A journey towards an extrahepatic islet delivery device with a tissue engineering toolbox in hand

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A JOURNEY TOWARDS AN EXTRAHEPATIC ISLETS DELIVERY DEVICE WITH A TISSUE ENGINEERING TOOLBOX IN HAND

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Summary

Type one diabetes affects 542'000 children per year worldwide and heavily worsens the quality of life of these young patients and their families. In addition to this, diabetes also accounts for substantial costs on national healthcare systems.

An emerging treatment for managing unbalanced glucose metabolism in type one diabetes patients is clinical islet transplantation. This procedure has been developed as a less invasive alternative to total pancreas transplantation with the aim of reaching independence from insulin injections in patients.

Islet transplantation into the portal vein however has heavy limitations and research has focused on the creation of alternative transplantation site that can overcome these limitations and provide a more favorable environment for islets to reside in.

In most cases, devices are used to provide a vehicle for islet transplantation and to contain them *in situ*. These devices also offer the possibility of providing proteins and growth factors for increasing islet viability and functionality after implantation, to ameliorate revascularization and oxygen supply and in general to recreate the most optimal condition for islet metabolism.

In this thesis, several strategies and scaffold design have been investigated for islet transplantation. The performance of the embedded islets has been evaluated *in vitro* and some critical parameters of the scaffold design have been identified as potential predictors of islet functionality *in vitro*.

The **general introduction** provides background information about this disease, its complications and the currently available methods for managing hyperglycemia.

Chapter 1 develops a model system resembling human islet functionality that will be used as a pseudo-islet substitute in some experimental procedures requiring large amount of islets. In the “**hydrogel section**”, two strategies involving the use of an hydrogel matrix for islet embedding are investigated. **Chapter 2** is focused on the fabrication of a two-layer hydrogel construct with specific functionalization of the two layers to achieve islet embedding and induction of blood vessels ingrowth.

Chapter 3 describes the use of a 3D printing device for creating porous hydrogel scaffolds for islet transplantation.

In the “**polymer section**” the approaches described are focused on the use of thermoplastic polymers as main constituents of the scaffold for islet transplantation. In **Chapter 4** a porous structure for islet embedding is obtained by salt leaching processing. **Chapter 5** describes a scaffold design for the creation of an extra-hepatic islet transplantation site based on the combination of hydrogel core and polymeric outer structure in a hybrid configuration. In **Chapter 6** a proof of concept is given for the functionalization of the polymeric surface with a layer-by-layer strategy to induce cell transfection on the scaffold surface.

Finally, in the **General Discussion** overall conclusions about the main findings of this thesis are drawn and future recommendations are given about important parameters to consider in scaffold design.

Nederlandse samenvatting

Ongeveer 542000 kinderen worden jaarlijks wereldwijd getroffen door Diabetes type 1. De ziekte heeft een grote invloed op het kwaliteit van leven van zowel de patient als hun familie. Hiernaast brengt de levenslange behandeling van deze aandoening een aanzienlijke toename van de gezondheidszorg kosten mee.

Een opkomende behandelingsmethode om de ongecontroleerde bloedsuiker spiegels weer in balans te brengen is transplantatie van de eilandjes van Langerhans. Deze procedure is ontwikkeld als een minimaal invasieve alternatieve interventie voor een totale alvleesklier transplantatie. Het heeft als doel om patienten die normaal gesproken dagelijks insuline moeten inspuiten weer insuline onafhankelijk te maken.

Eilandjes transplantatie via de poortader in de lever heeft echter een aantal behoorlijke beperkingen. Onderzoek op dit gebied richt zich vooral op het vinden van een alternatieve transplantatie lokatie welke de nadelen kan verminderen, door een optimale omgeving voor de eilandjes te creëren. In de meeste gevallen worden implantaten hiervoor gebruikt die als een drager voor de cellen kunnen functioneren. Deze dragers bieden de mogelijkheid om specifieke eiwitten, zoals groeifactoren, die de overlevingskansen en functie kunnen verhogen, of nieuwe bloedvat vorming en daarmee zuurstof toevoer kunnen stimuleren, en om in generieke zin de nabije omgeving van beta cellen te optimaliseren na implantatie.

In dit proefschrift, worden verschillende ontwerpen voor implantaten voor eilandjes transplantatie bestudeerd. De werking van de eilandjes in deze implantaten wordt onderzocht in een gecontroleerde laboratorium omgeving en belangrijke parameters voor het implantaat ontwerp worden geïdentificeerd als mogelijke voorspellers voor eilandjes functie.

De **algemene introductie** bevat achtergrond informatie over de aandoening, de bijkomende complicaties, en de huidige behandelingsmethoden voor het controleren van hoge bloedsuikerwaardes. **Hoofdstuk 1** beschrijft een model systeem dat gebruikt kan worden als vervanger voor humane eilandjes in

experimenten waar grote hoeveelheden eilandjes nodig zijn. In de **hydrogel paragraaf**, worden twee strategieën onderzocht voor eilandjes inkapseling met behulp van een hydrogel matrix. **Hoofdstuk 2** richt zich op het maken van een 2-laags ontwerp waarin elke lag een specifieke functie heeft als drager voor eilandjes en voor het stimuleren van nieuwe bloedvatvorming. **Hoofdstuk 3** beschrijft het gebruik van een driedimensionale printer voor het opbouwen van poreuze hydrogel implantaten voor eilandjes transplantatie.

In de **polymeer paragraaf** zijn verschillende manieren beschreven gericht op het gebruik van thermoplastische polymeren als de basis voor implantaten voor eilandjes transplantatie. In **hoofdstuk 4** wordt een poreuze structuur gemaakt met behulp van het uitwassen van zoutkristallen. **Hoofdstuk 5**, beschrijft een ontwerp waarin een hydrogel wordt gecombineerd met een thermoplastisch polymeer als een hybride ringstructuur die gebruikt kan worden voor extrahepatische eilandjes transplantatie. In **hoofdstuk 6** wordt een werkend ontwerp beschreven voor het activeren van polymeer oppervlaktes door middel van een gelaagde opbouw die transfectie van cellen kan bewerkstelligen door de drager.

Als laatste, in de **algemene discussie**, worden de algemene bevindingen en aanbevelingen voor de toekomst en de belangrijkste parameters voor het ontwerp van een implantaat besproken

General Introduction and Thesis Outline

Diabetes mellitus

Causing 5 million death in 2015, diabetes is and remains one of the major cause of death worldwide and one of the most critical healthcare emergencies of 21th century. Only in Europe, it is estimated that 59.8 million people are affected with this disease (1). To these, an additional 23.5 million people are estimated to have an undiagnosed form of the disease, while other 31.7 million are estimated to live with an impaired glucose metabolism condition (1), which can be preliminary to the development of diabetes.

In Europe, diabetes prevalence in 2015 is of 9,1%, leading to an expenditure of 158 billion dollars (1). The problem is also increased by the progressive ageing of worldwide population, which will be responsible for a further increase in the diabetes prevalence in the coming years.

Overall, diabetes is a metabolic disease that impairs glucose metabolism mechanisms. Glucose cannot be used as an energy source for cells, resulting in too high glucose level in the blood (2). Aetiologically, diabetes can be subdivided in different types: type 1, type 2 and gestational diabetes (2).

Type 1 diabetes is an autoimmune disease where insulin and insulin producing β -cells are destroyed by a T-mediated autoimmune mechanism (3). T-cell infiltration in islet of Langerhans has been widely documented in type one diabetes and T-cell directed immunosuppressive drugs has been shown to delay the progress of the disease. T-cell damage to β -cells occurs both through a direct and indirect mechanism. In the direct process, a specific region of the HLA class II responsible for antigen presentation is involved in susceptibility to the disease. In the indirect mechanism, polarized T-helper lymphocytes secrete a panel of cytokines which can turn the balance towards the pro-inflammatory state or a non-destructive (benign) insulinitis in case the equilibrium is shifted towards Th2 cells (3). 5 to 10% of all diabetes cases fall in the type one diabetes. In addition to T-cell reactivity and secretion of pro-inflammatory cytokines, also autoantibodies are developed against insulin, insulin producing beta-cells and some of the enzymes involved in glucose metabolism, like glutamic acid decarboxylase and subunit IA-2 and IA-3 β of

tyrosine phosphatase (4). Being an auto-immune disease, type one diabetes is mainly developed already at young age.

Type 2 is the most common form of diabetes, accounting for almost 90% of the total cases (5) and it occurs mainly in adults. Type two diabetes is more influenced by risk factor than by genetic predisposition. Type two patients are characterized by the development of resistance to insulin (4). Over time, insulin production becomes insufficient to sustain a proper glucose metabolism.

Gestational diabetes causes an elevated blood glucose level during pregnancy, with onset between the 24th and the 28th week of pregnancy (6). Woman affected by gestational diabetes have higher risk of high blood pressure and foetal macrosomia, moreover babies born to mother with gestational diabetes show a higher risk to be affected by type 2 diabetes in their adulthood (6).

Diabetes poses a higher risk for affected people to develop a variety of systemic, health-threatening complications, mainly caused by a sustained high blood glucose level.

Common complications involve retinopathy and blindness (7), cardiovascular diseases like myocardial infarction, angina, peripheral artery disease and heart failure (7,8). Diabetes leads to damage to arteries and small blood vessels (7) and in most cases also leads to impaired renal functionality and chronic kidney disease (9). Moreover, constantly elevated blood glucose levels are also responsible for neuropathies, leading to loss of sensation in the peripheral nerves (7), that together with a compromised blood flow to the limbs lead to ulcerations and infections, in the worst cases requiring amputation (diabetic foot) (10).

Most frequently, type 1 diabetes is managed by a palliative therapy consisting in insulin administration via injections or insulin pumps (11). In these cases the compliance of the patients in carefully screening their glycaemia is of pivotal importance for the success of the therapy. In the most severe cases, pancreas transplantation restores proper insulin secretion in type 1 diabetes patients (12).

Clinical islet transplantation

Being islets the active unit in the pancreas for insulin secretion, an alternative procedure to pancreas transplantation has been developed, consisting in transplanting isolated allogeneic islets of Langerhans by injecting them into the portal vein of the recipient (13). The first attempt was performed in 1972 by Lacy, which successfully reversed diabetes in rodents (14). In 1990 insulin independence for one month duration was achieved by Scharp and co-workers (15) after the first allogeneic islet transplantation. Clinical islet transplantation procedure became significant after 2000, when Shapiro and co-workers reported the case of seven patients which remained insulin independent for 11 months after islet infusion (16). This remarkable result was achieved also by the introduction of a specific glucocorticoid-free immunosuppression regimen, so called Edmonton protocol (17), and a method for isolating islet from the donor pancreas using a dedicated enzymatic and mechanical digestion of the donor pancreas (18), followed by islet injection in the portal vein of the recipient.

Figure 2: schematic of clinical islet transplantation procedure into the portal vein.

Limitations of clinical islet transplantation

Although initially regarded as very promising, the results of Shapiro and co-workers have shown to be less than optimal in a long term post-transplantation evaluation

(13). In a five years post-transplantation period, only 10% of the treated patient remained insulin independent and in 64.3 % of all cases multiple islet injections were required to maintain insulin independence (13,19).

Several reasons have been appointed as responsible for islet loss of activity after transplantation, but some of them already affect islet viability and functionality immediately after the procurement of the donor pancreas (20). Cold ischemia time is critical in determining the viability of the retrieved tissue, and must be kept to a minimum (21). During isolation, islets are exposed to collagenase and the digestion of the extracellular matrix, depleting them of those peculiar proteins which are essential in defining islet environment and their functionality (20). In addition to this, also vascular network within the islet is lost during isolation (22). The vast majority of the transplanted islets are lost in the immediate phases after transplantation because of instant blood mediate immune response (IBMIR) and complement activation (23-25). In addition, also specific immune mechanisms mediated by the re-activation of T-cells against insulin epitopes and specific pro-inflammatory cytokines can cause a decrease in the beta-cell mass in a long term (26).

The loss of vascular network within an islet is the major cause of ischemia, which is most likely the most important contributor to islet loss of functionality after transplantation (22). Islet transplanted into the portal vein remain avascular for several weeks after transplantation. Eventually most of the transplanted islet are incorporated within the vein walls, but this process may last even weeks. During this variable period of time, islets have to solely rely on nutrient and oxygen diffusion to survive. An hypoxic environment stabilizes the transcription factor HIF-1 α , involved in the apoptosis cascade and causes impaired glucose sensing and islet death (27).

To overcome this major limitation in islet transplantation, strategies to accelerate transplanted tissue revascularization or to increase nutrient and oxygen supply to the islets have been investigated. These strategies are based a) on supply of growth factors to increase revascularization or b) on co-transplantation of islet with

pro-angiogenic growth factors (28-31), endothelial cells (32-34) or mesenchymal (stem) cells (35) to increase revascularization rate .

In addition to the aforementioned reasons, islet transplantation in the liver is also hampered by the drug metabolism which takes place in the liver parenchyma and can additionally damage the metabolic activity of the transplanted tissue. For these reasons, the option of transplanting islet of Langerhans in alternative, extra-hepatic sites and the creation of protective environments by using polymeric bioengineered scaffolds has gained increased attention in the community.

Extrahepatic transplantation sites

Although well vascularized thanks to a double arterial and a venous blood supply, liver is still not the most optimal transplantation site (36-38) and oxygen tension remains below the one of pancreas (37). In order to avoid the limitations associated with islet transplantation into the portal vein, several authors have proposed the investigation of alternative sites. The site selection has become of increasing importance since the site selection can determine the efficiency of transplantation.

Kidney capsule: widely used transplantation site in rodents, requires a limited amount of endocrine mass to reverse diabetes, about 12.5 to 25 % islet mass (37). Additional advantage is that in rodents the kidney capsule can be easily retrieved and processed for histological evaluation. However the oxygen tension in the renal capsule is limited compared to pancreas. In contrast to the rodent model, islet transplantation into the kidney capsule in human is very difficult and associated with co-morbidities, which can lead to nephropathy (37,38). Overall, islet transplantation in the kidney capsule remains an experimental model suitable for rodents, but with difficult translation to clinical human applications.

Spleen: offers a very comparable environment to islets native environment. Islets can be either injected directly into the spleen or infused via the splenic vein, in a similar manner to intra-hepatic infusion. Overall, however, this site does not offer any clear advantage on hepatic parenchyma (37,38).

Pancreas: being the native environment for islets, pancreas would offer an ideal oxygen saturation to transplanted islet (37). Islet injection in the pancreatic parenchyma showed a lower critical islet mass to be required to reverse diabetes in comparison with other transplantation sites (37). However, important limitations need to be considered for clinical translation in human such as the liberation of pancreatic enzymes during the procedure, which could hamper pancreatic functionality and the recurrence of autoimmunity towards transplanted islets.

Intraperitoneal transplantation and omentum pouch: gives the main advantage to allow transplantation of tissue in large volumes and it is ideal in case scaffolds or devices are used to provide for islets containment (37). However, a higher islet critical mass is required to reverse hyperglycaemia (14,37,38). Also, this site suffers the lack of parasympathetic innervation, which results in abnormal glucose tolerance tests.

Gastrointestinal wall: newly suggested and not yet thoroughly studied location that offers several advantages as it is easily accessible in laparoscopic procedure and it offers fast glucose sensing, being the intestine the physiologic entry point of glucose in the body. Studies of Tchervenivanov et al (39) demonstrated that islet were extensively revascularized in two week time after transplantation.

Intramuscular and subcutaneous site: intramuscular islet transplantation is a convenient site which offers the possibility of easy monitoring the transplanted tissue by means of biopsy (37,38). This site is routinely used in case of parathyroid autotransplantation (40). the main limitation of this site is the limited oxygen tension, which led to unsuccessful transplantation even when hyperbaric oxygen post-transplantation treatment was applied (41). From an immunological perspective, in human transplantation some authors claim an higher leucocyte infiltration if compared to other locations (42). To overcome low oxygen tension and slow revascularization the transplanted islets needed to be associated with growth factors to induce faster revascularization of the tissue (43,44). Although published with good results in rodents this approach has not been yet thoroughly investigated in primates and humans.

Epididimal and mammary fat pads: the main advantages in transplanting islet into the fat pads is the extensive vascularization of these sites and the comparability to vascularization of the omentum in human (37). Moreover, given the copious vascularization of the site, a lower critical islet mass is necessary to reverse diabetes in mouse (37) .

Bone marrow: represents a favourable site for islet transplantation, providing abundant revascularization and showing promising results in rats and mice. Some authors also claimed it to be an immunoprivileged transplantation site, since isografts and allografts survived for 21 days post-transplantation in a rat model (45).

Figure 2: overview of possible transplantation sites for islet of Langerhans. Adapted from Merani et Al. (37)

Strategies for inducing vascularization in extra-hepatically transplanted islets of Langerhans

A recurrent limitation that arises in bioengineered scaffolds for the transplantation of islet of Langerhans and, more in general, of any other cell type of interest, is the lack of fast revascularization of the construct. This problem is more relevant if the

scaffold is meant to host a clinically relevant amount of cells and it affects particularly the cells embedded in the most inner area of the scaffold, because it is the last one to be reached by newly formed blood vessels. Given the high metabolic rate of islet of Langerhans, this problem is of particular importance in case of clinical islet transplantation.

An increasing attention emerged in the last years on this aspect and several strategies have been investigated in order to increase revascularization of transplanted islets as such or by embedding them in bioengineered constructs.

An approach by Johansson (46) consists in coating islet of Langerhans with human mesenchymal stromal cells and with endothelial cells and improving in this way their ability to initiate neovascularization *in vivo*. The same approach has been tried by other authors using a combination of endothelial progenitor cells and/or bone marrow derived hMSC with similar promising results (32,47,48).

Another common strategy for inducing faster islet revascularization is to attract and enhance proliferation of endothelial cells by using growth factors. Several approaches have used VEGF, bFGF and PDGF both as bolus administration or bound to scaffold for a controlled release to attract endothelial cells ingrowth (28).

Phelps showed in 2013 a peg-maleimide functionalized hydrogel presenting VEGF and adhesive peptides which could improve hydrogel revascularization and restore normoglycemia in diabetic mouse (49). This already promising result was even more significant because normoglycemia was achieved with a reduction of 40% on the required number of islets (49). Kaufman and co-authors have shown a porous PLGA/PLLA scaffold for subcutaneous transplantation of islet together with HUVEC and human foreskin fibroblasts. In this study, the scaffold was pre-vascularized *in vitro* before implantation for a period of 5 day (50). Stendhal *et al.* have used a similar approach using self-assembling heparin binding peptides to form nanofibers capable of binding VEGF and FGF2 and increasing significantly the amount of blood vessels in mouse omentum and islet engraftment (51).

Balamurugan *et al.* have fabricated a polyethylene terephthalate bag loaded with gelatin microspheres for the control release of VEGF and normoglycemia was achieved for more than 35 days in Sprague-Dawley rats (43).

A final, less common strategy that poses consistent concerns in terms of safety, is transfection of cells for induction of stable expression of growth factors in a more physiological rate in an attempt to increase revascularization (52,53).

Regardless of the strategy used to increase islet revascularization, most authors have acknowledged the importance of extensive and fast revascularization of the transplanted islet as an essential factor to preserve their functionality after transplantation.

Aim of the thesis

This thesis investigates possible strategies for creating a device for extra-hepatic islet transplantation, improve their nutrient supply, revascularization and oxygenation by using a panel of tissue-engineering tools. The leading idea behind this project was the creation of a more favourable extra-hepatic transplantation site for islet of Langerhans which could offer some improvement to the current state of the art.

During this research work, important intrinsic characteristics of the scaffold design have been found to be essential parameters that determine islet viability and functionality during *in vitro* studies and after *in vivo* implantation. These parameters dictated boundaries and directed the design of the concepts presented in this thesis work.

Chapter 1 presents the creation of a functional pseudo-islet β -cell cluster to be used as a model for studying islet of Langerhans response to glucose stimulation when embedded in three dimensional scaffolds.

The first section of the thesis is dedicated to the use of hydrogel material as an embedding matrix for islet confinement and transplantation: **Chapter 2** investigates the creation of a hydrogel scaffold based on PEGDA and extracellular matrix molecules for embedding of islets and faster induction of vascularization. A layer by layer structural approach separates the two functionalities in distinct zones of the same structure. **Chapter 3** explores the use of a 3D fiber deposition device for the fabrication of a porous hydrogel construct, with the aim of increasing oxygen and nutrient diffusion to the transplanted tissue.

The next section of the thesis has its main focus on the use of solid thermoplastic polymers and their functionalization as a strategy to facilitate blood vessels ingrowth in close proximity to the transplanted islets. In **Chapter 4** a porous salt leached polymer disk act as a matrix for islet transplantation. Also in this case a double layer approach separates areas of the scaffolds in which islet are embedded and areas where blood vessels ingrowth should occur. This strategy

allows the matrix to specifically present peculiar properties favourable to the specific cell type embedded. **Chapter 5** explores a similar concept, but in this case a combination of thermoplastic polymers and hydrogel is used in an hybrid device for islets embedding in hydrogel and for faster induction of blood vessel ingrowth on a thermoplastic polymer surface. **Chapter 6** presents a concept in which surface functionalization of the polymer structure is used to create a transfection system. Here a pilot version is presented, where cells are transfected to induce the production of green fluorescent protein, but the same strategy can be applicable to any protein or growth factor capable of stimulating blood vessel ingrowth toward the graft area.

In the **conclusion section**, a general overview of the general recommendations found in each study is given which can help identifying the most important parameters in the scaffold design which must be taken into consideration when engineering an extra-hepatic transplantation site.

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Chapter 1

Theophylline Improves Glucose Induced Insulin Secretion in MIN6 and INS1E Pseudo-Islets

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Abstract

In vitro research in the field of Type I Diabetes is frequently limited by the availability of primary cells. In order to avoid the limitations arising by the use of human donor material, cell lines represent a valid alternative. In literature many different beta-cell lines have been reported, but the lack of reproducible response to glucose stimulation remains problematic. In this work, we present an *in vitro* protocol for the functionalization of MIN6 and INS1E beta cells in pseudo islets demonstrating high and reproducible response to glucose stimulation by addition of theophylline to assay buffers. This response was dose- and cell line dependent resulting in a minimal stimulation index of 5 and rapid return to base-line insulin secretion by reducing glucose concentrations after a first high glucose stimulation. In conclusion, complementing glucose stimulation buffers with theophylline is an effective strategy to obtain reproducible and physiologically relevant glucose responses in INSE1 and MIN6 beta cell lines.

Introduction

In vitro research in the field of Type I Diabetes is frequently limited by the availability of primary cells due to donor shortage. In addition, islet isolation and their transport to the research facility affect the viability and functionality of the donor material. Moreover, another common problem for the *in vitro* testing of human islets is represented by the donor variability, which limits the comparison between different donors and different sets of experiments. All these limitations suggest the need of a reliable and easily available method to produce pseudo-islets for *in vitro* research purposes. For this reason many different cell lines have been created over the last decades (1). Of all these beta cell lines, mouse insulinoma MIN6 and rat insulinoma INS1E cell lines best reflect the physiological conditions as both cell lines are responsive to glucose stimuli and they both express glucokinase (1). The MIN6 cell line originates from a transgenic C57BL/6 mouse insulinoma (2), while INS1E cells were generated from rat insulinoma induced by X-ray irradiation (3). One major issue still, is the reproducible responsiveness to glucose stimulation. In particular a low-high-low insulin release profile needs to be detected in response to low-high-low glucose stimulation, as seen in functional islet of Langerhans and depicted in figure 1, top left graph (4). This is often not the case for both INS1E and MIN6 cells, as their insulin secretion does not return to basal level after stimulation with low glucose for the second time (figure 1, bottom left). Therefore either just the stimulation index or only the amount of insulin secreted in the first low glucose stimulation is shown in literature (5-8). The stimulation index is a measure to express islet functionality. It is defined as the amount of insulin secreted under high glucose stimulation, divided by the basal insulin secreted in low glucose conditions. For islets of Langerhans, a threshold stimulation index of at least 2 defines a functional response and often these cell lines do not reach this threshold level nor display a reproducible behavior (9).

In adult islets insulin secretion is a complex process and a schematic version is presented in figure 1. When the extracellular glucose rises, glucose enters the beta cell via the GLUT2 glucose transporter. Glucose is phosphorylated by a glucokinase, enters in the glycolysis and leads to an increased concentration of

ATP. This increase of ATP closes the K^+ channels and consequently depolarizes the plasma membrane of the beta cell. This makes an influx of Ca^{2+} possible via the voltage dependent L-type Ca^{2+} channel. The influx of Ca^{2+} induces insulin secretion from the secretory granules (1,10-13).

Mostly in the field of fetal and neonatal islets ways to functionalize cells to secrete insulin upon glucose stimulation are explored. It is known that these cells hardly secrete insulin upon glucose stimulation (14-16). Different components have been tested which act on different molecules/channels in the insulin secretion pathway, like leucine, glipizide, theophylline, nicotinamide, and sodium butyrate (14-17). Theophylline, a methylxanthine, is already described to enhance insulin secretion by stimulation of cAMP (14,15,17-23). Theophylline inhibits phosphodiesterase activity and thus increases intracellular cAMP (14,19,22,23). cAMP binds protein kinase A (PKA), thereby inducing a conformational change and releasing the two inhibitory subunits. Such an activated PKA opens the voltage dependent Ca^{2+} channel and allows increased Ca^{2+} flux within the cytoplasm. Adding theophylline to glucose buffers has already been applied in primary, fetal and neonatal islets as well as for the administration in Type I Diabetes patients, to enhance their responsiveness to glucose stimulation (14,15,17,19,20) but the effect of theophylline has not been examined using INSE1 and MIN6 cell lines. In this study, theophylline was applied to increase functional response of MIN6 and INS1E beta cell lines, both in monolayer and in an aggregate configuration to mimic islets of Langerhans response.

Research has already shown that the response to glucose stimulation differs between monolayers and cell clusters (4,24-27). In the case of primary islets there is an impaired insulin secretion in dispersed islets compared to intact islets. However, upon reaggregation insulin secretion is enhanced again (4,24,27). Research on beta cell lines, like MIN6 cells, also show significant enhanced insulin secretion when cell clusters, or so-called pseudo-islets, are formed compared to monolayers (4,24,27,28).

With the addition of theophylline, we show that MIN6 and INS1E aggregates cells become responsive to glucose stimulation in a reproducible manner and show stimulation indices > 5 and a low-high-low insulin secretion profile. Additionally, we show a dose dependent and cell-line dependent response. Since previous research has already shown that insulin secretion is enhanced in pseudo-islets compared to cells in monolayer, our protocol was developed on MIN6 and INS1E pseudo-islets.

Materials and Methods

Cell culture

INS1E rat insulinoma cells (provided by Dr. B. Guigas, LUMC, Leiden, the Netherlands and Dr. P. Maechler, University Medical Center, Geneva, Switzerland) were cultured in RMPI (Gibco) with 2.05 mM Glutamax (Invitrogen) supplemented with 5% (v/v) fetal bovine serum (FBS, Lonza), 100 U/mL penicillin and 100 mg/mL streptomycin (Gibco), 10mM HEPES, 1 mM sodium pyruvate, and 50 μ M freshly added beta-mercaptoethanol (Gibco) at 37°C and 5% CO₂. Mouse insulinoma MIN6-B1 cells (provided by Dr. P. Halban, University Medical Center, Geneva, Switzerland) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% (v/v) FBS (Lonza), 100 U/mL penicillin and 100 mg/mL streptomycin, and 70 μ M freshly added beta-mercaptoethanol (Gibco) (37°C, 5% CO₂).

Agarose microwell fabrication and controlled pseudo-islet formation

For controlled pseudo-islet formation, cells were cultured in sterile agarose microwells. These agarose microwells were fabricated as described previously (29). In short, polydimethylsiloxane (PDMS) negative molds containing micro pillars (200 μ m) were sterilized using 70% ethanol. A 3% agarose (UltraPure™ Gibco Invitrogen) solution in PBS was heated to 100°C in a microwave oven. PDMS molds were placed inside 6 wells plate and filled with 6 mL of 3% agarose solution. Air bubbles were removed by centrifuging the plates at 300g for 1 min. Solidification of the agarose was established by storing the plates at 4°C for at least

30 min. Next, the molds were removed from the agarose using a sterile spatula. The agarose chips were punched out leaving a thin agarose wall on all sides to fit into a 12 wells plate. Stable pseudo-islets were then created based on the work of Hilderink et al (30). MIN6 cells or INS1E cells were then seeded onto the agarose chips (250 cells per aggregate). The plates were centrifuged at 150g for a maximum of 1 min and 1 mL of medium was carefully added to the chips. After 48h at 37°C pseudo-islets were flushed out of the chips.

Glucose induced insulin secretion test

A tailor made Krebs buffer (115 mM NaCl, 5 mM KCl, 24 mM NaHCO₃ Sigma) supplemented with 2.2 mM CaCl₂, 20 mM HEPES (Gibco), 2 mg/mL bovine serum albumin, and 1 mM MgCl₂ was prepared at pH 7.4 with different concentrations of theophylline (Sigma) (concentration range from 0.1 mM to 20 mM)). Subsequently, the buffer was split into low (1.67 mM) and high glucose (16.7 mM). Cells were washed three times in low glucose buffer followed by a pre-incubation of 90 min in low glucose buffer. Cells were stimulated for 45 min in subsequent low, high and low glucose buffer with a three time wash step in low glucose between the high and second low. Samples were taken after each incubation, spun down (300g, 3 min) and the supernatant was stored at -20 degrees Celsius. Samples were analyzed using an insulin ELISA (Mercodia) specific for rat insulin in case of INS1E samples or mouse insulin in case of MIN6 samples.

The effect of theophylline on single cells insulin secretion

MIN6 cells were seeded in a 12 well plate at a cell density of 30.000 cell/cm². After 48h, to allow cell attachment, insulin secretion upon glucose stimulation with the addition of theophylline (10 mM) in both low (1.67 mM) and high glucose (16.7 mM) Krebs buffers or only the high glucose (16.7 mM) Krebs buffer was tested. Insulin secretion was quantified by means of ELISA assay, as described above.

The effect of theophylline on MIN6 and INS1E viability

MIN6 and INS1E pseudo-islets were prepared as described above. Per condition approximately 1400 pseudo-islets were placed in an ultralow attachment 48 well

plate (Corning). They were washed three times with a low glucose Krebs buffer (1.67 mM). Placed for 5h in a low glucose buffer with different theophylline concentrations (0, 5, 10, and 20 mM). After 5h the pseudo-islets were washed and stained with live/dead staining (Invitrogen). The 5 hours incubation time was chosen to match the average duration of a glucose induced insulin secretion test. Phase contrast and fluorescent images were taken by an EVOS fluorescent microscope.

The effect of theophylline on the metabolic activity of MIN6 and INS1E cells

MIN6 and INS1E pseudo-islets were prepared as described above. Per condition approximately 950 pseudo-islets were seeded in a 96 transwell plate (40 μ m, Millipore). A Presto blue assay (Invitrogen) was performed following manufacturer's protocol (1.5h incubation) to determine their basal level of metabolic activity, before theophylline treatment. After the first presto blue, the pseudo-islets were washed three times in low glucose buffer with different theophylline concentrations (0, 5, 10, and 20 mM) and incubated for 5h in the same low glucose buffer with added theophylline. After these 5h of incubation, a second presto blue assay was performed (1.5h reagent incubation).

