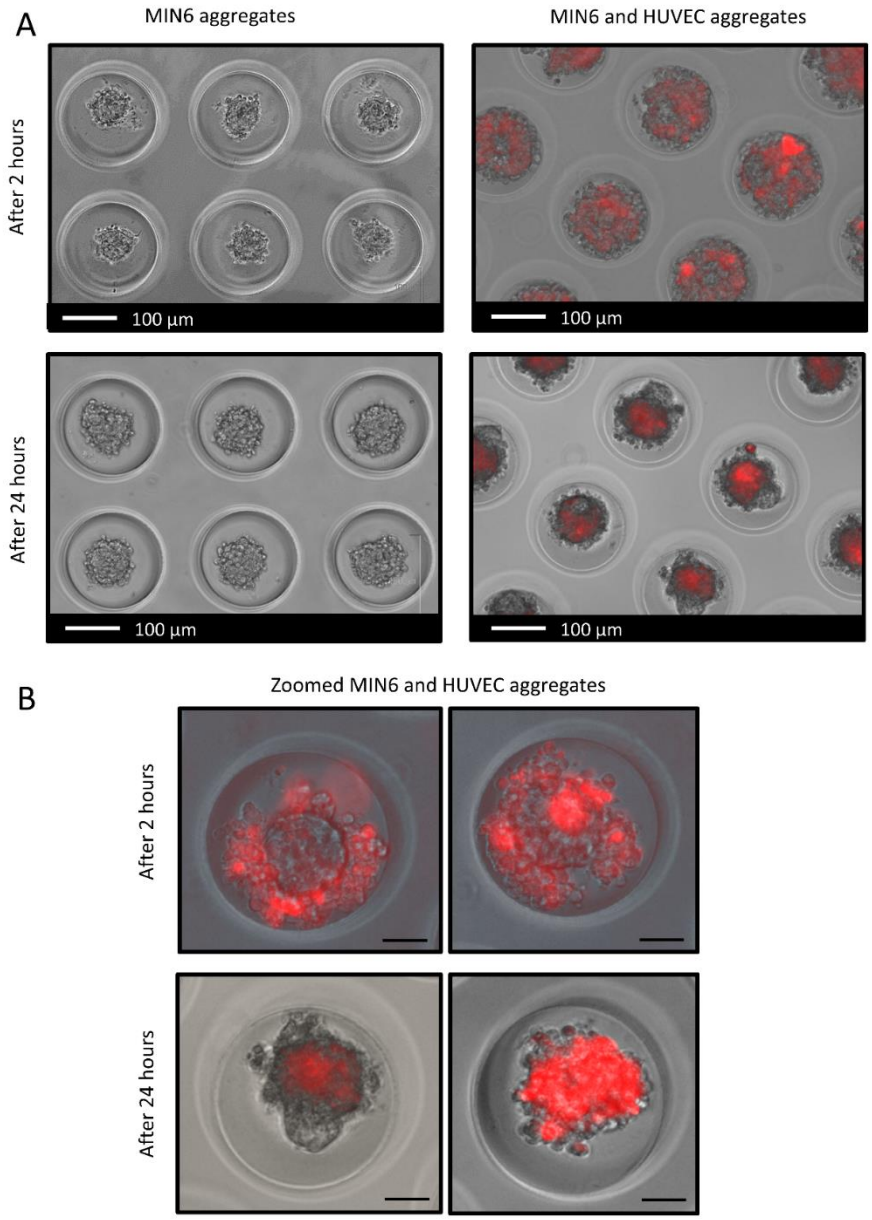

Figure 1. Aggregates formation. A) MIN6 aggregates and composite aggregates consisting of MIN6 cells and HUVECs (red) in agarose chips after 2 hours and 24 hours of culture, B) zoomed composite aggregates consisting of MIN6 cells and HUVECs (red) after 2 and 24 hours, scale bars - — P

3.2. Functionality of free-floating aggregates

In order to perform a functionality test (GIIST) on free-floating aggregates, the MIN6 and composite aggregates were flushed out of the agarose chips. Figure 2A shows that both types of aggregates remained intact and cell integration was preserved during and after harvesting from the chips. Figure 2B compares the functionality of free-floating MIN6 aggregates and of the composite aggregates.

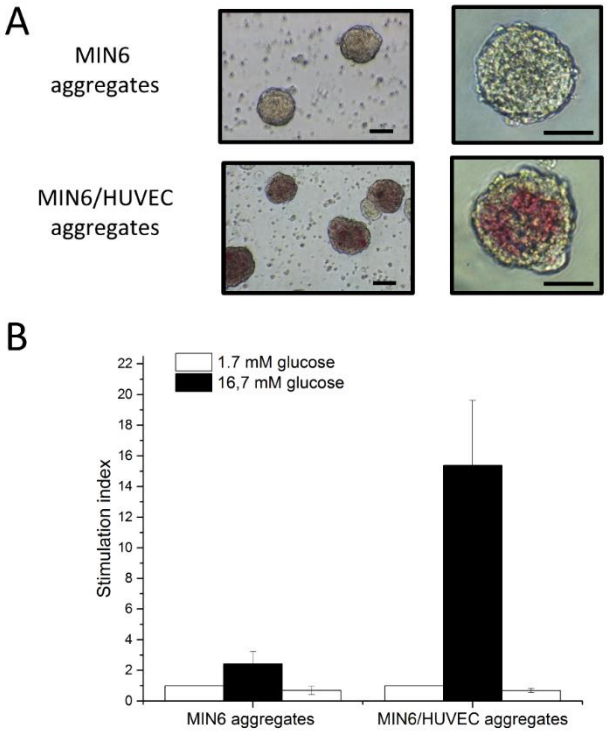

Figure 2. Free-floating aggregates: A) after flushing from the agarose chips. Scale bars ± — B) functionality after 1 day of culture. Insulin secretion is normalized to the first low glucose stimulation and presented as a stimulation index. Error bars indicate standard deviation (n=3).

Both secreted insulin in response to glucose concentration changes. However, we observed a clear increase in the response to high glucose stimulation for the composite aggregates in comparison to the MIN6 aggregates, used as positive control. In fact, the MIN6/ HUVEC composite aggregates have stimulation index of "VL[WLPH s higher than the index of control MIN6 aggregates, clearly indicating better functionality of the composite aggregates.

3.3. Functionality of encapsulated aggregates

The MIN6 aggregates and MIN6/HUVEC composite aggregates were further encapsulated within our microwell sealed device and their functionality was studied after 1 day of culture. Figure 3 compares the stimulation index obtained for both types of aggregates, used as free-floating controls and when encapsulated within our device. In all cases, the aggregates function well and respond clearly to glucose concentration changes. Moreover, the free-floating composite aggregates performed better (stimulation index of 6) than our positive control-MIN6 aggregates (stimulation index of 2) in terms of insulin secretion upon high glucose stimulation, as previously observed. MIN6 aggregates remained functional after encapsulation within our device and responded to glucose concentration changes in a similar manner to free-floating positive controls. Moreover, we observed a clear increase in the stimulation index when composite aggregates were encapsulated, confirming the positive effect of the addition of HUVECs to MIN6 aggregates on their insulin secretion also after encapsulation.

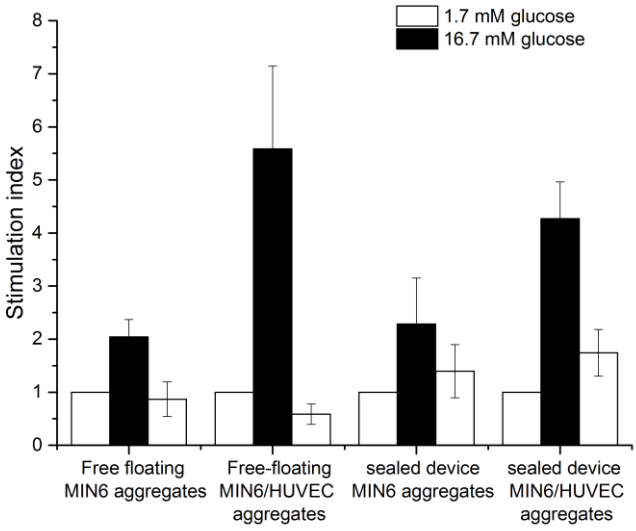

Figure 3. Functionality of aggregates encapsulated within sealed flat device in comparison to free-floating aggregates after 1 day of culture. Insulin secretion is normalized to the first low glucose stimulation and presented as a stimulation index. Error bars indicate standard deviation (n=3).

4. Discussion

Endothelial cells play an important role in maintaining pancreatic islet viability and HQKDQFLQJWKHLUIXQFWLRQRZHYHUWKHLVRODWLRQSURFHGXUHGLVUXSV vasculature, negatively affecting the -cell connection with the islet endothelial cells, which is required for proper cells signaling and promoting insulin secretion [3, 4]. Therefore, to improve islet transplantation outcomes using islet encapsulation devices, it is important to provide islets with close proximity to host vasculature. However, the revascularization of large implants still needs a prolonged time period while the lack of factors produced by endothelial cells contributes to insufficient insulin release and loss of beta cell function [23]. Kaufman-Francis et al. cultured mouse islets together with HUVECs and human foreskin fibroblast on highly porous

and biodegradable PLLA/PLGA scaffolds. Their results show that endothelial cells promote upregulation of ECM-associated genes in islet culture, improving islet survival and function in vitro, as well as, in vivo [24]. Therefore, we can hypothesize that the encapsulation of islets with supportive endothelial cells would allow for cell-cell contact and necessary signaling within the encapsulation device, improving beta cell function.

In this study, we investigated whether the co-culture of -cells with endothelial cells could improve -cell function within membrane based encapsulation devices. We used mouse insulinoma MIN6 cells as a -cell model, as they closely resemble primary -cells and reflect physiological conditions [15]. Since islet -cells require cell-cell contact to survive and properly function in vitro [25], we created stable MIN6 cell aggregates mimicking pancreatic islets and used them as our positive controls. The viability and functionality of MIN6 cells has been shown to improve in three-dimensional cell aggregates compared to two-dimensional monolayer culture, due to enhanced cell-cell contact [26].

We obtained MIN6 aggregates of 80- P L Q GLDPHWHU XVLQJ D QRQ-adhesive agarose microwell platform, based on the work of Hildering et al., [21]. The agarose chips served us also as a platform to create MIN6/HUVECs composite aggregates, where HUVECs, as an endothelial cell model, were attached and incorporated with the MIN6 aggregates. Buitinga et al. used in their study a similar agarose platform to create composite human islets with proangiogenic support cells for improvement of islet revascularization at the subcutaneous transplantation site [27]. Our composite aggregates were similar in size to MIN6 aggregates, however, not all spheroidal shaped as our controls but closer mimicking the native islets [28]. The islets of Langerhans are three-dimensional (3D) structures which contain insulin-producing beta cells in direct contact with islet endothelial cells. Therefore, recreating a more native structure of islet -cells by the formation of the -cell aggregates co-cultured

with endothelial cells is an important tool for the study of -cell physiology in a 3D conformation [29].

