Microencapsulation of pancreatic islets for cell transplantation in type 1 diabetes without immunosuppression

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LIST OF ABBREVIATIONS

Ba Barium

Ca Calcium

DTT Dithiolthreitol

EFP Epididymal fat pad

GSIR Static glucose stimulated insulin release

G blocks Guluronic acid

IEQ Islet equivalent

IgG/M Immunoglobulin G/M

IP Intraperitoneal

IL-1β Interleukine 1beta

M blocks Mannuronic acid

MCP-1 Macrophage colony promoting factor 1

MG blocks Mixed mannuronic/guluronic acid

MWCO Molecular weight cut off

NHP Non human primate baboons

NO Nitric oxide

NOD Non Obese Diabetic mice

Peg-Mal Polyethylene glycol-Maleimide

PDGF Platelet derived growth factor

PLL Poly (L-Lysine)

PLO Poly(L- Ornithine)

STZ Streptozotocin

TGF-β Transforming growth factor beta

TNF-α Tumor necrosis factor alpha

T1D Type 1 Diabetes

UP-MVG Ultra Pure medium viscosity G sodium alginate

VEGF Vascular endothelial growth factor

WOP Whole pancreas transplantation

1. ABSTRACT

Type 1 Diabetes mellitus (T1D) is a chronic autoimmune disease caused by the attack of autoreactive T lymphocytes on pancreatic β-cells, leading to absolute insulin deficiency. For patients with T1D, exogenous insulin injections to control blood glucose are a lifesaving treatment. However, exogenous insulin administration does not prevent daily risk of hypoglycemic episodes, and does not guarantee a tight control of blood glucose. Pancreatic islet transplantation through the hepatic portal vein has recently emerged as one of the therapeutic approaches for improving blood glucose control in T1D patients with severe hypoglycemic unawareness. Despite promising results, most patients lose insulin-independence and graft function in variable times after transplantation. Among the causes is early graft loss due to immunological, anatomical, physiological and metabolic limitations of the transplant site. Also, islet transplantation requires life-long systemic immunosuppression (SI) to prevent graft rejection. SI causes lymphopenia and several side effects, included tumor development, frequent infections, and general toxicity. In addition, in order to overcome the shortage of allogeneic islets from cadaveric donors, islets from xenogeneic donors, such as pigs, represent an unlimited source. Islet encapsulation with biocompatible and non-degradable hydrogels may represent a valuable alternative to systemic immunosuppression as it provides a physical barrier that immunoisolates and protects the graft from the cytotoxic attacks of the host immune system. In the past thirty years, alginate encapsulation has been evaluated in pre-clinical and clinical settings. The successful use of alginate microcapsules has, however, been hampered by 1) the large diameters (600 and 1000μm), and 2) the mechanical instability, followed by the lack of immunoprotection. The large capsule size constitutes a diffusion barrier, impairing oxygen and nutrient exchanges, and limits

the choice of transplant sites to areas that are not conceived for cell survival. Several alternative sites have been proposed to minimize early inflammatory reactions, promote vascularization and easy-accessibility, mimic physiological insulin release and protect from immune responses. Among all, the omental pouch, or the equivalent epididymal fat pad (EFP) in mice, is well vascularized and can accommodate large volumes. Conventional alginate microencapsulation has been optimized by minimizing capsule size (450-550μm in diameter), increasing the cell loading density (nearly 3%), and by using highly biocompatible Ultra-Pure medium viscosity sodium alginate (UP-MVG). This allowed for transplantation of microencapsulated islets in the EFP, engineered with a novel fibrin matrix to promote angiogenesis, decrease early graft loss, and improving islet engraftment. Under physiological conditions the capsules are also exposed to a combination of destabilizing forces, leading to swelling, increased pore size, dissolution, and capsule rupture. To protect the cells from the host immune system, the capsule must therefore be carefully designed, especially with respect to stability and porosity. Thus, novel alginate-based capsules have been designed with the goal to improve *in vivo* stability of alginate: 1) hybrid microcapsules (MicroMix) using an electrostatic droplet generator method by mixing UP-MVG with Polyethylene Glycol functionalized with Maleimide groups (Peg-Mal) 2) UP-MVG microcapsules double coated (Double) with Peg-Mal through an emulsification process. One of the main challenges of this part of the work has been to make alginate capsules stable under physiological conditions over extended periods of time. The hope is that the great, and still growing, knowledge about alginate-based capsule biocompatibility, mechanical properties and permselectivity will be useful for successful clinical transplantation.