Theophylline concentration dependent insulin secretion of MIN6 and INS1E pseudo-islets

MIN6 and INS1E pseudo-islets were prepared as described above. Per condition approximately 285 pseudo-islets were seeded in a 96 transwell plate (40 μ m, Millipore). Six theophylline concentrations were tested (0 mM, 0.1 mM, 0.5 mM, 1 mM, 5 mM, and 10 mM) by dissolving the theophylline in both low (1.67 mM) and high (16.7 mM) glucose Krebs buffers. Insulin secretion upon glucose stimulation was tested as described above.

Statistical analysis

One-way ANOVA statistical analysis was performed followed by a Bonferroni post-hoc test. Data is expressed as mean $\pm$ standard deviation and significant

differences are indicated with * ($p \leq 0.05$). The analysis was performed using IBM SPSS statistic 20 software.

Results

The effect of theophylline on insulin secretion

Figure 2 shows the insulin secretion of MIN6 cells when stimulated with glucose. Cells were either stimulated with standard glucose buffers (1.67 mM (low) and 16.7 mM (high)) or with the same glucose buffers with addition of 10 mM theophylline. Theophylline was added either in all buffers (T) or just in the high glucose buffer (T(hg)). Figure 2B shows the differences in stimulation indices between the different groups. The control group appeared to be non-functional whereas both theophylline groups exhibited functional insulin secretion profiles. Furthermore, no difference was seen in insulin secretion patterns between the two different theophylline groups. Although the low-high-low profile was visible when using theophylline compared to the control, only a marginal improvement in stimulation index was seen. As these experiments were performed on monolayers, and the beneficial effect on function of cell aggregation has already been described in literature, further experiments were conducted with pseudo-islets (4,24,27).

Figure 1: cartoon illustrating the expected insulin release profile upon glucose stimulation of islet of Langerhans (top left) and what is usually obtained by stimulating INS1E and MIN6 aggregates (bottom left). The insulin release pathway upon glucose stimulation is depicted. Theophylline inhibits the phosphodiesterases, thus increasing intracellular cAMP concentration and stimulates insulin secretion. The resulting insulin secretion profile of INS1E and MIN6 aggregates after theophylline addition to the glucose buffer is illustrated in the right graph.

Figure 2: The effect of theophylline on insulin secretion. Insulin secretion (ug/l) (A) and stimulation index (B) of MIN6 cells cultured on tissue culture plastic (monolayer) without theophylline, with theophylline 10 mM in all the incubation buffers (T) and with theophylline 10 mM added only in the high glucose buffer (T(hg)). After addition of theophylline MIN6 monolayer exhibited a functional insulin release profile in response to glucose stimulation, regardless of whether theophylline was added to all the buffers, or only in the high glucose buffer.

The effect of theophylline on MIN6 and INS1E viability

Aggregation of cells has shown to enhance functionality for both primary islet cells and beta cell lines (4,24,27). Stable pseudo-islets of both MIN6 and INS1E cells were created following the protocol recently published by Hilderink et al (30). Each pseudo islet contained 250 cells on average. The pseudo islets were homogeneous in size (figure 3). Pseudo-islets of MIN6 or INS1E cells were incubated with glucose buffers containing different concentrations of theophylline for the duration of a glucose induced insulin secretion test (5h). Afterwards cells were stained with calcein and ethidium homodimer to assess cell viability (figure. 3). In both MIN6 and INS1E pseudo-islets aggregation did not influence cell viability and no differences were seen between the different concentrations of theophylline. In all the conditions viability was overall higher than 95%, comparable with 0mM control condition.

Figure 3: The effect of theophylline on MIN6 and INS1E viability. Live/dead images of MIN6 (left) and INS1E (right) pseudo-islets incubated with different concentrations of Theophylline. No effect on cell viability was reported also at the highest concentration used. Scale bar 400 μ m.

The effect of theophylline on the metabolic activity of MIN6 and INS1E cells

In addition to the effect of theophylline on cell viability, the effect on metabolic activity was assessed on both MIN6 (figure 4A) and INS1E (figure 4B) pseudo-islets for the duration of a glucose induced insulin secretion test. Figure 4 shows the fold change of the metabolic activity after 5h incubation compared to the initial basal activity. Again no significant effect of theophylline was seen in both cell types

and for none of the concentrations tested, confirming that theophylline exerted no toxic effect on MIN6 and INS1E pseudo-islets, for an incubation time of the duration of a standard function test.

Figure 4: The effect of theophylline on the metabolic activity of MIN6 and INS1E cells. Metabolic activity of MIN6 (A) or INS1E (B) pseudo-islets cultured for 5h in presence of different concentrations of theophylline. Data are presented as percentage of the metabolic activity at $t=0$. No significant difference was seen between the different theophylline concentrations in both MIN6 and INS1E cells.

Theophylline concentration dependent insulin secretion of MIN6 and INS1E pseudo-islets

After establishing that theophylline did not have a negative effect on both MIN6 and INS1E cells with respect to viability and metabolic activity, the effect on insulin secretion was tested in MIN6 pseudo-islets (figure 5) and INS1E pseudo-islets (figure 6). Pseudo-islets were stimulated with different concentrations of theophylline to find the optimal concentration. Functionality is expressed as stimulation index and it is defined as the amount of insulin secreted under high glucose stimulation, divided by the basal insulin secreted in low glucose conditions. Pseudo-islets were considered functional when stimulation indices were above 2, and a decrease in insulin secretion was seen when stimulated with low glucose buffer for the second time (9). Figure 5 shows pseudo-islets functionality of MIN6 cells when stimulated with different concentrations of theophylline. Both the insulin secretion in $\mu\text{g/l}$ (figure 5A) and stimulation indices (figure 5B) are depicted. To compare the efficiency of different theophylline concentrations on cell stimulation, statistical analysis in figure 5 compares the stimulation indices in high glucose

conditions. The MIN6 pseudo-islets clearly showed a dose-dependent insulin secretion pattern. When looking at the insulin secretion ($\mu\text{g/l}$), the maximal effect appeared to be between 0.5 and 5 mM. However, when looking at the stimulation indices, the optimal concentration seems to be 0.1 mM. This was due to a lower insulin secretion when stimulated with low glucose for the 0.1 mM condition compared to the other concentrations of theophylline rather than a lower insulin production under high glucose stimulation, therefore resulting in a higher stimulation index. Despite the remarkable increase in insulin secretion by the addition of theophylline, insulin secretion returned to base line after replacing the high glucose buffer with a low glucose buffer, mimicking physiological response.

*Figure 5: Theophylline concentration dependent insulin secretion of MIN6 pseudo-islets. Dose-response results of the glucose induced insulin secretion test on MIN6 pseudo-islets at different concentrations of theophylline. (A) Secreted amount of insulin ($\mu\text{g/l}$) is shown, in (B) data are normalized by the amount of insulin secreted in low glucose condition (stimulation index). Remarkably, the stimulation index of MIN6 pseudo-islets at higher theophylline concentrations decreases due to a higher insulin secretion in low glucose conditions rather than because of a lower production under high glucose stimulation. Data are expressed as mean $\pm$ standard deviation and significant differences are indicated with * ($p \leq 0.05$).*

Hilderink et al showed an average stimulation index of 1.86 ± 0.7 for INS1E pseudo-islets (30). This is comparable to our control group (figure 5). Compared to the MIN6 pseudo-islets, INS1E pseudo-islets showed a similar insulin secretion pattern as MIN6 pseudo-islets. However, they seemed to be less sensitive to theophylline as they secreted less insulin when stimulated with 0.1 mM of theophylline compared to the MIN6 pseudo-islets (figure 5A). Additionally, no detectable

difference in the stimulation index was seen in the 0.5mM to 10mM range of theophylline concentration. Compared to MIN6 pseudoislets, insulin secretion in INS1E pseudoislets did not decrease as effectively to base line when stimulated with the second low glucose buffer particular at the highest concentrations used.

Figure 6: Theophylline concentration dependent insulin secretion of INS1E pseudo-islets. Dose-response results of the glucose induced insulin secretion test of INS1E pseudo-islets at different concentrations of theophylline. (A) Secreted amount of insulin ($\mu\text{g/l}$) is shown, (B) data are normalized by the amount of insulin secreted in low glucose condition (stimulation index). INS1E pseudo-islet show a higher stimulation index at higher theophylline concentration, but a residual stimulation is seen also in the second low glucose, showing a slower return to basal levels. Data are expressed as mean $\pm$ standard deviation and significant differences are indicated with * ($p \leq 0.05$).

Discussion

In this work, we demonstrated that the addition of theophylline to the glucose stimulation buffer is necessary to achieve a proper stimulation index both for MIN6 and INS1E pseudo-islets.

The generation of functional pseudo-islets for *in vitro* testing has been widely addressed in literature and many authors report aggregation as a way to increase insulin secretion and functionality. Both islets and beta cell lines aggregates showed an increased glucose responsive in an aggregate configuration when compared to their dispersed counterpart (4,24-27). This enhancement in insulin secretion is provided by a specific cell-cell contact mediated by many components such as the ephrin-A5 receptor and the adhesion molecule E-cadherin

(24,25,27,31). Aggregation of pancreatic beta-cells ensured that the basal level of insulin secretion in starvation conditions is reduced, while the glucose stimulated secretion is enhanced (31). An improvement in both INS1E and MIN6 functionality is therefore already provided by the aggregation procedure (5,24). In this work, we show that aggregation itself is not sufficient to generate a functional pseudo-islet, with a stimulation index comparable to native human tissue. As it was shown in figure 5, the stimulation index in control condition, without theophylline stimulation, is below 2 both for INS1E and MIN6 aggregates, which is considered the lower threshold for defining an islet as functional (9). Reproducible stimulation indices and consistent return to low basal level of insulin production upon low glucose 2 condition is still a major issue. Many authors only publish first low and high stimulation, but neglect the importance of return to basal level, which is essential for defining an islet functional (5,7,8). In other papers only the amount of insulin secreted in different conditions were compared among each other, but this does not give any indication about the proper functional response (low-high-low) of the tested cells (6).

With the addition of theophylline, we show that these cells are responsive in a reproducible manner with stimulation indices around 5 and proper return to basal level in low glucose conditions. This return to baseline was more effective in MIN6 pseudo islets than in INS1E pseudo islets. Theophylline, a phosphodiesterase inhibitor, increases the intracellular cAMP levels by blocking its degradation and making it longer available for the increase of cytosolic calcium concentration (32). A mechanism proposed by Malaisse, suggests that theophylline acts by mobilizing an intracellular source of calcium, originally located into the vacuolar system into the cytosol. In absence of glucose, most of the calcium still escapes from the cytoplasm, but the simultaneous presence of glucose blocks this outward flux of calcium (32). This mechanism explains why theophylline's action is dependent on glucose stimulation and does not exert its effect in low glucose conditions, at least at low concentrations (32). In fact, we show a dose dependent response of MIN6 pseudo-islets to theophylline stimulation. In particular for MIN6, the stimulation at a lower dose, induced insulin secretion only in high glucose conditions and did not

induce insulin release in basal medium (low glucose). On the contrary, a higher theophylline concentration (10mM) stimulated insulin release also in low glucose condition, thus lowering the overall stimulation index. For this reason, the action of theophylline on insulin secretion stimulation is glucose-dependent. Our results show that the addition of theophylline has no detrimental effect on cell viability, also when cells are aggregated in pseudo islets and that it has no effect on the cell metabolic activity. This behavior was relatively different for INS1E which showed a lower sensitivity to theophylline action during glucose stimulation and highlighting a cell-line dependent response. More research would be necessary to understand in depth the different behavior of MIN6 and INS1E pseudo-islets: the difference could reside in species-specific characteristics or in a distinct fine-tuning regulation in the cAMP level in insulin secretion mechanism. This difference might be explained by the higher glucokinase activity in MIN6 cells compared to INS1E cells. As shown by Arden and Co-workers, the glucokinase activity in MIN6 cells is higher because of the higher insulin granules content in MIN6 cells (33). Based on these results, we propose the use of 0,1 mM theophylline in glucose induced insulin secretion test of MIN6 aggregates, since in these conditions the functional behavior of islets of Langerhans is best resembled. Theophylline stimulated MIN6 aggregates showed a higher stimulation index and a better return to basal level, upon second low glucose conditions. For INS1E pseudo islets a higher theophylline concentration (5mM) was necessary to significantly increase the stimulation index compared to control but its subsequent return to base line was sub optimal. For in vitro purposes we therefore recommend the use of pseudo islets of MIN6 cells.

Conclusions

This study illustrates an effective method to obtain functional and physiologically relevant responses to glucose in MIN6 and INS1E pseudo-islets by treating them with theophylline. This treatment provided functional pseudo-islets with a stimulation index comparable to islets of Langerhans. We demonstrated that the aggregation procedure and the treatment of the pseudo-islets with theophylline do not influence their viability and metabolic activity. Theophylline's effect was still dependent on glucose stimulation. We show a cell-dependent response to

theophylline and dose-response behavior at different concentrations, where the optimal concentration has been identified. Addition of 0.1 mM Theophylline to MIN6 and 0.5 mM of Theophylline to INS1E pseudo-islets during glucose induced insulin secretion test provides a reliable model for studying islets of Langerhans physiology.

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Hydrogel Section

Chapter 2

Layered PEGDA hydrogel for islet of Langerhans encapsulation and improvement of vascularization

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Abstract

Islet of Langerhans need to maintain their round morphology and to be fast revascularized after transplantation to preserve functional insulin secretion in response to glucose stimulation. To maintain a round morphology, a not cell-adhesive environment is preferable for their embedding. Conversely, nutrient and oxygen supply to islet is guaranteed by capillary ingrowth within the construct and this can only be achieved in a matrix that provide cues for cells to adhere to. In this chapter, two different approaches are explored, which are both based on a layered architecture, in order to combine these two opposite requirements. A non-adhesive islet encapsulation layer is based on polyethyleneglycole diacrylate (PEGDA). This first layer is combined with a second hydrogel based on thiolated-gelatin, thiolated-heparin and thiolated-hyaluronic acid providing cues for endothelial cell adhesion and that additionally acts also as a growth factor releasing matrix. In an alternative approach a conformal PEGDA coating is covalently applied on the surface of the islets. The coated islets are subsequently embedded in the previously mentioned hydrogel containing thiolated glycosaminoglycans. The suitability of this approach as a matrix for controlled growth factor release has been demonstrated by studying the controlled release of VEGF and bFGF for 14 days. Preliminary tube formation has been quantified on the growth factor loaded hydrogels. This approach should facilitate blood vessel ingrowth towards the embedded islets and maintain islet round morphology and functionality upon implantation.

Introduction

The major limitation encountered so far in tissue engineered constructs is the ingrowth of a suitable vascular bed, able to sustain in short time after transplantation the metabolic needs of the newly implanted tissue. This restriction becomes more stringent for clinically relevant implants, containing sufficient amount of cells for restoring a lost function in the body, and when a highly metabolically active organ is considered (1). Islet of Langerhans are organules in the body having one of the highest blood supply. Although islets account only for 1-2% of total pancreatic mass, they receive up to 5-15% of the total pancreatic blood flow (2). In clinical islet transplantation (CIT), islets are injected into the portal vein of the recipient, where they settle and start producing insulin in response to glucose stimulation (3-5). Before injection, islets must be isolated from the pancreatic matrix by an enzymatic digestion that disrupts the extracellular matrix and the associated dense network of capillaries (6). The loss of the islets' vascular bed limits their ability to functionally respond to glucose stimulation and to ultimately achieve a successful transplantation (7,8). Due to islet loss of functionality, long term outcome for islet transplantation in the portal vein is suboptimal and less than 50% of the patient still remain insulin independent 5 years after treatment (9). In addition to poor revascularization, other factors like instant blood mediated inflammatory reaction and complement activation (IBMIR), recurrent autoimmunity (10) and allo-immune response (11) account for islet loss of functionality in the first phases after transplantation.

Many strategies for the creation of vascular beds have been developed by several authors in the past few years. In many of these approaches complex networks are created using 3D printing or microfluidic devices (12). The presented results are remarkable, but these approaches are characterized by difficult applicability to clinical strategies. Some other approaches used elegant but technically complex microlithography techniques (13) which are only applicable to small scale devices.

A more simple, but equally effective approach has been attempted by stimulating blood vessel ingrowth using growth factors embedded in hydrogels (14-18). By

tuning their controlled release in time and their spatial distribution, researchers aimed at recreating the initial phases of physiological angiogenesis. It has been extensively proven that this simple but effective technique is able to induce blood vessels in growth in vivo (17,18). Such an approach has been tried with different hydrogels (15,19-22) but the most effective ones has been demonstrated to be those introducing in their structure heparin, hyaluronic acid or binding sites for growth factor stabilization and controlled release (17,18).

Hydrogels have shown their potential not only for growth factor delivery purposes but have also been extensively used for islet encapsulation. Alginate has historically been the first choice for islet encapsulation, both in micro capsules (23,24) as well for macro chamber devices for artificial pancreas (25). The main purpose addressed by these studies is to have a delivery vehicle for islet transplantation and containment, together with some degree of immunoprotection. The leading hypothesis behind these studies is that a properly tuned hydrogel mesh around the islet could prevent antibodies and cytokines to interact with the embedded tissue and prevent recurrent attack of the immune system to allogeneic transplanted islets (26). Such an approach has the overall aim to dismiss conventional immunosuppression regimen by substituting them with a shielding capsule which could prevent antibodies and cytokines to interact with the embedded tissue (24). Finding a balance between proper insulin and nutrient diffusion to the islets and impeding antibodies and cytokines to permeate through the protection capsule has proven to be a very challenging task. PEGDA hydrogels have the advantage of a very defined and tunable molecular weight and chemical functionalization. Immunoprotection strategies using PEGDA functionalization have been attempted by creating a conformal coating of PEGDA on the islet surface (27-29). Reactive groups like N-hydroxy-succinimide (NHS) or succinimidyl-valeric-acid (SVA) have the potential to react with aminogroups exposed on proteins on islet's surface and create a conformal, single molecule thick coating, on the islet itself (30).

Literature has demonstrated the immunoprotective properties of conformal islet coating by PEGylation, without the need for embedding them in micrometer sized

hydrogel capsules (28). This approach could minimize limitations arising from nutrient diffusion impairments to the islet while maintaining the immunoprotection concept.

This study presents a combination of all the aforementioned concepts, merged into a single multifunctional construct. A combination of PEGDA hydrogels for islet embedding would act as a vehicle for islet delivery and retention, while a PEGDA based layer enriched with thiolated-gelatin and extracellular matrix proteoglycans would offer a cell adhesive environment for enhanced attraction of blood vessels by controlled release of pro-angiogenic growth factors. In a similar approach, the PEGDA layer for islet embedding is reduced to a conformal coating of PEGDA covalently bound to the islet surface proteins. This second approach would attempt to reduce nutrient diffusion limitations by using a thinner PEGDA layer and could at the same time provide immune-protective properties to the embedded tissue. These coated islets are then embedded in the previously mentioned hydrogel containing thiolated glycosaminoglycans for the induction of vascularization.

Distinctive feature of such a construct is the fact that in both cases, the two layers are covalently bound to each other by mean of free acryl groups on the surface of both hydrogels (first approach, figure 1A) or to the islet surface (second approach, figure 1B) creating in this way a unique, multilayered and multifunctional construct for revascularization of transplanted islets.

Materials and methods

Islet of Langerhans and MIN6 cell culture and pseudo-islets formation

Isolated human islets were kindly provided by the LUMC Leiden (after informed donor consent and only if the material had no sufficient activity to be used for clinical transplantation). Islets of Langerhans were cultured in CMRL medium (Cellgro) supplemented with 10% FBS and 1% Pen/Strep (31), 10 mM Hepes (Gibco), 10 mM Glutamax (Gibco) and 10 mM Nicotinamide (Sigma-Aldrich).

MIN6-B1 cells, derived from a mouse insulinoma, were kindly provided by Dr. P. Halban, University Medical Center, Geneva, Switzerland. Cells were cultured in

Dulbecco's Modified Eagle's Medium (DMEM, Gibco) with 10% (v/v) FBS (Lonza), and antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin, Gibco). 70 μ M beta-mercaptoethanol (Gibco) was freshly added at every medium change. Cells were cultured in standard culture conditions at 37 degrees, 5% CO₂ in humidified atmosphere.

MIN6-B1 cells were used for the preparation of cell aggregates as previously reported in our group (32) and as explained in chapter 1 of this thesis. Briefly, cells were cultured in sterile agarose microwells (200 μ m diameter) fabricated by pouring sterile agarose (3% w/v UltraPure™ Gibco Invitrogen) on top of a pre-sterilized, pre-fabricated polydimethylsiloxane (PDMS) mold with micropillars of 200 μ m in diameter. One million MIN6 cells were seeded on each agarose chip and cultured for two days to allow for aggregate formation. So formed pseudo-islet could be easily removed from the chip by flushing it with some medium and used for further experiments.

Islet of Langerhans / MIN6 pseudo-islets embedding in polyethylene glycol diacrylate gels

MIN6 pseudo-islets were resuspended in a sufficient amount of 5% w/v poly(ethylene glycol diacrylate) (PEGDA) with a molecular weight of 5000 (Laysan) in PBS (Gibco). A 10% w/v solution of Irgacure 2959 (BASF) in 70% Ethanol was prepared and 10 μ l added to 1 ml of PEGDA solution prior to crosslink. Each sample was prepared using 50 μ l of the precursor PEGDA additioned with photocrosslinker and contained on average about 1'400 MIN6 pseudo-islets.

For samples containing human collagen IV (Merck Millipore) or human laminin (Sigma-aldrich), a 20 μ g/ml stock solution of protein was prepared in sterile PBS (Gibco) and diluted to the working concentration of 10 μ g/ml by using 10% w/v concentrated PEGDA solution prior the addition of Irgacure (BASF). MIN6 pseudo-islets were resuspended in the solution and 50 μ l samples were pipetted in a 96 wellplate and exposed to 356 nm UV light for 10 minutes, at 5 cm distance from the light source, with an intensity of 8.2 mW/cm².

In case a bi-layered construct was prepared, the 50 μ l PEGDA sample containing aggregates was crosslinked on top of a pre-gelled 50 μ l vascularization gel.

Glucose induced insulin secretion test

Insulin secretion after glucose stimulation was tested on either islets of Langerhans or MIN6 pseudo-islet by incubating them in Krebs buffer (115 mM NaCl, 5 mM KCl, 24 mM NaHCO₃ Sigma) supplemented with 2.2 mM CaCl₂, 20 mM HEPES (Gibco), 2 mg/mL bovine serum albumin, and 1 mM MgCl₂ at pH 7.4. In case of testing MIN6 pseudo-islet functionality, Theophylline was added to the buffer to a final concentration of 10 mM (Sigma). This buffer was split into low (1.67 mM) and high glucose (16.7 mM) buffer. Cells were washed and pre-incubated for 90 min in low glucose buffer. Subsequently, cells underwent a series of 45 minutes incubation in 250 μ l of either low glucose, high glucose and finally again low glucose buffer, alternated by washings. 230 μ l samples were taken after each incubation and the amount of insulin secreted by the cells upon glucose stimulation was quantified by using either a human (in case of human islet) or mouse (in case of MIN6 pseudo-islets) insulin ELISA (Mercodia).

Islet of Langerhans PEGylation and imaging of the conformal PEGDA coating

Islets (1'250 islet equivalent per sample) were washed 3 times in PBS and incubated for 1 hour in a 12.5% w/v solution of NHS-PEG-Biotin (Laysan) in PBS (Gibco) pH 8 additioned with 5 mM glucose. Islets were washed, fixed with 10% w/v formalin solution for 3 minutes and subsequently incubated for an additional hour with a 20 μ g/ml solution of Streptavidin-FITC (Sigma-aldrich) protecting from light (30). PEGylated and control islets were imaged using an EVOS digital microscope (EMS) equipped with an EVOS light cube (EMS) for GFP imaging.

Islet of Langerhans PEGylation and embedding in the vascularization hydrogel

500 islet equivalent for each sample were washed with PBS supplemented with 5 mM glucose and incubated for 1 hour in 350 μ l of a 5% w/v solution of PEG-SVA-Acryl (laysan) in PBS additioned with 5mM glucose (30). At the end of the incubation period, islets were washed and sorted into 96 wells for subsequent

steps in sample preparation. Free floating PEGylated islets were left as such and further cultured in CMRL complete medium. PEGylated islets were also embedded within the vascularization gel by resuspending them in the gelin/heprasil/extralink mixture according to the specification by the manufacturer of the hydrogel kit and allow for spontaneous crosslinking at room temperature prior to medium addition. For functionality testing, PEGylated free floating islets and PEGylated islet embedded in the gel have been compared to untreated free floating islets and to untreated islet embedded in the gel as described before in this section.

Vascularization hydrogel

A commercially available product HyStem®-HP Hydrogel Kit (ESI BIO) was used as a base for the vascularization gel. The kit provides thiol-modified gelatin (Gelin), and a combination of thiol-modified heparin and thiol-modified hyaluronic acid (Heprasil) to be crosslinked in an hydrogel network by a Michael type reaction upon addition of polyethylene glycol diacrylate (Extralink). The hydrogel can be used as a matrix for growth factors delivery and cell attachment (17,18).

Kit components were reconstituted using the provided sterile water, according to the manufacturer's instruction (1ml sterile water in each Gelin and Heprasil vial). Only the Extralink vial was reconstituted at a double concentration than the recommended one (250 µl instead of 500 µl). All components were mixed according to Table 1, taking care of minimizing light and air exposure and adding Extralink as last. Typically, 400 µl of hydrogel precursor were prepared for each condition and 75 µl samples were used in each experiment. Basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) were purchased from Peprotech and reconstituted according to the manufacturer's instruction. Aliquots of 100 µg/ml were further diluted in PBS (Gibco) with addition of 1% Bovine Serum Albumin (BSA, Sigma) to a working solution of 10 µg/ml and used for further dilution upon hydrogel preparation. Hydrogel gelation began immediately after addition of the Extralink crosslinker, and gels were left undisturbed for approximately 1 hour to allow for complete gelation. Final concentration upon gel formation is 1% w/v for all the kit components.

	Extralink (μ l)	Gelin (μ l)	Heprasil (μ l)	PBS (μ l)	bFGF (μ l)	VEGF (μ l)
200ng/ml VEGF	40	160	160	32	-	8
500ng/ml VEGF	40	160	160	20	-	20
100ng/ml bFGF	40	160	160	36	4	-
200ng/ml VEGF+ 100 ng/ml bFGF	40	160	160	28	4	8
500ng/ml VEGF+ 100 ng/ml bFGF	40	160	160	26	4	20

Table 1: hydrogel preparation at different growth factor concentration according to the manufacturer's instructions. bFGF and VEGF working solution are both 10 μ g/ml.

Human umbilical vein endothelial cells culture (HUVEC) and tube formation assay

Human umbilical vein endothelial cells (HUVECs) (Lonza) were used as a model for testing *in vitro* activity and vascularization potential of growth factor loaded hydrogels. HUVECs were cultured in EBM-2 basal medium (Lonza) supplemented with the provided growth factor bullet kit (EGM-2, Lonza). Cells from passage 2 to passage 5 were used for tube formation assay. Cells were stained with Dil (Invitrogen) by resuspending the cells in serum free medium, the dye was added according to the manufacturer's specifications (5 μ l dye for each ml of cell suspension) and incubated for 20 min at 37 degrees. After incubation, cells were washed 3 times with medium supplemented with 2% serum. Cells were seeded on top of a preformed hydrogel at a density of 15'000 cells/construct in a 96 well plate (33). Growth factor concentration in the hydrogel were: 100 ng/ml basic fibroblast growth factor (bFGF, Peprotech), 200 and 500 ng/ml vascular endothelial growth factor (VEGF, Peprotech), combination of 100 ng/ml bFGF with either 200 or 500 ng/ml VEGF. Control conditions were 75 μ l hydrogel without additional growth factors and 75 μ l growth factor reduced Matrigel (BD). EGM-2 medium used for tube formation assay was prepared without the addition of VEGF or bFGF, so that the only source of growth factor was the hydrogel only. Constructs were imaged after 24h using a BD pathway (BD) at 4X magnification to measure the extent of the tube network formed on the surface of the gel using ImageJ software (NIH). The ImageJ software defines and quantifies parameters of the tubular network.

Master segments are defined as the elements of the network delimited by two junctions or branching points in the network and calculates their length in pixels. Nodes are defined as the branching points connecting two segments, of which one of those is not involved in the formation of another junction (dead end), while junctions refers to branching points connecting two or more master segments. Moreover, two more elements that describe the tubular structure formed are the mean mesh size (in pixels) of area delimited by master segments; and the number of isolated segments, meaning the number of isolated segments on the picture, which are not incorporated within the network.

Growth factor release study

Hydrogels with different growth factor concentrations as mentioned in Table 1 have been prepared according to the method previously described. 400 μ l hydrogel precursor were prepared for each condition and 75 μ l samples (quadruplicate for each condition) were allowed to gelate in an Eppendorf tube. After gelation 500 μ l of PBS (Gibco) supplemented with 0.1% Bovine Serum Albumin (BSA, Sigma) were added to each sample and incubated at 37 degrees. Samples have been taken daily for the first three days and subsequently every other day for a total of 14 days and the PBS/BSA 0.1% refreshed upon sample withdrawal. Growth factor release was measured by using a bFGF or VEGF ELISA kit (R&D) according to the manufacturer's protocol.

Statistical analysis

Statistical significance between groups was analyzed by performing a One-way ANOVA followed by a Bonferroni post-hoc test using IBM SPSS statistic 23 software. Data is expressed as mean $\pm$ standard deviation and significant differences are indicated with * ($p \leq 0.05$).

Results

Bi-layered structures could be easily fabricated by crosslinking PEGDA gels on top of a preformed vascularization gel. The presence of free thiol groups on the surface of the first hydrogel layer allowed for a covalent crosslinking of the islet

encapsulation layer on top of the vascularization layer. Such bi-layered hydrogels were mechanically stable and could be easily handled as a single construct. Similar results were achieved in the second approach where islets were modified with a conformal coating of bifunctional PEG, where the SVA group reacted with free amino groups exposed on the surface of the islets and the acryl group on the other extremity of the PEG chain was available for the reaction with acryl groups in the hydrogel matrix. Both approaches, schematically represented in figure 1, combine the favorable properties of a non-adhesive and cell adhesive layers, and can be independently tuned on the specific cell type that will be embedded within the gel. In both cases it was possible to combine the dual layer approach in a single construct, mechanically stable, in which the two layers were able to crosslink with each other by establishing covalent binding.

Figure 1: concept of the bilayered constructs (A) Vascularization hydrogel based on Michael type reaction of acrylate groups on PEG with thiol-modified heparin, thiol-modified gelatin and thiol-modified hyaluronic acid. These ECM molecules are able to provide an adhesion cues for endothelial cells and act as a controlled release compartment for growth factor delivery. This layer can be covalently crosslinked with a PEGDA based embedding layer for islet of Langerhans encapsulation. Collagen IV or laminin can be mixed within the hydrogel mesh to create a more favorable environment for islet of Langerhans. (B) Conformal coating model where free amino groups exposed on proteins on the surface of islets are covalently modified by PEG chains. The free acrylate group on the other extremity can react with the thiol groups present in the vascularization hydrogel via Michael type reaction.