Our earlier developed device, consisting of porous polyethersulfone based membranes (Chapter 3), was used for the encapsulation of the MIN6 aggregates and cell functionality was compared with encapsulated composite aggregates. As expected, after 1 day of culture, free-floating MIN6 aggregates showed a response to glucose concentration changes. In the case of the free-floating composite aggregates, we observed a significant increase in insulin secretion after high glucose stimulation (stimulation index six times higher than for the MIN6 aggregates), which can be associated to the presence of HUVECs in co-culture with MIN6 aggregates. Moreover, both types of aggregates function after encapsulation and, importantly, an increase in insulin secretion was observed for the composite aggregates in comparison to encapsulated MIN6 aggregates, indicating the positive effect of HUVEC addition on MIN6 cells functionality. Kusamori et al. in their study also observed improved insulin secretion when multicellular spheroids were created consisting of MIN6 cells co-cultured with aortic vascular endothelial cells [30]. Although factors produced by endothelial cells have been shown to significantly enhance glucose-stimulated insulin release [3, 10, 31], their beneficial effect on -cell functionality within encapsulation devices, presented in this work, have not been previously studied and represents a novel finding in the area of bioartificial pancreas.

5. Conclusion

In this study, we created composite cell aggregates consisting of co-cultured MIN6 cells and HUVECs, which mimic the -cell relation with endothelial cells in native islets. By addition of HUVECs, we achieved improved MIN6 aggregates functionality in terms of glucose-stimulated insulin secretion. Importantly, these composite aggregates maintain their function after encapsulation within our membrane-based sealed device and show better insulin release than encapsulated

pure MIN6 aggregates, indicating that providing -cells connection with endothelial cells within an encapsulation device is beneficial in terms of improved cells functionality and better device performance.

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Chapter 6

A new multibore hollow fiber device for macroencapsulation of islets of Langerhans

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The manuscript of this chapter has been submitted for publication.

Abstract

The macroencapsulation of islets of Langerhans is a promising strategy for extrahepatic transplantation of high number of islets needed to treat type 1 Diabetes. The encapsulation devices should protect islets from the immune system while providing an optimal microenvironment for islets in order to maintain their function and survival.

Hollow fiber membranes are interesting for macroencapsulation because they can offer a large surface-to-volume ratio and can potentially be retrieved or refilled. However, the application of hollow fibers with suboptimal morphology and transport properties often contributed to graft failure, due to a limited exchange of nutrients, oxygen, and insulin. Especially single-bore hollow fiber membranes suffer from performance instability during long-term use.

In this work, we developed multibore hollow fiber membranes tailored for islets encapsulation. The fibers consist of seven bores suitable for seeding a high number of islets. They are prepared using non-degradable, poly(ethersulfone)/polyvinylpyrrolidone polymer blend and they have high mechanical stability, and low cell attachment properties. Human islets encapsulated within the bores of the fiber retain their glucose responsiveness, similar to non-encapsulated islets, during 7 days of cell culture. Moreover, insulin secretion increases with increasing number of encapsulated islets within the fiber bores. These multibore hollow fiber membranes have higher islet encapsulation capacity than single fibers and allow for easier up-scaling, which are important factors for the development of a clinically applicable bioartificial pancreas.

1. Introduction

Clinical islet transplantation (CIT) in the liver via the infusion of islets into the portal vein has been explored as a potential therapy for patients with type 1 Diabetes [1, 2]. However, CIT is associated with a high degree of islet loss due to their exposure to several stress factors within the first two weeks after intervention [3, 4]. Extrahepatic islet transplantation using biomaterials as an islet carrier could improve the outcome of the transplantation by providing a more optimal environment [5-7]. In fact, the encapsulation of pancreatic islets, or -cells, within semipermeable membranes represents a promising strategy to immobilize transplanted islets in one location outside the liver and provide optimal spatial and functional support, which could ultimately lead to enhanced survival [8-10]. Intravascular systems often require a complex surgical intervention with varying successful outcome, while extravascular systems have the great advantage of relatively easy implantation and potential retrieval. Additionally, extravascular devices can be reloaded and replaced when necessary [9, 11]

In general, the optimal encapsulation device needs to provide proper transport properties of nutrients and oxygen to the islets and, at the same time, protect the encapsulated islets from the immune system of the patient [11, 12]. The configurations proposed for a membrane based macroencapsulation device include flat and hollow fiber membranes [9]. In comparison to the flat membranes, the hollow fibers are attractive, offering a combination of high surface area with a compact design, which is desirable for implantable devices [13, 14].

In order to achieve sufficient encapsulation in the fiber, several parameters need to be taken into consideration. The membrane material should preferably have low cell adhesive properties to avoid cell attachment onto the membrane surface, which could lead to loss of phenotype and subsequently their endocrine function [15, 16]. Additionally, the fiber diameter should be designed to tightly fit pancreatic islets,

while the wall thickness should be low and the membrane porosity high to decrease the diffusion distance and provide optimal mass transport [9].

In the past the development of hollow fibers for renal dialysis, stimulated also their application for cell encapsulation including intra and extravascular macrodevices [9]. These fibers have thin walls to achieve optimal mass transport. However, when applied to islet encapsulation, they often suffer from insufficient material biocompatibility or mechanical damage. Additionally, due to their inadequate dimensions, the oxygen and nutrients diffusion can be limited and occlusion can occur contributing to shorter graft survival [17-19]. Another important issue is the need for accommodating a high number of islets to be able to restore normoglycemia [2]. The modeling study of Dulong and Legallais has shown how challenging is the fiber optimization to achieve this [20]. In order to increase the total amount of islets in single bore hollow fiber devices, the length of the fibers needs to be increased unrealistically making them not suitable for a clinical application [21].

In this work, we aim to overcome all aforementioned challenges by proposing the development and application of a robust multibore hollow fiber system for islet encapsulation. In comparison to a single bore fiber, the multibore fiber offers more space for islet encapsulation, for the same fiber length, without significant increase of volume. Additionally, the multibore fibers are mechanically more stable and easier to handle during implantation.

Here, we develop multibore fibers with small bore diameter — Tailored to fit the broad size range of pancreatic islets. Non-degradable polyethersulfone (PES) is used as a membrane forming material, blended with the hydrophilic additive polyvinylpyrrolidone (PVP). The PES/PVP blend is used for hemodialysis membrane fabrication and has minimal cell interactions which is very important to islet encapsulation [22]. The characteristics of the new multibore fibers is tailored to achieve efficient nutrient delivery to the cells and insulin delivery by the cells. In

fact, the walls of the bores are fabricated as thin as possible without compromising membrane stability. The developed fibers are evaluated by analyzing the glucose responsiveness of encapsulated human islets using various cell numbers and islets from multiple donors. Non-encapsulated, free-floating islets are used as positive control, and our results are compared to those obtained for islets encapsulated into FRPPHUFLDOO\ DYDLODEOH PXOWLERUH ILEHUV ZLW previously used as hepatocyte bioreactors and three-dimensional tissue engineering applications [23, 24].

2. Materials and methods

2.1. Multibore hollow fiber fabrication

The multibore hollow fibers were fabricated by dry-wet spinning via immersion precipitation using a specially designed spinneret (supplemental figure 1A). The polymer dope solution was a blend of 15wt% polyethersulfone (PES, Ultrason E6020) and 10wt% polyvinylpyrrolidone (PVP K90, Sigma Aldrich) dissolved in N-methylpyrrolidone (NMP) (Acros organic). After 24 hours mixing on a roller bank, the solution was filtered using a 1 ~~m~~ filter (Bekipor ST AL3, Bekaert) into a stainless steel syringe and left to degas overnight. The following day, the syringe and the bore solution were mounted in the high-pressure syringe pumps and connected to a specially designed spinneret (supplemental figure 1B). Subsequently, the spinneret was placed above the coagulation bath at a fixed height (air gap). The polymer dope and bore solution were pumped through the spinneret and after a 6 cm air gap, the nascent multibore hollow fiber was immersed into the water coagulation bath, where phase separation occurred and the fiber was formed. The multibore hollow fibers were collected in a free falling way. During spinning, several parameters were varied, which are described in Table 1. The collected hollow fiber membranes were washed with demineralized water in order to remove remaining solvent traces and stored in demineralized water until further use.

To increase the membrane porosity, the PVP of some of the developed fibers was washed with 4000 ppm sodium hypochlorite aqueous solution (NaClO, Fluka) for 24 hours. Subsequently, the membranes were washed and stored in demineralized water. Prior to drying, the membranes were immersed in a 25 vol.% glycerol solution for 24 hours to protect the hollow fiber structure and morphology during air drying.

2.2. Scanning electron microscopy

The multibore hollow fiber membrane morphology was visualized using scanning electron microscopy (SEM). The membranes were dried in air followed by fracturing in liquid nitrogen to reveal the cross section. Subsequently samples were clamped in a cross section holder and sputter-coated with nm-thick gold layer prior to imaging.

2.3. Water transport through the membrane

Multibore hollow fibers were dried in air and modules were prepared by potting the fiber inside the 10 cm long tube with a Kartell T-connection (VWR) in the middle. Both ends were glued using two-FRPSRQHQW HSR[\ JOXH *ULIIRQŠ and cut open after the glue had hardened, opening the bores of the multibore hollow fiber. Before testing, the modules were washed with ultra-pure water and pre-pressurized at 0.7 bar for 1 hour, then transmembrane pressures of 0.7, 0.5 and 0.3 bar were applied and the flux of the permeated ultrapure water was measured over time (in L/m^2/h). The clean water permeability (Lp, in L/m^2/h/bar) was measured in a dead end mode and determined by calculating the slope of a linear fit of the flux versus transmembrane pressure graph.

2.4. Cell culture

+XPDQ LV/DQJHUKDQVIURPRUJDQGRQRUVHSDQFUHDWD SXULW\
DQG UHVSHFWLYHO\ ZHUH SURYLGHG E\ WKH +XP
/HLGHQ 8QLYHUVLW\ 0HGLFDO &HQWHU6WXG\ LQ WKH 7
SHUIRUPHG RQ LVOHWV QRW EH XVHG IRU FOLQLFDO WU

LQIRUPHG FRQVHQW E\ WKH GRQRUV $OO SURFHGXUH
'XWFK ODZ DQG WKH UHOHYDQW
&05/ PHGLXP PPROH
P8 P/ SHQLFLOOLQ DQG PJ P/ VWUHSWRP\FLQ *LEF
PJ P/ QLFRWLQDPLGH WR GHWHUPLQH LVOHW QXPE
ZLWK GLWKL]RQH '7= —
ZHUH FRXQWHG WZLFH LQ WULSOLFDWH

2.5. Cell seeding

Prior to the cell seeding, the multibore hollow fibers were cut into 1.3 cm pieces and the middle bore of the fiber was closed with glue using two-component epoxy glue * U L I I F Q Š (Figure 1 A and B). The fibers were then carefully placed at the inlet of the 1ml syringe and fixed with elastic tubing and glue (see Figure 1C). One end of the fiber was closed using sterile, surgical staples (Teleflex Medical, HORIZON, Ligating clips). The syringe with the attached multibore hollow fiber was sterilized with 70% ethanol, washed in PBS and pre-incubated in culture medium overnight. The relevant number of human islets (1000, 3000 or 6000) for the experiments was V X V S H Q G H G in medium and placed inside the syringe held in perpendicular position with the attached hollow fiber pointing downwards. The islet suspension was then carefully injected inside the bores of the fiber with minimal pressure applied on the piston. After injection the multibore hollow fiber was closed with a surgical staple near the inlet and cut off from the syringe leaving 1 cm of fiber with encapsulated islets. Afterwards, the fiber was placed in 1 ml of medium and cultured for 1 and 7 days. Culture medium was changed every day.