2. INTRODUCTION

2.1. Type 1 diabetes and exogenous insulin requirements

The β-cells within the islets of Langerhans produce and secrete insulin, a hormone that is essential for normal glucose homeostasis. T1D is a chronic autoimmune disease, characterized by selective destruction of pancreatic β-cells by the immune system (**Figure 1**).
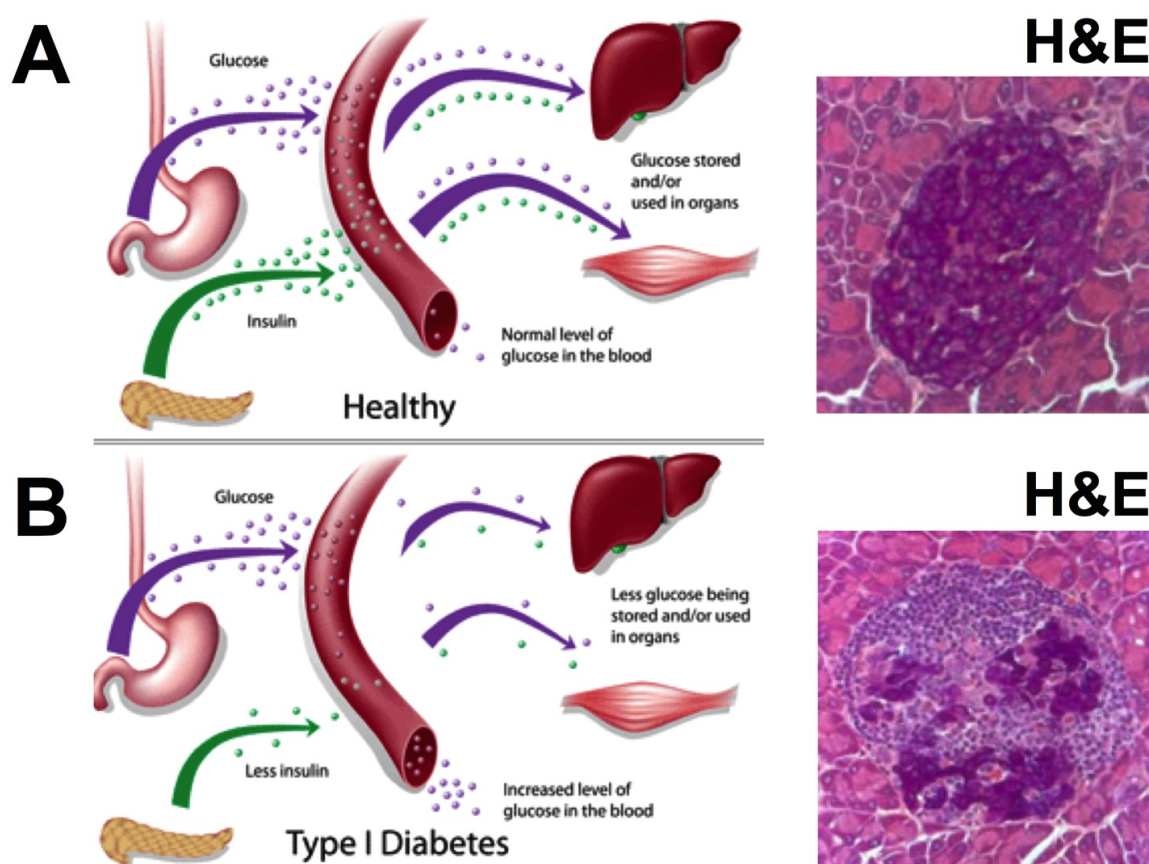
Figure 1. Schematic of insulin release regulating glucose metabolism, and histological image (H&E) of a pancreatic islet in a healthy person (A) and in a person with T1D (B). In diabetic patients pancreatic islets present heavy insulitis (B).