The vascularization layer allows for embedding and sustained the release of growth factors for a period of 14 days (figure 2). No burst release was shown in the first day of incubation both for bFGF and VEGF. The combined release of two growth factors from the same hydrogel did not show any difference in the release kinetic compared to the same growth factor being released alone. In a period of 14 days, only about 1 ng was released from the 100 ng/ml bFGF loaded hydrogels, accounting for about a seventh of the total growth factor load. In case of VEGF loaded hydrogels, about 4 ng were released from the 200 ng/ml VEGF or 200 ng/ml VEGF + 100 ng/ml bFGF, accounting for a fourth of the loaded amount. In case of the 500 ng/ml VEGF and 500 ng/ml VEGF + 100 ng/ml bFGF a third (~13 ng) of the originally loaded amount was cumulatively released in a 14 days investigation time.

Figure 2: release profile of VEGF and bFGF (different concentrations and combinations) from the PEGDA/thiolated-Hyaluronic acid/gelatin/heparin hydrogels for a release period of 14 days.

HUVEC seeded on the surface of the hydrogels adhered to the material in about 4 hours, showing a spread morphology and cellular organization within 24 hours. The degree of cell organization with respect to preliminary tubular network formation on the surface of the gels was dependent on the growth factor dose embedded within the gel layer (figure 3). In particular, while in the control without growth factor cobblestone-shaped cells are organized in clusters, when growth factors are present cells are more spread on the gel surface, display a more elongated shape and connect to each other with their cytoplasmatic extensions. In growth factor loaded gels, polygonal areas delimited by cells are visible, similar, although much

less defined, than the ones formed on Matrigel control gels. Although in none of the conditions we were able to achieve the same degree of tube formation obtained on Matrigel (positive control), it was possible to quantify cellular organization in preliminary tubular structure using ImageJ software (figure 4).

Figure 3: tube formation assay of HUVEC seeded on vascularization hydrogels at different growth factors loads and combinations thereof. Matrigel is used as positive control.

Figure 4: quantification of master segments number, master segments length, nodes, mean mesh size, isolated segments and junctions on tube formation assay of HUVEC seeded on vascularization hydrogels. Significant differences between groups are indicated with an (*) ($p \leq 0.05$).

In all the conditions tested, Matrigel scored the highest in parameters as master segments and master segments length, nodes, junctions and mean mesh size, indicating the highest degree of network organization. It also obtained the lowest score in isolated segments, meaning that the great majority of the cells is integrated in the network. Interestingly negative control images (0 ng/ml growth factors) have also been submitted to software analysis, but the program was not able to run the quantification algorithm and detect a pattern on these images. Overall, a quite comparable trend emerged from the picture analysis in all the

investigated parameters. 100 ng/ml bFGF alone and in combination with the 200 ng/ml VEGF being the conditions that showed an increased tubular network formation in comparison to the 500 ng/ml VEGF alone and in combination with bfgf 100 ng/ml. Gels with 100 ng/ml bFGF and 100 ng/ml bFGF+200 ng/ml VEGF are the ones that scored no significant difference in comparison to matrigel regarding nodes, mean mesh size and junction formation. By comparing the combination (bFGF+VEGF) vs bFGF alone, it seems that bFGF is the mayor responsible for HUVEC organization on the gel.

The functionality of MIN6 pseudo-islet embedded in the bilayered constructs was tested and compared to control free-floating aggregates. MIN6 aggregates functionality was heavily reduced compared to the control when aggregates were embedded in the single layer hydrogel (PEGDA only) and completely absent when the embedding layer of PEGDA was crosslinked on top of the vascularization layer. Addition of extracellular matrix proteins as collagen or laminin was not sufficient to rescue pseudo-islets functionality when embedded in the double layer construct (figure 5).

Figure 5: Stimulation index of free-floating MIN6 pseudo-islets, MIN6 aggregates embedded in the single layer PEGDA hydrogel and in a double layered hydrogel without protein addition and in presence of collagen 4 or laminin. Constructs have been incubated in low (1.67 mM) and high (16.7 mM) glucose concentration. Stimulation index is expressed as the ration between the amount of insulin secreted in high glucose condition divided by the basal insulin secretion level (in low glucose condition).

Successful surface modification with attachment of polyethylene glycole chain to the aminogroups of proteins exposed on the islet of Langerhans extracellular matrix was confirmed by a fluorescent staining appearing on the surface of the islets (figure 6).

Figure 6: control and pegilated islet showing a NHS-PEG-biotin layer covalently crosslinked on islet surface proteins and visualized using a streptavidin-FITC conjugate. Scale bar 1000 μm in the upper row and 400 μm lower row.

Functionality of islets of Langerhans from two human donors was analyzed at two time points and compared to the functional response of free floating islets (untreated), PEGylated free floating islets, islets embedded in PEGDA hydrogels and PEGylated islet embedded in the gel. For all donors and at both time points functional insulin secretion in response to high glucose stimulation was drastically reduced compared to the free floating control. Donor 2 performed overall better with a higher stimulation index. At day 1 PEGylated islets embedded in the gel show still a functional response, although reduced in comparison to the control. At day 7 all the conditions showed a borderline functional behavior, but the embedding and PEGylation procedure seem to exert a detrimental effect on islet functionality.

Figure 7: stimulation index of islet of Langerhans from two donors in basal condition (1.67 mM glucose) and high glucose stimulation (16.7 mM). Free floating islet have been compared to pegylated free floating islet, islet embedded in the construct and islets embedded in the gel after PEGylation. Both at day 1 and 7 functional response is reduced in all the experimental groups.

Discussion

PEGDA is a commonly used matrix for islet of Langerhans encapsulation (34-38). PEGDA has been widely used in tissue engineering for cell encapsulation in cartilage regeneration purposes (39-41), heart valves regeneration (42), islet (36) or beta cells encapsulation (34,35,38) and for controlled delivery systems of pro-angiogenic growth factors (17,18,43) with promising results.

Main advantages of this synthetic matrix are that it is highly defined, with tunable molecular weight, hydrogel mesh size, mechanical properties, water content comparable to soft tissue in the body (44), and ease of functionalization thanks to tunable free binding groups within the matrix (44).

Although widely used, this very versatile system for cell encapsulation also poses strong limitations. Being PEGDA matrices inert and non-cell adhesive, many authors have spent considerable efforts in functionalizing PEGDA hydrogels with peptides and molecules of the extracellular matrix in order to confer cell adhesion properties to the material (45,46). Although chemical functionalization is pretty straightforward, finding the right cocktail and concentrations of peptides for that specific cell type, is not a trivial subject. Weber and coworker (37) functionalized PEG gels with collagen IV, laminin, a combination thereof, or peptides derived from these proteins and found an increase in insulin secretion. Many other adhesion peptides like RGD sequences have been incorporated in PEG gels to improve their adhesion properties (45,46). Also matrix metalloprotease sequences have been integrated in the mesh to allow cells to rearrange the synthetic matrix (47). Another way to increase cell adhesion on PEGDA based hydrogels is to include extracellular matrix molecules like heparin, hyaluronic acid and collagen (or gelatin). Hyaluronic acid and collagen molecules can enhance cell binding via specific receptors like integrins (48) and CD44 (49). Heparin is a common constituent of the extracellular matrix and is widely used for binding and stabilizing growth factors. These modifications to otherwise inert PEGDA hydrogels makes them more similar to native extracellular matrix in the body, create a more favorable environment for embedded cells and controls the release of growth factors in a more physiological way.

In this chapter, a bi-layered scaffold concept offers the advantage of combining all the aforementioned tunable properties of PEGDA hydrogels in a unique, multifunctional construct. In this construct, the versatility of PEGDA matrices is exploited to create distinct areas in the scaffold presenting different fouling properties, proteins, and growth factors to the embedded cells. Non-cell adhesive PEGDA layer mixed with collagen and laminin can be used for islet of Langerhans encapsulation, in the attempt of reducing cell attachment and maintaining islet round morphology. PEGDA layer with the addition of thiol-modified extracellular matrix molecules like heparin, hyaluronic acid and gelatin can serve to increase cell adhesion cues on the hydrogel surface, act as a controlled release system for pro-

angiogenic growth factors and ultimately increase blood vessels ingrowth in the construct.

In an alternative approach the PEGDA layer has been reduced to a conformal coating around the islet surface and embedded into the vascularization layer. In both approaches, both layers are covalently crosslinked to each other in a single multilayered construct using free acryl groups present either at the surface of both gels or on the surface of the islet.

Thiol functionalized heparin, gelatin and hyaluronic acid react spontaneously in mild, physiological conditions with acryl groups on PEGDA forming a stable gel network via a Michael-type addition reaction.

This ECM enriched PEGDA gel is able to sustain dual controlled release of VEGF and bFGF for at least 14 days *in vitro*. The presence of negatively charged glycosaminoglycans assure a slow release of the growth factors from the gel and preservation of their three dimensional structure and activity. The ability of the released growth factor to induce primitive organization of endothelial cells into elongated structures is still lower than Matrigel, but the advantage of this semi-synthetic system is that this is a fully characterized matrix, in which known growth factor cocktails and proteins at known concentration can be embedded. Further work is required to find an optimal growth factor combination but results reported in literature with similar systems showed promising outcome (17,18). In these papers, an increased vessel density with erythrocytes confined in the capillary was found when growth factor loaded hydrogels were implanted in mouse ear. However, in these studies, only the induction of vascularization is tested, but no application to an engineered tissue or organ is presented. *In vivo* studies are necessary to further study growth factor dose and release kinetic on attraction of endothelial cells and revascularization of the embedded islets. Moreover *in vivo* degradation studies of this semi-degradable matrix would provide more insights in the release kinetic of the different growth factors and in the timing of the revascularization process.

MIN6 pseudo-islet were used as a model for islets, embedded in the PEGDA layer with or without the addition of collagen IV or laminin. A reduced but still functional

response was detected in the single layer gels, while functionality was completely lost when a double layer was used, suggesting a limitation in nutrient diffusion through the construct when its thickness is doubled. A higher molecular weight PEGDA could increase the mesh size of the hydrogel and help diminishing nutrient diffusion limitations.

In an attempt to reduce nutrient diffusion limitations in a bi-layered construct, a second strategy was developed and consisted in the conformal coating of islets using a SVA-functionalized PEGDA. SVA would react to free amino groups exposed on proteins on the islet surface. The other acryl functionalized end would react with thiol groups exposed on the vascularization hydrogel.

Conformal coating on the surface of an islet would only account for a few nanometers in thickness, which should not significantly hamper nutrient diffusion. Moreover, nutrient and oxygen diffusion should be facilitated also by the fact that islets are directly embedded in the vascularization gel. This gel presents weaker mechanical properties compared to UV-crosslinked PEGDA hydrogels. A lower concentration of PEGDA crosslinker and the higher molecular weight of the ECM molecules crosslinked within the mesh account for a larger pore size in the gel mesh and consequently weaker mechanical properties. All these factors account for a hydrogel mesh with bigger distance between crosslinking points resulting in larger mesh size, facilitating in this way nutrient diffusion through the matrix. With regard to PEGDA conformal coating, Lee and coworkers also claim the possibility to achieve immune-protective properties by using this kind of strategy (27-29).

PEGDA binding to amino groups on islet was confirmed by using a NHS-PEG-biotin. NHS-PEG-biotin was used in this case instead of the SVA-PEGDA, because the available biotin functionalization would allow for streptavidin-FITC detection of the successful functionalization. The reactivity of NHS and SVA groups has been reported to be similar (50). Experimentally, effective PEGylation with SVA-PEGDA was shown by the faster crosslinking speed and increased stiffness of the vascularization gels where PEGylated islets were embedded compared with gels

where non-PEGylated islets were embedded. This fact indirectly confirms the effectiveness of SVA-PEGylation.

Functionality of embedded and PEGylated islets in the vascularization layer remains borderline. Functionality is reduced compared to the free floating control condition and seems that only highly functional islets (donor 2, stimulation index > 4 at day1, >20 at day7) are able to still retain a functional profile after PEGylation and embedding. This fact is indicative for a quite harsh PEGylation reaction. Nutrient diffusion limitation is believed to be the main responsible for islet's reduction in functionality in the bi-layered constructs, but in case of PEGylated islets it might not be the only reason for islet reduced functionality. Although conformal coating creates a very thin layer, SVA-PEGDA can still impair antibodies to interact with islets surface antigens. It might be that a similar limitation occurs for insulin diffusion from the islet. In this case, a concentration curve of different PEGDA/islet ratios during PEGylation or lower SVA-PEGDA molecular weight might help defining more friendly conditions for conformal coating of islet of Langerhans.

Another possible explanation lies in the fact that crucial proteins for glucose sensing, like GLUT1 receptors might have undergone a conformational change during PEGylation or that the attachment of PEG chains would impede glucose recognition by the channel and its transport into the cytoplasm.

Conclusions

PEGDA based hydrogels represent a versatile matrix for cell embedding. The addition of extracellular matrix proteins (thiolated-gelatin) and thiol-modified glycosaminoglycans (heparin and hyaluronic acid) provided cell adhesion properties and growth factor binding and release capability to the otherwise inert PEGDA matrix. These results show the potential of the vascularization layer to attract and induce organization of an endothelial cell layer in close proximity to islets of Langerhans.

More research is required in tuning mesh size properties in the PEGDA layer for islet of Langerhans embedding, since limited nutrient diffusion still seems to remain the biggest limiting factor in islet functionality. The alternative PEGylation approach reduces the thickness of the PEG layer surrounding the islets, thus limiting nutrient limitations. However the PEGylation procedure might irreversibly change the three dimensional conformation of proteins exposed on the surface of the islets.

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Chapter 3

Fabrication of three-dimensional bioplotting hydrogel scaffolds for islets of Langerhans transplantation

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Abstract

In clinical islet transplantation, allogeneic islets of Langerhans are transplanted into the portal vein of patients with type 1 diabetes, enabling the restoration of normoglycemia. After intra-hepatic transplantation several factors are involved in the decay in islet mass and function mainly caused by an immediate blood mediated inflammatory response, lack of vascularization, and allo- and autoimmunity. Bioengineered scaffolds can potentially provide an alternative extra-hepatic transplantation site for islets by improving nutrient diffusion and blood supply to the scaffold. This would ultimately result in enhanced islet viability and functionality compared to conventional intra portal transplantation. In this regard, the biomaterial choice, the three dimensional (3D) shape and scaffold porosity are key parameters for an optimal construct design and, ultimately, transplantation outcome. We used 3D bioplotting for the fabrication of a 3D alginate-based porous scaffold as an extra-hepatic islet delivery system. In 3D-plotted alginate scaffolds the surface to volume ratio, and thus oxygen and nutrient transport, is increased compared to conventional bulk hydrogels. Several alginate mixtures have been tested for INS1E β -cells viability. Alginate/gelatin mixtures resulted in high plotting performances, and satisfactory handling properties. INS1E β -cells, human and mouse islets were successfully embedded in 3D-plotted constructs without affecting their morphology and viability, while preventing their aggregation. 3D plotted scaffolds could help in creating an alternative extra-hepatic transplantation site. In contrast to microcapsule embedding, in 3D plotted scaffold islets are confined in one location and blood vessels can grow into the pores of the construct, in closer contact to the embedded tissue. Once revascularization has occurred, the functionality is fully restored upon degradation of the scaffold.

Introduction

Islet transplantation is usually performed in a selected group of patients with type 1 diabetes with unstable glycemia characterized by recurrent low blood glucose levels and hypoglycemia unawareness (1, 2). However, the efficiency of this procedure is limited: it is estimated that a large proportion of the transplanted islets is destroyed shortly after transplantation (1, 2). In addition long term survival is not optimal as less than 50% of the patients remain insulin independent after a 5 years follow-up (3). Several reasons are relevant for the adverse outcome of islet transplantation, like: (i) the instant blood mediated inflammatory reaction (IBMIR) caused by complement activation and innate immune response (4); (ii) the disruption of cell-extracellular matrix interaction during the isolation phase from the donor pancreas (5-7); (iii) the loss of islet vasculature, resulting in a reduced nutrient and oxygen supply after transplantation (8, 9); (iv) toxins and drugs which are commonly processed in the liver and further cause islet mass decay (10,11); (v) alloimmune response; and (vi) recurrent autoimmunity. It is likely that a combination of all these factors is responsible for the reduced graft survival in the long term.

The creation of an extra-hepatic transplantation site, aided by the use of bioengineered implants in which islets are combined with scaffolds, could help overcoming the aforementioned disadvantages. Scaffolds can provide a favorable and protective environment for islets of Langerhans, including the possibility of designing the shape of the construct and functionalizing the material to specifically match the requirements of the embedded cells. Recently, a number of polymeric biomaterials have been used for the creation of porous islet-containing implants. Salt leached porous scaffolds of Poly(DL-lactide-co-glycolide) acid (PLGA) (12-14), plotted PLGA scaffolds (15), polydimethylsiloxane salt-leached scaffolds (16, 17) and thermoformed microwell scaffolds of poly(ethylene oxide terephthalate)-poly(butylene terephthalate) (PEOT/PBT) block copolymer (18) are examples of the variety of materials and scaffold shapes used for extra-hepatic islet entrapment and transplantation. Although successful in small animal models, the main disadvantage of such tissue-engineered constructs is the difficulty in scaling them

up to a clinically relevant size, containing a therapeutic dosage of islets, without compromising nutrient and oxygen diffusion.

A different approach has been tried by encapsulating islets in hydrogels. Hydrogels are particularly attractive for cell encapsulation and have been used in a variety of applications for soft tissue engineering (19). Hydrogel encapsulation can be used for immune-protection by preventing contact of the encapsulated cells with the host immune cells. Their mechanical properties and water content closely match the ones of soft tissues in the body (20). Furthermore, hydrogels can be easily mixed or covalently functionalized with extracellular matrix proteins, peptides and growth factors (21-23). Among different hydrogel formulations, alginate is a commonly used material for islet encapsulation and has been widely used for immunoprotection of allogeneic transplanted islets from the attack of antibodies and cytokines after transplantation (3, 24, 25). Islets are either embedded in small alginate beads or mixed in bulk alginate hydrogels, and injected subcutaneously or into the peritoneal cavity (3, 26). A study by Ludwig et al reports the fabrication of an oxygenated and immunoprotective alginate-based macro-chamber for islets transplantation in a male patient (27). In all these cases, the mesh size of the surrounding alginate hydrogel has to be carefully tuned to assure optimal insulin and nutrient diffusion to the embedded islet. At the same time, the hydrogel mesh needs to be tight enough to prevent antibodies and cytokines to interact with the embedded islets (24). The disadvantage of encapsulation is that the embedded islets cannot be regarded as a single construct but more as a multitude of self-standing micro organs, which are difficult to implant and be retrieved all at once, if needed.

We studied three dimensional (3D) bioplotting for the fabrication of an alginate-based, islet-laden 3D construct. Islets of Langerhans are mixed in the precursor alginate solution and this is plotted in a predefined 3D fashion. Islets are embedded in the hydrogel strands constituting the construct. Such construct can combine the advantages of a tailor made shape and porosity with the beneficial properties of hydrogels in an innovative construct for islet of Langerhans delivery. In contrast to what happens with other constructs or with islet encapsulation in

beads, one of the main advantages of this approach is that this type of construct can host a clinically relevant amount of islets in a defined tridimensional, porous construct. In addition, the fabrication of a cell-laden alginate construct can also combine the advantages of a porous hydrogel scaffold with a defined three dimensional geometry with the immunoprotection properties provided by alginate embedding.

A variety of different techniques has already been developed to tackle the problem of three-dimensional cell-laden hydrogel constructs for several other tissues. Strategies as 3D plotting and inkjet printing are the most widely used and have been applied to the regeneration of different tissues (28, 29). 3D deposition of cell-laden hydrogels has been previously investigated for hepatic tissue regeneration (30) and for bone tissue engineering applications (31), showing that a plotted porous structure can be beneficial to enhance cell viability and metabolic activity compared to bulk constructs. 3D hydrogel deposition has been also applied to the fabrication of aortic valves (32), micro-vessels and vascularized tissue fabrication (33), for cell transfection strategies (34) and for cell culture of neural stem cells (35), fibroblasts and keratinocytes (36, 37).

The main advantage of plotting is, therefore, the possibility of fabricating large hydrogel constructs, capable of containing a clinically relevant amount of islets, with a porous structure that might improve oxygen and nutrient supply to the embedded cells. In this study, plotting of cell-laden hydrogels constructs is extended to large cell aggregates of 50-300 μm in diameter, such as islets of Langerhans.

We hypothesized that islets embedded in a porous plotted structure could have a better glucose and insulin exchange, compared to bulk hydrogels. We show that the material choice and construct shape are of crucial importance in defining the viability and functionality of the embedded cells.

Materials and methods

INS1E cell culture and islet culture

INS1E β -cell line, derived from rat insulinoma, were kindly provided by Dr. B. Guigas, LUMC, Leiden, The Netherlands and Dr. P. Maechler, University Medical Center, Geneva, Switzerland. Cells were cultured in RPMI 1640 (Gibco) supplemented with 5% (v/v) FBS (Lonza), 1% Pen/Strep (Gibco), 1% sodium pyruvate (Sigma) and 1% HEPES 1M (Invitrogen). For cell expansion, β -mercaptoethanol (Invitrogen) was added fresh to the culture to a final concentration of 1 μ l/ml medium. Medium was refreshed every 2 to 3 days.

Human cadaveric donor pancreata were procured via a multi-organ donation program. Isolated human islets were used in this study if they could not be used for clinical transplantation, according to the national law, and if research consent was present. Human islets were kindly provided by the LUMC Leiden and cultured in CMRL medium (Cellgro) supplemented with 10% FBS and 1% Pen/Strep according to Fraga et al (38). Mouse islets were isolated from double heterozygous crossbreed mice between Tg(Ins1-eGFP), Jackson Laboratory strain #006864, and B6(CAG-DsRed*MST), Jackson Laboratory strain #005441 and kindly provided by Hubrecht Institute Utrecht. Mouse islets were cultured in ultra-low attachment plates with RPMI 1640 (Gibco) supplemented with 10% FBS and 1% Pen/Strep.

Preparation of alginate mixtures and cell-containing bulk hydrogels

Alginate powder (Sigma) was sterilized by 5 minutes exposure to UV light (Labino Duo spotlight) with an intensity of 45 mW/cm² at 38 cm and dissolved at a 4% w/v concentration in PBS (PAA). Pre-crosslinked alginate was prepared by mixing a 5.3% w/v alginate solution with a 102 mM CaCl₂ solution (Sigma) in HEPES 10 mM in a 3:1 volume ratio, resulting in a final alginate concentration of 4% w/v. Two syringes connected with a T-shaped connector were used to mix the calcium chloride solution with alginate and after mixing this precursor solution was used for plotting. After scaffold preparation the resulting construct was definitively crosslinked by using 102 mM CaCl₂ solution. Similarly, an alginate 4%/Matrigel

25% w/v mixture was also prepared, by pipetting. Additionally, alginate 4%/gelatin 5% w/v and alginate 4%/hyaluronic acid 0.5% w/v mixtures were prepared by dissolving 0.8 g of alginate in 20 ml of a 5% w/v bovine gelatin solution (gelatin type B, Sigma), or in 0.5 % w/v Hyaluronic acid solution (Fluka) at 37 degrees. Gelatin and Hyaluronic acid solution were filter sterilized using an 0.22 mm filter unit (Millipore) attached to a 10 ml syringe (Norm-Ject HSW). All the different alginate mixtures are summarized in table 1. Ultrapure alginate was kindly provided by Paul De Vos (UMCG Groningen) and used at a concentration of 2% w/v in PBS after purification according to P. De Vos et al (26).

Viscosity of the different alginate compositions was quantified using an Anton Paar Physica MCR 301 rheometer with flat plate geometry (20 mm diameter, 1 mm gap). Shear rate ranged from 0.01 to 100 1/s. Given the Non-Newtonian behavior of alginate solutions, viscosity measurement at a low shear rate of 0.03 1/s was selected.

When islets or cells were added to the different gel mixtures, $10 \cdot 10^6$ cells/ml were resuspended in the less viscous component of the mixture (CaCl_2 , gelatin, hyaluronic acid solution or in Matrigel) to obtain a homogeneously dispersed cell suspension and mixed with a 5.3% w/v alginate solution, in a 1:3 ratio, to a final 4% w/v alginate concentration. Both plotted and bulk constructs (0.3 ml) were crosslinked with a 102 mM CaCl_2 in 10 mM HEPES solution for 15 minutes and then washed for 5 minutes with tris buffered saline (TBS) (31). Other crosslinking solutions used in this study were 20 mM CaCl_2 in 10 mM HEPES and 20 mM BaCl_2 (Sigma) in 10 mM HEPES to study the effect of crosslinking strength and ion on islet activity.

3D hydrogel plotting

Constructs were plotted using a 3D bioplotter (SysENG, Germany). Briefly, a cell suspension in hydrogel is laden into a syringe. The hydrogel is then extruded from the syringe in a controlled layer by layer fashion by a XYZ moving arm, which deposits the material into a predefined 3D structure according to a computer aided design model (CAD-model).

Plotting settings were set accordingly to the viscosity of the material. Plotting speed ranged from 750 to 1100 mm/min, needle diameter ranged from 0.41 mm to 0.2 mm. Alginate 4%/gelatin 5% w/v 2x2 cm construct was plotted with a XY speed of 750 mm/min, a spindle speed of 1.75 mm/min and a needle diameter of 0.41 mm for a total of 17 layers. A 0.41 mm needle diameter was selected to be compatible with an islet size, which ranges between 50 and 300 μm . Plotting settings for the other compositions are reported in detail in table 1. The spacing between each individual strand of the porous constructs was set on 3 mm to assure open pores in the constructs, whereas solid constructs were plotted with an offset of 1.16 mm to avoid strand superposition in following layers and create a compact structure. Control bulk samples were prepared by pipetting the same volume of hydrogel precursor on a petri dish, and they were then immediately crosslinked.

3D plotted construct characterization

Blue coloured polystyrene beads of 100 μm diameter (Phosphorex) were used as a model for islets and embedded in a plotted alginate 4%/gelatin 5% w/v construct (referred from now on as alginate/gelatin) in a concentration of 45'000 beads/ml. Constructs were plotted and pictures were taken with a stereomicroscope (Nikon SMZ-10A, equipped with Qcaputre software). The bead/pore distance of circa 400 beads (20 images) was measured with Image J.

Glucose diffusion measurement

An 80 μl bulk alginate/gelatin hydrogel was prepared as previously described and placed in the rubber insert of a custom made chamber to measure glucose diffusion through the gel. The 80 μl hydrogel volume resulted in a gel thickness of 1mm. The device is composed of two chambers. One of the compartments has been loaded with 50 ml of 12 mM glucose, 1% pen/strep 102 mM CaCl_2 in 10 mM HEPES; the other compartment was loaded with the same solution, without glucose. Samples were taken from both compartments at defined time points and the concentration of glucose in time was measured using a VITROSS DT60 II chemistry system (Ortho-Clinical diagnostics).

The “lag time” method was used for the calculation of glucose diffusion coefficient (cm^2/s) in alginate 4%/gelatin 5% (w/v) gel according to the method described by Hannoun (39). This method is based on Fick’s law of diffusion, with constant diffusion coefficient. Total glucose flux through the gel was calculated and plotted as a function of time. A constant flow/time ratio, represented by the linear part of the graph, is reached only after a certain period of time, and this period of time is referred to as the lag time. The lag time is the time a glucose molecule needs to diffuse through the whole thickness of the gel (l), before the glucose flux becomes constant in time. The lag time is calculated as the intercept of the linear function on the x axis and it is given by $t=l^2/6D$, where D is the diffusion coefficient, t the time and l the thickness of the gel layer. The diffusion coefficient can be then calculated as $D=l^2/6t$. The lag time corresponds to the time difference in which the diffusing molecule, glucose in this case, starts penetrating the hydrogel and the time at which the glucose flow rate into the acceptor chamber reaches a steady state (40).

Cell viability and metabolic activity

Cell viability was measured with a Live/Dead viability assay (Invitrogen) according to the provided protocol and images were taken with a fluorescence microscope (Nikon Eclipse E600). Before imaging, hydrogels were cut in half using a scalpel. The resulting slice had a thickness of about 850 μm , the plotted morphology was maintained and four pictures per scaffold, in a random area, were analyzed to score the percentage of live cells to total cells.

To measure cell metabolism in the three different constructs shapes, medium samples of 3×10^6 cells/construct cultured in bulk, plotted porous or plotted solid hydrogels were taken every second day and glucose and lactate concentrations in the medium were measured with a VITROSS DT60 II chemistry system (Ortho-Clinical diagnostics). After every analysis, medium was refreshed for further culturing.

Lentiviral transduction of human islets

Islets were overnight transduced by incubating them in 3 ml DMEM medium containing 1% pen/strep, 10% FBS, 8 ng/mL polybrene (Sigma-Aldrich), and pRRL-CMV-GFP virus (1000 ng/ml). After transduction, islets were extensively washed in PBS and cultured again in RPMI supplemented with 10% FBS and 1% pen/strep. Circa 500 of the brightest transduced islets were selected and used for plotting and for bulk hydrogel embedding.

Islet plotting and imaging

1000 IEQ of transduced human islets were mixed with alginate 4%/ gelatin 5% w/v and alginate 4% w/v. Islets were plotted according to the procedure or embedded in bulk hydrogel and imaged at day 1, 4 and 7 with a Leica TCS SP5 AOBS two-photon microscope. The microscope was equipped with a 20x dry objective, NA 0.40. The laser lines used were 488 nm for GFP and the emission filter used was a 495-540 nm. Mouse islets were similarly plotted and imaged using a 488 nm excitation line/495-540 nm emission filter for GFP and a 561 nm excitation line/570-620 nm filter for DsRed. Non-transduced islets were imaged in the bright field mode.

INS1E cell sample preparation for glucose induced insulin secretion test (GIIST)

Glucose responsiveness of INS1E cells embedded in alginate 4%/gelatin 5% w/v bulk hydrogel was compared to INS1E cells cultured on tissue culture plastic. 3×10^6 cells/sample were seeded in a T25 flask and an equal amount of cells was embedded in 0.1 ml of bulk hydrogel. Cells were mixed with alginate 4%/ gelatin 5% w/v solution at a density of 30×10^6 cells/ml and crosslinked with 102 mM CaCl_2 in 10 mM HEPES for 15 minutes. Cell laden hydrogels were also crosslinked with 20 mM CaCl_2 in 10 mM HEPES for 20 and 30 minutes, and with 20 mM BaCl_2 in 10 mM HEPES for 10 minutes. The previous conditions were also compared to INS1E embedded in 1% w/v Agarose (Invitrogen).

Islet sample preparation for glucose induced insulin secretion test (GIIST)

25 islets per sample were embedded in 0.1 ml of alginate 4%/ gelatin 5% w/v and crosslinked with 102 mM CaCl_2 in 10 mM HEPES for 5 or 15 minutes, with CaCl_2 20 mM in 10 mM HEPES for 20 minutes or with BaCl_2 20 mM in 10 mM HEPES for 10 min. The same number of islets were also embedded in 2% w/v ultrapure alginate and crosslinked for 15 min with 102 mM CaCl_2 in HEPES. Free floating islets in ultra-low attachment plate were used as control. After gel crosslinking, all the samples were washed with TBS and cultured in CMRL medium. At day one a glucose induced insulin secretion test was performed. After the function test, the alginate hydrogels (102 mM CaCl_2 crosslinking for 15 min) were solubilized by incubating them in 1 ml of dissolving buffer containing 0.15 M NaCl (Merck), 30 mM disodium-ethylenediamine tetra acetic acid (EDTA disodium salt) (Calbiochem) and 55 mM sodium citrate (Sigma-Aldrich) (41) for 40 min. Islets were retrieved from the gel and after 2 days the function test was repeated again comparing free floating untreated islets, free floating islets treated with the dissolving buffer and islets previously embedded in the gel and treated with the dissolving buffer for their retrieval.