2.6. Human islets functionality in vitro

To assess the function of the encapsulated islets, a glucose induced insulin secretion test (GIIST) was performed after culture under static conditions for 1 day and 7 days.

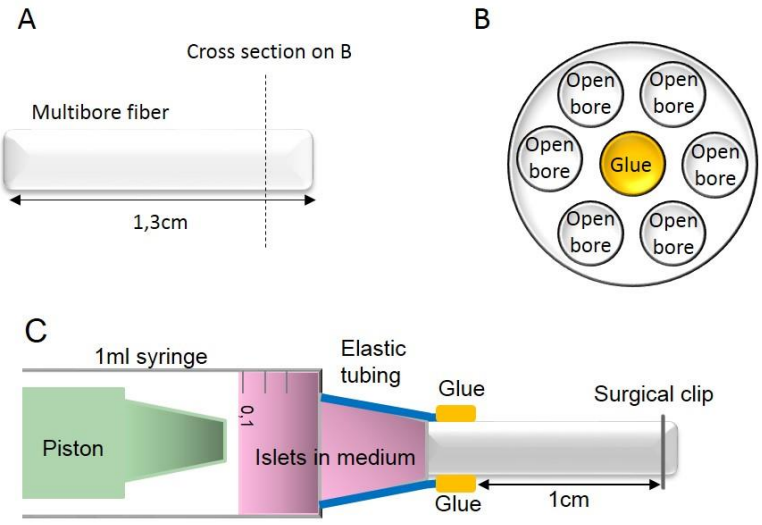

Figure 1. Schematic representation of seeding procedure: A) 1,3cm long multibore hollow fiber, B) cross section of the multibore hollow fiber, where middle bore is closed with the glue and side bores left open, C) fiber is placed in the outlet of the 1ml syringe and fixed with elastic tubing and the glue and the end of the fiber is closed using surgical clip, islets resuspended in 100 µL of medium are injected inside the open bores.

Free-floating islets in a transwell system (MilliPore) (n=3) were used as positive controls. Fibers with encapsulated islets and free-floating non-encapsulated islets of the same donor, as controls, were first pre-incubated in a modified Krebs buffer (115 mM NaCl, 5 mM KCl, 24 mM $NaHCO_3$, 2.2 mM $CaCl_2$, 20 mM HEPES, 1 mM $MgCl_2$, 2 P J P / E R Y L Q H V H U X P D O E X P L Q S + I R Ų. P L Q Subsequently, the samples were then incubated for 1 hour in low (1.67 mM), high (16.7 mM), low, and again high and low glucose buffer, with three times 5 min washing in Krebs buffer between each high and low glucose incubation step. Two additional GIIST steps (second high and third low glucose incubation) in total 5, instead of the 3 step often used in the literature [16, 25], were performed to determine if there is delay in islet response. Samples were taken after each incubation step, spun down (300 G for, 3 min) and the supernatant was stored at - Û & 6 D P S O H V

were analyzed using a human insulin ELISA kit (Mercodia). The functionality of human islets was determined by determining the amount of insulin secreted and displayed as the glucose induced insulin stimulation index. For the calculation of the stimulation index, the insulin secretion of all samples was normalized to the insulin secreted during the first low glucose incubation (1.67 mM glucose). 150 islets were used as free-floating positive controls, representing the quality of the islets used for encapsulation. Stimulation index of at least two defines a functional response.

3. Results

3.1. Development of new multibore hollow fiber membranes

Various batches of multibore hollow fibers with seven bores were produced by dry-wet spinning method. The spinning conditions were tuned in order to obtain mechanically stable fibers with thin walls, suitable for pancreatic islet encapsulation. Figure 2 shows representative SEM images of the developed fibers with small bores R I D E R X W(Figure 2B-D) in comparison to the commercially available PESM multibore fibers with bores R I — P G L and rather thick membrane walls (Figure 2A). The first batch of produced membranes (MF1, Figure 2B) had round bores and rather thick outer and inner walls (200- — P with asymmetric membrane pore morphology. Besides, thick dense selective layers containing small pores were present on both sides of the fiber, while in the fiber cross section, we can observe the presence of macrovoids.

In order to decrease the fiber wall thickness, we adapted the spinning conditions by applying 50% v/v NMP in water, as a bore solution. The new fibers, MF2 (Figure 2C), had thinner walls, however, all the bores were deformed and the membrane mechanical stability was much lower in comparison to MF1 fibers. To improve this while having thin walls, we applied during the spinning process a coagulation bath temperature of 48-50 |C which resulted in stable multibore hollow fibers (MF3) with thin walls and rounded bores (Figure 2D).

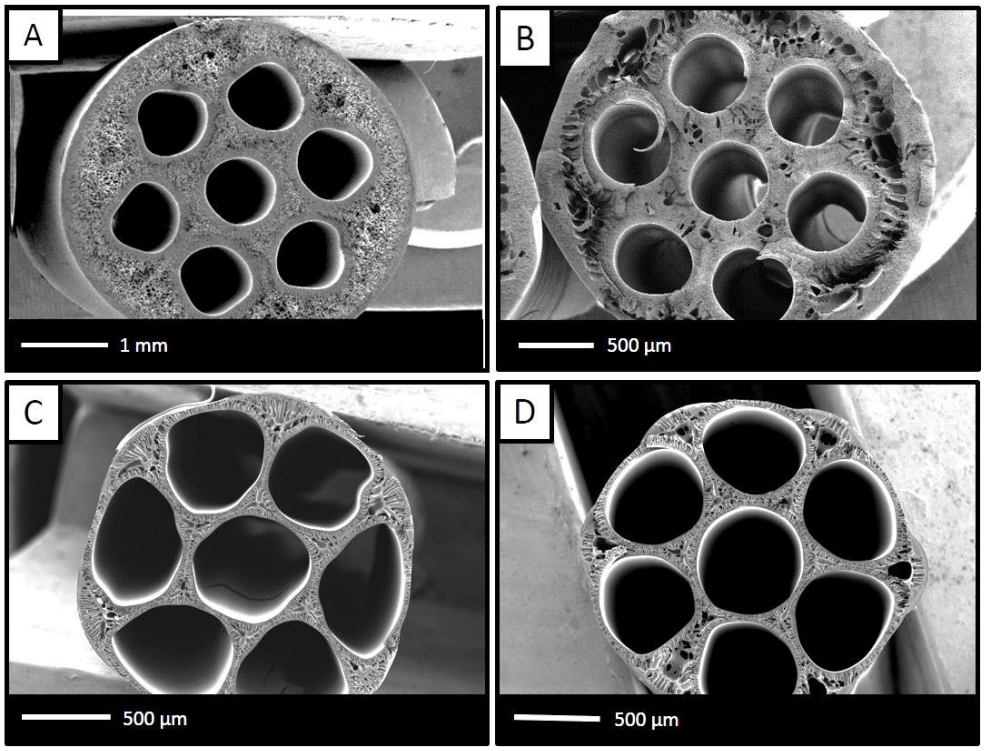

Figure 2. Scanning electron microscopy images of multibore hollow fibers: A) Commercial Multibore® membrane – PESM, B) MF1, C) MF2, D) MF3.

Figure 3 shows the detailed morphology of the MF3 membrane cross section at higher magnification. The membrane walls between the bores consist of a very thin dense selective layer with small pores, while a finger-like, porous sub-layer is SUHVHQW LQ EHWZHHQ 7KH RXWHU ZDOO RI WKH VL curved resulting in bores slightly flattened on one side. Additionally, the surface of the outer wall of the bores has small pores which are not present at the connections between the bores. Based on the structural stability and morphology, the MF3 membrane was selected for further characterization and islet encapsulation studies.

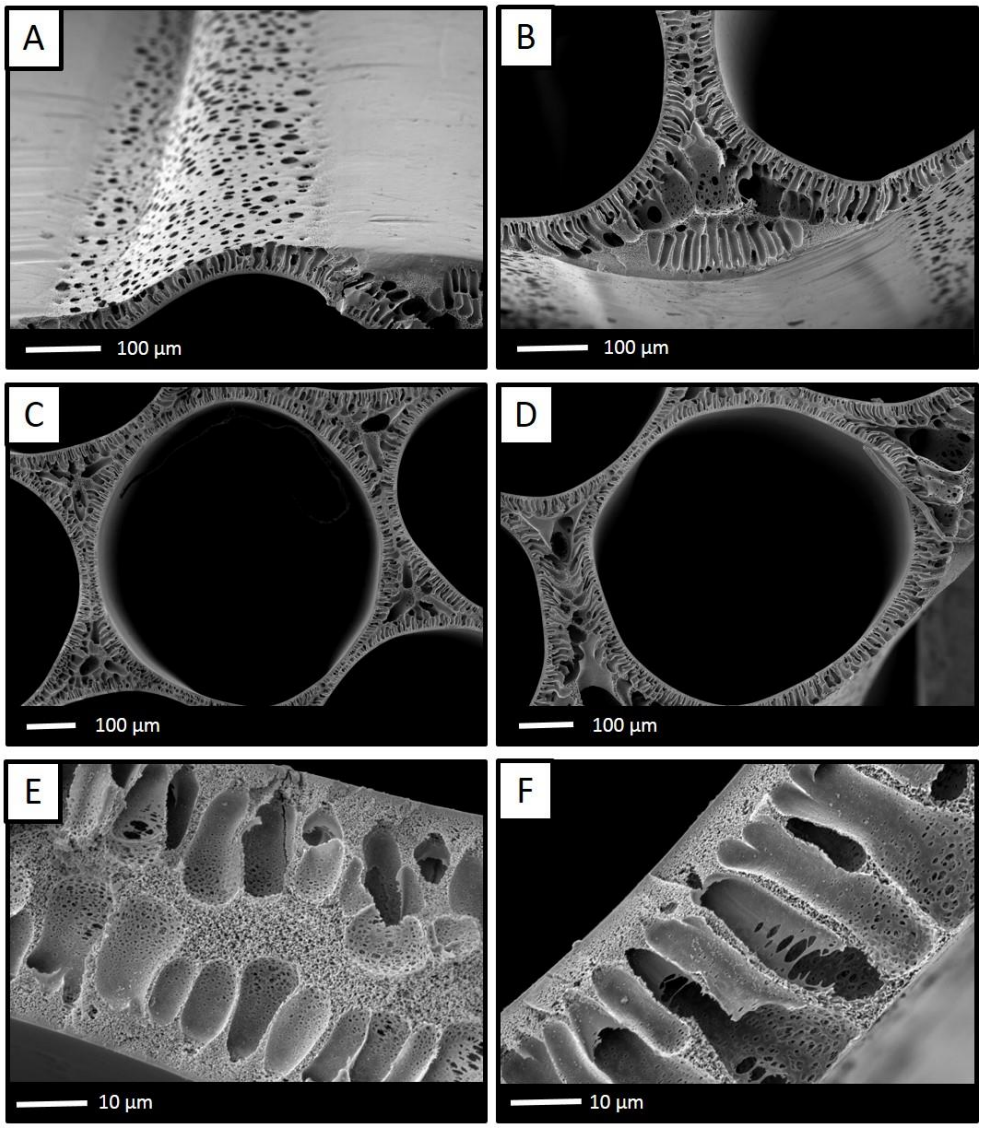

Figure 3. Scanning electron microscopy images of multibore hollow fiber – MF3: A) outer bore surface, B) cross section of outer connection between two bores, C) middle bore cross section, D) side bore cross section, E) cross section of the wall between two bores, F) cross section of the bore outer wall.