Individuals with T1D lose their capability to secrete insulin and develop hyperglycemia and related complications in several organ systems. T1D was a lethal disease until 1920s, when Banting and Best identified insulin as the hormone in the pancreas responsible for maintaining blood glucose homeostasis [1]. Both the regulation of several genes and environmental factors have been implicated with the development of T1D [2]. The incidence of T1D varies among different countries, further confirming that genetic and environmental factors play a critical role of the etiology of the disease.

For patients with T1D, exogenous insulin injections to control blood glucose are a lifesaving treatment. However, the requirement of exogenous insulin has a negative impact on personal and social functioning. More importantly, administration of exogenous insulin does not allow a tight control of blood glucose and it does not prevent daily hypoglycemic episodes. In addition, secondary complications, such as retinopathy, neuropathy, nephropathy and cardiovascular diseases, can occur despite administration of exogenous insulin because controlling the blood glucose level is sometimes difficult, even with intensive insulin therapy.

2.2. Whole pancreas transplantation (WOP)

Despite significant improvements in monitoring of blood glucose and administration of exogenous insulin, insulin therapy cannot guarantee glucose homeostasis. Therefore, curative therapies for the disease have relied on biological replacement of the β-cell mass by transplantation.

During the last 35 years, whole organ pancreas transplantation has evolved gradually into a possible therapy for T1D [3-7]. A successful whole pancreas

transplant provides a closed-loop system to achieve tight blood glucose control without hypoglycemic episodes [8]. As a result, the number of pancreatic transplants has increased dramatically and the graft survival rate has improved reaching a survival rate of 76% and 62% at one year and three years post-transplant, respectively. Long-term normoglycemia and insulin independence was achieved with a 5- year graft survival rate of 50-70% [9]. However, because the risks of severe complications associated with the WOP procedure, the American Diabetes Association's Clinical Practice Recommendations states that a pancreas transplant is an acceptable procedure only for T1D patients undergoing renal transplantation [10], or (for pancreas transplantation alone) when the patient experiences frequent and acute metabolic complications, incapacitating clinical or emotional problems with insulin injection or failure of exogenous insulin to prevent acute complications. Drawbacks of whole pancreas transplantation consist in the major surgical intervention, the morbidity from the procedure, associated with the need to drain the exocrine fluids, for example, and the thrombosis of the graft. In addition, the patients need chronic immunosuppression with anti-rejection drugs. Side effects associated with chronic immunosuppression include the increased susceptibility to infection, renal dysfunction, hyperlipidemia, anemia, mouth ulcers, and increased risk of cancer [11].

2.3. Pancreatic islet transplantation

The exocrine tissue constitutes the bulk of the pancreas (>95%). Exocrine tissue is responsible for producing digestive enzymes, but it does not contribute to insulin production. The endocrine component of the pancreas constitutes only ~1% of the pancreas, suggesting that transplantation of just the endocrine component of the pancreas may be a simpler approach than whole pancreas

transplantation. In specific, transplantation of the endocrine pancreas component should minimize complications arising from transplanting the exocrine component of the pancreas.

The pioneering experiments by Lacy and Kostianovsky provided the fundamental means to introduce islet transplantation as an effective therapy to correct hyperglycemia through the ability to isolate a sufficient number of metabolically active and intact islets from rodent pancreata [12]. While several authors reported correction of hyperglycemia in diabetic mice using varied islet doses and success via the intraperitoneal route, Reckard et al in 1973 were the first to effectively cure diabetes in a chemically induced model [13]. Yet despite these successes, the same principles of isolation and purification could not be applied to larger animals or humans whose glands are more dense and fibrous [14]. Refinements in the methods of islet isolation and purification for islet transplantation continued for decades with improved success in isolating significant quantities of highly pure islet preparations. However, the development of the Ricordi® Chamber (BioRep, Miami, FL, USA) in 1988 introduced a semi-automated process that was instrumental in consistently isolating and purifying large islet quantities [14,15]. This method of islet isolation, in conjunction with improvements in islet purification and transplantation techniques, was paramount in the translation of islet transplantation from an experimental concept to an efficient clinical treatment modality for a selected group of patients suffering from T1DM [14].