For testing islet functionality in the plotted constructs, the plotted scaffold and the bulk samples were prepared with circa 125 islets/sample and crosslinked with a 102 mM CaCl_2 solution in 10 mM HEPES for 15 minutes, followed by a wash with TBS. Both bulk and plotted constructs final volume was 0.3 μl . Insulin secretion test was performed as following and compared with the same amount of free floating islets.

Islet and INS1E glucose induced insulin secretion test

Islet functionality was tested as reported elsewhere (18). The GIIST protocol was slightly modified for testing INS1E functionality. Briefly, islets were pre-incubated in Krebs-Ringer low glucose buffer (1.67 mM glucose) for 1.5 hours while INS1E samples were incubated for two hours in RPMI medium without glucose (Invitrogen). Samples were then exposed for 1.5 hours in case of islets, or for 45 minutes in case of INS1E, to a low glucose Krebs-Ringer buffer containing 1.67

mM glucose. Subsequently, samples were incubated for an equal period of time respectively in high glucose medium (16.7 mM glucose) followed by a last incubation in low glucose medium (1.67 mM). Samples were collected at the end of each incubation step for further analysis. Released insulin was quantified by using an ELISA kit (Mercodia). Results were presented as low-high-low profiles, both as actual amount of insulin secreted (mU/l) and as stimulation index, which is the ratio of insulin secretion between the high and low glucose condition.

Statistical analysis

Experiments were performed in triplicate. All the quantitative data are expressed as mean $\pm$ standard deviation. Data were analyzed using a one-way ANOVA, followed by Bonferroni post-hoc test. The analysis was performed using IBM SPSS statistic 20 software. Significant difference between groups is indicated with * ($p \leq 0.05$).

Implantation and in vivo imaging

Circa 500 of the brightest transduced islets were plotted in the scaffold following the usual procedure. A 1 cm circular section of the plotted scaffold was punched out and glued on one side of a pre-sterilized abdominal imaging window by using H-butyl-cyanoacrylate glue. Two other equal sections of the same scaffold were used as a subcutaneously implanted control and as an *in vitro* control, respectively. The window was implanted on the back of NSG mice (Jackson Laboratory), the second part of the scaffold was implanted subcutaneously as a control.

The islet graft was monitored at day 1, 4 and 7 (figure 8). Intravital imaging was performed by placing the mouse in a custom designed imaging box. The imaging window was fixed in a ring-opening on the bottom part of the box. The box was also equipped with a nose cone for anesthesia, air ventilation, and a MouseOx system (starr lifescience Corp) to monitor vital signs. The imaging was performed keeping the mouse at a controlled temperature of 32°C. A Leica TCS SP5 confocal microscope was used and equipped with a 20x dry, NA0.40 objective, a 488nm laser line for GFP and a 495-540 nm emission filter. After seven days the mouse

was sacrificed with isoflurane anesthesia and the graft was explanted and processed for histology.

Explants were fixed with 4% w/v paraformaldehyde, dehydrated, and embedded in paraffin. Sections 4-8 μm thick were cut and stained with hematoxylin and eosin. Alternatively, immunostainings for insulin (Dako; 1:200) and glucagon (Vector Laboratories; 1:100) were performed. Images were acquired on a Leica TCS SPE with a 20x oil-immersion objective.

Results

Scaffold fabrication by 3D plotting and characterization

Different alginate-extracellular matrix components blends have been successfully plotted. As shown in table 1, only some of these blends had sufficient mechanical properties to allow plotting and handling of the final construct. The plotting speed and spindle speed were adjusted according to the viscosity of the material, being slower for highly viscous materials (table 1). Among all the other composition tested, alginate 4%/gelatin 5% (w/v) composition was found to be the most suitable for scaffold plotting, because of the high viscosity of the blend compared to all the other materials tested (figure 1). This assured better plotting performances in terms of handling and stability of multilayered cell-laden constructs (table 1).

	Needle $\varnothing$ (mm)	F_{xy} Speed (mm/min)	Spindle Speed (mm/min)	Layer Thickness (mm)	Layer Number	Viscosity (Pa/s)
Alginate	0.41	1000	1.75	0.20	10	11.2 +/- 0.85
Pre-crosslinked alginate	0.41	500	1.75	0.20	15	2923.33 +/- 958.45
Alginate 4%/ Gelatin 5%	0.41	1100	1.75	0.10	17	163.67 +/- 26.72
Alginate 4%/ Hyaluronic acid 0.5%	0.41	1500	1.50	0.10	7	9.13 +/- 1.48
Alginate 4%/ Matrigel 25%	0.41	1250	2.00	0.10	10	25.8 +/- 1.51

Table 1: parameters used for plotting different alginate mixtures, number of layers that could be plotted and viscosity values for each alginate composition.

Alginate 4%/gelatin 5% mixture was selected for future experimentation. Handling and plotting of the alginate/gelatin mixture was further improved by the partial gelation of gelatin at room temperature, compared to the lower viscosity of its initial state (37 C, required for solution preparation), which increased the viscosity of the mixture during plotting.

Figure 1: viscosity of the different alginate mixtures at different shear rate values. The non-newtonian behavior at different shear rates is evident from the graph.

Alginate/gelatin blend allowed plotting up to 17 layers, resulting in scaffolds with open pores in the X-Y direction (figure 2). The number of layers defines how many times the strand deposition process was repeated on top of each other according to the template (CAD model) to achieve the final construct. In a 2x2 cm scaffold, the pore diameter was 1.88 ± 0.18 mm and the strand thickness of the construct 1.55 ± 0.13 mm (figure 2). The diameter of the needle used was 0.41 mm, resulting in a final scaffold 3.7 times thicker because of the superpositioning of 17 layers on top of each other, which showed a partial collapse of the viscous hydrogel precursor before crosslinking. Similarly, the spacing between strands decreased during plotting to 0.5 mm instead of the initial 3 mm programmed into the CAD model. In contrast to conventional thermoplastic polymers extrusion, the hydrogel construct had no pores along the cross-section (z direction). This fact is explained by the low viscosity of the hydrogel precursor compared to molten polymers and

also by the fact that the crosslinking procedure was performed after the plotting of the last layer.

Polystyrene beads of 100 μm in diameter were used as a model for islets of Langerhans to study their distribution in the plotted construct. After plotting, the distance between each bead and the closest pore of the scaffold was measured (figure 2D). Beads were homogeneously distributed in the scaffold, in close proximity to the pores. The layer of gel surrounding them had a reduced thickness compared to conventional bulk structures. The great majority of the beads were at less than 200 μm from the closest pore (figure 2D), which was considered still close enough to the external environment to allow oxygen diffusion to the islets (42).

Glucose diffusion measurements

In order to determine mass transport in this material, a two compartment glucose diffusion assay was performed. Scaffolds were placed in between a compartment containing 12 mM glucose and a compartment containing no glucose. Glucose diffusion through the gel was found to be limited by the alginate/gelatin mesh. Only after 24 hours the glucose concentration in both 50 ml compartments of the chamber reached the equilibrium, accounting for a delay in glucose diffusion through the gel. The graph in figure 2F shows the total flow of glucose in function of time through the gel layer. The graph shows a significant lag time before the glucose flow to time ratio becomes constant (steady state condition). This lag time allows for the calculation of the diffusion coefficient according to $t = l^2/6D$, as explained in the materials and methods. The resulting glucose diffusion coefficient in alginate 4%/gelatin 5% (w/v) gels was estimated to be $D = 1.13 \times 10^{-6} \text{ cm}^2/\text{s}$. In literature, the diffusion coefficient of glucose in water and in a 4% alginate solution has been reported to be $6.7 \times 10^{-6} \text{ cm}^2/\text{s}$ (43) and $5 \times 10^{-6} \text{ cm}^2/\text{s}$, respectively (39). Both coefficients are significantly bigger compared to glucose diffusion values in our system.

Figure 2: (A) 3D printing scheme e concept cartoon (B) Alginate 4%/ Gelatin 5% 2x2 cm plotted scaffold: the pore diameter is 1.88 ± 0.18 mm and the strand thickness 1.55 ± 0.13 mm (scale bar 1 cm). (C) Image of plotted islets in the construct (scale bar 1 mm). (D) Distance islet-pore (μm), polystyrene beads were used as a model for islets distribution in the scaffold. (E) Percentage of glucose diffused through the gel from a 50 ml donor compartment to the acceptor compartment of a custom made diffusion chamber. Equilibrium is reached after circa 24 hours (1440 min). (F) Total flow of glucose through the gel layer in time. There is a significant lag time before the system reaches the steady state. Based on the simplified Fick's law of diffusion (see materials and methods) the diffusion coefficient can be estimat

INS1E viability screening in bulk materials

INS1E insulinoma cells were used as a model for islets of Langerhans and encapsulated into the different alginate-ECM blends to study the effect of hydrogel composition on cell viability over 21 days of culture (figure 3 and table 2). INS1E viability in alginate 4% w/v was suboptimal, with a mean value of 80% of viable cells 24 hours after embedding. Viability dropped to 50% after 3 days of culturing.

In order to further improve hydrogel biocompatibility and cell viability, alginate 4% (w/v) was also mixed with other components such as 5% (w/v) gelatin, 0.5% (w/v) hyaluronic acid or Matrigel 25% (w/v). INS1E cells embedded in alginate 4%/gelatin 5% (w/v) performed the best in terms of cells viability, having a 95% viable cells up to 21 days of culture. Cell viability in alginate 4%/ hyaluronic acid 0.5% (w/v) and alginate 4%/ Matrigel 25% (w/v) also showed improved cell viability, compared to alginate 4% only. However, the handling properties of the alginate/hyaluronic acid and alginate/Matrigel mixtures were less favorable than alginate/gelatin. Constructs made with these mixtures collapsed easily, were less stiff, and the plotting performances were less consistent to the CAD-model.

Figure 3: (A) viability of encapsulated INS1E cells in 4% w/v alginate gel, alginate 4% w/v/gelatin 5% w/v, alginate 4% w/v/hyaluronic acid 0.5% w/v, and alginate 4% w/v /Matrigel 25% w/v. (B) Live/Dead pictures of INS1E cells in the different alginate compositions at day 1, 3 and 7 (scale bar 200 μ m).

	Day 14	Day 21
Alginate 4%	81.7 ± 21.8	91.7 ± 7.8
Alginate 4% Gelatin %5	91.8 ± 7.8	95.2 ± 5.4
Alginate 4% /Hyaluronicacid 5%	88.5 ± 9.4	87.4 ± 8.8
Alginate 4% /Matrigel 25%	88.0 ± 10.9	86.9 ± 10.6

Table 2: viability of INS1E in the different hydrogel mixtures at day 14 and 21. Alginate 4% gelatin 5% supports high viability also at later time points.

Construct shape influence on cell viability and metabolic activity

INS1E viability and metabolic activity were not influenced by the shape of the alginate/gelatin construct in which the cells were embedded (figure 4). INS1E cells were embedded in bulk constructs, plotted porous structures and plotted solid hydrogel scaffolds. The volume (0.3 ml) and the cell concentration (10×10^6 cells/ml) were kept constant to avoid differences in cell density in the three constructs shapes. Cell viability was assessed by live/dead assay and no difference was observed among the three different construct shapes analyzed (figure 4A). The same result was also obtained by analyzing the metabolic activity of the cells. The quantification of glucose and lactate concentrations in the medium resulted in no differences among the three constructs, but in an overall low metabolic activity, suggesting that embedded cells were alive but not functional in the gel (figure 4 B and C), in line with results previously reported by other authors (44).

Figure 4: (A) INS1E viability in bulk constructs, plotted porous and plotted solid constructs. (B and C) metabolic activity of INS1E cells embedded in constructs with different geometries measured as glucose (B) and lactate (C) concentrations in the culture medium. Viability, glucose consumption and lactate production are not influenced by the plotting process or construct shape. (D) live/dead images of the INS1E cells in the different constructs at day 1, day 7 and day 14 (scale bar 200 μm).

Responsiveness of hydrogel embedded INS1E cells

In all the conditions tested, INS1E insulin secretion was drastically reduced or totally absent in response to high glucose conditions, when cells were embedded in hydrogel constructs (figure 5). To detect a functional profile, the insulin secreted in the three low-high-low glucose conditions needs to be compared. The only condition in which a functional (low-high-low) insulin profile in response to the correspondent glucose stimulation was detected, are INS1E seeded on tissue culture plastic. In all the hydrogel compositions, no functional response was seen. Irrespectively of the type of hydrogel or the crosslinking conditions (Ca^{2+} vs Ba^{2+}), cells showed limited responsiveness to glucose stimuli. As reported in literature, calcium ion gradients over the cytoplasmic membrane are involved in regulating insulin secretion mechanism. In our experiment, the loss of functionality was independent on the type or on the concentration of the divalent cation used for crosslinking and was also observed when a non-ionic gel, as agarose, was used for embedding. For this reason, we exclude that interfering with Ca^{2+} equilibrium during the crosslinking procedure could be responsible for the loss of insulin secretion (45, 46). Once the influence of the crosslinker ion was excluded, nutrient diffusion limitations are considered the major reason to explain the loss of functionality when INS1E cells are embedded in bulk hydrogels.

Figure 5: INS1E cells functional response in glucose induced insulin secretion test. Adherent INS1E cells on TCP have been compared to INS1E embedded in bulk agarose gel 1% w/v, bulk alginate 4% w/v /gelatin 5% w/v crosslinked with 102 mM CaCl₂ for 15 minutes (same crosslinking condition used for plotting) alginate/gelatin crosslinked with 20 mM BaCl₂ for 10 minutes, or with 20 mM CaCl₂ for 20 or 30 minutes. In none of the hydrogel composition analyzed, INS1E cells showed a functional response.

Islet transduction, embedding and functionality in the construct

Human islets were plotted in the construct and their morphology, aggregation, viability and response to glucose challenges were analyzed and compared with bulk constructs. Before plotting, viral transduction was used to induce stable expression of GFP in the islets and allow direct visualization in the plotted construct *in vitro* as well as *in vivo* with intra-vital imaging techniques.

GFP transduced human islets plotted in the alginate/gelatin constructs showed comparable morphology and GFP expression as the islets embedded in the same alginate/gelatin composition in bulk form, in 4% w/v alginate only, and in ultrapurified alginate (24-26, 47). Ultrapure alginate was used to compare islet morphology in the new alginate/gelatin mixture to an already used material in the islets encapsulation field (figure 6).

Figure 6: Plotted fluorescent islets in alginate 4% w/v /gelatin 5% w/v, islets in bulk material, islets in bulk alginate and islets in bulk ultrapurified alginate. Images have been taken at day 0, 4 and 7 (scale bar 50 μ m).

The embedding and the plotting procedure did not induce any change in cell morphology. Islets were still round shaped and the shear stress experienced during plotting did not cause islets to disaggregate or decrease in viability. According to the morphological analysis of the embedded islets, the presence of gelatin in the hydrogel mixture did not induce islet spreading or cell adhesion. Hydrogel embedding also prevented aggregation of individual islets (figure 6 and figure 7).

Figure 7: human islet plotted in alginate 4% gelatin 5%, embedded in bulk alginate/gelatin, in alginate only and compared to free floating islet. Islets were imaged at 1, 4 and 7 days. Scalebar 100 μ m.

The retained morphology of the islets was confirmed also after plotting of murine islets (figure 8). These islets were isolated from double heterozygous crossbreed mice Tg(Ins1-eGFP) and B6(CAG-DsRed*MST). In this mouse strain, all cell types are characterized by the expression of DsRed protein under the control of the ubiquitous chicken b-actin promotor. Insulin production by β -cells within the plotted islets is visualized by the specific expression of GFP, under the control of the mouse insulin promotor. The GFP signal in β -cells is much stronger than DsRed, allowing for the visualization of the insulin producing β -cells by the green GFP signal.

Figure 8: murine islet isolated from double heterozygous crossbreed mice Tg(Ins1-eGFP) and B6(CAG-DsRed⁺MST). DsRed protein is expressed under the control of the ubiquitous chicken β -actin promoter. Insulin production by β -cells within the plotted islets is visualized by the specific expression of GFP, under the control of the mouse insulin promoter. Islet maintain their round morphology after plotting in alginate 4% gelatin 5% mixture. Scalebar 100 μ m.

Islet functionality in plotted constructs was compared to islets in bulk hydrogels and to free floating islets. Islets are regarded as functional when their stimulation index is higher than 2 (61). As shown in figure 9A, islets in bulk hydrogels lose their functionality already after one day, being not responsive to glucose stimuli. In figure 6B one can see that the normal low-high-low insulin response to glucose is still present in the plotted construct while it is not visible anymore in the bulk construct. The diffusion of oxygen and insulin is slightly improved in the plotted construct compared to the bulk construct. Functionality in both bulk and plotted constructs is lost after seven days in culture. Interestingly, the reduction in the functional response of plotted islets was not caused by a reduced insulin secretion in high glucose, since this was comparable to the response of the control islets, but was due to an increased insulin secretion in low glucose condition, as shown in (figure 9 B).

Figure 9: glucose induced insulin secretion test on free floating islets, islets embedded in bulk hydrogel and islets in plotted constructs. At day 1, islets are functional in the plotted constructs but not in the bulk hydrogel. (A) stimulation index normalized to insulin secreted in low glucose 1, (B) actual values of secreted insulin (mU/l). Islets are considered functional if the stimulation index in high glucose condition is higher than 2 (61).

Insulin secretion is dependent on the extracellular calcium concentration (44, 45). In an ionic crosslinked hydrogel such as alginate, the extracellular environment is rich in calcium and this fact could interfere with insulin secretion mechanism and explain the apparent loss of functionality of islets when embedded in the gel. To further investigate if islet loss of functionality is related to the divalent ion used for crosslinking, islets were embedded in different hydrogels, using different crosslinking conditions (figure 10A) Irrespectively of the crosslinking ion used (calcium or barium) or the crosslinking time used for gelation, none of the conditions, were able to sustain islet functionality. Islets in ultrapure alginate, that is often used for islet encapsulation (24-26, 47), were crosslinked under the same conditions used for plotting. Also in this case, islets were not responsive to glucose stimulation (characterized by a stimulation index lower than 1).

Since the crosslinking ion appeared not to be the limiting factor for reestablishing islet functionality, we showed that the loss of functionality of embedded islets is caused by a decreased glucose diffusion through the gel. In fact, the glucose diffusion coefficient in these gels ($1.13 \times 10^{-6} \text{ cm}^2/\text{s}$) has been calculated to be significantly smaller than glucose diffusion in water or in a 4% alginate solution ($6.7 \times 10^{-6} \text{ cm}^2/\text{s}$ (43) and $5 \times 10^{-6} \text{ cm}^2/\text{s}$ (39) respectively). If this hypothesis would be

correct, the inhibition of islet response to glucose challenge should be transient and the islets should recover after retrieval from the gel. To further prove this point, embedded islets were retrieved from the gel and their functionality tested again 2 days after retrieval. As shown in figure 10B, islets functionality was fully restored when retrieved from the hydrogel, statistical test was performed and no significant difference was found among the stimulation indexes of the three conditions. This finding confirmed that islets remained viable in a plotted hydrogel despite losing glucose responsiveness. The lack of such responsiveness was likely due to limitations in glucose and nutrient diffusion.

Figure 10: (A) function test on islets cultured in different hydrogels with various crosslinking conditions. Islets have been embedded in alginate 4%/gelatin 5% or ultrapure alginate and crosslinked with CaCl₂ 102 mM for 5 and 15 min, with CaCl₂ 20 mM for 20 min and with BaCl₂ for 10 min. None of the conditions tested were functional. (B) alginate/gelatin gel crosslinked with 102 mM CaCl₂ for 15 min was dissolved and islets were retrieved from the gel. Functionality was tested and compared to free floating islets and free floating islets treated with buffer. After retrieval from the gel, functionality was restored. Statistical test was performed and no significant difference was found among the stimulation indexes of the three conditions.

Intravital imaging results

The alginate/gelatin (4%/5% w/v) plotted hydrogel scaffolds containing islets were mounted on the imaging window and transplanted subcutaneously in nude mice. A schematic drawing can be seen in figure 11. With this *in vivo* imaging technique, we were able to non-invasively image the islets in the hydrogel construct up to 7 days after transplantation. This imaging method allowed us to longitudinally monitor a specific islet in consecutive imaging sessions. We were able to detect GFP expression and image the islets in the construct at different time points (figure 11G), where single cells within an islet are clearly visible.

At subsequent time points, islets remained visible in the construct and the same islet could be easily traced, although appearance of GFP expression changed and single cells were not as clearly visible as before. This observation can be seen in figure 11G by a more speckled appearance as the fluorescent signal of the cells decreased over time. This fact correlates with the diminished metabolic activity and functionality already demonstrated in the *in vitro* experiments. The decreased GFP signal and metabolic activity at day 4 and 7 after transplantation can be again correlated with the fact that nutrient and oxygen diffusion in the construct are limited to the lower layers of the construct, which are in direct contact with the surrounding tissue and blood vessels. The diffusion in the upper layers of the construct is restricted by the presence of the imaging window. Moreover, the intravital technique is limited to 450 μm imaging depth, which allows only for the upper layers of the scaffolds to be studied. These layers are more affected by a lack of nutrients diffusion from the underlying tissues. This hypothesis was confirmed by the fact that islets in the *in vitro* control were still bright fluorescent up to day 7, with no sign of cell death or disaggregation. At day 7 both the mice with a subcutaneous transplant and the mice with window implants were terminated and the alginate/gelatin scaffolds were explanted and processed for histology. Hematoxylin and eosin staining confirmed the presence of islets in the scaffold, which showed no sign of apoptotic nuclei, but no insulin or glucagon was detected by immunostaining (figure 12D) which might indicate a reduced metabolism due to the embedding in hydrogel and lack of sufficient nutrient supply.

Figure 11: human islets (A) were transduced with CMV-GFP virus, handpicked (B), and plotted into a 2x2 alginate construct (C). A 1 cm circular sample was glued to the abdominal imaging window, as showed in D and schematically depicted in F. Figure E shows GFP transduced islets in a plotted construct. The same islets could be imaged at day 1, 4 and 7 (G) (scale bar 100 µm).

Figure 12: In vitro islet fluorescence was bright (A), and the morphology of the islets was preserved (B). After explant of the in vivo samples, islets were imaged with hematoxylin/eosin staining (C), however no expression of insulin or glucagon was detected by immunostaining (D) compared to pancreas sections (E) (red: insulin, green: glucagon, blue: DAPI. Scale bar 100 µm).

Discussion

3D bioplotting is a well described technique in tissue engineering field, mostly applied to the extrusion of thermoplastic polymers, to create custom engineered scaffolds for the regeneration of bone and cartilage (48, 49). Printing allows tailoring of shape, porosity and mechanical properties: these parameters can be finely tuned in order to steer cells response to match the properties of the specific tissue to be regenerated. The only application of printing in pancreas tissue engineering is reported in a paper of Daoud et al (15), with islets being seeded in a printed poly(DL-lactide-co-glycolide) construct, a material whose mechanical properties are not comparable with those of the soft tissues in pancreas.

Combining hydrogels with the 3D bioplotting for the regeneration of soft tissues and in particular for the encapsulation of islets of Langerhans should result in a clinically relevant size construct for islet delivery. This construct will provide a protective environment for the embedded tissue, with mechanical properties better mimicking those of soft tissues, if compared to solid thermoplastic materials. In contrast to conventional embedding in bulk hydrogels, a plotted structure can increase the available surface area for nutrients and catabolites exchange, avoiding the formation of a necrotic core which can affect bulk hydrogels. This would ultimately result in the possibility of up-scaling the construct to a size that can contain a clinically relevant amount of islets.

This study demonstrates that the plotting of large cell aggregates of 50-300 μm like islets of Langerhans in a hydrogel is feasible, without compromising islet viability, morphology and aggregation.

Material selection was performed using the model cell line INS1E and high viability values were observed for INS1E and islets in the alginate/gelatin composition, but the metabolic activity of cells embedded in the hydrogel is reduced and these findings are in line with what has been also reported by other authors (44, 50, 51). Our data suggest that islet plotting per se is not causing the impairment in functionality, but it is caused instead by impaired nutrient diffusion in such a

viscous hydrogel precursor. This loss of metabolic activity cannot be rescued only by improving nutrient diffusion by means of a macro-porous structure.

Opposite results in terms of metabolic activity were reported for human mesenchymal stem cells (hMSC) encapsulated in similarly plotted constructs (31). In this case, embedding the cells in the porous plotted scaffold had a beneficial effect on their viability compared with solid or bulk constructs (31).

The comparison with previously published results highlights that different cell types react oppositely to the embedding in the printed construct and might suggest that INS1E β -cells and islets of Langerhans are more sensitive than mesenchymal stem cells (MSCs) to hydrogel embedding. *In vivo*, islets in the pancreas are extensively vascularized in order to support their high metabolic activity, requiring constant and abundant glucose and oxygen supply for their normal functioning. On the contrary, MSCs normally reside in hypoxic environments in the body and are less metabolically active. This fact could explain why embedding in a porous hydrogel construct is beneficial for MSCs viability and metabolic activity (31), yet not enough for a more sensitive and metabolically requiring cell type as β -cells and islets of Langerhans.

The low INS1E metabolic activity and islet reduced functionality in the gel are indications that their activity is somehow limited by the material in which they are embedded. This is confirmed by the restored islet functionality when retrieved from the gel. According to these data, the major limitations that the plotting of cell-laden constructs still encounters is the availability of a suitable material capable of proper mass transport properties to maintain cell functionality and having at the same time sufficient mechanical properties for a layer by layer processing.

The optimal material for plotting has to meet various requirements: high viscosity, fast crosslink, biocompatibility, mechanical properties comparable with soft tissues and, once crosslinked, permeability to metabolites and catabolites. For plotting purposes the material has to be viscous enough to be plotted on subsequent layers on top of each other without collapsing under its own weight: such a highly viscous material restricts mass transport in the material itself, resulting in a limitation for

islets functionality. To date, limited materials are available for cell-laden plotting purposes (52-54), and finding a compromise between mechanical properties for plotting, matrix stiffness, nutrient diffusion and functionality of embedded cells is still rather challenging (55). The main limitations in the hydrogel used for this study are given by nutrient diffusion in such a viscous and highly concentrated gel. This study shows that the viability and functionality of embedded islets could be limitedly improved by a porous construct shape, if compared to solid bulk hydrogels. Islets showed functional response, although reduced, at day one in the porous constructs while no functional behavior was observed in the bulk constructs at this time point (figure 9B). This shows that the plotted porous design can offer an improvement, although still limited, for nutrient diffusion in the gel. The reduced mass transport still remains a major limitation to the applicability of this technique to a scaffold of a clinically relevant size.

This is particularly true for islets of Langerhans, since they have very peculiar and demanding metabolic requirements and thus, they are more sensitive to the surrounding environment. In their native state, islets of Langerhans extensive vascularization provides for proper glucose sensing and insulin secretion and this characteristic is essential for their proper functionality. If this exchange is limited by the tight surrounding matrix, as it happens when islets are embedded in the alginate/gelatin mixture, islet functionality is hampered. Islets are viable in the hydrogel construct, but their ability to detect changes in glucose concentration and respond with proper insulin secretion is reduced by the tight matrix in which they are embedded. Islets are fully functional again when retrieved from the hydrogel construct and this confirms that the apparent loss of functionality is caused by limited mass transport through the gel.

The retrieved islet functionality after dissolution of the gel also excludes that the islets were damaged by the shear stress experienced during printing. It is reported in literature that a mechanical stress could permanently damage islet functionality, but in that case the functionality could not be retrieved simply by dissolving the surrounding gel.

As shown by the bead distribution in the plotted gel, the great majority of the beads is located at less than 200 μm from the interface, less than the oxygen diffusion limit through tissues. Yet, the glucose diffusion coefficient measured in our system ($1.13 \times 10^{-6} \text{ cm}^2/\text{s}$) is significantly lower than glucose diffusion in water ($6.7 \times 10^{-6} \text{ cm}^2/\text{s}$) (43) and in 4% alginate solution only ($5 \times 10^{-6} \text{ cm}^2/\text{s}$) (39). Such limited glucose diffusion in the construct is explained by a crosslinking obtained in saturation condition and by the presence of gelatin. The mesh size of a 4% w/v alginate solution has been reported to be around 7 nm (56), but the presence of gelatin as a interpenetrating network decreases consistently the number, diameter and length of the pores in an alginate hydrogel. This accounts for a considerable delay in glucose supply to the islets and a corresponding delay in insulin secretion from the hydrogel. Moreover, given the higher molecular weight and bigger hydrodynamic radius of an insulin molecule, the limitations experienced in insulin diffusion through the gel would be even more severe. Limited diffusion coefficients can explain the low metabolic activity, together with the apparent lack of functionality of the embedded cells. Moreover, the diffusion coefficient D increases with the second power of the gel thickness ($D=l^2/6t$). This means that for a small decrease in the gel thickness, a bigger increase in the diffusion coefficient is expected. This would explain why islets embedded in the plotted construct show some reduced functionality at day one, which is already completely absent in bulk constructs. This emphasizes the importance of construct design for islets functionality.

The effect of calcium ions used for crosslinking on insulin secreting pathways was also investigated as one of the possible reasons to explain INS1E and islet loss of functionality in the construct. As showed by many authors, voltage gated calcium channels, and consequently trans membrane calcium ion gradient are involved in insulin secretion mechanism (45, 46, 57). Since the extracellular gel environment is quite rich in calcium ion concentration (102 mM crosslinking), the increased calcium concentration of the extracellular compartment might have caused the increased insulin secretion in low glucose condition. Although no difference in

functionality was shown when hydrogels were crosslinked with other divalent cations, different concentrations or with reduced exposure times.

To further study whether encapsulated islets maintained their viability *in vivo*, we implanted plotted constructs subcutaneously in nude mice and monitored their viability by intravital imaging. This is a powerful technique for *in vivo* follow up of transplanted tissue, enabling the imaging of the same transplanted islet in consecutive imaging sessions and study the process of revascularization of the scaffold. Intravital imaging has been applied to transplanted islets implanted under the kidney capsule (58) or in the anterior chamber of the eye (59, 60), which is a highly vascularized location. Here we show for the first time the applicability of this technique for the imaging of subcutaneously transplanted tissue in an implantable scaffold. In this kind of application, the presence of the window on top of the construct and the poorly vascularized location can furthermore limit oxygen diffusion to the transplanted tissue and islets are not anymore surrounded by tissue and blood vessels from all directions but only from the bottom. Consequently, nutrient diffusion to the islets located in the upper parts of the scaffolds has to rely solely on nutrient supply from the bottom part of the construct. This is even more important if we consider that the imaging depth of this intravital imaging model is also limited to 450 microns, which allow us to monitor only the upper layers of the hydrogel, the ones which suffer the most for lack of oxygen diffusion. Despite these limitations, we were still able to detect GFP expression and image islets for seven days in the construct, although islets showed signs of reduced GFP expression at later time points. This result correlates with our assumption of a reduced metabolic activity of the embedded cells, caused by an increased nutrient diffusion limitation in our model which was even more pronounced when an imaging window was used. Thus, both *in vitro* and *in vivo* results show that the plotting of islets is possible but the material optimization for this application is crucial to allow proper metabolite exchange with the embedded tissue.