3.2. Clean water transport

Figure 4 presents the clean water fluxes of the new MF3 multibore membranes at various transmembrane pressures in comparison with commercial multibore hollow fibers. In all cases, the graph is linear indicating good mechanical stability of the membranes in this pressure range. The MF3 membrane has higher water hydraulic permeability (1824 L/m^2/h/bar) than the commercial membranes (1023 L/m^2/h/bar). Additional treatment with NaClO solution for 24 h, which removes part of the PVP, results in membranes with 40% higher permeability compared to untreated membranes, and more than 100% higher compared to commercial membranes (MF3 washed, 2590 L/m^2/h/bar). Therefore, the MF3-washed membranes were chosen as the most suitable for cell encapsulation.

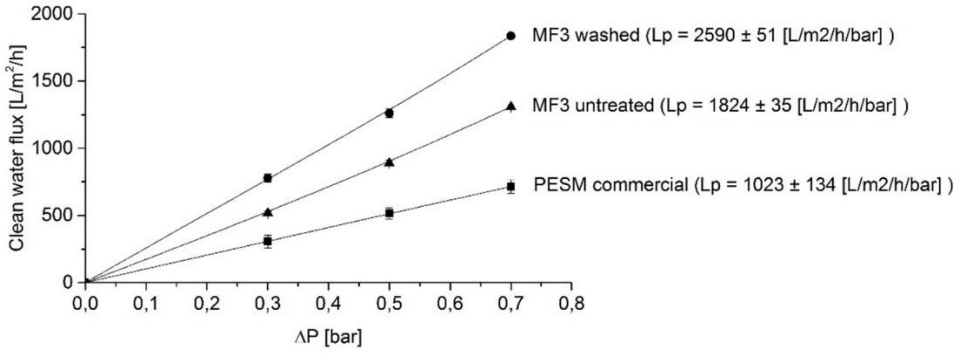

Figure 4. Clean water flux vs. transmembrane pressure for commercial PESM membranes, MF3 – untreated new multibore hollow fiber and MF3 washed with NaClO for 24h.

3.3. Human islets functionality

In order to study the endocrine function of islets encapsulated within the multibore hollow fiber, we performed glucose induced insulin secretion tests. Figure 5 A and

B compare the stimulation indices of islets encapsulated in the developed fibers in comparison to commercial multibore fibers, PESM, with larger bores.

In all cases, free-floating islets responded well to glucose concentration changes. The islets encapsulated within the PESM fibers do not function neither after 1 day nor after 7 days of culture (stimulation indices for high glucose concentration below 2, see Figure 5A). In contrast, islets encapsulated within the new multibore MF3-washed fibers respond to changing glucose concentrations and retain their endocrine function during the 7 days culture period (Figure 5B).

We also studied the potential of the multibore fibers to encapsulate various numbers of human islets from one donor. The islets were seeded in the multibore using the procedure described in Figure 1. We performed various experiments where in total of 1000, 3000 or 6000 islets per 1 cm fiber were encapsulated. With the applied seeding method, it was not possible to estimate the number of cells seeded per bore.

Figure 5C compares the amount of insulin secreted from the islets encapsulated in the MF3 washed membranes to free-floating islets after 7 days of culture. In all cases, the free-floating islets (Figure 5C and zoom on Figure 5D), as well as, the islets encapsulated within the fibers function well showing a clear response to glucose concentration changes during all 5 glucose incubation steps. In fact, the amount of secreted insulin increases with the number of islets encapsulated in the membrane. Basal insulin concentration after first low glucose simulation was 1500 pmol/L for 1000 islets and almost double (2741 pmol/L) when 3000 islets/fiber were encapsulated, reaching 10657 pmol/L for 6000 islets/fiber. Furthermore, upon the first high glucose stimulation, the insulin concentration for 6000 encapsulated islets/fiber is more than double than for 3000 islets/fiber and about 20 times higher than for 1000 encapsulated islets/fiber. The following glucose concentration changes resulted in an adequate response, namely a decrease in insulin secretion for low

glucose concentration and an increase of insulin secretion for high glucose concentration, indicating that islets remain functional within the multibore fibers.

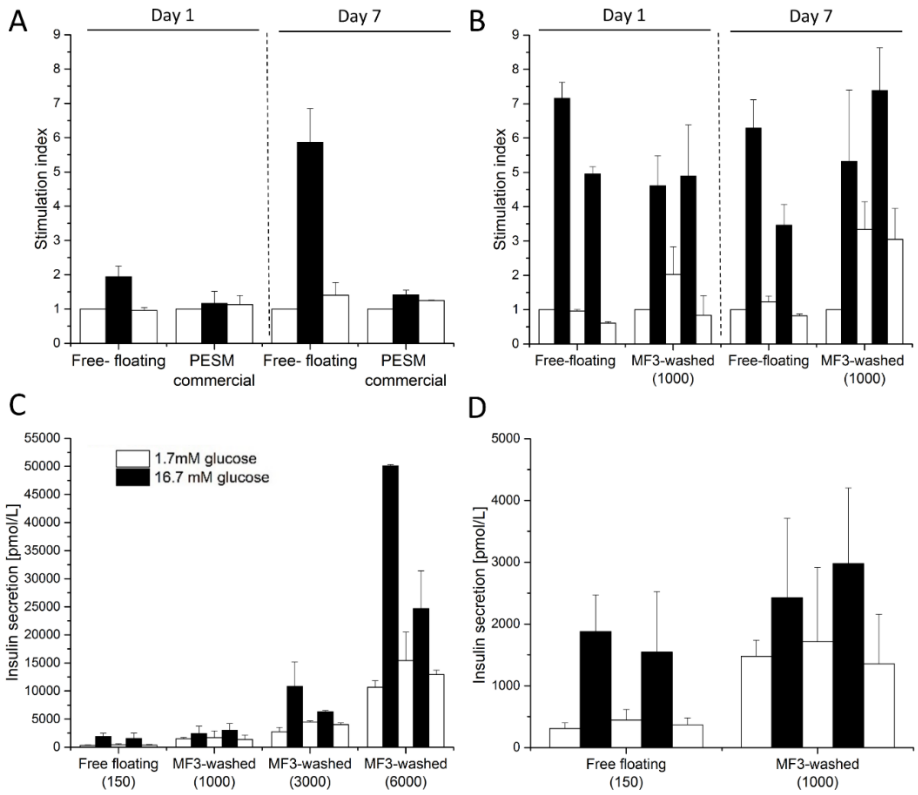

Figure 5. Functionality test of encapsulated islets: A) stimulation index after day 1 and 7 for islets encapsulated within commercial PESM membranes (3500 islets) (reproduced with permission from [26]), B) stimulation index after day 1 and 7 of encapsulated islets from donor 1 within MF3-washed (1000 islets, n=3), C) insulin secretion of different number of human islets from donor 2 after 7 days of culture, encapsulated within MF3-washed (n=3), D) zoomed in insulin secretion of free -floating islets and 100 islets encapsulated within MF3-washed.

Figure 6 shows the stimulation index of islets from two other donors, encapsulated within the new multibore fibers, compared to the free-floating islets. The islets from both donors secrete insulin upon glucose stimulation after one day of culture and they retain their endocrine function after 7 days of culture, similar to free-floating non-encapsulated islets. In all cases, the stimulation index of the first high glucose stimulation is more than double compared to basal insulin release. Again, even when a high number of islets is used, the encapsulation device remains functional for cells of all used donors. Since there is often variation between donors, we also found that, the free-floating islets from donor 3 showed higher response to glucose concentration changes (Figure 6A) in comparison to less responsive islets from donor 4, where stimulation index was less than 3 for high glucose stimulation (Figure 6 B and C). The insulin secretion was also higher for the islets from donor 3.

Importantly, we also observe an increase in stimulation index after the second and third low glucose stimulation for all number of encapsulated islets within the fibers in comparison to basal insulin release. Perhaps, insulin produced during the previous high glucose incubation step is slowly released during the next low glucose stimulation steps. Another reason for this could be that the glucose was not completely removed from the device after high glucose incubation and, therefore, the final concentration used for the next incubation step was higher than 1.67 mM, causing an increased islet response. Despite this, for all number of encapsulated islets we observe a clear response to the first increase of glucose concentration over 7 days of culture.

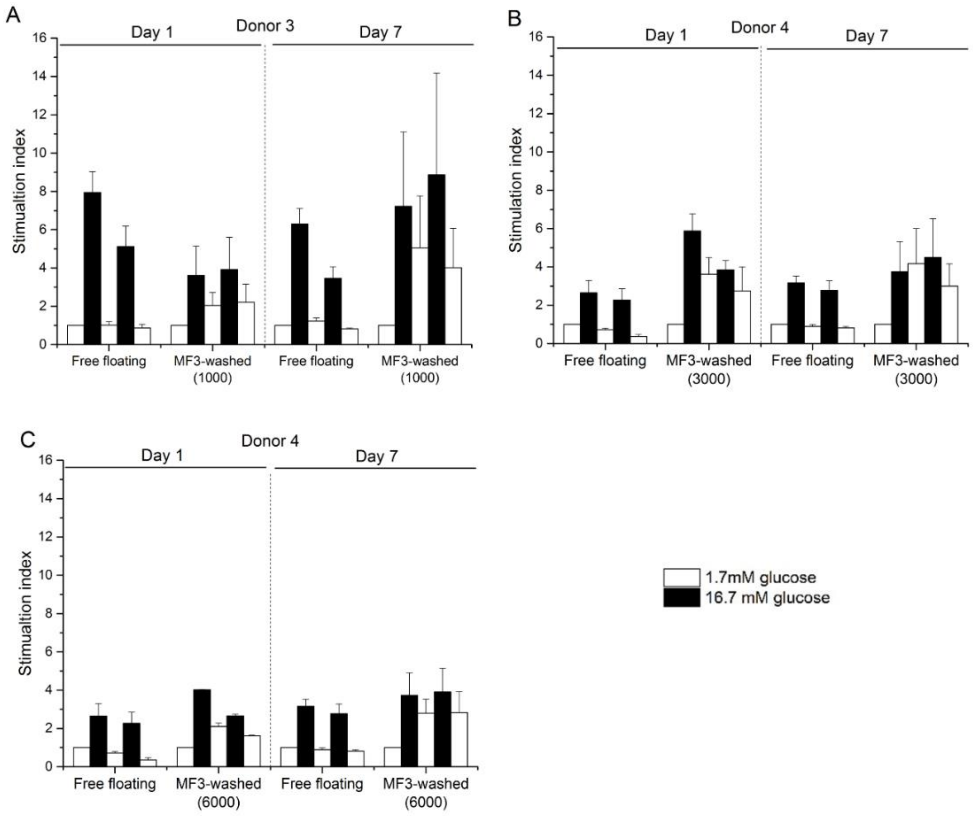

Figure 6. Human islets functionality within new multibore hollow fiber membranes (MF3-washed) in comparison to free-floating islets after 1 and 7 days of culture: A) encapsulated 1000 islets from donor 3, B) encapsulated 3000 islets from donor 4, C) encapsulated 6000 islets donor 4.