Outcomes in clinical islet transplantation have progressed significantly since its inception, also coupled with the more effective immunosuppression (induction and maintenance) to protect against both auto- and alloreactivity [16]. Islet-transplantation has recently become an accepted practice to stabilize frequent

hypoglycemia or severe glycemic liability in highly selected subjects with poor glycemic control [17]. In spite of these advancements, 9% of the 267 patients that received islet transplantation since 1999 were insulin independent for only 1 year [18]. It was not until 2000 that the application of the Edmonton Protocol for immunosuppression reported insulin independence in seven consecutive T1D patients over a median follow-up of 11.9 months with sustained C-peptide [16]. Of particular importance, patients had received at least two different intraportal islet transplants, as well as a steroid-free immunosuppressive regimen.

Intrahepatic transplantation is a minimally invasive portal infusion that results in islet entrapment within hepatic sinusoids. This vascular space provides nutritional and physical support for islets, which is an essential role given that the islet isolation process strips the islets of their dense vasculature and specialized extracellular matrix [19,20]. However, research efforts to improve intrahepatic islet delivery have identified multiple mechanisms that limit islet engraftment and long-term function. Portal vein infusion results in embolization of islets in the liver that exposes the cells to a relatively hypoxic environment since the liver has a parenchymal oxygen tension below that of the pancreas [21,22]. Furthermore, infusion into the portal vein exposes patients to additional risks of hemorrhage, thrombosis, biliary puncture, transient rise in serum aminotransferase, and arterial-venous fistula [23],

Nowadays, clinical islet transplantation through the hepatic portal vein and with chronic immunosuppression is currently indicated only to treat individuals with severe hypoglycemic unawareness.

The systemic immunosuppression and the associated lymphopenia trigger homeostasis of T lymphocytes, including alloreactive and autoreactive memory

cells and recurrence of autoimmunity. Moreover, it decreases the safety of the islet transplantation procedure. Insulin independence is not durable long-term, as most patients returned to modest amounts of insulin, despite the elimination of recurrent hypoglycemia by 5 years post-transplant, clearly indicating room for improvement [24].

Another critical aspect to be considered is the limited availability of the current islet source [25]. Allogeneic islets from cadaveric donors, xenotransplantation, regeneration therapy (generation of pancreatic β-cells from pre-existing β-cells or from stem cells) and development of insulin-producing cell-lines are being extensively studied to overcome this problem [14,26]. In addition to immunoisolation, recent progress in the induction of donor specific tolerance [27], and the development of pancreatic β-cells from human stem cells [14] gives hope for transplantation without immunosuppression. However, as diabetes is an autoimmune disease, the need for protection of the cells from the immune system may still be needed in order to avoid immune reaction to the insulin-producing β-cells.

2.4. Encapsulation of cells (macrodevices vs. microcapsules)

The necessity to apply immunosuppression to prevent islet rejections can be by-passed by immunoisolation. The general concept of immunoisolation is to prevent rejection by separating the transplanted cells from the hostile immunological environment in the host by a selectively permeable artificial membrane. The small pores of the membrane prevent the passage of high-molecular weight substances such as large antibodies and cytotoxic immune cells. At the same time, the small pores of the membrane have to allow for the free passage of nutrients,

electrolytes, oxygen, bio-therapeutic agents, and smaller molecules, i.e. insulin which has to be easily released from the capsules.

Thus, immunoisolation by encapsulation allows for both successful transplantation of cells in the absence of immunosuppression [28,29], and transplantation of cells from non-human origin, i.e. xenografts, which could be a mean of overcoming the obstacle of limited supply of donor tissue [30,31].

Encapsulation of living cells may be achieved through mainly two geometries: macro and microcapsules. In the first case, pancreatic islets are entrapped within macroscopic devices with semipermeable properties [32-34]. In general, since macrodevices involves the encapsulation of the whole islet graft, issues related to cell density, adequate nutrition and hypoxic condition in the center of the device are frequently experienced [35]. Macrodevices can be used as intra- or extravascular device. Intravascular devices provide artificial capillaries enclosed by immunoisolating membranes, inside of which blood flows, and outside of which cells are distributed. The presence of blood flow provides constant and evenly distributed oxygen supply and a fast exchange of nutrients, but with a great risk of thrombosis as drawback. In the extra vascular devices, cells are enveloped within semipermeable diffusion chambers and they are implanted under the skin or in the peritoneal cavity without direct access to the blood supply. Implantation of extra vascular devices requires a minor surgery. Further, replacement of extra vascular devices is easier, in case of failure. Unfortunately, macrodevices require high amounts of nutrients to guarantee an adequate diffusion gradient to supply a large number of cells, due to the relatively large surface to volume ratio of such devices. Finally, macrodevices generally induce heavy fibrotic responses [33,36].