In contrast to conventional islet encapsulation, here islets are confined in one location and after transplantation, blood vessels might grow through the pores of the construct, in closer contact with the embedded islets compared to the inner part

of a bulk hydrogel scaffold. Potentially, an immunoprotective scaffold could be developed by using a non-degradable hydrogel, with a proper mesh size capable to block antibodies and cells of the immune system and at the same time large enough to allow insulin diffusion.

Conclusions

This study demonstrated that 3D hydrogel plotting can be a valuable technique to couple islet embedding in hydrogel strands with a pre-defined three-dimensional scaffold architecture. 4% alginate/ 5% gelatin was found to be a suitable hydrogel mixture for plotting of islets and β -cells, without compromising their viability and morphology. However, the high viscosity of the material needed for plotting, resulted in a dense mesh size, which impairs glucose diffusion and limits islet functionality. Since a highly viscous biomaterial is needed for plotting, a compromise has to be established between material properties allowing scaffold plotting and sufficient nutrient diffusion. Plotted hydrogels could effectively increase nutrient diffusion compared to the same bulk hydrogel, but the macro porous structure is not enough to overcome limitations caused by an insufficient diffusion of nutrients in this specific gel composition.

Despite the limitations in islet functionality when embedded in the construct, islets restored full functionality when retrieved from the hydrogel. In conclusion, plotting can be a powerful technique to engineer complex constructs, containing a clinically relevant number of islets. Although promising, the potential of the technique is currently hampered by scarce availability of suitable biomaterials. Further research is needed to develop hydrogels with sufficient viscosity and mechanical properties suitable for plotting but also with the capacity to support sufficient nutrient and secretory product diffusion for a physiologically relevant islet functionality.

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Thermoplastic polymer section

Chapter 4

Salt-Leached Porous Scaffolds functionalized with VEGF for Islets of Langerhans Transplantation

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Abstract

Lack of revascularization is a common problem affecting the great majority of constructs for tissue engineering applications. Cells located in the innermost core of the scaffold may undergo apoptosis and this holds particularly true for large constructs, which can host a large amount of cells for clinical use. An example of a clinical application involving cells which require a dense vascular network to properly function is islet transplantation in type 1 diabetes treatment. The use of islets in combination with three dimensional scaffolds is limited by a scarce blood supply to the transplanted tissue, thus hampering the beta cell's viability and functional response to glucose stimulation. In this study we developed a double layered, salt leached polymeric scaffolds. This scaffold combines an open porous structure to retain islets, to a porous polymer layer which allows for biofunctionalization with vascular endothelial growth factor (VEGF) to stimulate revascularization.

The individual layers in these scaffolds were composed of poly(D,L-lactide-co- ϵ -caprolactone) (PDLLCL), commercially know as Neurolac or Vivosorb (Polyganics BV, NL), and polycaprolactone (PCL), both made porous by means of salt leaching technique and assembled in subsequent layers. The porous PDLLCL layer, having minimal interaction with islets, serves as a protective environment for islets of Langerhans. The porous PCL layer was covalently bound to heparin, which can electrostatically bind VEGF and act as a reservoir to attract blood vessels to the construct. VEGF immobilization increased in heparinized scaffolds compared to non-treated samples ($91.2 \pm 6.7\%$ vs $71.4 \pm 6.1\%$ of the total amount of VEGF used for biofunctionalization). In addition, we also observed considerably reduced burst release profile in case of heparinized scaffolds. After seeding islets and MIN6 Insulinoma cell aggregates into the porous PDLLCL layer, we observed no noticeable cell outgrowth or attachment to the material resulting in maintenance of their rounded morphology. The results show that the porous scaffold can serve as a protective environment for islets and can increase the number of endothelial cells in the construct *in vitro*.

Introduction

Neovascularization within large sized tissue engineered scaffolds is a crucial factor for the survival, proliferation, differentiation and function of transplanted cells and to provide a good integration with the surrounding host tissue. Cells located in the most central part of large transplants often suffer from a lack of revascularization (1-3), and their survival is negatively affected by an insufficient oxygen and nutrient supply (2,3). Several studies have shown that poor nutrient diffusion leads to the formation of a necrotic core in the center of the implant, resulting in a lack of functional cells in the center, while most cells initially seeded into these implant can be found at the outer rim. In the creation of large scaffolds used for cell therapy, one should take into account the limitations in nutrient and oxygen diffusion and design the construct geometry in such a way that revascularization and neoangiogenesis can take place rapidly and in the most efficient way possible (4).

Rapid revascularization is particularly important in case of islet transplantation. The Islet's natural physiological environment is characterized by an abundant blood supply: islets of Langerhans receive 5-10% of the total pancreatic blood flow, although their mass only accounts for 1-2% of the total pancreatic mass (5). This abundant blood supply is needed in order to provide enough oxygen to the metabolically very active beta cells, quickly sense variations in blood glucose concentrations and properly respond with appropriate insulin secretion. During islet isolation from the pancreas the islet's extensive capillary network is lost due to mechanical and enzymatic digestion (6-8). It is important that the vascular network of the transplanted islets and their connection to the host vasculature is re-established as soon as possible after transplantation to not only preserve the potency of the transplanted tissue, but also its viability and functionality in the long term as well (9). Besides the instant blood mediated inflammatory reaction after intrahepatic clinical islet transplantation (CIT), poor revascularization is often one of the main reasons limiting the outcome of CIT. Commonly only 10% of the transplanted patients remain independent from insulin injections 5 years after transplantation (10), In most patients, this transplant failure in the long term is associated with poor engraftment and poor reperfusion.

Several islet delivery strategies and alternative transplantation sites have been investigated (11,12), most of the times with the use of a biomaterial to help confine the transplanted islets to one location, and provide a more favorable environment in comparison to the intrahepatic milieu used in CIT (13-16). The use of growth factors is an established method for enhancing neoangiogenesis (17-19). Over the years Vascular Endothelial Growth Factor (20-23) and basic Fibroblast Growth Factor (21,24-27) have been identified as important biomolecules which can be used to boost the formation of new blood vessels. Injecting these growth factors alone is not enough to achieve proper revascularization leading to mature blood vessels. Several authors showed that a controlled delivery of these growth factors *in vivo* is necessary to obtain properly formed and stable capillaries (20,24).

Besides the use of growth factors, the scaffold design is crucial for guiding and enabling the formation of a dense vascular network. A relatively straightforward method for the creation of an open porous structure is particle leaching using salt crystals to create porous scaffolds from different polymers (28-30). This rather simple and inexpensive technique is based on mixing salt crystals into a polymer solution. After solvent evaporation and solidification of the polymer, the salt can be dissolved by immersion in water. This technique can be used with a large variety of polymers and leads to highly porous constructs containing interconnected pores with variable pore size distribution. Modifications of this technique can involve the use of gelatin particles (31,32), paraffin spheres (31,32) or a combination of thermal processing methods and salt leaching techniques (33,34). Other more sophisticated methods for obtaining porous scaffolds involve also the use of casting porous polymer sheets on pillared wafers, laser drilling (35) and the printing of 3D porous polymeric scaffolds (36). The main advantage is that particulate leaching can be applied without the need for expensive and dedicated equipment. Moreover, changing simple parameters such as the concentration of salt crystals or their size gives good control over parameters such as scaffold porosity, interconnectivity and pore size, without extensive adaptations to the fabrication method.

Here we report on a novel scaffold design for an islet delivery device based on a porous double layered biofunctionalized scaffold. The scaffold is composed of a combination of two polymers, polycaprolactone (PCL) and PDLLCL. PDLLCL is not cell adhesive and can be used for containing islets of Langerhans, efficiently, preventing their clustering and keeping their rounded morphology (37). PCL allows the formation of an amine binding to heparin: the negatively charged sulfate groups on heparin electrostatically interact with the positively charged amino groups in lysine residues of VEGF. This interaction can be exploited for controlled release of VEGF from heparin coated PCL scaffolds. We found that in the double layered system PCL acts as a VEGF containing depot, allowing for efficient binding and delivery of VEGF, while PDLLCL acts as an islet retainment compartment.

Materials and Methods

Fabrication of porous multi-layer scaffolds

Porous PCL-PDLLCL layered scaffolds were created by solvent casting combined with salt leaching technique. PCL (Sigma, Mw 45.000) and PDLLCL (Vivosorb Polyganics BV, NL) were separately dissolved in chloroform (Sigma) at 10% and 4% (w/v) respectively. Sodium chloride was sieved to select a particle size of 250-425 μm to create an homogeneous size distribution and added to both solutions. This crystal size was selected because compatible with the islet size, ranging from 50 to 300 μm . A 1:10 (w/v) salt ratio was used in PDLLCL solution and a 1:25 (w/v) salt ratio was used in PCL solutions. Mixtures were mixed for 15 minutes to obtain a homogenous slurry. Subsequently, PCL and PDLLCL slurries were loaded in syringes (Becton Dickinson) and deposited in 4 cm wide dishes in case of PCL, and 50 ml beakers in case of PDLLCL. These slurries were left to dry for 48 hours. NaCl particles were dissolved in demi water for 24 hours under constant stirring after which the scaffolds were allowed to dry. Scaffolds were cut in squares (PCL 12x12 mm and PDLLCL 10x10 mm). An 8 mm circular hole was punched into of the PCL scaffolds to create an inlet port for islet seeding and the resulting circle was kept to serve as a lid (see figure 2). The PCL layer is placed on top of the

PDLLCL layer and the two are annealed by heating up the rim of the two layers to 65 °C with a point heating device (Weller wtcpt).

Heparin cross-linking on scaffolds

Heparin was covalently immobilized on the PCL layer of the scaffolds by using N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide (EDC, Sigma) and N-hydroxysuccinimide (NHS, Sigma). Heparin coating creates a binding layer for vascular endothelial growth factor (VEGF). Scaffolds were soaked for 15 minutes in 0.05M 2-(N-Morpholino)ethanesulfonic acid MES buffer pH 5.5 (2ml/scaffold). Porcine derived heparin (Sigma) 1% w/v was dissolved in fresh MES buffer together with 0.5M EDC and 0.5M NHS. The scaffolds were then added to the solution (2ml/scaffold) and stirred for 15 hours at room temperature. Sterile water was used to extensively wash (5 times) the scaffolds afterwards to remove any unbound heparin (38).

Static attachment of Vascular Endothelial Growth Factor

VEGF 165 (Peprotech) was dissolved at 1000, 500 and 200 ng/ml in 0,1% PBS/BSA (Gibco, Sigma-Aldrich). Sterile scaffolds were added to 1ml of VEGF solution and incubated for 24 hours at 4 °C.

Scanning electron microscopy

The surface morphology of the heparin and VEGF functionalized scaffolds was assessed using a scanning electron microscope (SEM) (Philips XL30 ESEM-FEG). Samples were mounted on aluminum stubs with carbon tape and gold-sputtered (Cressington sputter coater 108 auto). Samples containing cells or islets were first fixated with 2.5% glutaraldehyde in cacodylate buffer. After serial dehydration in ethanol, samples were immersed in hexamethyldisilazane series (Merck Millipore) and left to air dry.

Pore size distribution

Salt particles were sieved to a size between 250 and 425 μm . SEM was used to measure the resulting pores after salt leaching. 240 measurements were made on 10 samples which were subdivided into groups of 50 μm increments.

Azure staining of scaffolds

To check heparin binding, a 0.01 mg/ml solution of Azure II (Sigma-Aldrich) was prepared in distilled water. Scaffolds, with and without heparin, were incubated in 1 ml of the solution for 10 minutes. All samples were washed 3 times with distilled water washing away excess or unbound dye.

Quantification, release and activity of VEGF

Human Umbilical Vein Endothelial Cells (HUVEC) were cultured in EBM-2 medium (Lonza) at 37 °C and 5% CO₂. Cells between passage 2 and 6 were used in all the experiments.

VEGF content determination

VEGF was electrostatically bound to the heparin-coated scaffold surface with initial concentrations of 200, 500 and 1000 ng/ml in PBS/BSA (0.1%). To determine the amount of VEGF immobilized on the scaffold, the remaining VEGF amount in the initial immobilization solution after 24h incubation was quantified by using a VEGF Elisa kit (R&D Systems). To assess the release kinetic, scaffolds were incubated in PBS/BSA (0,1%) solution at 37 °C and samples were taken every 24 hours for a period of 14 days. Collected solutions were analyzed for their VEGF content using an ELISA kit (R&D Systems). To show whether VEGF was still present on the scaffolds after two weeks they were stained using a staining ELISA kit (Peprotech).

Migration assay

To assess whether VEGF was immobilized without affecting its biological activity, an in vitro endothelial migration assay was performed. HUVECs were seeded at a density of 45.000 cells/cm². After adhesion to tissue culture plastic, cells were

treated with 10 µg/ml mitomycin (Sigma-Aldrich) for 1,5h to block proliferation and subsequently washed with PBS. A porous PCL scaffold of 5mm in diameter, either blank, with loaded VEGF (200, 500 and 1000 ng/ml) or with heparin and VEGF (same concentrations) were laid onto the cell layer. Samples were left to incubate for 2 days to allow for migration of the HUVECs in the porous layer. After three days the scaffolds were collected and the cells were lysed by repeated cycles of freeze-thawing and the DNA content of each sample was assessed by using a Picogreen assay (Invitrogen).

Proliferation assay

The capacity of VEGF to induce HUVEC proliferation was assessed by means of a PrestoBlue® assay (Invitrogen). Polymer samples (circular 5mm diameter) were prepared as described before and a test series of non treated controls, heparin-coated, or heparin-coated samples with 200, 500 and 1000 ng/ml VEGF was created. On top of the porous PCL sample 3×10^5 HUVECs were seeded, in a droplet of 50 µl, and left to adhere for 4 hours at 37°C. After cell attachment, 1 ml of cell culture medium was added for subsequent cell culture. A Presto Blue assay (Invitrogen) was performed according to the manufacturer's protocol to determine the viability and proliferation of the cells after day 1, 3, 5, 7 and 10 of cell culture. After the last time point total DNA was determined using a Picogreen assay (Invitrogen).

Cell culture and Aggregate formation

Human islets of Langerhans were isolated from donor pancreata and kindly provided by LUMC Leiden's islet isolation center. The Dutch Government has approved human islet isolation by this institution for clinical and research purposes. We used human islets which were not eligible for clinical transplantation. Only islets of donors with a signed informed consent were used. All anonymous donors were coded in accordance with the Code of Proper Secondary Use of Human Tissue in The Netherlands by the Dutch Federation of Medical Scientific Societies. Islets were cultured in CMRL medium (Cellgro) supplemented with 10% FBS and 1% Pen/Strep according to Fraga *et al.* (39).

Mouse insulinoma MIN6 cells (P29-38) were cultured in DMEM (Gibco) with 10% FBS (Lonza), 1% penicillin-streptomycin (Invitrogen) and 1.4 μ l/ml 2-mercaptoethanol (Sigma, Gibco). Serving as a representative model for islet of Langerhans, MIN6 cells were cultured in micro-aggregates using microwell agarose chips containing microwells of 200 μ m in diameter according to Hilderink *et al.* (40). In short to fabricate the microwell-containing agarose chips, a 3% agarose (Invitrogen) solution in PBS was poured on a PDMS mold with pillars of 200 μ m in diameter. After solidification the molds were peeled away and agarose chips incubated overnight in cell culture medium. Onto each microwell chip 10^6 MIN6 cells were seeded and centrifuged for 1 minute at 150 g to disperse the cells into the microwells in an homogenous manner. Cells were allowed to aggregate for 2 days before they were retrieved from the chips by mild centrifugation at 150 g for 1 minute (40).

Aggregate seeding and glucose induced insulin secretion test

The MIN6 aggregate's capacity to secrete insulin in response to glucose stimulation was assessed by using a static glucose induced insulin secretion test. MIN6 aggregates were seeded in porous PCL-PDLLCL scaffolds, with or without Heparin coating and/or VEGF (1000 ng/ml).

Prior to islet seeding, the scaffolds were put in a T25 flask (Thermo-Scientific) with 10 ml of medium and were placed in a vacuum desiccator. Vacuum was applied for two times 10 minutes to remove any air trapped in the porous scaffold to facilitate the entrance of medium in the pores of the construct. The scaffolds, with lids removed, were placed in non-adherent 24 well plates (Corning) after which seeding of the cell aggregates was performed with the help of stainless steel rings (Aix-scientific) placed on top of each scaffold. About 1000 aggregates, suspended in 75 μ l, were seeded in the ring on each scaffold. Samples were centrifuged for 1 minute at 150 g to drive the aggregates into the porous architecture of the PDLLCL layer. The stainless steel ring was removed, and the round lid was placed into the entry hole after which an additional 825 μ l of medium was added. Scaffolds were cultured for a period of 7 days while medium was refreshed daily. After 7 days the

aggregate containing samples were incubated in Krebs buffers containing consecutively low (1.67mM), high (16.7mM) and a low concentration of glucose for 45 minutes. At the end of each incubation step, medium samples were taken and the insulin content was quantified by ELISA (Mercodia). The stimulation index was calculated from each sample by dividing the amount of insulin released at high glucose by the amount of insulin released at low glucose and displayed in bar graphs for comparison. Samples for testing Islet of Langerhans functionality were prepared in a similar manner and glucose stimulation was tested in a similar way. The amount of insulin secreted was quantified using a human insulin ELISA kit (Mercodia).

Statistical analysis

IBM SPSS statistics 20 was used for statistical analysis of the data. A one-way ANOVA followed by a Bonferroni post-hoc test was performed and data were considered significant when p values were below 0.05. When experiments with multiple time points were executed, statistical significance was determined between samples taken at the same time point to compare the effect of different scaffold types.

Results

Fabrication of porous multi-layer scaffolds

Porous PCL was obtained using a salt leaching technique. An inhomogeneous structure was obtained after evaporation of the solvent, with the upper side (air interface) being more dense than the side in contact with the glass mold (Fig 1 A, B). To remove the outer dense layer and therefore obtain an open homogeneous porosity throughout the thickness of the scaffold, the dense part was removed with a scalpel.

Scanning electron microscopy was used to study the structure of the cross section more closely, as shown in figure 1C. The PCL structure presented a very open and interconnected porosity. The porous PDLLCL layer was created in the same way as the porous PCL layer, but with a different polymer:salt concentration; 1:10

instead of 1:25. Similarly to PCL, also PDLLCL showed an inhomogeneous porosity at its two interfaces. In this case, the dense side was intentionally left and used as a barrier to prevent that islets would flush out during seeding and cell culture. Figure 1D to F show macroscopic light micrographs of both sides of the porous PDLLCL sheet and electron micrograph of the cross section showing the internal structure of the sheet (fig 1F).

To fabricate the final bi-layered construct, the porous PDLLCL (on the bottom) and PCL (on top) were cut in squares and joined together by using a point heat source. Islets were seeded in homogenous fashion via the circular entry port in the top PCL construct to minimize islet clumping. After seeding of aggregates or islets, the circular entry ports could be easily closed by placing the circular lid into the hole in a press fit manner (see figure 2).

Figure 1: A) Glass-PCL interface showing a porous and rough structure while the air-PCL interface (B) is more smooth but closed. C) 20X SEM image of PCL cross section displays both its pores and its interconnectivity. PDLLCL shows, like PCL, an open porous side on the glass-PDLLCL interface (D) and a dense layer at the air-PDLLCL interface (E). 50X magnification of the PDLLCL's cross section with distinct open pores and openings connecting pores to one another (F).

Figure 2: 'Open and Close' setup. (A) schematic representation and (B) picture. The scaffold displays the two parts individually while on the right the plug is inserted in the bottom portion, forming a barrier to keep the islets in the scaffold.

Pore size distribution

By measuring the PDLLCL pore dimensions we found a normal distribution, with a median in the 200-250 μm range (figure 3). The initial salt particles used range between 250 and 425 μm , suggesting that PDLLCL shrinks after salt leaching and drying, or the pores collapse slightly during this process. The resulting porosity matches the average size distribution of human islets which ranges between 100 to 300 μm .

Figure 3: Pore size distribution in porous PDLLCL sheet. Measured pores are divided into groups of 50 μm increments showing a normal distribution.

Functionalization of multi-layer scaffolds

Heparin was covalently bound to PCL by EDC/NHS chemistry. We found that the heparin coated PCL surfaces display a submicron roughness after the coating process, while untreated PCL samples showed a smooth surface (figure 4). Positive azure blue staining further confirmed the presence of covalently bound heparin to the PCL layer, which remained even after extensive washing of the scaffold with distilled water (figure 4 C and D). Initially an unspecific background staining was present on the PDLLCL layer which disappeared during washing.

Figure 4: SEM image of PCL scaffolds uncoated (A) or coated with heparin by means of EDC/NHS chemistry (B). Both images taken at similar magnification. Representative image of a bilayered scaffold stained with azure blue. The blue coloration indicates the presence of heparin. PCL cover and plug show a clear blue staining after extensive washing with distilled water, confirming covalent coupling of heparin (C). PDLLCL bottom becomes white after washing with distilled water, demonstrating its inability to bind heparin (D).

Electrostatic binding of Vascular Endothelial Growth Factor

The presence of VEGF after 14 days was confirmed by immunolabeling. Scaffolds onto which VEGF was adsorbed via heparin showed a considerable more intense coloration compared to samples with VEGF only. (figure 5 D). Control scaffolds

with or without heparin to which no VEGF was adsorbed showed no specific staining.

Quantification, release and activity of VEGF

To assess the immobilization efficiency of VEGF on heparin coated scaffolds, the amount of VEGF still present in the immobilization solution after overnight incubation was quantified by using an ELISA kit. Figure 5 provides the different amounts of VEGF that were bound on porous PCL coated scaffolds. As expected, samples with heparin showed a higher binding affinity, respectively 167 ± 3 ng, 480 ± 1.8 ng and 941 ± 12.1 ng of VEGF for the 200 ng, 500 ng, and 1000 ng concentrations initially used. Non-heparinized PCL showed lower binding affinity for VEGF, with measurements of 155.6 ± 12.9 ng, 353 ± 22.6 ng and 657.5 ± 12.4 ng VEGF. Respectively, the percentage of bound VEGF from the total amount of administered VEGF was also higher for heparinized PCL than uncoated PCL. The three concentrations showed $77.8\% \pm 6.5\%$, $70.6\% \pm 4.5\%$ and $65.8\% \pm 1.2\%$ attachment on non-coated porous PCL while $83.5\% \pm 1.5\%$, $94.1\% \pm 1.2\%$, $91.2 \pm 6.7\%$ for heparin-coated PCL, thus showing an almost complete binding of the loaded VEGF.

Cumulative release of VEGF from heparinized and non-coated scaffolds was also tested for a period of 14 days (figure 5 C). The two highest concentrations of VEGF (500 and 1000 ng/ml) immobilized on non-heparinized porous PCL showed a burst release in the first 5 days. This same trend could also be seen in samples of PCL with 200 ng/ml VEGF, but to a smaller extend. After this initial period, the release reached a plateau, releasing on average 40 pg/day for all three concentrations. In the heparinized samples the burst release was reduced showing a constant release rate of 30 pg/day on average.

Figure 5: VEGF immobilized on porous PCL scaffolds (grey) or to heparinized porous scaffolds (black) shown as concentration of VEGF left in the immobilization medium (ng/ml) (A) or as percentage of the initial load (B). Values represent means and standard deviations, $n=3$. In vitro release of VEGF for varying doses of VEGF either on bare PCL scaffolds or on PCL scaffolds with immobilized heparin. The graph shows the cumulative release of VEGF up to 14 days. (D) Staining of the VEGF still absorbed on the scaffolds after the release study. From left to right: PCL 0, 200, 500 and 1000 ng VEGF and PCL/heparin 0, 200, 500 and 1000 ng VEGF. * represents statistical significance with $p<0.05$. Values represent mean and standard deviation ($n=3$).

Metabolic activity assay

The metabolic activity of 3×10^5 HUVECs seeded on the scaffolds was determined using a Prestoblu[®] assay. Metabolic activity appeared to be higher in samples without heparin treatment compared to the ones with heparin. The presence of VEGF in both conditions significantly improved cell metabolic activity and a clear increasing trend for increasing VEGF concentration is seen in the heparinized surfaces (figure 6).

Figure 6: Presto blue assay quantifying proliferation per sample over 5 time points. Heparin coated samples all show a lower proliferation of HUVECs. Symbols indicates a significant difference ($p < 0.05$). Statistical significance has been evaluated comparing heparinized and non-heparinized scaffolds at the same time point. Values represent means and standard deviations ($n=3$).

To discriminate if the higher metabolic activity signal was effectively correlated to cell proliferation, the DNA content of all samples was evaluated at the final time point (figure 7). Heparinized scaffolds showed overall less DNA amount compared to non-heparinized scaffolds, thus indicating slightly reduced cell proliferation in the heparinized group.

Figure 7: DNA quantification of samples at day 10 of the Presto blue assay. PCL with immobilized heparin (black bars) exhibited lower DNA content than untreated samples (grey bars), although no statistical significant difference can be seen, except for the group with no VEGF immobilized. Values represent means and standard deviations ($n=3$).

Migration assay

Porous PCL, either with or without heparin, was coated with different concentrations of VEGF (0, 200, 500 and 1000 ng/ml). VEGF activity was assessed by evaluating its ability to attract HUVECs on the scaffolds. The migration was evaluated by quantifying the amount of DNA per sample after one day of culture. Samples with heparin showed on average 2.8x more DNA than samples without heparin (figure 8). The highest cell migratory potential was found in samples with heparin functionalization.

Figure 8: DNA quantification of migrating HUVECs towards different samples. Blank PCL and blank PCL loaded with 200, 500 and 1000 (ng/ml) of VEGF. Heparinized PCL scaffolds with the same loads of VEGF. An * denotes significant difference ($p<0.05$), values represent means and standard deviations ($n=3$).

Islets and MIN6 aggregates morphology and function in hybrid scaffolds

Islets and MIN6 aggregates were seeded via the entry port onto scaffolds and stained with DTZ (figure 9 A-D). Aggregates and islet showed bright red staining due to DTZ, indicative of the presence of insulin (figure 9 A and C). SEM micrographs showed that both islets and aggregates seeded into the PDLLCL layer of the bi-layered scaffold did not adhere to the surface and cell clusters maintained a round shape without signs of cellular outgrowth (figure 9 B and D). 7 days after seeding, MIN6 aggregates showed a functional response in all the conditions tested (stimulation index of 1.8 ± 0.04). Although reduced in comparison to free floating aggregates stimulation index at day 7, functionality was still comparable to the stimulation index of free floating aggregates at day 1 (figure 9E). The positive free floating control performed better, showing on average a 5.2x increase. Islet functionality was tested in PDLLCL scaffolds PDLLCL/PCL composite bi-layered structures and compared to free floating controls, exhibiting good functional response when seeded in the porous construct 1 day after seeding (figure 9F).

MIN6 beta-cell functionality was tested 7 days after seeding to allow recovery of the aggregates from the seeding procedure. Functionality at day 1 was not always similar between samples (data not shown), which might have been caused by the stress experienced by the aggregates in the centrifugation step of the seeding procedure. Although this was supposed to be a mild centrifugation step at low speed, the fact that a porous, semi-rigid network was present could have constituted an additional stress factor for the beta-cells and could have caused insulin vesicles to degranulate and insulin to be secreted. This hypothesis might be validated by the fact that 7 days after seeding aggregates showed again a functional response to glucose stimulation, meaning that they had enough time to reconstitute a functional insulin vesicles pool.

Being islets of Langerhans generally more robust than the respective aggregate model based on cell lines, figure 9F shows that islet maintained their functional response to glucose stimulation also 1 day after seeding in the construct.

Figure 9 Picture of Islet of Langerhans (A) or a MIN6 aggregate (C) seeded in the porous scaffolds and stained with DTZ, (B) Scanning electron micrograph of an islet (B) or a MIN6 aggregate (D) The porous scaffold prevented clustering of individual islets or pseudo-islets (MIN6 aggregates) up to 7 days in culture. No attachment can be seen in case of islets or MIN6 aggregates onto the scaffold surface. (E) Stimulation index of insulin secretion of MIN6 aggregates in hybrid scaffolds. Stimulation index is calculated as the ratio of insulin secreted in high glucose conditions (16.7 mM) normalized by the basal insulin secretion (low glucose 1). (F) stimulation index of human islet of Langerhans seeded in PDLLCL scaffolds, PDLLCL/PCL scaffolds and free floating control. Values represent means and standard deviations ($n=3$).

Discussion

This study investigated the salt leaching technique as a simple method for fabricating a porous device for islet of Langerhans transplantation. A bi-layered porous scaffold was designed in order to combine a PDLLCL, which has a favorable interaction with islets, and PCL which allows for biofunctionalization and can act as a drug delivery depot.

A coating technique based on covalent immobilization of heparin on the PCL layer via EDC NHS chemistry facilitates the subsequent electrostatic binding of VEGF to induce neoangiogenesis in and around the scaffold after transplantation (38). The organization of the construct in two different areas, each with specific physical and chemical properties in terms of cell interaction and protein absorption, allow for the creation of an optimal islet niche and attraction of endothelial cells towards a positive VEGF gradient.

The fabrication technique allowed the production of highly porous scaffolds with interconnected pores in both area of the scaffold, with the great majority of the pores (75%) smaller than 300 μm . Although the pore size is in the desired range for islet of Langerhans, a 30% shrinkage was observed if compared to the particle size of 250-425 μm initially used. This can be explained by the solvent evaporation and by the mechanical properties and elasticity of PDLLCL. SEM images showed that islets were retained by the porous PDLLCL scaffolds. No sign of cell adhesion was found both for MIN6 aggregates and for islet of Langerhans.

The vascularization layer is based on pure PCL. In this layer cell adhesion is desired and is improved by the functionalization with heparin. Our results showed that heparinization occurs only on PCL and not PDLLCL. An explanation for this lies in the different molecular composition of each polymer: since heparin functionalization can only occur at the terminal free carboxyl groups, given the molecular composition of PDLLCL in comparison to PCL, the number of available carboxyl groups on PDLLCL is lower than on PCL. Heparin was cross-linked on porous PCL using EDC/NHS chemistry. This reaction enables the amino groups of heparin to react with the terminal carboxylic groups of PCL. The presence of NHS

increases the efficiency of the EDC mediated coupling. Multiple materials have been cross-linked using this mechanism including collagen (41,42), alginate (43), PLGA (44) and electrospun PCL fibers with hyaluronic acid (45). It was therefore considered to be a suitable mechanism for porous PCL sheets as well. Data demonstrated that the fraction of immobilized VEGF on heparin coated scaffolds was significantly higher than uncoated PCL scaffolds.

The electrostatic binding of VEGF to the covalently bound heparin on the scaffold's surface will increase the availability of growth factor at the biomaterial surface (38,41,46). The PCL compartment in each scaffold acts as a growth factor reservoir and promotes VEGF controlled release to stimulate vascular ingrowth, thanks to its ability to induce neoangiogenesis (24). VEGF release from our scaffolds was measured for a period of 14 days *in vitro* and the release profile was comparable to other studies, in which a similar technique was applied for the immobilization of different growth factors (38,47,48). At the end of the test period of 14 days a considerable amount of VEGF was still bound on the surface of the scaffold, confirming the high affinity of VEGF for heparinized surfaces (49,50).