4. Discussion

One of the advantages of hollow fibers over flat membranes is their large surface area to volume ratio which is desirable in order to encapsulate a high number of islets in a relatively small volume [27]. However, single fibers applied as macroencapsulation devices often have low mechanical stability. They tend to bend and break, which consequently could lead to islet loss after transplantation [28]. In

this study, we propose a novel islet macroencapsulation device based on porous multibore fiber membranes. They consist of seven equally spaced bores, where the bore diameter is optimized to host a range of sizes of human islets. The membrane structure provides one of the best geometries for hollow fiber membranes and ensures both large porosity and excellent mechanical properties [29]. High stability is a great advantage of the multibore membranes, important during the handling process and in the period before, during and after transplantation.

In order to provide long term islet encapsulation, the fibers were fabricated using non-degradable PES/PVP polymer blend. PES is a widely used biomaterial for hemodialysis membranes, with excellent chemical and mechanical properties [22]. The PVP improves material biocompatibility and introduces non-cell adhesive properties to avoid cell attachment onto the membrane surface, which are important for islets transplantation [30, 31]. In the literature, other materials have been used for hollow fibers fabrication, such as modified polyacrylonitrile polyvinyl chloride [32], regenerated cellulose and polyamide [14], acrylic copolymer (XM-50 Amicon)[33] and polysulphone [34]. However, the use of large volume of islets encapsulated in hollow fibers with relatively big diameter (0.6 ± 3 mm [35-37]) led to limited transport of nutrients and oxygen, and consequently islet death.

The new multibore hollow fibers were fabricated using dry-wet spinning method via immersion precipitation [38]. By changing the spinning parameters: composition of bore liquid and coagulation bath temperature, we obtained highly porous membranes with bores of 500 –m D Q G Y H U \ W K L Q ZDOOV. This was achieved by adding solvent (NMP) in the bore liquid which reduced the rate of phase separation; the exchange between solvent and non-solvent (lower concentration gradients during solvent/non-solvent exchange). As a result, we obtained a relatively spongy membrane structure with small voids and high porosity (MF2). The fiber wall thickness was decreased and the pore morphology was improved in comparison to MF1 membranes (Figure 2B). When pure non-solvent (water) was used as a bore

liquid, the formation of dense inner surface occurred. The addition of NMP in the bore solution can increase the porosity of the inner surface and can prevent the formation of a dense skin layer [39, 40]. However, the exchange of solvent and non-solvent on the inner surface of our fiber lumen was slower than on the outside surface of the fiber, resulting in deformation of the bores. Mousavi et al. studied the effects of various temperatures of the coagulation bath on the formation of polyethersulfone membranes and found out that increase in the temperature of the coagulation bath leads to an increase in the solvent ±non-solvent exchange rate [41]. Subsequently a more porous structure is formed resulting in higher membrane permeability [42]. When we increased the coagulation bath temperature, we obtained the MF3 membranes with improved outer surface porosity. The combination of tailoring the bore liquid composition and the temperature of coagulation bath resulted in a proper exchange rate during the phase separation process, resulting in stable and highly porous multibore fibers with desired bore dimensions and thin walls. The latter is a very important requirement for optimal mass transport during islet encapsulation, since the cell survival depends on the diffusion distance of nutrients to the cells. :KHQ WKLV H[FHHGV —P LW LV NQRZQ WKDW FHOO [9].

The development of multibore hollow fiber membranes is known in literature in various applications. Wand and Chung designed and fabricated a lotus-root multibore hollow fiber membrane for membrane distillation process [29]. This concept has also been adopted for inorganic ceramic membranes [43, 44]. In recent years, a seven-bore ultrafiltration hollow fiber membrane has been fabricated using a specially modified polyethersulfone material (PESM) by Inge GmbH. Here, we compared these membranes to our new developed multibore fibers in terms of islet encapsulation. Our optimal hollow fiber (MF3 washed) is two times smaller than these commercial PESM membranes considering both outer membrane and bore diameter. Besides our fibers have high permeability (2590 L/m^2/h/bar), more than

double in comparison to the permeability of commercial membranes and others with similar pore size [23, 24].

The tailor made MF3 membranes were used for encapsulating pancreatic islets and were compared to commercial PESM membranes. De Bartolo et al. had shown earlier that highly permeable PESM membranes allow for transport of bovine serum albumin (66.5 kDa), which is a much bigger molecule than insulin or glucose [24]. However, human islets encapsulated within PESM membranes did not respond to glucose concentration changes, which could be attributed to suboptimal dimensions of the fiber. The large bore diameter (0.9 mm) and the thick walls of the fiber, probably limit the diffusion of nutrients to cells, which negatively affects islet survival and function. In contrast, our multibore hollow fibers, especially designed for this application, succeeded and the encapsulated islets remained functional over 7 days of culture.

Often, a single bore fiber allows for the encapsulation of a low number of islets. Lembert et al. encapsulated 50 islets within 1 cm hydroxyl-methylated polysulphone fiber (0.9 mm inner diameter) [34]. Here, we were able to encapsulate up to 6000 islets inside a 1 cm long fiber and we observed that the concentration of secreted insulin increases with the number of encapsulated islets, indicating that the membrane porosity is sufficient to provide nutrients to the cells and achieve good insulin delivery by the cells. We showed that the human islets encapsulated within the bores of our MF3 membranes secret insulin in response to glucose concentration changes and function well after 7 days of culture, despite the variability is the quality of human islets obtained from various donors.

Finally, in encapsulation studies, separation factors such as particles and / or gels are used to avoid islets aggregation. Here, we avoided the use of separation factors during islet seeding to prevent additional diffusion transport barriers [45, 46]. Perhaps, the lack of separation factors creates empty space between the membrane

walls and the encapsulated islets where secreted insulin or glucose could be trapped and then is slowly released. Nevertheless, the insulin response to glucose concentration changes is clear indicating the potential of the application of these multibore hollow fibers for islet macroenacapsulation.

5. Conclusions

In this study, we have developed new PES/PVP multibore hollow fiber membrane for islet macroencapsulation. The membranes are non-degradable, mechanically stable, offer good protection of encapsulated islets and allow the encapsulation of a high number of islets, crucial for device upscaling and clinical application. Moreover, the optimized bore dimensions and membrane porosity provide sufficient glucose and insulin transport, important for maintaining islet function.

Acknowledgments

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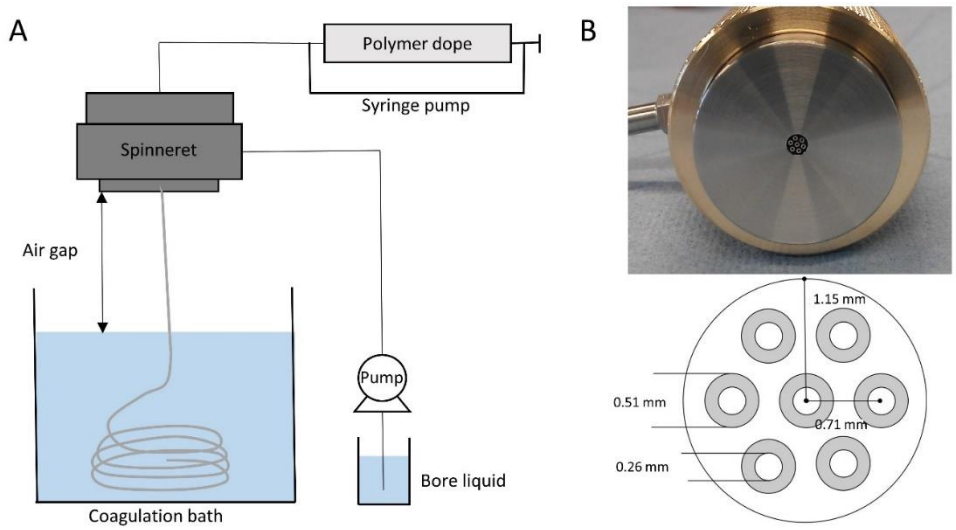
Grant title: New islet encapsulation method for ideal mass transport and immune-protection

Grant key: 17-2013-303

Supplemental material



Supplemental figure 1. Spinning set-up. A) Schematic representation of the hollow fiber spinning set-up, B) photograph of multibore spinneret and its dimensions.

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Chapter 7

Conclusions and Outlook

1. Conclusions

Encapsulation of pancreatic islets or βcells is a promising strategy to improve islet transplantation outcomes in the treatment of type 1 Diabetes by providing an immune isolated environment and allowing for transplantation in a different location than the liver. The use of porous membranes for macroencapsulation devices gives the opportunity to optimize the device properties regarding the transport of nutrients and the exchange of glucose and insulin. An important advantage of these devices is that they can be relatively easily retrieved, replaced or reloaded. Additionally, they can contain a high density of islets, which can be fixed in one location. Besides, since the cells in macroencapsulation devices are in close proximity to each other, their communication and synchronization regarding insulin secretion is improved. However, a high number of islets placed in one location may lead to their clustering, which negatively affects islet native structure and results in limited nutrient diffusion. Therefore, in **Chapter 3,** we have developed a novel device for macroencapsulation, in which islets are physically separated in microwells and closed by a membrane lid, without compromising their function. Non-degradable poly(ethersulfone) (PES) / polyvinyl pyrrolidone (PVP) with low cell adhesive properties is used for the membrane preparation. Additionally, the tailored membrane porosity allows for sufficient glucose and insulin transport, crucial for maintaining islet viability and function.

Since the islets are highly vascularized in the pancreas, they also require large amounts of oxygen and access to nutrients to function properly within the encapsulation devices. However, the isolation procedure. Prevascularization of the devices in vitro can improve the connection between the device and the host vasculature after implantation, providing the encapsulated islets with sufficient blood supply and oxygenation. However, during in vitro generation of microvascular networks, it is important to guide their formation, since native vasculature is a highly-organized tissue. Here, the membrane

surface topography/pattern has a potential to support and guide prevascular network formation. In **Chapter 4**, we fabricated porous, micropatterned PES/PVP membranes, which can be applied as a lid for our microwell islet encapsulation device, and established the co-culture of HUVECs and fibroblast grown there without addition of hydrogels. By using membranes with surface patterns of bricks, as well as, channels and bricks, we achieved interconnected HUVEC branch-like structures oriented in the direction of the patterns. Additionally, the presence of intermittent bricks allowed for communication between cells and the connection of HUVEC branch-like structures. This is an important step towards obtaining a stable endothelial cell network for the in vitro prevascularization of our flat encapsulation device.