On the other hand, in microencapsulation, each islet is individually encapsulated,

offering several advantages over other types of encapsulation. Microcapsules are not associated with surface to volume ratio issues because of their spherical geometry. Also, microcapsules allow efficient exchange of nutrients and oxygen. An important point to consider is that, unlike macrodevices, the risk of device breakage is spread over a large number of capsules, i.e. if one capsule breaks the whole graft is not lost. Moreover, the spherical geometry of the microcapsules minimizes foreign body reactions, because of the absence of corners, which are known triggers of host inflammatory reactions [37] . Since Chang first described microencapsulation in 1964 [38], microencapsulation has been used with a variety of cell types including PC12 cells [39]for the treatment of Parkinson's disease, and hepatocytes [40] for the treatment of liver diseases, as well as for the encapsulation of genetically modified cells producing factor IX for the treatment of haemophilia B [41], and growth hormone for the treatment of dwarfism [42].

Since 1980, when Lim and Sun first microencapsulated islets of Langerhans in alginate beads for the treatment of T1D [28], the alginate microencapsulation technology has been evaluated in several pre-clinical (in rodents, dogs, pigs, non-human primates) trials and in few human pilot trials [43-46].

2.5. Alginate–based microencapsulation
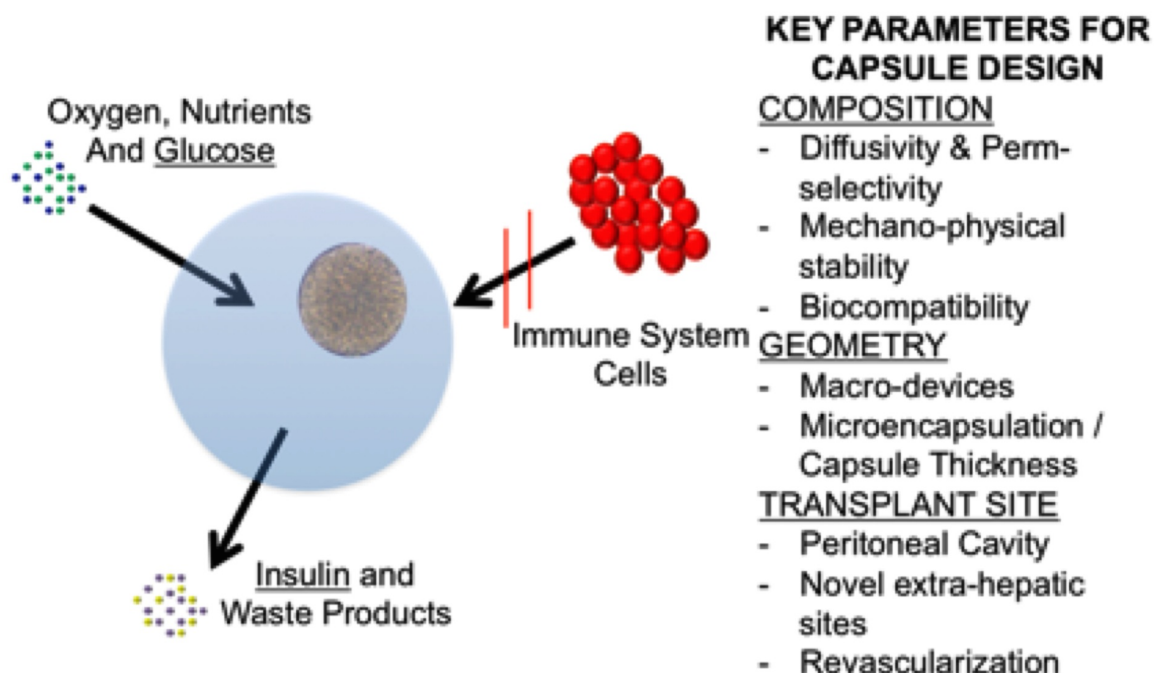
Figure 2. Schematic of cell immunoisolation through microencapsulation for transplantation without immunosuppression. Key parameters that directly affect capsule performances are indicated.