In vitro migration tests were aimed to show that VEGF retained its original three dimensional conformation after immobilization on the heparin layer, and was able to attract endothelial cell towards the functionalized surface. However, the effect of different concentrations of VEGF on cell migration was masked by heparin itself and the highest amount of migrated cells was found on heparinized surfaces regardless of the VEGF concentration used. In the migration test, HUVECs migrated 2.2 to 3.3 times more towards heparinized surfaces compared to non-coated samples, indicating that the heparin coated scaffold exhibit a more favorable surface for cell adhesion compared to uncoated PCL. A possible explanation might lie in the capacity of heparin to bind other molecules than VEGF. In this specific case, it might have bound serum proteins present in culture medium and facilitated the adhesion of cells to the scaffold surface (53) .

VEGF immobilization on heparinized surfaces did not reach complete saturation of all the available binding sites. This is demonstrated by the fact that the VEGF

immobilization (figure 5A) does not reach a plateau value at the highest concentration. Additional surface is available for more protein absorption from culture medium (*in vitro*) or from extracellular fluids (*in vivo*). This would create a cell adhesive surface capable of facilitating endothelial cells adhesion independently on the amount of VEGF previously immobilized. Additionally, heparinized surfaces could bind supplementary growth factors present in the HUVEC medium, like basic fibroblast growth factor, which would increase cell migration towards the surface.

Surprisingly, in the heparinized surface group, a higher VEGF dose results in lower cell migration to the construct. This indicates that a low VEGF concentration in combination with a heparinized coating is more beneficial for HUVEC migration than a high dose (17) and that

a slowly raising VEGF gradient is more favorable for cell attraction than a steep gradient (17). Ozawa *et al.* showed that a low but steady release of VEGF promotes the growth of mature and healthy vessels *in vivo*, in contrast to a higher burst release which is known to cause bulbous glomeruli bodies and hemangiomas (20). The same authors assert that the steepness of the developed gradient of VEGF is more important than the actual quantity. A similar result was also shown when a VEGF gradient was created in porous collagen scaffolds (54).

VEGF release and its effect on cells was further characterized by HUVEC proliferation. It was hypothesized that the increased VEGF dose in heparin coated samples would increase proliferation.

If heparinized and non-heparinized surfaces were compared, a higher signal was given by cells proliferating on non-heparinized surfaces. This was also confirmed by DNA quantification after 10 days proliferation.

A possible explanation for this is a consequence of the VEGF release from the non-heparinized samples. In non-heparinized scaffolds, a burst release of VEGF in the first 3 days is shown in comparison to heparinized scaffolds. A burst release of VEGF can lead to a initially increased cell proliferation, while if the growth factor is

electrostatically bound to a negatively charged layer, a modulation in its release kinetic can be achieved.

In a study by Anderson *et al*, the effect of electrostatically or covalently immobilized VEGF on endothelial cells was investigated. Surfaces with electrostatically bound VEGF on heparin, covalently bound VEGF on modified heparin and soluble VEGF were compared. A reduced HUVECs proliferation was seen when cells were grown on surfaces with immobilized VEGF compared to the soluble counterpart (51). The effect was more marked when the electrostatic immobilization approach was used compared to the covalent binding (51).

Moreover, also the molecular weight and structure of heparin could play a role on HUVEC behavior on a heparinized surface: as reported in a research by Khorana and co-authors, high molecular weight heparin is capable of inhibiting HUVEC proliferation, metabolic activity and their arrangement into a tube-like network (52).

However, at day 3, 5, 7 and 10 of the proliferation study an increased cell proliferation is seen for increasing VEGF concentrations, which confirms that VEGF retains its tertiary structure and activity after immobilization. As shown in other studies utilizing a similar immobilization system and growth factors (22, 41) increasing the total dose of growth factor increases HUVEC proliferation potential and blood vessels density *in vivo* (38).

However, It should be noted that the interplay of factors involved in *in vivo* vascularization is extremely complex and *in vitro* tests cannot mimic this complexity. An ultimate proof of the capacity of VEGF to induce vascularization can be given only by *in vivo* tests.

While PCL was chosen as the backbone material for the vascularization layer of the construct, PDLLCL was chosen for retaining aggregates or islets of Langerhans. PDLLCL differs from PCL in terms of mechanical properties, being PDLLCL softer and more elastic than PCL alone. For this reason a lower polymer:salt ratio (1:10) was used for scaffold fabrication. This condition allowed us to fabricate porous sheets, with interconnected porosity but still able to support

itself and preventing collapse of the highly porous structure. PDLLCL is known to minimize tissue attachment (37) a condition that must be fulfilled in order to allow islets to maintain their round shape and, consequently, their functionality. Islets seem to lose their responsiveness to glucose when they are cultured for either a prolonged period or placed on a material that promotes cell attachment (55).

MIN6 aggregates showed an adequate functional response to glucose stimulation, but the stimulation index was not as good as the free floating control. Islets of Langerhans also showed a functional release profile, more comparable to free floating control islets. For islets of Langerhans to be regarded fully functional, the stimulation index should be at least show a twofold increase (56). In case of the MIN6 pseudo-islet, the decrease in insulin secretion, compared to the control, might be explained by the stress experienced by the islets during the centrifugation step performed in the seeding procedure and their penetration in such an intricate lattice structure. Mechanical stress and penetration into an intricate polymeric porous structure might have induced the unspecific release of insulin vesicles. If this should be the case, it highlights the importance of considering parameters such as pore size and interconnectivity not only for islet retention in the scaffold but also for efficient and effective seeding prior to implantation. A bigger pore size, higher interconnectivity and more regular geometrical location of the pores together with optimization of the seeding conditions, might help in reducing the stress experienced by the islet during the seeding procedure.

Another hypothesis which could explain islets and MIN6 aggregates reduced functional response in diffusion limitation through the porous construct. Removing the thick bottom layer in the salt leached PDLLCL layer could help overcoming this design limitation. Further work is required for investigating more in depth the causes of a reduced functionality of the islets (or model aggregates) in the scaffold and perhaps different geometries must be tested in order to indicate the most effective one in terms of nutrient and oxygen diffusion. *In vivo* studies will eventually prove the efficacy of the VEGF release pattern on the induction of vascularization and investigate whether this is an essential factor contributing to the functionality of the transplanted islets in the scaffold.

Conclusions

A bi-layered approach has proven to be an effective way to combine multiple functionalities into a composite scaffold. By mean of a suitable material choice differential functionalization in the different areas of the same scaffold can be achieved and this allows to create an optimal microenvironment for different cell types and combine opposite requirements in a single implantable construct. Such a scaffold design offers the possibility to culture different cell types with different requirements in terms of physico-chemical and biological environment. In this case, the scaffold presented in this chapter combines a non-adhesive cell type as islet of Langerhans together with endothelial cells, requiring adhesion cues to provide their function. Moreover the PCL layer of the scaffold can also be used as a growth factor delivery depot for controlled release of VEGF.

Salt leaching has been proved to be a suitable technique to fabricate porous scaffolds, having a pore size distribution in the correct size range to entrap islets of Langerhans and MIN6 cellular aggregates of similar dimensions, and used as a pseudo-islet model. Scanning electron microscopy showed that islets and MIN6 aggregates were separately embedded in the pores of the scaffolds and maintained their natural rounded morphology during prolonged cell culture. Scaffolds fabricated in this way showed a versatile construction and functionalization with bioactive molecules to induce rapid endothelial cell migration, thus holding the potential to be beneficial for vessels ingrowth *in vivo*. VEGF maintained its structure and activity and heparin coating was shown to be an effective method for obtaining a controlled release of the growth factor from the polymeric surface.

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Chapter 5

Hybrid Polycaprolactone / Alginate Scaffolds Functionalized with VEGF to promote de novo vessel formation for the Transplantation of Islet of Langerhans.

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Abstract

Although regarded as a promising treatment for type 1 diabetes, clinical islet transplantation (CIT) in the portal vein is still hindered by a low transplantation outcome. Alternative extra-hepatic transplantation sites have also been proposed, but the survival of extra-hepatic transplanted islets of Langerhans critically depends on the pace of connection of the islets to the systemic host vasculature, which can be very limited in case of large constructs. This study presents a strategy for the fabrication of a scaffold that can actively boost vascularization of the construct and may find an application for extra-hepatic islet transplantation. The construct consists of a three-dimensional doughnut-shaped polycaprolactone (PCL) scaffold with heparinized surface for the controlled delivery of vascular endothelial growth factor (VEGF) and a hydrogel core embedding the islets. Results showed that heparin immobilization improves the amount of VEGF retained by the construct, up to 3,6 fold compared to untreated PCL scaffolds. In a chicken chorioallantoic membrane (CAM) assay model, VEGF immobilized on the construct showed enhanced blood vessel density in close proximity to and at the surface of the scaffolds. After 7 days islets encapsulated in the alginate core of the constructs showed full functional response to glucose stimuli, comparable to free-floating islets. We conclude that a combination of a ring-shaped macroporous PCL construct, functionalized with VEGF, containing a core of alginate encapsulated islets supports rapid vascularization and islet endocrine function.

Introduction

The ability of Islets of Langerhans to maintain their endocrine function is greatly influenced by a sufficient supply of oxygen and nutrients via the pancreatic vasculature (1). Specifically, islets of Langerhans receive 5-15% of the total pancreatic blood flow, although their mass only accounts for 1-2% of the total pancreatic tissue (2). Clinical islet transplantation (CIT) involves the isolation of islets from the pancreas by extensive enzymatic digestion and mechanical disruption from the surrounding exocrine tissue (3, 4). During this procedure, the pancreatic islet cells can lose their connection to the surrounding islet extracellular matrix and the unique islet microvasculature is lost (5). After their isolation and transplantation, islets are avascular for several days (1). Initial inadequate blood supply and delayed revascularization causes islet necrosis after transplantation, which impairs islet functionality and leads to a loss of transplant mass (1). Together with the instant blood mediated inflammatory reaction (IBMIR), high concentrations of immunosuppressive drugs, high lipid and glucose concentrations, and mechanical stress in the liver, the lack of proper revascularization is one of the reasons for which most islets are lost within the first few days after transplantation(6, 7).

To overcome the limitation of CIT, alternative transplantation sites have been suggested. The renal subcapsule, the omentum pouch, and the intraperitoneal and subcutaneous locations have been used among others in many studies as reviewed by Merani and Cantarelli (8, 9). Drawbacks are associated with each of these locations. The renal subcapsule has a low blood supply and lower oxygen tension compared to other locations, moreover surgical access is difficult and not easily translatable to human clinical applications (8). The omentum pouch and the peritoneum require a higher number of islets to be transplanted (8, 9). In this location, islets are not confined in a restricted area and the lack of parasympathetic innervation is responsible for an abnormal glucose tolerance test (8). The subcutaneous location lacks proper vascularization and issues like long term oxygen and nutrient diffusion need to be addressed (9). To further optimize islets transplantation in these alternative sites, therefore, scaffolds are often needed to provide confinement and mechanical protection to the transplanted islets (10, 11).

Furthermore, the use of such scaffolds can provide a supporting environment to convert inhospitable locations to suitable implantation sites (11). The main limitation of these scaffolds, however, is that they need to be quickly revascularized in order to provide the necessary supply of nutrient and oxygen to the transplanted tissue.

The capacity to induce construct revascularization is critical for engineering of complex tissues and organs of a clinically relevant size. In diabetes, and in general for the scaffold-based regeneration of a variety of tissues, different strategies have been investigated. Revascularization of tissue-engineered constructs has been attempted by seeding of endothelial cells (ECs) (12), endothelial progenitor cells (13), or embryonic stem cells (14) in a variety of scaffolds. An array of polymeric materials like PLLA and PLGA have been used in association with salt leaching techniques to create porous sponges to support endothelial cell proliferation and ingrowth of blood vessel (14, 15). Polyethylene glycole (PEG) based hydrogels in combination with glycosaminoglycans (16-18), and other synthetic and natural hydrogels were used as growth factor delivery vehicles. These growth factor loaded hydrogels were shown to sustain a controlled release of growth factors in time, to support *in vitro* cell proliferation and new blood vessels formation *in vivo*. Due to its interaction with several growth factors, fibrin glue gel was also used as a controlled release system for VEGF, basic fibroblast growth factor (bFGF) and transforming growth factor beta (TGF β) (19). Gold (20), glass, and silica substrates (21) have been used for micro contact printing, photolithography and laser writing to provide patterned substrates capable of inducing directional growth of endothelial cells (22). Despite having showed promising results in terms of capillary and micro vasculature formation, these approaches may be limited when aiming at the regeneration of complex vessel networks for whole organs due to a limited control over the spatial presentation of growth factors in three-dimensions. Other surgical techniques have also been proposed for the pre-vascularization of implanted tissue, like pedicle models (23) or the creation of vessels loops in or around the scaffolds (24-26). These techniques consist often of a two-step surgical

procedure and might be difficult to apply in patients with diabetes or other comorbidities because of impaired wound healing (27, 28).

Another easy applicable method to enhance vascularization involves the use of growth factors. Growth factors can be directly injected *in bolus* into the area of interest, but this often leads to local overdoses of growth factors, and consequently to leaking vessels with an abnormal vessel structure (29, 30). Therefore, several strategies to control the release of growth factors from biomaterial systems have been developed to prevent such abnormal tissue reactions. Growth factors can be adsorbed (31), incorporated (16-18), or covalently bound to biomaterials (32), which enables tuning of the release kinetics depending on the scaffold's degradation time. Spatial presentation of the growth factor, dose and gradients are all characteristics that can play an important role in the establishment of a stable vascular network and, therefore, need to be carefully tuned to ensure proper vascularization (22). The controlled delivery of angiogenic growth factors, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) (33, 34) and PDGF (31) has been explored for enhancing the revascularization of implanted constructs. In particular, VEGF has been demonstrated as one of the critical regulators of neovascularization and has been also used in clinical studies (35). VEGF has been shown to stimulate the mobilization and recruitment of ECs, and to initiate and accelerate the process of angiogenesis (36). VEGF-mediated activation of the endothelial cells stimulates the production of matrix metalloproteinases (MMPs), the subsequent degradation of the surrounding extracellular matrix, migration of ECs, and formation of buds and sprouts. Subsequently, the newly formed sprouts are elongated by the cell proliferation (31). For therapeutic angiogenesis to be effective, the growth factor dose, as well as the temporal and spatial presentation to the cells, need to be carefully tuned.

Controlled growth factor delivery systems have been developed using hydrogels (37, 38), microspheres (39, 40), covalent functionalization of biomaterials (41) and electrostatic binding to heparin (42, 43) or heparan-sulfate molecules. Heparin presents a natural affinity for electrostatic binding of growth factors such as FGF-2, HGF and VEGF (44, 45). This specific property of heparin plays an important role

in many physiological processes, in which growth factors are required (44). Heparin is a natural component of the extracellular matrix: its ability to bind growth factors in the matrix allows the storage of a large quantity of these signaling molecules in a readily available form, prevents their degradation (46), and facilitates their “on-demand” and controlled release (44, 47). Of particular importance in case of angiogenesis, a tuned release of growth factors can generate highly localized gradients that provide faster activation of signal transduction pathways (44).

In this study we created a macroporous ring-shaped PCL scaffold to deliver VEGF-A in a localized and controllable manner, thus stimulating neo-angiogenesis in and around the implant, while retaining human islets of Langerhans in an alginate core. The macroporous PCL ring was fabricated by 3D fiber deposition, heparin was covalently bound to the polymer using EDC-NHS chemistry and the scaffold was functionalized with electrostatically bound VEGF. The core of the scaffold consists of alginate encapsulated islets. We showed that with a relatively low amount of VEGF, neovascularization could be efficiently induced, around and throughout the pores of the scaffold. Heparin that was covalently bound to the surface of the scaffolds enabled an efficient absorption and controlled release of VEGF, while islets encapsulated in the alginate core were viable and able to maintain their endocrine function over time.

Materials and methods

Scaffold fabrication

Polycaprolactone (PCL) scaffolds (Sigma, Zwijndrecht, the Netherlands) were fabricated using a rapid prototyping machine (sysENG, Germany). PCL (mW 45000) was heated to 100 °C. Plotting speed and pressure were set respectively to 175 mm/min and 5 bars. A needle of 200 µm in diameter was used for extrusion. Scaffolds were plotted in a 0-90 fiber deposition configuration in a toroidal shape with an 8 mm outer diameter, a 4 mm inner diameter and 1 mm height. For each layer, fiber thickness was set to 150 µm and fiber spacing to 600 µm. The central open core of the scaffold was filled with 2% w/v ultrapure alginate in PBS (courtesy of Paul de Vos, UMCG, Groningen) and cross-linked for 5 min with a 20 mM

calcium chloride solution (Sigma, Zwijndrecht, the Netherlands) in 10 mM HEPES (Invitrogen, Grand Island, USA). Scaffolds were then washed with Tris-buffered saline (TBS).

PCL flat disks production

Polycaprolactone (M_w 45000, Sigma, Zwijndrecht, the Netherlands) flat disks were fabricated by placing the PCL grains in a metal mold in between two hydrophobic silica wafers. The PCL was heated in a press at 100 degrees and pressure was applied to the molten polymer. After cooling, the metal mold was removed from the press and disks of 10 mm in diameter were punched out.

Heparin and vascular endothelial growth factor (VEGF) immobilization

Heparin coating on PCL scaffolds was performed by dissolving 1% w/v heparin (Sigma, Zwijndrecht, the Netherlands) in 0,05 M 2-(N-morpholino)ethanesulfonic acid (MES) buffer (Sigma, Zwijndrecht, the Netherlands) at a pH of 5.5. 0,5 M 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 0,5 M N-Hydroxysuccinimide (NHS) (Sigma, Zwijndrecht, the Netherlands) were added to the heparin solution. Scaffolds were previously equilibrated for 15 min in MES buffer and subsequently immersed in the reaction mixture. The reaction was stirred for 15 hours at room temperature. The reaction was stopped by extensively washing the scaffolds with sterile H₂O to remove all the residues of unbound heparin (13, 48).

Vascular endothelial growth factor (VEGF Peprotech, London, UK) was diluted to the loading concentrations of 200, 500 and 1000 ng/ml in phosphate buffer saline solution (PBS) (Gibco, Grand Island, USA) supplemented with 0,1% wt/v bovine serum albumin (Sigma, Zwijndrecht, the Netherlands). Each scaffold was immersed in 1 ml VEGF solution for 24 hours at 4 degrees to allow absorption of the growth factor on the scaffold surface.

Heparin staining and heparin quantification

Staining of the heparin coating on PCL scaffolds was performed by immersion of the treated scaffolds in a 0,01 mg/ml Azure II solution (Sigma, Zwijndrecht, the Netherlands). Before imaging, scaffolds were extensively washed with water (49). The heparin left in the reaction mixture after 15 hours immobilization was quantified with a colorimetric assay based on Azure II, as reported by Gutowska et al.(49). Briefly, 0,5 ml of the reaction blend was mixed with 4,5 ml of 0,01 mg/ml Azure II solution. The absorption of the heparin-Azure II complex was measured after 1 minute at 500 nm, and compared with a reference curve. The amount of heparin immobilized on the scaffolds, was calculated by difference with the initial heparin loading.

Scanning electron microscopy (SEM) and Energy Dispersive X-ray analysis(EDAX)

PCL scaffolds and heparin-coated PCL scaffolds were gold sputtered (Cressington sputter coater 108 auto) and analyzed by scanning electron microscopy (Philips – XL 30 ESEM-FEG). SEM images were taken at different magnifications (33X, 200X and 5000X) with an accelerating voltage of 5 kV. Scaffold features such as fiber diameter, fiber spacing, and layer thickness were analyzed using Image J software.

Surface coating of the scaffolds was investigated using SEM equipped with an Energy Dispersive Analysis X-Ray detector (EDAX, Ametek, USA). Magnification was kept the same as before while voltage was increased to 10 kV. Point analysis for the chemical composition at various random spots of the scaffolds was performed and the quantification of Carbon (C), Oxygen (O) and Nitrogen (N) was obtained. The nitrogen content was related to the amount of immobilized heparin.

VEGF immobilization efficiency and release study

VEGF-A immobilization efficiency on PCL and PCL/HEP scaffolds was quantified by ELISA (R&D, Minneapolis, USA) by measuring the amount of VEGF left in the immobilization medium after 24 hours absorption and calculating the difference with the total amount placed in solution. Similarly, the amount of VEGF released daily in 0,5 ml PBS/0,1% BSA was also quantified by ELISA. The amount of VEGF

left on the scaffold after 15 days of release was stained with an adaptation of the ELISA kit from Peprotech (Peprotech, London, UK). Briefly, VEGF still bound on the scaffold was stained with a biotinilated-anti-VEGF antibody, which was subsequently detected with avidin-peroxidase and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) substrate.

Chicken chorioallantoic membrane assay

Neovascularization potential of the PCL and PCL-HEP coated scaffolds with VEGF (loading concentration 0, 200, 500 and 1000 ng/ml) was tested using a chicken chorioallantoic membrane (CAM) assay. Fertilized leghorn chicken eggs were purchased from a local farm and incubated for 3 days at 37 degrees at an 80% humidity and regularly turned. On the 4th day, a window of circa 1x1 cm was opened in the shell and the next day samples were implanted. Scaffolds were prepared as previously explained and implanted after 4h incubation in a PBS solution with 0,1% BSA to get rid of the burst release of weakly bound VEGF. PCL/VEGF and PCL/HEP/VEGF scaffolds were implanted in the eggs for 4 days, before the chicken embryos were sacrificed with an ethanol injection. The membrane with the scaffold was fixed with formalin and excised for further imaging (50).

Migration assay

To assess the potential of the PCL/HEP scaffolds loaded with VEGF to attract endothelial precursor cells for the improvement of vascularization *in vivo*, the migration of human umbilical vein endothelial cells (HUVECs) towards the scaffold was tested *in vitro*. HUVECs (Lonza, Basel, Switzerland) were used as a model and cultured at 37 °C, in a 5% CO₂ incubator, in EBM-2 basal medium (Lonza, Basel, Switzerland) supplemented with 2% FBS and growth factors (bullet kit EGM-2, Lonza, Basel, Switzerland).

85,500 cells/well were seeded in a 24 well plate. One day after seeding, cells were treated for 2 hours with a 0,5 mg/mL solution of Mitomycin C (Sigma, Zwijndrecht,

the Netherlands) in culture medium to block cell proliferation, followed by a washing step with PBS.

We prepared PCL and PCL-HEP coated scaffolds loaded with VEGF concentrations of 0, 200, 500 and 1000 ng/ml. Immediately after VEGF immobilization, samples were incubated in complete EGM-2 medium for 2 hours, to increase serum protein absorption on the scaffold surface to enable cell attachment. After this pre-incubation step, samples were removed and placed on top of the previously cultured HUVECs monolayer in EGM-2 medium without addition of VEGF. After 2 days, the scaffolds were cut, immersed in water and sonicated to lyse the HUVECs which had migrated onto the scaffold surface. After cell-lysis, DNA was quantified using a Pico Green assay (Invitrogen, Grand Island, USA) and related to total cell number.

Methylene blue staining

3×10^5 Cells seeded on PCL printed scaffolds were washed in PBS, fixed with formalin and stained by incubating the samples in a 1% methylene blue solution (Sigma, Zwijndrecht, the Netherlands) for 2-5 minutes. The samples were then extensively washed with distilled water and imaged with a Nikon SMZ-10A stereomicroscope, equipped with Qcaputre software.

Islets of Langerhans culture

Human islets of Langerhans were provided by the islet isolation and transplantation centre at the Leiden University Medical Centre with informed consent. Human islets were cultured in CMRL medium (Cellgro, Manassas, USA) supplemented with 10% FBS (Lonza, Basel, Switzerland) and 1% Pen/Strep (Gibco, Grand Island, USA) (51).

For islet encapsulation in the scaffold core, islets were handpicked and resuspended in a 2% w/v ultrapure alginate solution in PBS. Subsequently, islets were mixed in alginate (1'330 islets/ml) and a droplet (15 μ l) of islet-alginate mixture were deposited on a hydrophobic polystyrene surface. This resulted in alginate constructs containing 20 islets in average. The PCL or PCL-HEP coated

scaffold was placed around each droplet. Alginate was crosslinked with a 20 mM calcium chloride solution for 5 minutes and the resulting hybrid constructs were subsequently washed in tris buffered saline (TBS) solution. The above mentioned fabrication method resulted in encapsulation of circa 20 islets per hybrid scaffold or in alginate droplets only. Glucose responsiveness was evaluated by a glucose induced insulin secretion test (GIIST). The functional response of islets embedded in alginate only and in the hybrid construct was compared to the same number of free floating islets in non-adherent tissue culture plates.

Glucose induced insulin secretion (GIIST)

Human islets were incubated for 1,5 hours in a Krebs-Ringer buffer with 1,67 mM glucose, to ensure stabilization of beta cell insulin secretion. Subsequently, islets were incubated for 1,5 hours in a low glucose Krebs-Ringer buffer (1,67 mM glucose) after which medium samples were taken for ELISA. Islets were then incubated for an equal amount of time in a high glucose (16,7 mM) containing medium, followed by low glucose (1,67 mM) medium, while medium samples were taken after each step for ELISA. All medium samples were analyzed for insulin content using ELISA kits (Mercodia, Uppsala, Sweden). Plates were scanned using a Multiskan GO Microplate Spectrophotometer (Thermo scientific, Breda, The Netherlands) and insulin release profiles were calculated according to the manufacturer's protocol.

Results

PCL plotted scaffolds resulted in a toroidal construct with an outer diameter of about 0.8 cm, an inner diameter of 0.5 cm, and a height of 1 mm (figure 1). The fiber diameter of the uncoated PCL fibers was $210 \pm 25 \mu\text{m}$ and the fiber spacing was $457 \pm 63 \mu\text{m}$. After coating of the PCL constructs with HEP the fiber diameter was $192 \pm 14 \mu\text{m}$ while the fiber spacing was reduced to $380 \pm 29 \mu\text{m}$.

Figure 1: Schematic of the hybrid scaffold concept. 3D plotted polycaprolactone rings were covalently functionalized with a heparin layer. Heparin was used as an active linker to bind VEGF and protect it from degradation. A) Islets of Langerhans were encapsulated in the inner part of the structure using alginate hydrogel, crosslinked with 20 mM CaCl_2 . B) Multiple constructs can be printed one next to the other in a honeycomb configuration increasing the available surface for islets embedding and revascularization of the scaffold.

Azure staining proved complete and uniform heparin coating both on the 2D model surfaces (figure 2) and on 3D plotted scaffolds (figure 3). The uniformity of heparin distribution on the scaffold surface was further confirmed by SEM (figure 3). After covalent binding of heparin to PCL the surface texture changed from a smooth surface into a rougher surface containing sub-micrometric features (figure 3 I-K).

Heparin content was analyzed by quantifying the amount of nitrogen present on the scaffold by EDAX analysis. The percentage of nitrogen after heparin immobilization was 15.5 ± 4.1 % of the elements composing the analyzed surface area, while in the non-coated scaffolds no nitrogen was detected on its surface (figure 3 B to E). Efficacy of covalent binding of heparin to PCL via EDC-NHS chemistry was first proved on 2D surfaces and was determined by quantifying the amount of heparin left in the immobilization solution after 15 hours reaction when using standardized PCL flat films (1 cm diameter). However, we were not able to detect any heparin left in the immobilization solution, or the amounts left were below the detection limit

of the Azure assay used (Figure 2). This indicates that all heparin molecules, used at a concentration of 1% w/v per 1,6 cm² PCL surface, were able to covalently bind to the polymer.

Figure 2: (A) PCL disks before and after heparin immobilization and staining with Azure II. (B) Heparin quantification in the reaction medium, after 15h. The arrow indicates a sample of the reaction medium. Heparin amount left after 15h reaction was undetectable with this method. Table: VEGF immobilization on heparin-coated 2D PCL disks. After 4 °C overnight immobilization, the substrate was functionalized with 130 ng of growth factor, accounting for the 65% of the starting amount.

Figure 3: A) PCL and PCL/heparin plotted scaffolds stained with Azure II. B and C) Nitrogen quantification on heparin-coated versus bare PCL scaffolds. Nitrogen accounted for the 15.5% (w/v) of the total isotope mass. D and E) Representative spectra for element quantification on heparin coated (D) versus uncoated PCL scaffolds (E). F–K) SEM pictures at different magnifications of the bare PCL scaffolds (F–H) and of the heparinized constructs (I–K) where the change in surface topography can be appreciated.

Three different concentrations of VEGF (200, 500 and 1000 ng/ml) were allowed to absorb on PCL-heparinized scaffolds overnight. For all the three different VEGF loading amounts used, heparinized PCL scaffolds retained a much higher

percentage of absorbed VEGF compared to the uncoated PCL scaffold (figure 4A). The percentage of immobilized VEGF was $77 \pm 3.9\%$ in case of the 200 ng initial loading and $61 \pm 4.6\%$ in case of the 1000 ng initial loading for heparinized scaffolds. The maximal percentage immobilized on uncoated PCL scaffolds was $30 \pm 4.3\%$, confirming the beneficial role of heparin in the electrostatic binding of VEGF onto the scaffold's surface. Doubling the initial VEGF load applied on the scaffold resulted in a double amount absorbed onto the scaffold (figure 4B) implying that complete saturation of the available binding sites has not been reached yet.

Figure 4: A) percentage of the initial amount of VEGF and B) total VEGF amount (ng) immobilized on the scaffold. Complete saturation of all the binding sites was not reached yet. C) Cumulative release of VEGF from PCL scaffolds compared to heparin coated PCL scaffold. The release kinetic was consistently decreased by the heparin layer in all the concentrations tested. D) Staining of the VEGF left on the scaffold after 15 days of release; the color intensity was higher in the heparin coated scaffolds, confirming a higher amount of VEGF retained by the coated scaffolds.

Heparin coated scaffolds were not only able to retain a higher amount of VEGF compared to PCL scaffolds, but heparin functionalization also plays a role in reducing the initial burst release of VEGF from scaffolds. The cumulative release from PCL uncoated scaffolds was higher than the corresponding concentration immobilized on heparin coated ones. After an initial burst release of VEGF, both coated and uncoated scaffolds displayed a comparable release profile (figure 4C).

In all the conditions tested, at the end of the test period of 14 days the cumulative release was still very low, with the majority of the immobilized growth factor still bound on the scaffold surface. To investigate whether VEGF was still present on the scaffold after 15 days of release, the scaffold bound VEGF was stained. Heparin coated scaffolds displayed a higher color intensity, indicative of a higher VEGF amount present (figure 4D). The staining was consistently higher than the correspondent VEGF loading concentration on uncoated scaffolds.

Covalently bound heparin also increased cell attachment to the scaffold surface. Seeding efficiency of HUVECs on heparin coated PCL scaffolds increased compared to non-coated PCL scaffolds (figure 5). The migration of HUVECs cultured on tissue culture plastic onto the ring-shaped PCL scaffold increased on all heparin coated surfaces (figure 6).

Figure 5: Methylene blue pictures of HUVECs seeded on PCL and PCL/HEP 3D scaffolds with different loads of VEGF. Heparin coating seems to increase cell adhesion on the construct.

In both experiments, it seemed that the addition of increasing amounts of VEGF, both to heparin coated and non-coated scaffolds, didn't influence the migratory movement or attachment of HUVECs in vitro.

Figure 6: DNA quantification of HUVECs migrated from a monolayer culture to the 3D scaffolds. Heparin coating increased the amount of migrated cells, regardless of the VEGF concentration.