In the pancreas, islet endothelial cells, which form capillaries, are an important source of signals that enhance the survival and function of the insulin-producing cells. However, the isolation procedure damages islet endothelial cells, therefore, islets used for encapsulation often have lower functionality. In **Chapter 5,** we created stable composite aggregates by co-culture of MIN6 cells with HUVECs to mimic cell-cell relations with endothelial cells in native islets. The composite aggregates have improved functionality in terms of glucose-stimulated insulin secretion that the aggregates of MIN6 cells alone. Moreover, composite aggregates encapsulated within the microwell PES/PVP device maintain their high performance and secrete more insulin than encapsulated aggregates consisting of only MIN6 cells. This indicates that providing cells with a connection to endothelial cells within an encapsulation device is beneficial in terms of improved cell functionality and better device performance.

An alternative for the flat configuration of extravascular macroencapsulation devices is the use of hollow fiber membranes, which offer a large surface-to-volume ratio, preferable for implantable devices. However, the suboptimal fiber bore dimensions and membrane morphology often contribute to graft failure, due to a limited

exchange of nutrients, oxygen, and insulin. Additionally, single-bore hollow fiber membranes often suffer from performance instability during long-term use. Therefore, in **Chapter 6**, we developed new PES/PVP multibore hollow fiber membranes for islet macroencapsulation. The fiber consists of seven bores and has high mechanical stability offering good protection to the encapsulated islets. Human islets encapsulated within the fiber bores retain their glucose responsiveness and the insulin secretion increases with the increasing number of encapsulated islets. Our new multibore hollow fiber membranes have higher islet encapsulation capacity than single-bore fibers and allow for easier up-scaling, which are important factors for the development of a clinically applicable bioartificial pancreas.

2. Outlook

2.1. Device design

Flat membranes

The flat PES/PVP macroencapsulation device consisting of a microwell membrane sealed with a flat membrane lid was developed with the aim to avoid islet aggregation within the device, improving nutrient diffusion and therefore islet function. In other preliminary studies, the microwell PES based membranes were also created by microthermoforming method (Figure 1 A-C) based on the work of Builtinga et.al. [1], where heated polymer porous film was stretched into a negative mold using backing material. The created microwells had dimensions suitable for islet encapsulation and separation, however, the high temperature and pressure applied during the fabrication process caused membrane pore deformation and pore collapsing leading to decrease of membrane permeability (Figure 1D). Obtaining microwell porous membranes by microthermoforming with suitable transport properties for islet encapsulation can be challenging as it requires tight control of the membrane pore morphology changing during the microwell array formation. However, the microwells obtained using this method could greatly mimic curved

microanatomy of the alveoli and be used for the development of chip-type lung models helping better understanding issues related to the lung diseases [2, 3].

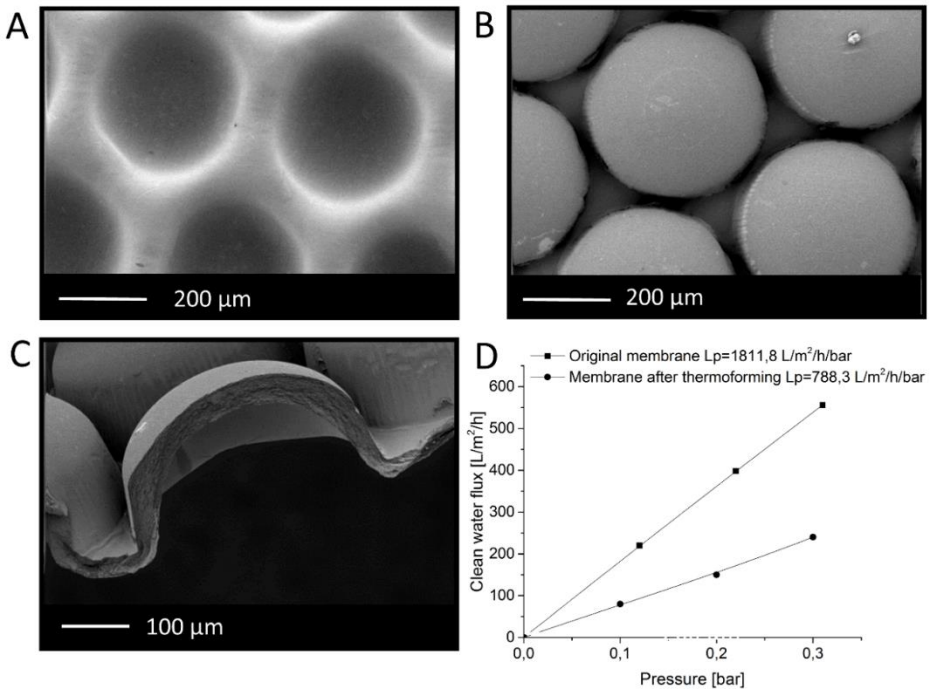
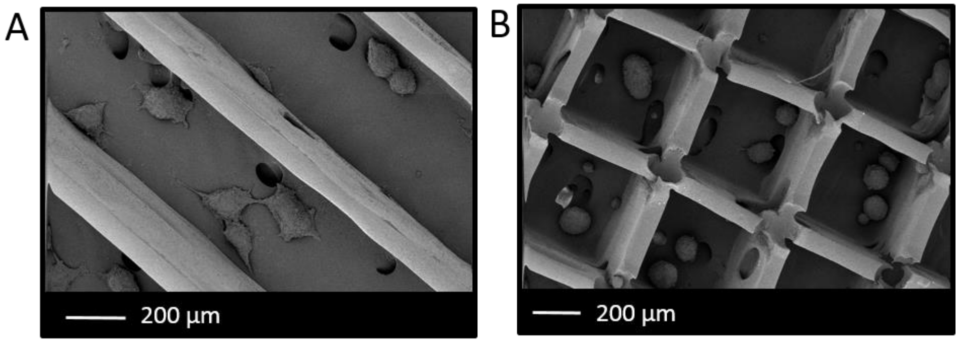


Figure 1. Microwell membranes obtained by microthermoforming method. A) top, B) bottom, C) cross section, D) water permeability.

Our goal was to develop islet separation system with sufficient transport properties for islets encapsulation. Therefore, we used the [45], where, through immersion precipitation of the polymer on a micropatterned mold, we obtained in one step a highly porous material with controllable micrometer-scale pores and good quality microwells, (see **Chapter 3)**. Besides the round shape wells mostly presented here, we also fabricated the PES/PVP microwell membranes with rectangular walls as well membranes with only channels (Figure 2). The channel design did not assure

good cell separation and cells could attach to each other, therefore in the future, additional separation factors needs to be applied to avoid islets aggregation. However, it would be interesting to investigate the effect of the channels on organization of HUVEC branch-like structures studied in **Chapter 4** to compare them with bricks and combination of bricks and channels used there.



[illegible]
A) membrane with channels, B) membrane with squared wells.

The squared wells assured aggregates separation and could be potentially used for islets encapsulation, but it would also require a larger membrane surface to have the same number of wells in comparison to membranes with round microwells. In order to decrease the surface area covered by microstructures and in the same time provide islet separation, it would be interesting to develop membranes with regularly spaced pillars as a part of an encapsulation device.

In **Chapter 4**, we fabricated porous flat PES/PVP membranes with surface topography with bricks and channels and bricks, which assist the formation of organized cell networks over the membrane surface. In the future, the microwell membrane used in our encapsulation device could be sealed with the micropatterned membrane as a lid. Similar microstructured surface could also be created on the

bottom side of the microwell membrane, by polymer casting on one of the micropatterned molds and a second mold with the other microstructures applied on the top of the polymer layer. The micropatterned surface would support cell organization during the development of a prevascularized layer on the outside of the device. In order to prepare the macroencapsulation device, the micropatterned membrane will be sealed with microwell membrane on the edges, leaving small opening for seeding of the islets. Then, cells can be cultured on the outside surface of the device and, after organized microvessel-like structures are established in vitro, islets can be seeded inside the device, closed and transplanted. The presence of a highly interconnected prevascularized layer, closely mimicking native tissue, could reduce the time of construct reconnection to host vasculature and improve islet survival within our macroencapsulation device.

As an alternative strategy for prevascularization, we also investigated the use of the microstructured membranes developed in **Chapter 3** in combination with mesenchymal stromal cells (MSCs), which have endothelial potential [5]. The MSCs are known to have a positive influence on vessel formation and additionally have immune modulatory effect when transplanted in vivo [6-8]. The microstructured and non-patterned membranes with and without the addition of MSCs were implanted subcutaneously in female Lewis rats and the samples were explanted after 14 days (Figure 3). The membranes with MSCs seem to recruit more blood vessels than those without cells. Moreover, the membranes with brick surface topography in combination with MSCs have the highest rate of vessel formation in close proximity to the membrane in comparison to non-patterned membranes or to membranes with surface topography of channels and bricks.

In the future, both of our strategies to enhance vascularization should be applied for the microwell devices with encapsulated islets and further studied in vivo to reveal their actual effect on islet survival and function.

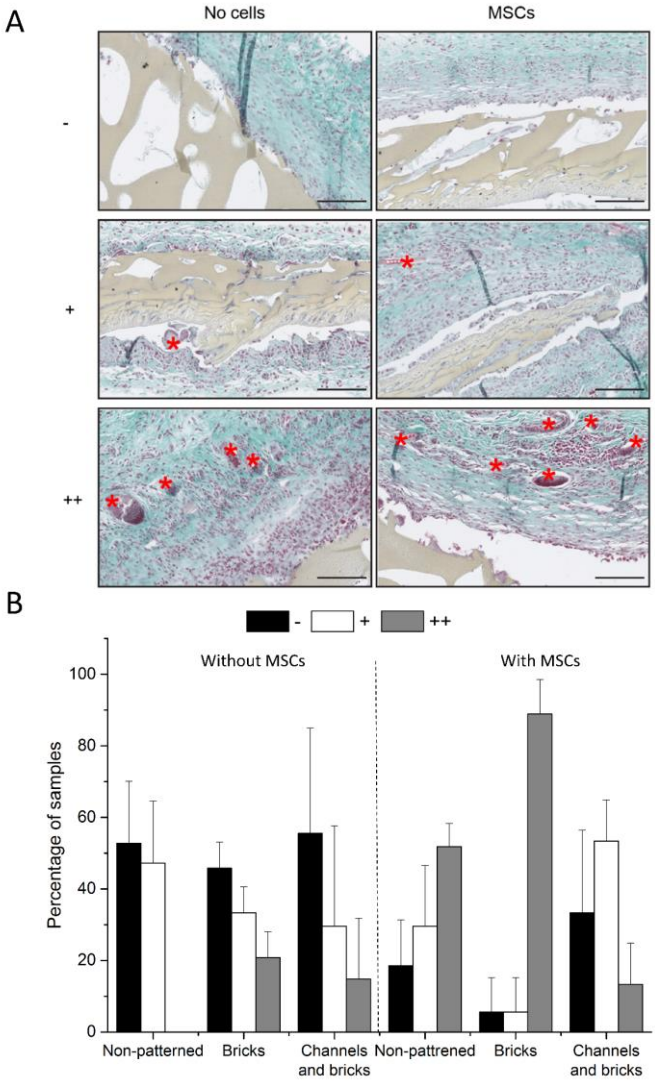


Figure 3. Analysis of vessel formation of in vivo samples, samples classified in three categories: hardly any vessels (-), some vessels (+) and a lot of vessel infiltration (++). A) Examples of Trichrome stained sections from membranes with bricks. The left panel shows the samples without MSCs and the right panel the samples with MSCs. B) The effect of non-patterned, bricks and channels with bricks on vessel formation in samples with and without MSCs.