Ideally, the material used for encapsulation should evoke minimal fibrotic tissue deposition, minimal macrophage activation, and minimal inflammatory cytokine release, if any. Moreover, the encapsulation material should not negatively impact the viability of enclosed cells. Capsules should allow for the transport of oxygen, nutrients, cytokines, glucose, insulin and waste products through the capsule, while preventing contact between the enclosed cells and the host immune cells. Also, the material should be flexible and soft while being mechanically stable (**Figure 2).**

A variety of materials have been investigated during the past thirty years [32]: poly (ethylene glycol) (Peg), polyurethane, polyacrylates, chitosan, cellulose, xanthan gum, and alginate. Polyacrylates such as hydroxyethyl methacrylate and methyl

methacrylate (HEMA-MMA) form stable capsules, but diffusion of water-soluble nutrients is limited, as well as long-term cell viability, due to the non-aqueous nature of these capsules [47]. A thin layer of Peg hydrogels has been used in the layer-by-layer, pegylation, and conformal coating technologies, with promising results and *in vivo* studies [48].

Due to their high water content and three-dimensional matrix structure, hydrogels of naturally occurring or synthetic polymers are the most commonly used materials for microencapsulation. Their hydrophilic nature reduces the friction between the capsule and surrounding fluids and tissues. In addition, hydrogels have a pliable consistency, which prevents damage to surrounding tissues. Although some water- insoluble materials, due to their high stability, have been preferred by some groups for encapsulation of living cells, these are often limited by the need for organic solvents which may influence cell viability. Transparent hydrogels also allow for an easy visualization of the encapsulated cells [47,49].

Of all hydrogels used for microencapsulation, alginate has been and will be one of the most important immobilization material [50]. In addition to being heat stable, alginates possess the ability to form hydrogels rapidly and under very mild (physiological) conditions [51]. Moreover, no alginate-degrading enzymes have so far been reported in humans. Alginates are highly characterized and well understood both in the liquid and gel phase making this biopolymer unique as an immobilization material.

Alginate is a natural anionic polymer, isolated from *Azotobacter vinelandii*, from several Pesudomonas species and from brown algae [32].The polysaccharide contains regions of M-blocks, regions of G blocks and regions of mixed sequences MG-blocks (**Figure 3**). The ratio and sequence of the uronic acid groups differ

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depending on the source of the alginate and they determine the properties of the alginate [52]. In the encapsulation field, alginates are classified as high G alginates, intermediate G alginates, and low G alginates. For polymerizing and forming microcapsules, alginate monomers need to get in contact with solutions containing high concentrations of cations, leading to gel formation. During this process, the uronic acid blocks bind to cations: the guluronate blocks of one polymer form junctions with the guluronate blocks of adjacent polymer chains in what is termed the egg-box model of cross-linking, resulting in a gel structure giving rigidity to the capsules.
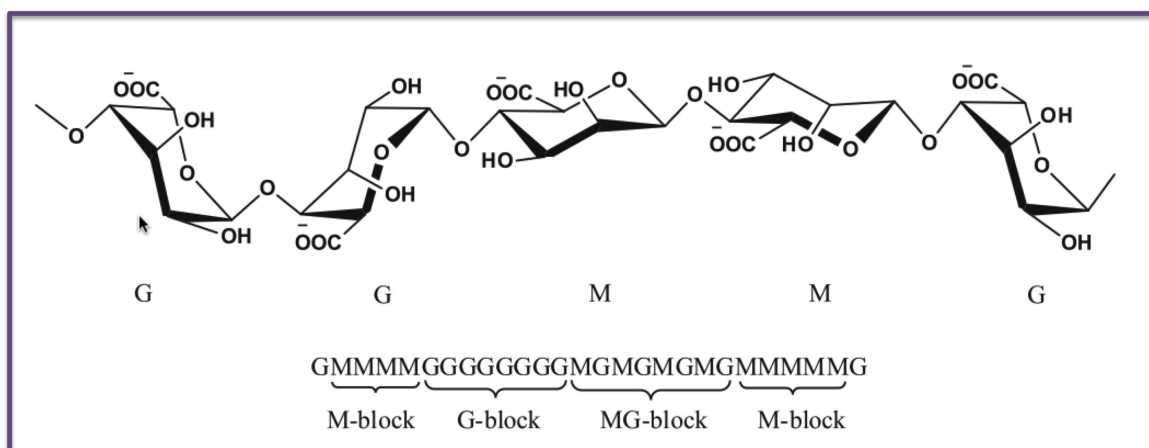
Figure 3. Structure of M and G chains in alginate