A chicken chorioallantoic membrane (CAM) assay demonstrated that VEGF retained its bioactivity after electrostatic binding via heparin to the scaffolds. This was confirmed by the induction of neovascularization in close proximity and on top of the scaffold in all of the VEGF concentrations used (figure 7). Differences in blood vessel morphology were observed for different VEGF concentrations: vessels induced after loading with 200 ng seemed leaky and had an immature appearance, whereas a lower induction of neovascularization, or even inhibition, was seen in the two other PCL conditions with a higher (500 and 1000 ng/ml) VEGF loading. Conversely, in the heparin coated scaffolds new blood vessels were formed also on the surface of the scaffold, in some cases guided by the fibers of the plotted 3D structure. The most effective VEGF concentration in inducing *de novo* vascularization on the construct was found to be 200 ng. Blood vessels with normal morphology were observed growing on the fibers or throughout the scaffold's macropores. A lower amount of blood vessels could be also observed at the two higher loading concentrations (500 and 1000 ng), comprising of leaky

vessels in some cases (figure 7). Unmodified PCL scaffolds showed no effect on *de novo* vessel formation, growth and morphology, which was comparable to the morphology observed in the control membrane with no implant. Interestingly, an increase in blood vessels ingrowth towards the scaffold, although with a thinner diameter, was also induced by the presence of heparin coated scaffolds without VEGF.

Figure 7: CAM assay performed with PCL and heparin coated PCL scaffolds with three different concentrations of VEGF. Leaking vessels were seen in the 200 ng VEGF load on PCL scaffolds, while a higher VEGF concentration seemed to inhibit vessels formation. On heparin coated scaffolds, 200 ng load VEGF seemed to induce the highest blood vessels formation, with normal morphology (Arrows indicate the blood vessel formation).

Islets encapsulated in the alginate in the central core of the scaffold showed a rounded morphology and could be easily stained with dithizone, indicating the presence of insulin (figure 8C). Islets embedded in the scaffold were responsive to glucose stimulation, with stimulation indexes comparable to free floating control islets (figure 8 A and B and figure 9). Alginate embedding, heparin and VEGF immobilization on the polymer backbone didn't cause reductions or inhibition in islets functionality.

Figure 8. Functional behavior of islets at day 1 and day 7 when embedded in alginate bulk gels, in PCL-hybrid scaffolds and in the scaffolds coated with heparin and functionalized with VEGF. The results are the average of three donors. A,B) Pictures of islets embedded in 2% alginate gels in the C) 3D PCL-construct and islets stained with dithizone embedded in the same scaffold, after 1 d of culture.

Donor 1

Day 1

Day 7

Donor 2

Day 1

Day 7

Donor 3

Day 1

Day 7

Figure 9: Functional behavior of islets at 1 and 7 days from three donors in alginate, in PCL scaffolds, and the scaffolds coated with heparin and functionalized with VEGF.

Discussion

One of the mayor challenges to improve currently applied clinical islet transplantation is to convert a poorly vascularized site into a more favorable one, which could significantly improve islet functionality. Fast revascularization of the transplanted islets and connection to systemic circulation will ultimately result in increased nutrient and oxygen supply, increased viability and enhanced islets response to glucose stimuli. In this study, we presented a novel scaffold fabrication strategy where islets can be encapsulated in an alginate core of the scaffold and surrounded by a structure decorated with angiogenesis inducing factors via electrostatic binding to heparin. We showed that efficient covalent binding of heparin on the scaffolds was possible through standard NHS-EDC chemistry. Covalent binding of heparin affected the scaffold's surface topography by increasing the surface roughness. Heparin coating increased cell attachment on the scaffold surface compared to bare PCL and allowed absorption of a 3,6 times higher amount of VEGF compared to untreated PCL scaffolds. As showed in figure 4B, a plateau value in the amount of VEGF immobilized was not reached yet, which means that additional growth factor can be loaded on the scaffold. If needed, in future studies one could envision to use these available heparin sites to bind a second growth factor (e.g. bFGF) to the scaffold to further modulate its vascularization potential. As also observed by other authors, this study showed that heparin can be efficiently used for the functionalization of biomaterials (48, 52, 53), for the reversible binding of growth factors and for their controlled release. In our case, the results clearly show that the amount of VEGF immobilized on the scaffold is sufficient to elicit a vascular response already at the lowest concentration (200 ng/ml loading). On the other hand, the excess in heparin immobilization still provides free binding sites for serum proteins to adsorb to the scaffold surface, thus increasing cell adhesion on the scaffold coated surface. A high heparin binding affinity to VEGF has been already proven, among others, by Robinson and Zhao (54, 55). This was also demonstrated in this study by the fact that after 15 days of release only a small percentage of the growth factor was released, while the great majority still bound on the scaffold itself. The burst release of VEGF

detected at day one was already reduced up to 5 times in case of heparinized constructs for all the tested loading amounts. In the following days VEGF was released at a concentration ranging between hundreds and tens of picomoles/l, in range with what has been reported in literature to be the optimal concentration to elicit cell response (10^{-9} - 10^{-11} M) (56).

VEGF functionalization of the scaffold via heparin binding was able to induce an improvement of the vascularization in each of the three concentrations analyzed. Although the newly formed blood vessels appeared leaky in some conditions, the lowest VEGF concentration on heparin coated scaffolds provided new vessel formation with normal morphology. The best response was obtained with the 200 ng/ml loading concentration, resulting in 154.0 ± 7.8 ng VEGF immobilized on the scaffold. It has been reported that a concentration threshold exists between normal and aberrant vascularization: too high VEGF production will lead to hemangiomas, while medium to low doses will lead to stable, not leaky capillaries (57). When this concept was applied to growth factor absorption on scaffolds, it was found that the most effective control of angiogenesis was not dependent on the total load of growth factor on the scaffold, but on the gradient that developed upon release from the construct into the surrounding environment (57, 58). As shown by Chen et al., the most effective control of angiogenesis was achieved when the highest concentration in the developed gradient from the source scaffold to the surrounding environment was 100 ng/ml (58). Not only the initial growth factor loading, but also the microenvironment gradient developed upon delivery could play a role in defining the directionality of the blood vessels (58). A 100 ng/ml growth factor concentration initiated sprouting, but the developed gradient was not so steep to lose control over directionality (58). Moreover, the released growth factor dose can also exercise an influence over a normal or aberrant morphology of the newly formed blood vessels (30). It has been reported in literature that matrix bound VEGF is more effective in comparison to its soluble form in stimulating endothelial cells proliferation and maturation (32). In our test model, heparin functionalization of the construct and the tested loading concentration of 200 ng/ml optimally meet

these requirements for the potential revascularization of the implanted construct and could be used for enhancing islet viability and functionality after implantation.

HUVECs were used as a cell model to show endothelial cell migration toward the scaffold. Surprisingly, in the used setup no difference in cell migration was detected among the different VEGF concentrations. Heparin coating on the scaffold, though, was by itself responsible for an increased cell migration towards the scaffold (figure 6), for an improved cell seeding efficiency and adhesion on the scaffolds (figure 5), and increased blood vessel ingrowth in the CAM model (figure 7). Considering the high protein binding capacities of heparin, a higher blood vessels ingrowth on the heparinized scaffolds might be caused by an increased serum protein absorption and thus increased cell adhesion on the heparin coated scaffolds. Moreover, the surface topography of the scaffolds increased upon heparin immobilization. This change in surface topography might increase the available surface for protein absorption. In addition, a change in the topography could provide better adhesion cues for the cells to adhere to, if compared to a smooth PCL surface.

Islet embedding in a hydrogel located in the inner core of the scaffold protected islets from losing their round morphology. Islets were stained with dithizone showing the presence of insulin and a functional response to glucose stimulation, which was comparable in all the conditions tested. A low alginate concentration (2%) could be used in such a construct, since weak mechanical properties of the hydrogel are compensated by the presence of the polymer ring around it, which provides mechanical support and keeps the islets confined in one location. A low alginate concentration is beneficial for islet encapsulation, because it prevents nutrients diffusion limitations that occur in a more concentrated hydrogel mesh (59). Diffusion limitations are also prevented by the presence of such a porous polymeric structure around them, which can provide mechanical support, without impairing oxygen, nutrients and insulin diffusion.

So far, available cell-based strategies for neovascularization focused on the delivery of ECs or endothelial progenitor cells (EPCs), but it is not clear yet if the improvement in revascularization is given by these cells or by the angiogenic

factors they secrete and research to identify the optimal cell source has still to be performed (27). Co-culture systems involving EPCs and other cell types of interest have been researched in an attempt to improve revascularization and are reviewed by Kirkpatrick (60). Other strategies involved the transplantation of genetically modified and transfected cells to induce stable expression of growth factor, but transfection also poses significant safety issues (27, 61, 62). More sophisticated approaches used advanced biofabrication techniques as photopatterning (63, 64) or micromachining (65) to fabricate substrates for capillary networks growth *in vitro*, which would subsequently be implanted via microsurgical techniques (27). Although elegant, these approaches are also characterized by difficult applicability and translation to the clinic.

In most of the scaffold-based strategies developed for enhancing vascularization growth factors still play a pivotal role. Many studies have been performed in order to identify the ideal growth factor dose, release profile (66), attachment (67) or release strategy and growth factors combination (17). Release systems have been developed using hydrogels (16, 17, 67-69) or other polymeric biomaterials (13, 22, 38, 70, 71). Covalent attachment of growth factors to the hydrogel matrix (67, 70) could hamper the protein functionality, because the covalent linkage could interfere with the protein folding. Other approaches, both on hydrogels and polymer, rely on the presence of heparin, hyaluronic acid or other glycosaminoglycans to mimic more closely the natural extracellular matrix, provide reversible binding of the growth factor to the gel or polymer matrix and tailor binding and release in time. In addition to these advantages, our VEGF-functionalized scaffold design can be easily produced and functionalized, upscaled to host a relevant amount of cells and implanted without posing significant safety concerns.

The scaffold concept that we developed in this paper can be in principle applied not only to islets of Langerhans implantation but also to the transplantation of other cell types of interest. The scaffold structure could be easily expanded to accommodate a clinically relevant amount of islets for transplantation, without impairing nutrient diffusion. Islet embedding in the alginate core of the scaffold did not impair islet functionality and could possibly provide immuno protection

properties. Heparin functionalization and VEGF immobilization did not affect insulin secretion from the scaffold or cause an adverse effect on islet functionality. Adjacent polymers rings can be plotted in a honeycomb configuration, increasing the spots available for islet embedding and still having a network of blood vessels surrounding these islet-specific areas from all sides (figure 1B). An alternative approach for using this scaffold in the clinics is given by a subcutaneous implantation of the heparinized-PCL ring only, followed by injections of the islets in a hydrogel in the central core. The pre-implantation of the ring would prepare a highly vascularized subcutaneous location for islet implantation, which would be a more favorable environment for the islets to reside in. Once the vascular bed is ready, approximately 15 days later, injection of the islet in the central core of the scaffold could take place.

Conclusions

This study demonstrates that heparin immobilization is a simple and convenient strategy for controlled delivery of VEGF and its protection from degradation. The absorbed VEGF was able to induce a normal angiogenic response in a CAM model at a loading concentration of 200 ng/ml. At this concentration, the 3D fiber configuration of the plotted scaffolds may provide a substrate where blood vessels ingrowth towards the islets is facilitated. Islets embedded in the scaffold were functional and responded normally to glucose stimulation. This scaffold can be regarded as a potentially new approach for subcutaneous islet transplantation and improvement of revascularization, addressing one of the most important limitations in the currently applied procedures for islet transplantation.

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Chapter 6

Poly(amido amine)-based multilayered thin films on 2D and 3D supports for surface-mediated cell transfection

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Abstract

Two linear poly(amido amine)s, pCABOL and pCHIS, prepared by polyaddition of cystamine bisacrylamide (C) with 4-aminobutanol (ABOL) or histamine (HIS), were explored to form alternating multilayer thin films with DNA to obtain functionalized materials with transfection capacity in 2D and 3D. Therefore, COS-7 cells were cultured on top of multilayer films formed by layer-by-layer dipcoating of these polymers with GFP-encoded pDNA, and the effect of the number of layers and cell seeding density on the transfection efficiency was evaluated. Multilayer films with pCABOL were found to be superior to pCHIS in facilitating transfection, which was attributed to higher incorporation of pDNA and release of the transfection agent. High amounts of transfected cells were obtained on pCABOL films, correlating proportionally over a wide range with seeding density. Optimal transfection efficiency was obtained with pCABOL films composed of 10 bilayers. Further increase in the number of bilayers only marginally increased transfection efficiency. Using the optimal multilayer and cell seeding conditions, pCABOL multilayers were fabricated on poly(ϵ -caprolactone) (PCL), heparinized PCL (PCL-HEP), and poly(lactic acid) (PLA) disks as examples of common biomedical supports. The multilayers were found to completely mask the properties of the original substrates, with significant improvement in cell adhesion, which is especially pronounced for PCL and PLA disks. With all these substrates, transfection efficiency was found to be in the range of 25–50% transfected cells. The pCABOL/pDNA multilayer films can also conveniently add transfection capability to 3D scaffolds. Significant improvement in cell adhesion was observed after multilayer coating of 3D-plotted fibers of PCL (with and without an additional covalent heparin layer), especially for the PCL scaffold without heparin layer and transfection was observed on both 3D PCL and PCL-HEP scaffolds. These results show that layer-by-layer dip-coating of pCABOL with functional DNA is an easy and inexpensive method to introduce transfection capability to biomaterials of any nature and shape, which can be beneficially used in various biomedical and tissue engineering applications.

Introduction

The layer-by-layer (LbL) fabrication technique has emerged as a versatile way not only to modify or improve surface properties, but also to add functionalities to the surface of various biomedical materials (1–4). For example, a substantial development in tissue engineering relies on scaffolds for mechanical cell support and many medical operations involve mechanical support devices such as stents, prostheses, and other implants that have specific shapes and sizes. Often these devices are made of specific materials chosen for their mechanical strength and biodegradability, but do not really provide optimal performance in their interactions with cells (5). LbL assembly offers an excellent option to add functionality to biomedical materials by coating various substrates with thin multilayers of functional macromolecules simply by dipping the substrates alternately into two aqueous solutions containing the desired macromolecules. The resulting surface can promote or reduce cell adhesion (6–8), deliver small drugs (9–11) or therapeutic proteins (12–15), induce differentiation (16–19) and cell transfection (20–22). Moreover, the concentration and variety of material incorporation and release can be adjusted simply by adding or reducing the deposition cycles. A promising application for LbL assembly that has recently received increasing attention is surface-mediated cell transfection (23). As early as 1993, Lvov et al. reported the preparation of a multilayer using DNA as a building block (24). Since then, several research groups, notably the groups of Lynn and Hammond, have studied the potential of LbLbased multilayers to provide localized delivery of transcriptionally active DNA (22,23,25–28). The DNA may be deposited between the layers (20,25), or pre-complexed with polymers into polyplexes (29,30). Notably, surface-mediated cell transfection has been attempted on intravascular stents (26,27), flexible stainless steel (20), poly(D lactic acid) film (31), and micro-needles (28) aimed for vaccination application (32,33). Multilayer coatings for surface-mediated transfection purposes on 3D scaffolds for tissue engineering have not been extensively reported. Attempts to introduce transfection capability to 3D scaffolds are mostly carried out before (34,35) or after (36–38) seeding into the scaffolds, by loading the plasmid or polyplex into the matrix of the scaffolds (39,40)

or adsorbing on the surface (41–43). Mineral coatings have also been reported, most notably CaP (44), which was incubated with lipoplexes to induce transfection upon cell seeding (45). Compared to these techniques, LbL dipping technique offers the possibility to design more intricate multilayer design for prolonged or scheduled release (46) of multiple components. Moreover, the aqueous conditions for formation of the LbL films enable to preserve native structures of functional macromolecules. As a recent example, Holmes et al. reported coating of 3D scaffolds with multilayers of glycol-chitosan and hyaluronic acid. The multilayer-coated scaffolds were found to enhance tissue growth relative to non-coated scaffolds, and additional transfection capability could be observed by depositing a layer of DNA-containing lipoplexes (47). Hammond and co-workers have reported the multilayer assembly on poly(lactic-co-glycolic acid) molded into micro needle arrays for transcutaneous delivery of plasmid DNA (28). The multilayer-coated micro needles successfully induced transfection on mice *in vivo*. To enable multilayer formation, a pair of macromolecules is needed that interact with each other. For negatively-charged DNA, a positively charged biocompatible polymer may serve as a good counterpart. Poly(β -amino ester) (PBAE) introduced by the group of Lynn, is one of the most extensively studied polymer for this purpose (22,25–28). In this paper we report on the preparation and properties of multilayers of DNA with bioreducible linear poly(amido amine)s (PAA). Poly(amido amine)s are a class of peptidomimetic polymers synthesized via Michael-type addition polymerization of amines and bisacrylamides. Through the presence of amide bonds, PAAs are inherently biodegradable through hydrolysis. Moreover, through the availability of various building blocks, these polymers can also be designed to incorporate various moieties for added functionality such as charge-shift, bioreducibility, ‘stealth’ properties and targeting moieties (48–50). Here, we report the fabrication, characterization and cell transfection properties of multilayered thin films of DNA with pCABOL and pCHIS, respectively. These linear, bioreducible PAAs were selected because these polymers have previously shown to be two of the best performing PAAs in polyplex systems for cell transfection (51). Cell transfection efficiency of the multilayers was optimized using flow cytometry as an analytical tool to determine transfection efficiency as a function of the PAA type,

cell seeding density, and layer number. Finally, with the optimized conditions, surface-mediated cell transfection was accomplished on poly(ϵ -caprolactone) (PCL), heparinized PCL (PCL-HEP), and poly(lactic acid) (PLA) substrates, both in 2D (disk shape) and 3D (fiber deposited PCL and PCL-HEP).

Materials and methods

Chemicals, syntheses and characterization

N,N'-Cystamine bisacrylamide (CBA, Polysciences), 4-amino-1-butanol (ABOL, Sigma-Aldrich, Zwijndrecht, The Netherlands), N-Boc-1,4-diaminobutane (NBDAB, Sigma-Aldrich, Zwijndrecht, The Netherlands), histamine dihydrochloride (HIS $\cdot$ 2HCl, Sigma-Aldrich, Zwijndrecht, The Netherlands), calcium chloride (CaCl₂, Sigma-Aldrich, Zwijndrecht, The Netherlands), triethylamine (TEA, Sigma-Aldrich, Zwijndrecht, The Netherlands), tert-butylamine (tBA, Sigma-Aldrich, Zwijndrecht, The Netherlands), sodium dihydrogen phosphate monohydrate (NaH₂PO₄ $\cdot$ H₂O, Merck, Darmstadt, Germany), disodium hydrogen phosphate dihydrate (Na₂HPO₄ $\cdot$ 2H₂O, Merck, Darmstadt, Germany), sodium chloride (NaCl, Sigma-Aldrich, Zwijndrecht, The Netherlands), dithiothreitol (DTT, Sigma-Aldrich, Zwijndrecht, The Netherlands), 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES, Sigma-Aldrich, Zwijndrecht, The Netherlands) and glucose (Sigma-Aldrich, Zwijndrecht, The Netherlands) were purchased in the highest purity available and used as received. Solvents were of reagent grade and used without further purification unless otherwise noted. Milli-Q water was obtained from a Synergy® water purification system (Millipore). PBS buffer was prepared by dissolving 1.54 g of Na₂HPO₄ $\cdot$ 2H₂O, 0.3 g of NaH₂PO₄ $\cdot$ H₂O, and 8.2 g of NaCl into 1 L of Milli-Q water and adjusting the pH to 7.4. HEPES buffered glucose (HBG) was prepared by dissolving 4.79 g of HEPES, and 50 g of glucose into 1 L of Milli-Q water and adjusting the pH to 7.4.

Polymer synthesis

The PAA polymers pCABOL and pCHIS were synthesized by polyaddition of cystamine bisacrylamide (C) with 4-aminobutanol (ABOL) or histamine (HIS)

according to modified literature procedures (51). Therefore, in a brown polymerization flask containing 2 mL methanol/water 3/1 and 200 mM CaCl₂ as catalyst, N,N'-cystamine bisacrylamide (1.04 g; 4.0mmol) was mixed with an equimolar amount of 4-amino-1-butanol (0.37 g) or histamine dihydrochloride (0.74 g), respectively (52). Polymerization was carried out under N₂ atmosphere for two days at 70 °C during which a gradual viscosity increase was observed. The polymerization was terminated by adding excess tertbutylamine into the mixture and stirring at 70 °C for two or three more days. After bringing the flask to room temperature, the solution was diluted and acidified to pH ~5 by addition of 4 M HCl and purified by ultrafiltration using a 1000 Da MWCO membrane. The purified polymer solution was then freeze-dried leaving white transparent solid as the final product in its HCl-salt format ~50% recovery. ¹H NMR spectroscopy confirmed complete termination and allowed determination of the number-average MW based on the tert-butylamine end-group. ¹H NMR spectra were recorded on an AVANCE III-400 MHz NMR (Bruker, Wormer, The Netherlands) spectrometer. Gel permeation chromatograms were recorded on a Polymer Labs GPC 220 in 0.1M NaOAc buffer pH 4 with 25% methanol as eluent and 0.7 mL/min flow rate against poly(ethylene glycol) (PEG) standards. pCABOL ¹H NMR (D₂O) δ(ppm) = 1.35 (s, 9H, (CH₃)₃R); 1.60 (m, 2H, CH₂CH₂NR); 1.77 (m, 2H, CH₂CH₂OH); 2.74 (t, 4H, CH₂CONHRNHCOCH₂); 2.85 (t, 4H, CH₂SSCH₂); 3.22 (t, 2H, HO(CH₂)₃CH₂NR); 3.44 (t, 4H, NCOCH₂CH₂NRCH₂); 3.53 (t, 4H, CH₂CH₂SSCH₂CH₂); and 3.62 (t, 2H, CH₂OH). ¹H NMR end group analysis Mw = 9 kg/mol. GPC Mw = 3.8 kg/mol (Mw/Mn = 1.18). pCHIS ¹H NMR (D₂O) δ (ppm) = 1.39 (s, 9H, (CH₃)₃R); 2.78–2.95 (m, 8H, CH₂CONHRNHCOCH₂ & CH₂SSCH₂); 3.23 (t, 2H, CCH₂); 3.48–3.80 (m, 10H, (CH₂)₃ N & CH₂CH₂SSCH₂CH₂); 7.45 (s, 1H, NC = CH); and 8.71 (s, 1H, N = CH). ¹H NMR end group analysis Mw = 5.5 kg/mol. GPC Mw = 4.6 kg/mol (Mw/Mn = 1.26).

Substrate preparation

Poly-D-lysine-coated 96-well plates (PDL–TCPS) for multilayer build-up for cell culture and transfection experiments were purchased from Greiner (Alphen aan den Rijn, The Netherlands). Poly(lactic acid) (PLA) sheets with a thickness of 120

μm were kindly provided by Sidaplast (Ghent, Belgium) and cut in 6 mm diameter disks with a biopsy puncher (Miltex, Rietheim —Weilheim, Germany). Poly(ϵ -caprolactone) (PCL) Mw 45,000 was purchased from Sigma, (Zwijndrecht, The Netherlands). 2D disks were fabricated by placing the PCL grains in between two hydrophobic silica wafers and heating the PCL grains in a press at 100 °C. Pressure was applied to the molten polymer. After cooling, the polymer sheet was removed from the press and disks of 6 mm in diameter were punched out from the polymer sheet with a biopsy puncher. Poly(ϵ -caprolactone) 3D plotted scaffolds were fabricated using additive manufacturing (sysENG, Germany). Scaffolds presented a toroidal shape and were 8 mm in the outer diameter and 4 mm in the inner diameter. The scaffold thickness was 1 mm. PCL with a molecular weight of 45,000 was heated to 100 °C before being extruded from a needle of 200 μm in diameter. Plotting speed and pressure were set to 175 mm/min and 5 bars, respectively. Scaffolds were plotted in a 0–90 fiber deposition configuration, with a fiber spacing of 600 μm and a layer thickness of 150 μm . Covalent immobilization of heparin (HEP) on the PCL sheets and 3D plotted PCL was performed by dissolving 1% (w/v) heparin (Sigma, Zwijndrecht, The Netherlands) in 0.05 M 2-(N-morpholino) ethanesulfonic acid (MES) buffer (Sigma, Zwijndrecht, The Netherlands) at pH 5.5. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-Hydroxysuccinimide (NHS) (Sigma, Zwijndrecht, The Netherlands) were added at a concentration of 0.5M to the heparin solution, vigorously stirred and added to the PCL disks. After 15 h reaction at room temperature, the disks were extensively washed with water (53,54).

Multilayer construction

Multilayers for cell culture were fabricated directly in the wells of poly-D-lysine-coated 96-well plates (PDL–TCPS, Greiner) by alternately dispensing 70 μL of plasmid DNA (1 mg/mL in sterile water) and poly(amido amine) (PAA) (2 mg/mL in PBS pH 7.4) with washing solutions in between, which consisted of the solvents of the respective deposition solutions (2 $\times$ 150 μL). The layer-by-layer (LbL) deposition was performed under sterile conditions. The DNA used to induce cell transfection was plasmid DNA encoding for green fluorescent protein (GFP) as a reporter gene

and its expression was controlled by a cytomegalovirus promotor (PlasmidFactory, Bielefeld, Germany). This construct will be referred as pCMV-GFP and it was used at a 1 mg/mL concentration in sterile water. The resulting ensemble is denoted by (pCMV-GFP#PAA)*n*, where PAA represents the identity of the poly(amido amine), and *n* represents the number of bilayer. Typically, the ensemble consists of 10 bilayers with the plasmid DNA as the first layer. Deposition started with pCMV-GFP (30 min for the first layer, 10 min next) to a total of 10 bilayers, ending with the PAA layer. No intermediate drying steps were applied. At the end of the fabrication process, the plates were briefly left inside the laminar flow hood to allow the films to dry. Coated plates were kept at 4 °C and used as soon as possible (typically overnight). LbL assembly on the 2D disks and 3D plotted constructs was carried out by alternate dipping of the PCL and PCL-HEP constructs in sufficient volume of 2 mg/mL pCABOL and 1 mg/mL pCMV-GFP solutions. Unlike for multilayer fabrication on PDL–TCPS, deposition on 2D and 3D constructs started with pCABOL layer as a precursor layer with 30 min deposition duration. Next deposition steps were 10 minute long followed by two washing steps for 1 min each. Typical multilayer on 2D and 3D constructs is denoted by pCABOL-(pCMV-GFP#pCABOL)₁₀ to indicate the presence of a pCABOL layer as a precursor layer that is excluded from the bilayer count. Multilayer-coated 2D disks were glued on the bottom of regular polystyrene 96-well plates using silicone glue. For 3D scaffolds, three scaffolds were simultaneously coated in the same 48-well as the container. The constructs were left to dry in the laminar flow hood or with nitrogen stream at the end of the assembly and kept at 4 °C until use. Scanning electron microscopy (SEM) of the 3D plotted PCL and heparin-coated PCL scaffolds, with or without multilayers were carried out following gold sputtering of samples (Cressington sputter coater 108 auto) on a Philips — XL 30 ESEM-FEG. SEM images were taken with an accelerating voltage of 5 kV.

Cell culture, transfection and metabolic activity

COS-7 cells (European Collection of Animal Cell Cultures (ECACC) Catalogue No. 87021302) were grown in DMEM containing 4.5 g/L glucose and GlutaMAX™ (Invitrogen, Breda, The Netherlands) supplemented with 1% (v/v) Pen/Strep

(Lonza, Breda, The Netherlands) and 10% (v/v) fetal bovine serum (Lonza, Breda, The Netherlands). Cells were cultured at 37 °C, in a 5% CO₂ incubator and trypsinized at about 80% confluency. Unless otherwise specified, cells were seeded in a 96-well plate at a density of 7360, 24,000 or 44,800 cells/well. On the 3D plotted constructs, cells were seeded at a density of 320,000 cells/construct in four small droplets of 80 µL each.

Cell imaging was performed using an EVOS digital inverted microscope (EMS, Wageningen, The Netherlands) equipped with a 4× objective and an EVOS light cube (EMS, Wageningen, The Netherlands) for GFP imaging.

Presto blue assay for the measurement of cell metabolic activity was purchased from Invitrogen (Breda, The Netherlands) and used according to the manufacturer's instructions. Fluorescence intensity was measured at 535–560/590–615 ex/em using an Infinite M200 PRO plate reader (Tecan, Giessen, The Netherlands). As positive controls, cells were also seeded on uncoated TC-treated polystyrene well plates (TCPS). All fluorescence intensities were corrected by subtracting the values with those of their respective no-cell control wells. Metabolic activity was calculated as the percentage of fluorescence intensity of the samples relative to cells cultured on TCPS (100% metabolic activity). Experiments were done in triplicates.

Determination of transfection efficiency by flow cytometry

After 48 h culturing, COS-7 cells on multilayer-coated PDL–TCPS and on the polymer disks were trypsinized (100 µL of 0.25% trypsin solution, Invitrogen, Breda, The Netherlands). Cell suspensions were then centrifuged (5 min, 300 g, 1200 rpm) and the pellets were resuspended into 100 µL of medium. Cells were kept on ice until measurement by the FACSCalibur (Becton-Dickinson, Breda, The Netherlands). Excitation of expressed GFP was performed at 488 nm and emission was detected via a 530 nm band-pass filter. At least 4000 and 10,000 events were measured for samples with lower, intermediate and higher seeding density, respectively. Data analysis was performed using the FACS Cellquest Software. The gate setting was equal for all samples within the same experiment. Dot plot

was applied to separate live cell population from dead cells and film residues. From the histogram obtained, marker was drawn to identify cells as live GFP-positive cells or GFP-negative cells.

PicoGreen assay and agarose gel electrophoresis

PicoGreen assay was performed to estimate the amount of DNA in the multilayers. Multilayered samples were prepared as described previously. At the end of the multilayer formation, pCMV-GFP and PAA deposition solutions were collected and diluted 500 times in sterile water. For calibration curve, clean unused pCMV-GFP solution of known concentration was first diluted 500 times in sterile water and then serially diluted. Finally, 10 μ L of diluted solutions was transferred into black 96-well plate containing 90 μ L of 1 $\times$ PicoGreen solution in TE buffer, incubated for 5 min at RT in the dark and read for fluorescence intensity (485/520 ex/em) using the InfiniteM200 PRO plate reader (Tecan, Giessen, The Netherlands). Direct method for pCMV-GFP concentration determination was carried out similarly, but each sample solution was retrieved from multilayer-coated well which had been incubated for 1 h in 200 μ L of 4 mM DTT in PBS or HBG at pH 7.4 in the incubator. For agarose gel electrophoresis, 200 μ L of complete cell culture medium was added directly on top of multilayer-coated culture wells (prepared as described in Section 2) and incubated for 1 h in the incubator. Following the incubation, 21 μ L of incubation medium was collected from each samples, mixed with 4 μ L of loading buffer and loaded into a 1.5wt% agarose gel (Bio-Rad) containing 1 $\times$ SYBR Safe (Invitrogen). Electrophoresis was run for 90 min at 90 V in TAE running buffer (40 mM tris(hydroxymethyl)aminomethane, 20 mM acetic acid, 1 mM EDTA, pH 8.2). Pictures were taken on a FluorChem M (ProteinSimple, Westburg, Leusden, The Netherlands) under UV illumination.