Hollow fibers

In **Chapter 6** we developed a PES/PVP multibore hollow fiber macroencapsulation device as an alternative to flat device geometry. The seven-bore fiber allows for the encapsulation of a higher number of islets than a single-bore fiber of the same length. Although we did not observe islet aggregation within the bores of the fiber, the high number used for encapsulation could lead to their clustering. Therefore, one could consider adding separation agents to the suspension of islets, such as microparticles which should be highly porous to avoid limitations of nutrient delivery to the cells and maintain their function within the bores of the fiber.

In order to provide islets encapsulated within multibore fiber with access to vasculature, various strategies can be considered (see Figure 4). In our experiments in **Chapter 6**, the middle bore of the fiber was closed. In the future, this bore could be used for vascular ingrowth since it has optimal position for providing nutrient and oxygen to the islets encapsulated within the other six bores. To achieve vessel ingrowth there, the surface of the bore needs to be coated, e.g. with fibronectin, to introduce cell adhesive properties (as it was shown in **Chapter 4**) and cells with angiogenesis potential like human endothelial vein cells in combination with supportive cells (smooth muscle cells, fibroblast) could be cultured in the bore (see Figure 4A). Such co-culture systems are beneficial as their better mimic the cell interaction in the native vasculature [9]. The same strategy could be applied on the outside of the device, as the established prevascular layer on the outer surface of the fiber could improve the connection between the device and the host vasculature after implantation (see Figure 4B). Hollow fibers have been previously used for the culture of endothelial cells and support cells on the inside as well as the outside of the fiber [10-12].

Another possibility would be the delivery of growth factors such as vascular endothelial growth factor or basic fibroblast growth factor, which have been shown

to enhance the formation of new blood vessels close to the implant in vivo [13, 14]. The growth factors combined with biodegradable hydrogel or another drug delivery system could be injected inside the middle bore of the multibore fiber aiding vessel formation there.

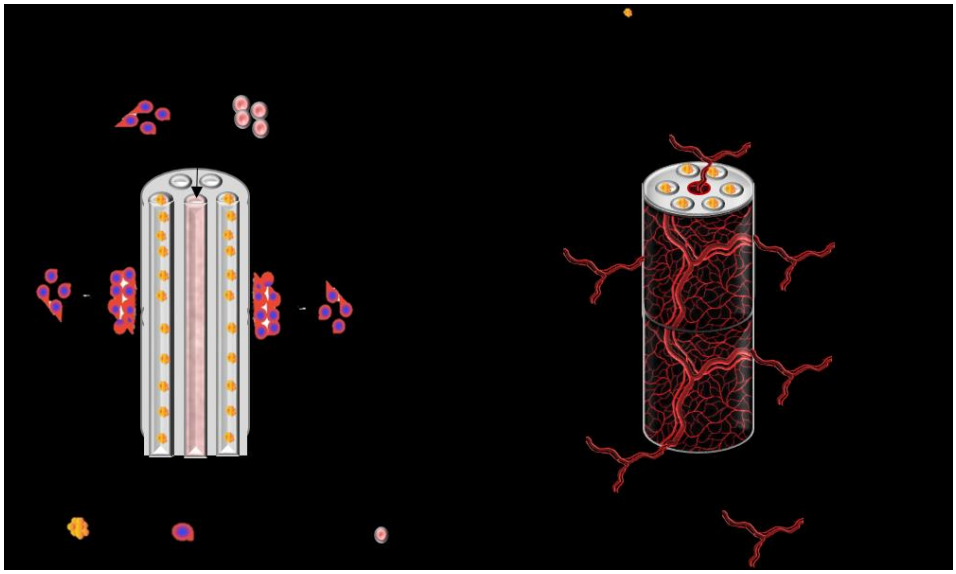


Figure 4. Strategies to induce vascularization of the multibore fiber. A) Culture of endothelial cells inside the middle bore and on the outside of the device or injection into the fiber bore of growth factors embedded within hydrogel in vitro. B) Prevascularized fiber improving the connection between the device and the host vasculature after implantation.

2.2. Immune protection

Membrane based islet macroencapsulation devices should allow for sufficient transport of nutrients, glucose, oxygen and insulin but also provide immune protection to the encapsulated cells to avoid harmful immunosuppressive drug therapy. In this thesis, the immune protective properties of the developed devices were not tested. However, membranes with 0.4 - 0 . 4 5 µ m p o r e s i z e h a
to successfully protect allogeneic cells from the immune system and, although IgG

and IgM were still able to pass through the membrane, the allogeneic tissue survived [15-18]. As the lid of our microwell device has 0.45 µm pore size, we expect that the device would be immune protective. Moreover, preliminary results of biocompatibility, where the microwell membranes were implanted in epididymal fat pads of a mouse indicate that the host cells cannot infiltrate through the selective layer of the microwell membrane (Figure 5). There, the microwell membrane was implanted without the lid and therefore cell infiltration is visible from the side of the microwells. However, combining our microwells with membranes of smaller pore size can have the potential to provide immune isolation to encapsulated islets.

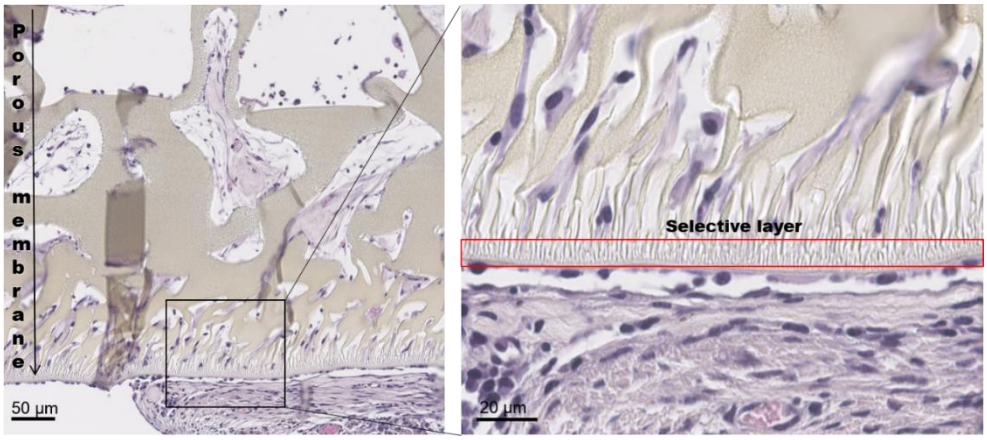


Figure 5. A high magnification of the hematoxylin and eosin stained membrane section shows cell infiltration up to selective layer (on the left). The selective layer in more detail is indicated by the red outlined area (on the right).

The ideal immune protection requires physically excluding immune cells, as well as, restricting diffusion of immune mediators such as cytokines that T-cells are [19]. Therefore, in vitro assays involving cytokine cytotoxicity, mixed lymphocyte reaction and reactive T-cells should be used in the

immune protective properties. However, the best indication would be the result of an allogeneic transplantation in immune competent animals.

The use of immune protective encapsulation devices can also solve the critical problem of the shortage of human islets by allowing for the transplantation of animal tissue or novel insulin-producing cells, as described in **Chapter 2** of this thesis. Moreover, the encapsulation of endothelial cell-cells would combined improve β-cell function and device performance, as we showed in **Chapter 5** by co-culture of HUVECs with MIN6 aggregates. Besides HUVECs, other cell types like adipose-derived stem cells could be used for improved islet cell functionality as it was shown in the study of Jun et.al [20].

2.3. Device implantation

The obvious next step is the implantation of the developed devices into an animal model. Here, we took this first step for microwell macroencapsulation device and preformed allogeneic and xenogeneic transplantation in the intraperitoneal cavity of mice (Figure 6A). During the implantation, the blood glucose level and weight of the animals were measured. The animals' weight remained implantation and, interestingly, the device with rat islets used for xenogeneic transplantation performed similar to the controls (non-encapsulated islets in epididymal fat pads) in terms of glucose regulation over time (Figure 6B). We achieved this by using 600 islet equivalents (IEQs) within a device of 10 mm in diameter, which was regarded as the minimum number of islets to reverse hypoglycemia in a mouse animal model. In order to use a higher number of islets, the device dimensions need to be optimized. In this thesis, we designed membranes with microwells of 500 µm and size ranged of islet ter available to us. However, for clinical implementation, smaller islets (50-150 µm) ar recommended as they show higher viability and function comparing to bigger islets

[21]. Therefore, in the future, membranes with smaller spacing between the we

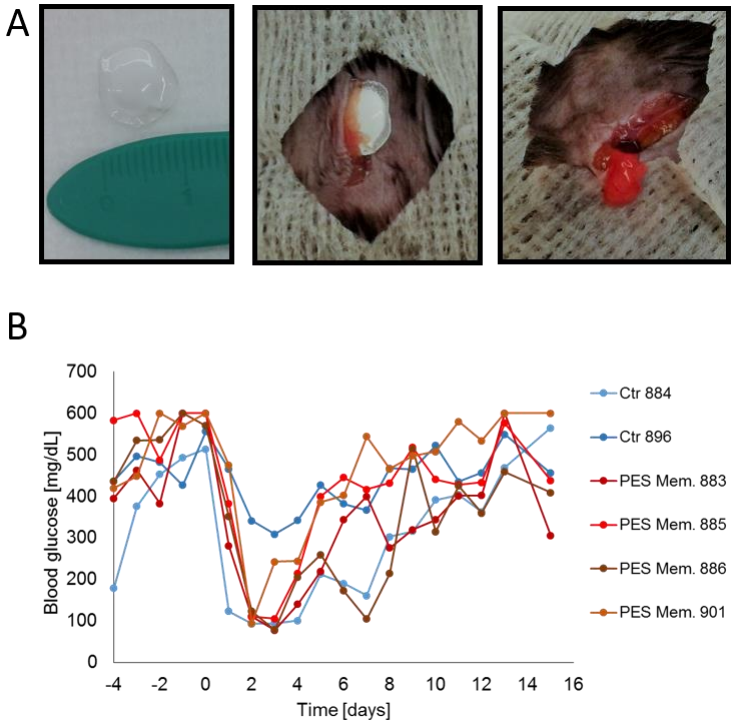


Figure 6. Implantation of microwell device with encapsulated islets in mouse animal model. A) Images representing: sealed device with encapsulated islets, implantation of the device into intraperitoneal cavity and exposed epididymal fat pad with free-floating islets sealed with fibrin glue respectively. B) Blood glucose measurements over time with implanted free-floating islets is represented by Ctr 884 and 896, while the PES Mem.: 883, 885, 886 and 901 represents blood glucose level of the mice with implanted microwell device with encapsulated islets.