Results and discussion

The PAA polymers pCABOL and pCHIS were obtained in ca. 50% yield by polyaddition of cystamine bisacrylamide (C) with 4-aminobutanol (ABOL) or histamine (HIS), respectively (51). Multilayered thin films of these cationic polymers with anionic plasmid DNA can be easily prepared by alternating exposure of

substrates to aqueous solutions of these components. The detailed physical characterization of these thin films is described elsewhere (55). In this study, the transfection capabilities of the multilayered systems were examined in relation to cell seeding density, type of PAA, and number of bilayers. Subsequently, the optimized conditions were used to build multilayers on several common biomedical materials, both in 2D and 3D, to endow cell transfection capabilities to these materials.

Effects of type of PAA and seeding density

To investigate whether cell seeding density influences cell transfection efficiency, COS-7 cells were seeded at three seeding densities, i.e. 7360 cells/well (23,000 cells/cm²), 24,000 cells/well (70,000 cells/cm²), and 44,800 cells/well (140,000 cells/cm²). Transfection efficiency increased slightly going from 1 day to 2 days of culture along with increasing cell number, but no further significant increase was observed upon longer culture duration (data not shown). Thus, 2 days was found to be the optimal culture duration to determine transfection efficiency. Overlaid brightfield and GFP images of COS-7 cells cultured in three different seeding densities on (pCMV-GFP#pCABOL)10 and (pCMV-GFP#pCHIS)10 multilayers after 2 days of culture are shown in figure 1A. Excellent cell attachment was observed for both multilayers. After 2 days of culture, cells at the lowest seeding density have just reached confluency. Cells at the medium seeding density were notably more crowded, while cells at the highest seeding density were overconfluent with significantly more detached dead cells. The similar confluency on both multilayers indicates that the differences in the PAAs have no influence on cell proliferation rate. Figure 1A also indicates qualitatively that the transfection efficiencies achieved by the two different multilayers depend on the seeding density of the cells on top of the multilayered films. For (pCMV-GFP#pCABOL)10, an optimum in transfection efficiency was observed at the intermediate seeding density while for (pCMV-GFP#pCHIS)10, the highest transfection efficiency was seen at the lowest seeding density.

Figure 1. (A) Overlaid brightfield and GFP images of COS-7 cells 48 h after being cultured on (pCMV-GFP#pCABOL)₁₀ (top) and (pCMV-GFP#pCHIS)₁₀ (bottom) at 7360 (left), 24,000 (middle), and 44,800 (right) cells/well seeding densities (scale bars=1000 μ m). Insets show the higher magnification to reveal the general cell morphology and confluency. (B) Transfection efficiency in percentage of total live cell population, and (C) absolute number of transfected cells irrespective to the total cell population. (*) Not significantly different from each other ($p>0.05$).

To get a quantitative estimation of the transfection efficiency on all studied samples, flow cytometry was employed to count GFP-positive cells from the rest of the cell population. Figure 1B shows the percentage of transfected cells in the total cell population in each sample. This graph agrees with the qualitative observation in figure 1A; at all different seeding densities used the pCABOL-multilayer provides higher transfection efficiency than the pCHIS-multilayer. Furthermore, the pCABOL-multilayer provides the highest transfection efficiency at intermediate seeding density (i.e. 24,000 cells/well), while the pCHIS-multilayer gives the highest transfection efficiency at the lowest seeding density (i.e. 7360 cells/well).

Figure 1C shows the transfection efficiency in terms of absolute number of transfected cells produced on the multilayers at the three cell seeding densities. It is clear from this figure that the highest yield of transfected cells can be obtained when pCABOL-multilayers at the highest seeding density are used.

In relation to cell growth phase, optimal transfection efficiency is normally reached when the cells are in the log phase (56). Therefore, the lowest seeding density which leads to ~95% confluency after 2 days of culture can be expected to provide the highest transfection efficiency, as is observed for the pCHIS system. Higher seeding density may lead to stationary or even death phase, where proliferation and subsequently production of GFP protein are no longer optimal.

Unlike the pCHIS system, pCABOL multilayers maintain optimal transfection efficiency up to the intermediate seeding density, before slightly reducing at the highest seeding density (figure 1B). The ability of pCABOL system to provide both higher transfection efficiency than pCHIS, and to maintain it up to the intermediate seeding density may be related to previous findings that pCABOL provides higher transfection efficiency than pCHIS when used as polyplex at the same polymer/DNA ratio (51). Thus, pCABOL acts as a better transfection agent for DNA than pCHIS under the same conditions. In a layer-by-layer multilayered thin film system, the ratio of polymer/DNA in a multilayer is largely determined by specific interactions between a given polymer–DNA pair. UV absorbance measurements at 260 nm on 10-bilayered films of both pCABOL and pCHIS indicated deposition of approximately similar amounts of DNA (55). The pCHIS multilayer, however, contains significantly more polymer than the pCABOL multilayer, as was evident from the significantly higher thickness based on AFM. Thus, at the same number of bilayers, the pCHIS system contains a higher polymer/DNA ratio compared to the pCABOL system. This difference may further influence DNA packing and transfection capability of the multilayers. In order to gain more insight into the amount of incorporated pCMV-GFP in the two multilayered systems and to qualitatively observe differences in the release of polyplexes into cell culture medium, PicoGreen assay and agarose gel electrophoresis were employed, respectively.

The PicoGreen assay was utilized to estimate the amount of incorporated pCMV-GFP in the multilayers by comparing the decrease in pCMV-GFP concentration in the deposition solution before and after multilayer formation (figure 2A). From the assay, it was estimated that (pCMVGFP#pCABOL)₁₀ and (pCMV-GFP#pCHIS)₁₀ multilayers coated on the surface of a 96-well plate contained 2.37 ± 0.13 and 1.96 ± 0.06 μg of pCMV-GFP, respectively. This leads to ~ 650 , and ~ 540 $\text{ng}/\text{cm}^2/\text{layer}$, respectively, which falls within the broad range of reported values of DNA-containing multilayers (20,26,46,57). It needs to be noted that this indirect method is based on the assumption that there is no change in the solution volume before and after the 10-bilayer deposition, and that there is no significant loss of pCMV-GFP during the washing steps. Nevertheless, both UV measurements at 260 nm and PicoGreen assay point to a slightly higher deposition of pCMV-GFP in the pCABOL multilayers than in the pCHIS multilayers.

Figure 2. pCMV-GFP quantification by determination of the remaining concentrations in the deposition solution after multilayer build-up (A), and by PicoGreen assay after multilayer degradation for 1 h in 4 mM DTT in PBS (B) or HBG (C).

Since both polymers contain repetitive disulfide linkages in the main chain, the polymers, and therefore the multilayers, can be easily degraded in the presence of the reducing agent DTT, which cleaves the disulfide bonds (51). In an attempt to get a more direct estimation of pCMV-GFP content, 4 mM DTT was employed to degrade the layers for 1 h and the degradation solution was analyzed on DNA

content by PicoGreen assay (figure 2B). Interestingly, using this approach the pCABOL multilayers were found to release at least twice as much pCMV-GFP as the pCHIS multilayers. The much lower amount of DNA detected after pCHIS multilayer degradation may be an underestimation due to less release of DNA in this case.

In order to gain further insight about the pCMV-GFP released into cell culture medium, multilayer-coated wells were incubated with cell culture medium for 1 h in the incubator, after which the incubation solutions were loaded into an agarose gel for electrophoresis. Free pCMV-GFP in water and in medium were used as controls. Lanes 1 and 2 of the photograph of the gel under UV illumination (figure 3) show that pCABOL and pCHIS multilayers disassemble into plasmid DNA mostly present in its open circular conformation. This finding is in agreement with the report from Zhang et al. (25) which also showed the preferential release of plasmids in the open circular form upon release from the multilayer. The absence of supercoiled pCMV-GFP may indicate the preferred state of pDNA during deposition on the surface. Indeed, an open circular conformation may lead to more multivalent electrostatic binding with the oppositely-charged surface, leading to stronger attachment. The visible bands in the wells of the gel also show the presence of possible polymer-bound pCMV-GFP. This band appears brighter for pCABOL than for pCHIS multilayers. A similar band was not observed for pCMV-GFP dissolved in the cell culture medium (lane 3), indicating that the remaining DNA in the wells is due to binding of pCMV-GFP with PAA polymer and not caused by binding with plasma proteins present in the cell culture medium. For a typical polyplex system, stronger DNA/polymer complexation, for example at higher polymer/DNA ratios, usually leads to less visible bands in the gel wells due to the lower accessibility of the dye to intercalate into the condensed DNA (51). The differences in fluorescence intensity in the wells in lanes 1 and 2 suggest stronger binding of pCMV-GFP with pCHIS than with pCABOL, in line with the previous discussions. However, a weaker but sufficient interaction between pCABOL and pCMV-GFP for internalization by cells may lead to more efficient unpacking of the pCMV-GFP cargo within the cytoplasm, resulting in better transfection (58). The

results from the PicoGreen assay and the agarose gel electrophoresis may serve as an indication that the difference in transfection capability between the two multilayers is due to the fact that pCABOL multilayers contain and release more pCMV-GFP, and that the pCABOL/pCMV-GFP polyelectrolyte complex is likely more efficient in inducing transfection. It can therefore be concluded that pCABOL based multilayers are more efficient than pCHIS-multilayers in facilitating surface-mediated cell transfections.

Figure 3. Agarose gel electrophoresis of cell culture media used to incubate the 10-bilayered pCABOL and pCHIS multilayers (lanes 1 & 2, respectively) and pCMV-GFP solutions in cell culture medium (lane 3) and in water (lane 4) as controls. O.c. = open circular, and s.c. = supercoiled.

Bilayer Number optimization and metabolic activity

Based on the previous results, the (pCMV-GFP#pCABOL) system was selected for further examination of the number of bilayers necessary for optimal cell transfection, using the intermediate seeding density of 24,000 cells/well. Cells were seeded on multilayers composed of 5, 7, 9.5, 10 and 15 bilayers and the percentage of transfected cells was quantified accordingly (figure 4A and B). Cell transfection efficiency increases with increasing bilayer number and reaches a plateau value at 10 to 15 bilayers (figure 4B).

Figure 4. (A) Overlaid brightfield and GFP images of COS-7 cells 46 h after being cultured on (pCMV-GFP#pCABOL) multilayers with various bilayer numbers at 24,000 cells/well seeding density. Bars = 1000 μ m. (B) Transfection efficiency and (C) cell metabolic activity relative to the number of bilayers. (*) Not significantly different from one another ($p>0.05$).

Build-up of the pCABOL and pCHIS multilayers proceeds linearly with increasing bilayer numbers (55). Therefore, it can be expected that a 5-bilayer film would contain and eventually release half of the amount of materials contained in a 10-bilayer film. Figure 4B shows, however, that a 5-bilayer film leads to only ~15% transfection efficiency of that achieved by a 10-bilayer film. Moreover, increasing the bilayer number from 10 to 15 leads only to very small increase in transfection efficiency. Multilayer films composed of only a few bilayers probably supply suboptimal amounts of transfection agent (59), while at bilayer numbers of 10 and

higher sufficient transfection agent is released to meet the uptake capacity of the cells growing on the surface, resulting in a plateau value. Figure 4B also shows only a minor, although significant ($p < 0.05$), difference in transfection efficiency between 9.5 and 10 bilayers, i.e. multilayers ending with pCMV-GFP and pCABOL layer, respectively. This indicates that the identity of the top layer, either being DNA or pCABOL, only marginally affects cell transfection, which is in agreement with various other reports on alternating DNA-based multilayer coatings (60,61).

Cell metabolic activity in relation to the bilayer number was also analyzed and expressed as a percentage of the metabolic activity of cells seeded on tissue culture plastic (TCPS) (figure 4C). Cells seeded on multilayers showed ca. 10% lower metabolic activity at lower bilayer numbers, whereas at bilayer numbers 10 and 15 the metabolic activity tends to decrease. However, cells maintained similar shapes throughout the various bilayer numbers studied and are similar to those cultured on TCPS. It has been shown in other studies, that pCABOL and its polyplexes exhibit no or only minor ($\leq 10\%$) metabolic toxicity to cell cultures (51). Therefore the further decrease in the metabolic activity at higher bilayer numbers can most probably be attributed to the high GFP production of the cells on these films. It is generally accepted that transfected cells have to spend more efforts towards biosynthesis of the recombinant proteins, leading to a decrease in metabolic activity (56).

Transfection efficiency of multilayers assembled on various substrates

To test the applicability of the (pCMV-GFP#pCABOL)₁₀ multilayer to add cell transfecting capability to several common biomaterials, these multilayers were assembled on poly(ϵ -caprolactone) (PCL), heparinized poly(ϵ -caprolactone) (PCL-HEP), and poly(lactic acid) (PLA) (figure 5A), in a similar way as was previously done with poly-D-lysine (PDL)-coated 96-well plates (PDL-TCPS), but with pCABOL as the precursor layer instead of PDL. The transfection efficiency of COS-7 cells, seeded at a density of 24,000 cells/well and cultured for 48 h on the coated disks is shown in figure 5B. The transfection efficiency was found to be slightly

higher for PLA disks than for PCL-based disks, and was about similar to the values obtained for PDL–TCPS substrates (figure 1B).

Despite the increased surface roughness and the presence of a negatively charged surface underneath the multilayer due to the heparin coating, these differences have little effect on the transfection efficiency. The results indicate that the multilayers were formed to a similar extent on the three different substrates and completely mask the properties of the underlying surface at the 10-bilayers utilized. Masking of the underlying substrate was very prominent for PCL and PLA disks, where cell attachment and cell shape were found to be significantly improved after multilayer coating compared to uncoated substrates (data not shown). The reported multilayers therefore not only add cell transfecting capability, but also improve cell adhesion for poor cell adhering biomaterials.

Figure 5. (A) Digital photograph of the three disks: PCL (white opaque), PCL-HEP (stained with Azure A), and PLA (transparent). (B) Transfection efficiency of COS-7 cells cultured on multilayer-coated disks after 48 h of culture. (*) Not significantly different from each other ($p > 0.05$).

3D plotted scaffold application

To further demonstrate the versatility of the multilayer coating to provide new functionality to various scaffolds and supports for tissue engineering applications, we prepared (pCMV-GFP#pCABOL)₁₀ multilayers on 3D fiber deposited PCL and PCL-HEP scaffolds. Using this approach, for example, cells can be transfected right at the spot of the implantation site. 3D fiber deposition technique was used for

the fabrication of three dimensional PCL scaffolds, which were subsequently heparinized (for PCL-HEP) and coated with multilayers (figure 6). Covalent attachment of heparin already caused a change in the scaffold topography, making the smooth PCL surface topography look rougher. This has been previously reported to facilitate cell adhesion on the material, both because of the increased nano-scaled topography, providing anchor points to cells and also because heparin increases protein adsorption on the surface (54). Higher magnification SEM images confirmed multilayer coating both on PCL and heparinized PCL, where slight changes can be observed in the scaffold topography with and without multilayer (figure 6). The multilayer, approximately 100 nm in thickness (55), conformed to the topography of the original substrates, thus, maintaining the rough feature of heparinized surface, but covering up smaller indentations observed on the surface of the substrates.

Figure 6. First three rows: SEM images of 3D fiber PCL and PCL-HEP scaffolds with and without multilayer coating at various magnifications. Two bottom rows: overlaid brightfield and fluorescence microscopy images of COS-7 cells cultured on the multilayer-coated scaffolds.

When multilayer-covered scaffolds were seeded with COS-7 cells, transfection was observed to occur both on PCL and heparinized PCL scaffolds. The positive transfection observed on the 3D scaffolds were confirmed through the co-localization of GFP-producing cells with the opaque scaffolds. Due to the relatively wide fiber spacing (~600 μm), a significant number of cells fell through the spaces/pores during seeding and subsequently attached and proliferated on the well surface. Some of these cells were also successfully transfected through the transfection agent released into the cell culture medium (data not shown). The availability and use of 3D scaffolds with smaller fiber spacing most likely enable higher seeding densities that can lead to even higher transfection efficiencies.

Conclusions

pCABOL and pCHIS, two peptidomimetic linear poly(amido amine)s with differing side groups, have been used to form multilayers with pCMV-GFP as a reporter plasmid. This layer-by-layer strategy was demonstrated to be an effective and inexpensive method to add biofunctionality to biomedical materials of various shapes and sizes. The pCABOL#pCMV-GFP multilayer system proved to be more effective in transfection of COS-7 cells cultured on top of these layers than the pCHIS#pCMV-GFP system, which is attributed to the higher pCMV-GFP/polymer ratio and higher release of pCMV-GFP in the former system. Transfection efficiency of the pCABOL#pCMV-GFP multilayer system was found to increase with increasing number of bilayers and reached a plateau level at 10–15 bilayers, in which range no significant further increase in transfection efficiency was observed. Cells on top of the multilayers show only small decreases in metabolic activity, which is consistent with the low toxicity of pCABOL and the high production of GFP in the transfected cells. Various biomedical materials such as PCL, HEP-PCL and PLA were covered with the pCABOL#pCMV-GFP multilayer transfection system and showed significant improvement of cell attachment compared to uncoated substrates. Transfection was found to be equally efficient as when using PDL-TCPS as substrates. Moreover, it was demonstrated that this simple method can also be applied to 3D plotted structures leading to successful transfection of cells adhering on these structures. Such surface-mediated transfection may prove

beneficial in various biomedical and tissue engineering applications and provide transfection of cells at the spot of implantation, before and after construct implantation.

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General Discussion and Future Perspectives

General discussion and future perspectives

Clinical islet transplantation is a promising alternative to total pancreas transplantation for reaching insulin independence in type 1 diabetes patients. Although promising, several aspects of this procedure still require improvements. In particular, in the case of the creation of an extra-hepatic transplantation site, several requirements need to be taken into consideration, mainly regarding nutrient and oxygen diffusion and the creation of a suitable environment to preserve and possibly improve islet viability and functionality.

In each of the chapter presented in this thesis, a different aspect of scaffold design has been unraveled: the material choice, the scaffold geometry, the size, the porosity are just some of the key parameters that an optimal construct has to fulfil in order to preserve functional grafts in a long term after transplantation. This thesis presents an overview of strategies for addressing some of these variables and gives some perspectives on possible future applications.

Islet availability

The availability of islet for research purposes is often a neglected bottle-neck that severely limits research possibility. Good quality and high purity islet of Langerhans from donor pancreas are understandably destined for clinical transplantation, but the scarce amount of human islet left for research sensibly limits the possibility of high-throughput screening of conditions. In **chapter 1** we present a protocol to fabricate pseudo-islets based on mouse and rat beta-cell lines with a controlled aggregate size. Other authors published earlier the importance of aggregation in establishing beta-cell proper functional response (1,2). Increasing cell-cell contact among beta-cells stimulates the adhesion via cadherine molecules (3), but was not sufficient to induce proper insulin secretion. We showed that inclusion of theophylline in the glucose buffer achieved specific insulin secretion only upon glucose stimulation and showed a stimulation index dependent on the theophylline concentration used. We showed that these pseudo-islet can be successfully used as model for islets since they are able to mimic the functional behavior of human islets in a more physiological and representative manner. This model has been

used through the thesis as a surrogate for human islets in experiments requiring a high number of islets.

Constructs for islet of Langerhans transplantation

Hydrogel based scaffolds

Hydrogels have always been considered an attractive alternative for islet of Langerhans transplantation, as a starting material for the fabrication of an artificial pancreas. Given their high water content and their mechanical properties they represent a valid alternative for mimicking the native extracellular matrix. Islet encapsulation in hydrogel beads is a known concept since 1984, when Lim made the first attempt of encapsulating islet for reversing diabetes in streptozotocin-induced diabetic rats and reverted hyperglycemia for up to 15 weeks (4). Since then, hydrogel encapsulation has been a widely exploited tool, in particular in diabetes research. Several works, in various stages of research or clinical experimentation, illustrate micro encapsulation in hydrogel or in macro hydrogel-based devices as a promising tool for islet encapsulation and diabetes treatment. Micro encapsulation is based mainly on alginate (5-9) or poly-ethylene glycol (3,10).

In **chapter 2**, synthetic PEG diacrylate hydrogels have been prepared with the aim of combining islet encapsulation and creating a controlled growth factor release system for increasing revascularization in proximity of the scaffold. The PEGDA system is specifically intended for combining islet encapsulation in a non adhesive hydrogel with the induction of blood vessels ingrowth in a cell adhesive hydrogel, based on extracellular matrix molecules for adhesion and on a controlled growth factor release system for neoangiogenesis stimulation. This chapter presents an innovative and promising concept, but nutrient diffusion limitations play an important role in defining islet functionality in the bulk construct. Also the conformal coating of PEG diacrylate on the surface of the islet and their embedding in the cell adhesive matrix drastically reduces islets functionality

In the following **chapter 3** a strategy based on the fabrication of a 3D porous structure is presented with the aim of overcoming the problem of nutrient diffusion in hydrogels for islet embedding. We show that a 3D plotted porous construct slightly improves nutrient diffusion compared to bulk hydrogels, but in this case the mayor limitation resides in the viscosity of the precursor material required for the plotting technology.

As the results of both studies highlighted, hydrogel encapsulation of islets can be performed with good results only if the selected hydrogel has specific characteristics in terms of mesh size, matrix thickness and porosity. The fabrication of a macroscopically porous hydrogel is not sufficient to obtain a proper oxygen and nutrient diffusion, if the microscopic pores within the hydrogel network are not sufficiently wide to allow metabolites to penetrate.

In few papers the thickness of the hydrogel has been investigated as one of the limiting factors in predicting islet functionality. Chicheportiche *et al* have reported a seven fold insulin secretion decrease from stimulated islets embedded in 650 μm alginate capsules if compared to similarly stimulated unencapsulated islets (11). In comparison to the microbeads presented in the previous study, the constructs of chapter 2 and 3 can be regarded as macro-encapsulation devices, making the limitations in insulin secretion even more pronounced.

Another factor involved in islet functionality is the density at which islet or beta-cells are seeded within the construct. In a paper of Weber and in another of La Flamme the influence of cell density within the hydrogel matrix has also been found as one of the factors involved in a proper insulin response towards glucose stimulation (12,13). In particular, a higher cell density is preferred for achieving proper insulin secretion. Nutrient diffusion in hydrogels might impair also paracrine signaling and cell sensing, thus limiting also cell-cell interaction and glucose secretion in a physiological manner. In the case of hydrogel embedded islets, a higher cell density might be beneficial for functionality, but nutrient diffusion limitations should be considered as they are more pronounced for cells embedded in the most inner part of the construct.

Another factor to consider is the implantation site, which needs to assure sufficient nutrient and oxygen diffusion to the embedded islet. Subcutaneous transplantation is a good alternative in terms of ease of implantation and retrieval of the device, in case of need, but scaffold design and functionalization need to specifically take this fact into consideration and propose solutions in order to overcome this limitation.

Polymer based scaffolds

Similar strategies for the development of extra-hepatic sites for islet of Langerhans transplantation have been elaborated using thermoplastic polymers. Although mechanical properties and compression moduli are consistently higher compared to soft tissues in the human body, thermoplastic polymers are attractive because of their ease of functionalization, defined chemical structure and easy processability.

In **chapter 4**, a simple and inexpensive technique as salt leaching has been used to fabricate a controlled and interconnected porous structure for islet to be seeded within a polymeric sponge. In comparison to hydrogels, the pore size within the matrix can be selected by tuning the size of the porogen. Also in this case nutrient diffusion plays a major role in defining islet functionality within the scaffold. Moreover, thermoplastic polymer scaffolds present a more stiff compression modulus than hydrogels: the procedure itself of seeding the islet within the scaffold could originate some mechanical stress in the seeded tissue and reduce islet functionality in a limited period of time right after the initial seeding.

The chemically defined structure of thermoplastic polymers also allows tight control on their physio-chemical characteristics and functionalization with other molecules in order to obtain coatings with defined characteristics. These superficial coatings are used to fine tune material interaction with cells. While bulk mechanical properties remain pristine, the superficial layer responsible for cell interaction can be modified to present a more favourable surface for cell adhesion, present ligands to interact with specific cell types or release molecules to exert specific functionalities.

This approach has been used in chapter 4, 5 and 6 for controlled release of molecules capable of inducing vascularization in close proximity to the scaffold and the embedded islets. In **chapter 5** heparin coating of the material has been shown to act as a controlled release system for vascular endothelial growth factor and induction of capillary ingrowth on a porous printed 3D structure of polycaprolactone fibers. The interesting concept developed in this chapter is a combination of the favorable properties of thermoplastic materials and hydrogels. Thermoplastic materials are able to provide ease of functionalization and suitable mechanical properties. In case a reinforcing element fabricated with a thermoplastic polymer is present, the thickness and the volume of the central hydrogel core can be drastically reduced, improving in this way nutrient and oxygen diffusion in the construct and assuring overall better performances of the embedded islets.

Modification of the surface properties of 3D printed PCL scaffolds was also explored in **chapter 6**, where a layer by layer coating was successful in inducing cell transfection on top of the scaffolds. This strategy can be used for transfecting cells *in situ* after scaffold implantation and prompting on demand production of the desired growth factor directly by autologous cells in the area of transplantation. Chapter 6 proved this to be a successful proof of concept using plasmidic DNA for green fluorescent protein synthesis, next step would be functionalization of the scaffold with a DNA plasmid encoding for VEGF and induction of vascularization after autologous cell transfection.

Concluding remarks

The work presented in this thesis encompasses a variety of strategies for the creation of an extra-hepatic transplantation device for homing islet of Langerhans. The re-creation of the physiological environment is particularly complex for such a metabolically requiring cell type and a variety of parameters need to be taken into consideration. Main determinants for islet functionality include geometrical consideration about the scaffold design like thickness/dimension, mesh size and porosity, nutrient diffusion and material choice to name a few. On the biological side, cell adhesion on the biomaterial, presence of extracellular matrix molecules or

growth factors which could enhance islet functionality. Moreover “helper” cells as endothelial or mesenchymal cells can be included in the construct to recreate the physiological environment of the transplanted islets. Interplay between those different factors also is important in defining islet functionality.

In the author’s opinion, future research should be focused in unraveling the role of all these parameters independently and in interplay with each other, reserving a special attention on how construct geometry, macroscopic and microscopic structure influences islet functionality in a long term. Not only structural architecture but also suitable material’s physio-chemical properties need to be matched with the most suitable fabrication technique. Last but not least, bioactive molecules can be incorporated in the construct to steer the biological response toward the desired result, in the chosen implantation site.

An optimal scaffold design has not been reached yet, however the experimental work presented in this thesis suggests possible amelioration to the scaffolds currently considered for pre-clinical islet transplantation. Although the use of hydrogels provides a water rich environment with mechanical properties similar to soft tissues in the body, their mesh size is often not optimal in terms of nutrient and oxygen diffusion to the embedded islets. In this regard, a low concentration hydrogel precursor can be beneficial for obtaining a mesh with pores wide enough to allow for sufficient nutrient diffusion. Being mesh size and resulting mechanical properties so tightly linked, mesh with sparse crosslinking points and wide pores might not present sufficient mechanical properties for islet retention and handling of the construct. Moreover the construct might not withstand physical forces and shear stress upon implantation. As a solution for this problem, a polymeric support structure presenting open pores might provide mechanical support to the hydrogel without impairing nutrient and oxygen diffusion.

We believe that a biphasic structured scaffold can be beneficial not only for tuning the different mechanical properties in the different zones of the scaffold but also to provide different cues to the embedded cells. In order to increase revascularization of the transplanted tissue, areas in the scaffolds providing adhesion cues and

growth factors for the attraction of endothelial cells can be coupled with non-adhesive areas designed for islet of Langerhans embedding. Specialized sets of proteins can be embedded or covalently linked to the specific area of the scaffold in an attempt to provide the desired properties in closer contact to the cell type of interest and optimizing in this way the performance of both functionalities in the scaffold. Also specific chemistry and material properties in different areas of the scaffolds allow for differential functionalization of the scaffold in an attempt to recreate a more favourable environment for the embedded cells.

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Here we are, finally thinking about the last pages of this work, and probably the most important ones. There has been moments, as maybe many other PhD students experienced, in which I thought I would never have reached this point and I need to acknowledge many of you if I finally did it.

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Curriculum Vitae

Giulia Marchioli was born on the 20th August 1985 in Monfalcone, Italy. She studied biotechnology at the University of Trieste, Italy, where she obtained her Bachelor degree in 2007, after an internship on Biocatalysis at the department of Pharmaceutical Sciences under guidance of Prof L. Gardossi. She continued her Master in Medical Biotechnology and graduated in 2011 with a thesis entitled “Bioactive Hydrogels for Biomedical Applications” under supervision of Prof. S. Paoletti. In November 2011 she started her PhD at the University of Twente under guidance of Prof. dr. Marcel Karperien, dr. ir. Aart van Apeldoorn and Prof. dr. Lorenzo Moroni. The results of her doctoral research are described in this thesis.

In July 2015 she started working as Biomaterial Scientist at Beta-cell NV, Brussel, Belgium for a period of 8 months. Since March 2016 she is enrolled in a post-doc position at the University of Eindhoven, the Netherlands, under supervision of Dr. Dr. Patricia Dankers.

List of Publications

Hybrid Polycaprolactone/Alginate Scaffolds Functionalized with VEGF to promote de novo vessel formation for the Transplantation of Islet of Langerhans.

Marchioli G, Di Luca A, de Koning E, Engelse M, van Blitterswijk C, Karperien M, van Apeldoorn A, Moroni L.

Advanced Healthcare Materials 2016

Fabrication of three-dimensional bioplotting hydrogel scaffolds for transplantation of islets of Langerhans

Marchioli G, van Gurp L, van Krieken P, Stamatialis D, Engelse M, van Blitterswijk C, Karperien M, de Koning E, Alblas J, Moroni L, van Apeldoorn A.

Biofabrication 2015

Poly(amido amine)-based multilayered thin films on 2D and 3D supports for surface-mediated cell transfection.

Marchioli G*, Hujaya SD*, Roelofs K, van Apeldoorn AA, Moroni L, Karperien M, Paulusse JM, Engbersen JF.

J Control Release. 2015 (* first shared authorship)

Theophylline improves glucose induced insulin secretion in MIN6 and INS1E pseudoislets

Groot Nibbelink M, Marchioli G, Karperien M, van Apeldoorn A.

International Journal of Molecular Sciences 2016

Cell-transfecting multilayered surfaces from poly(amido amine)s.

Hujaya SD, Marchioli G, van Apeldoorn AA, Paulusse JM, Karperien M, Engbersen JF.

Journal of Controlled Release 2015

Wettability influences cell behavior on superhydrophobic surfaces with different topographies.

Lourenço BN, Marchioli G, Song W, Reis RL, van Blitterswijk CA, Karperien M, van Apeldoorn A, Mano JF.

Biointerphases. 2012

Mechanical spectroscopy and relaxometry on alginate hydrogels: a comparative analysis for structural characterization and network mesh size determination.

Turco G, Donati I, Grassi M, Marchioli G, Lapasin R, Paoletti S.

Biomacromolecules 2011

Salt-Leached Porous Scaffolds functionalized with VEGF for Islets of Langerhans Transplantation

Marchioli G, Hertsig D, Engelse M, de Koning E, Karperien M, Moroni L, van Apeldoorn AA

Manuscript in preparation

Layered PEGDA hydrogel for islet of Langerhans encapsulation and improvement of vascularization

Marchioli G, Zellner L, Engelse M, de Koning E, Karperien M, van Apeldoorn A, Moroni L.

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