To be able to compare the volume of islets with different diameters and volumes, individual islets need to be converted to standard islet equivalents (IEQs) with a diameter [22]. For clinical application, it has been estimated that 9000

IEQs per kilo bodyweight of patient are needed to restore normoglycemia (540000 IEQ for 60 kg patient) [23]. Based on this, we estimated that we would need 4 microwell membranes (well diameter 200 a clinically relevant number of islets. Besides, the thin film design of our device allows for the creation of multilayer stacks of microwell membranes similar to those recently developed in our laboratory for upscaling of tissue engineering constructs [24], enabling possibly the development of a compact microwell device.

In case of multibore fibers with cells encapsulated within the 6 equally spaced bores, leaving the middle bore for possible vascularization, we would need 8 multibore hollow fibers of 20 cm (11250 IEQs per bore, 50% fiber loading capacity) in order to treat a 60kg patient. To minimize the volume of the device, the loading of the fibers could be increased, composite aggregates with endothelial cells could be applied and the fibers could be coiled (in a ring of about 6 cm in diameter) due to the superior mechanical properties of the mutibore fiber in comparison to single bore fibers.

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Summary

The research presented in this thesis is about the development of novel membrane based macroencapsulation devices for improved pancreatic islet survival and function. A general introduction on the topic of this thesis and its scope is presented in **Chapter 1**.

Chapter 2 presents a literature overview of the important factors considering the development of a bioartificial pancreas. Current encapsulation strategies are described and materials used for fabrication of membrane based macroencapsulation devices as well as their configurations are presented. The promising results obtained with macroencapsulation devices have led to first clinical studies, however, there is still room for improvement in order to develop a life-long, fully functional islet encapsulation device for type 1 Diabetes treatment.

To improve pancreatic islets functionality by avoiding their aggregation within macroencapsulation devices, in **Chapter 3**, we developed a novel microwell membrane based encapsulation device, where the islets are seeded in separate microwells avoiding their fusion and clustering. The membrane porosity is tailored to achieve shielding of the islets from the host immune cells without compromising their secretory responses. The non-degradable, microwell membranes are composed of poly (ether sulfone)/polyvinylpyrrolidone (PES/PVP) and manufactured via phase separation micromolding. Our results show that the device prevents aggregation and preserves the islet's native morphology. The encapsulated islets maintain their glucose responsiveness, comparable to free-floating non-encapsulated controls, demonstrating the potential of this novel device for islet transplantation.

In **Chapter 4**, we fabricated porous, micropatterned PES/PVP membranes and we investigated the effect of patterns (bricks and channels) on human umbilical vein endothelial cell (HUVEC) alignment and interconnection as a first step towards the development of a stable prevascularized layer in vitro. In contrast to non patterned membranes where HUVECs form typical randomly spread HUVEC branch-like structures, in the case of micropatterned membranes we achieved a clear alignment

of these structures in the direction of the patterns. Additionally, the presence of intermittent bricks allows for communication between cells and the connection of HUVEC branch-like structures creating a network over the membrane surface. We obtained this by co-culture of HUVECs on the monolayer of fibroblasts grown on the fibronectin coated membrane surface. The micropatterned surface, applied as lid for the microwell macroencapsulation device, would support cell organization during the development of a prevascularized layer on the outside of the device. Providing encapsulated islets with close proximity to blood vessels is important for their survival and function.

In order to mimic the β-cell relation with endothelial cells in native islets, in **Chapter 5**, we created stable composite aggregates by co-culture of mouse insulinoma MIN6 cells with HUVECs on a non-adherent agarose microwell platform. The presence of HUVECs there results in improved insulin secretion upon glucose stimulation in comparison to aggregates consisting of only MIN6 cells. Importantly, these composite aggregates maintain their function after encapsulation within our microwell PES/PVP device and show better insulin release than encapsulated pure MIN6 aggregates, indicating that providing the β-cells with a connection to the endothelial cells within an encapsulation device can improve the encapsulated cells' functionality.

In **Chapter 6**, we have developed a new PES/PVP multibore hollow fiber membrane for islet macroencapsulation as an alternative for the flat membrane configuration. The seven-bore fiber offers higher mechanical stability than common one-bore fibers and allows for the encapsulation of a high number of islets, crucial for device upscaling and clinical application. The bore dimensions and membrane porosity are optimized to provide sufficient glucose and insulin transport, important for maintaining islet function. In fact, human islets encapsulated within the new multibore fiber secret insulin in response to glucose concentration changes relative to their number used for encapsulation.

Chapter 7 presents the main conclusions of the work described in this thesis, as well as, the outlook for further improvement of the developed macroencapsulation devices described here.

Samenvatting

In dit proefschrift wordt de ontwikkeling van nieuwe membraan gebaseerde macroencapsulatie devices beschreven voor het verbeteren van de overlevingskans en werkzaamheid van de eilandjes van Langerhans. Een algemene introductie over het onderwerp van dit proefschrift wordt gepresenteerd in **Hoofdstuk 1**.

In **Hoofdstuk 2** wordt een literatuuroverzicht gegeven van de belangrijke factoren die een rol spelen bij de ontwikkeling van een bio-artificiële pancreas. Huidige encapsulatietechnieken worden beschreven en er wordt ingegaan op zowel de materialen als de configuraties die van belang zijn bij het vervaardigen van membraan gebaseerde macroencapsulatie devices. De veelbelovende resultaten van de macroencapsulatie devices hebben geleid tot klinische studies, maar verbetering van de devices is noodzakelijk. Voor de behandeling van diabetes type 1 patiënten is namelijk een volledig functioneel encapsulatie device voor de eilandjes van Langerhans, dat levenslang meegaat, noodzakelijk.

De werkzaamheid van de eilandjes van Langerhans kan verbeterd worden door aggregatie van de cellen in de macroencapsulatie devices te voorkomen. In **Hoofdstuk 3** worden daarom nieuwe microwell, membraan gebaseerde encapsulatie devices ontwikkeld waarin de eilandjes geplaatst worden in gescheiden microwells, zodat fusie en clustering van de cellen voorkomen kan worden. De porositeit van de membranen wordt zó gemaakt dat de eilandjes van Langerhans zowel afgeschermd worden van cellen van het immuunsysteem als hun functie van afscheiding kunnen behouden. De niet-afbreekbare, microwell membranen zijn gemaakt van poly (ether sulfone)/polyvinylpyrrolidone (PES/PVP) via de phase separation micro molding techniek. Onze resultaten laten zien dat de ontwikkelde devices aggregatie tegengaan en dat de eilandjes de oorspronkelijke morfologie behouden. Verder behouden de geëncapsuleerde eilandjes de gevoeligheid voor glucose, net zoals de vrije, niet geëncapsuleerde controle eilandjes. Dit demonstreert de potentie van het nieuwe microwell, membraan gebaseerde encapsulatie device voor de transplantatie van de eilandjes van Langerhans.

In **Hoofdstuk 4** hebben we poreuze PES/PVP membranen met microstructuren ontwikkeld en hebben we onderzocht wat het effect is van verschillende structuren (stenen en kanalen) op de groeirichting en interconnectie van human umbilical vein endothelial cellen (HUVEC) als een eerste stap in de richting van de ontwikkeling van een stabiele, gevascullariseerde laag in vitro. In tegenstelling tot de structuurloze membranen, waarbij de HUVECs typische willekeurig verspreide, vertakkingsachtige structuren aannemen, groeien de HUVEC cellen op de microstructuur membranen duidelijk in de richting van de structuren.

Bovendien draagt de aanwezigheid van een onderbroken stenen microstructuur bij aan de communicatie tussen cellen en de verbinding van de vertakkingsachtige structuren van de HUVEC cellen. Hierdoor wordt een netwerk van cellen op het membraanoppervlak gecreëerd. We hebben dit verkregen door middel van het gelijktijdig kweken van HUVEC cellen op een monolaag van fibroblasten, die groeien op een fibronectin gecoat membraanoppervlak. Het oppervlak met de microstructuren dient als deksel van het microwell macroencapsulatie device en ondersteunt de organisatie van de cellen gedurende de ontwikkeling van de gevascullariseerde laag aan de buitenkant van het device. Voor de overlevingskans en functie van de geëncapsuleerde eilandjes is het namelijk van belang dat de cellen in de nabijheid zijn van bloedvaten.

Om de relatie tussen β-cellen en endotheelcellen in de oorspronkelijke eilandjes van Langerhans na te bootsen, werden in **Hoofdstuk 5** stabiele, composieten aggregaten gemaakt door middel van het gelijktijdig kweken van HUVEC cellen en insulinoma MIN6 cellen afkomstig van muizen op een antikleef, agarose microwell platform. De aanwezigheid van HUVECs resulteert hier in verbeterde insuline afscheiding na glucose stimulatie, in vergelijking met aggregaten waarop alleen MIN6 cellen aanwezig zijn. Het is belangrijk te vermelden dat de composieten aggregaten hun functie behouden, nadat ze geëncapsuleerd zijn in ons microwell PES/PVP device en dat ze een betere insuline uitscheiding hebben dan de geëncapsuleerde, pure

MIN6 aggregaten. Dit wijst er op dat wanneer er een verbinding wordt gecreëerd tussen de β-cellen en de endotheelcellen in een encapsulatie device, de functionaliteit van geëncapsuleerde cellen verbeterd kan worden.

In **Hoofdstuk 6** hebben we nieuwe PES/PVP multibore hollow fiber membranen voor de macroencapsulatie van eilandjes ontwikkeld, als alternatief voor de platte membraanconfiguratie. De multibore fiber met 7 lumen biedt betere mechanische stabiliteit dan gebruikelijke fibers met één lumen en meerdere eilandjes kunnen in deze multibore fiber worden geëncapsuleerd. Dit is cruciaal voor het opschalen en de klinische applicatie van het device. De afmetingen van de lumen en de membraanporositeit zijn geoptimaliseerd, zodat er voldoende glucose and insuline transport is voor het behoud van de functie van de eilandjes. De humane eilandjes die geëncapsuleerd zijn in de nieuwe multibore fibers scheiden, in verhouding tot het aantal geëncapsuleerde cellen, inderdaad insuline uit als reactie op veranderingen in de concentratie van glucose.

In **Hoofdstuk 7** worden de belangrijkste conclusies van dit proefschrift gepresenteerd en daarnaast worden interessante richtingen voor toekomstig onderzoek beschreven voor de verdere verbetering van de ontwikkelde macroencapsulatie devices.

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List of publications

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Katarzyna Skrzypek, Yazmin Brito Barrera, Thomas Groth, Dimitrios Stamatialis. Endothelial and beta cell composite aggregates for improved function of a bioartificial pancreas encapsulation device. *Submitted*

Katarzyna Skrzypek, Milou Groot Nibbelink, Lisanne P. Karbaat, Marcel Karperien, Aart van Apeldoorn, Dimitrios Stamatialis. An important step towards a prevascularized islet macroencapsulation device - Effect of micropatterned membranes on development endothelial cell network. *In preparation*

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