Depending on the type of cations, alginate beads can have different rigidity and elasticity. In specific, the binding of ions is selective and the affinity strongly depends on the alginate composition. Ba^{2+} binds to G-G and M-M blocks, whereas Ca^{2+} binds to G-G and M-G blocks, and Sr^{2+} only to G-G blocks. A higher affinity of cations for the alginate residues is associated with a stronger gel. Also the permeability and, thus, the immunoprotective properties of alginate-based capsules are determined by the combination of type and concentration of alginate

with the type of cations.

As alginates are negatively charged polymers, they form strong complexes with polycations such as polysaccharides (e.g. chitosan) [53-55], polypeptides (e.g. poly(L-lysine) (PLL) [56], poly(L-ornithine) (PLO) [57] or synthetic polymers (e.g. poly(methylene-co-guanidine) [58] and poly(ethylene-imine) [59]. As these complexes are stable in the presence of non-gelling cations or calcium chelators, they have been extensively used to stabilize the gel and reduce the porosity of alginate beads, as it will be discussed in the following sections.

Allotransplantation with alginate coated capsules has been shown to work routinely in diabetic rodents and in a limited number of large animals often without the need for continuous immunosuppression [29,60-64]. Xenografts have also been shown to reverse diabetes in rodents [65,66] and non-human primates [67].

A more recent approach has been to omit the polycation layer using simple Ca- or Ba-alginate beads. This has given promising results in allotransplantation as well as in some xenotransplantation studies in animals [61,68-71].

The first human clinical trial in encapsulated islet allotransplantation was performed in a 38-year-old type 1 diabetic (T1DM) male. Cadaveric human islets were encapsulated in alginate microcapsules, and placed IP at a dose of 10,000 IEQ/kg, with a 5,000 IEQ/kg booster given six months later. The patient was able to discontinue all exogenous insulin at nine months. However, it is important to note that the patient was already on anti-rejection medications due to a renal transplant [72]. This initial success though did demonstrate that encapsulated islets were able to achieve glycemic control in a T1DM patient similar to unencapsulated islets placed in the portal vein. Almost 12 years later, Calafiore *et al*. carried out a phase 1 clinical trial using human islets encapsulated in calcium alginate-PLO

without immunosuppression [73]. Although this study proved that allografting of encapsulated islets is safe, only a minor clinical benefit was observed. There is a phase 1 clinical study conducted with barium alginate microcapsules by Tuch and his investigators [74]. In this study, four type 1 diabetic patients with no detectable C-peptide were infused with different infusions of human islets encapsulated within barium alginate microcapsules intraperitoneally without immunosuppression. C-peptide was detected on day 1 post-transplantation, and blood glucose levels and insulin requirements decreased, albeit transiently. C-peptide was undetectable by 1-4 weeks. In the patient receiving the higher infusion number, C-peptide was detected at 6 weeks after the third infusion and remained detectable at 2.5 years. Neither insulin requirement nor glycemic control was affected by the release of this small amount of insulin. To understand better what was occurring in the transplanted capsules, a laparoscopy was performed in the recipient of the four islet infusions at 16 months after the first infusion. Large numbers of capsules were found scattered throughout the peritoneal cavity in clusters attached to the peritoneum, spleen, omentum, and kidney. A biopsy showed that the loss of graft function was probably due to either ischemic necrosis or an inflammatory process, [75,76].

So far, the major pitfalls making difficult to interpret whether the alginate gel beads are suited for islet transplantation have been the large discrepancies in results from animal studies from the various groups working with alginate encapsulated islets. A system working in one animal model may not work in another slightly different model, depending on the capsule properties, animal model and transplantation site. It may be that ultimately the alginate beads are sufficient to provide immune protection in the case of allogeneic models, whereas the development of microcapsule materials for xenogeneic models remains a

challenge. In general, also the absence of standardization between laboratories has been a major contributor to the lack of consistent results.

As already said, outcome of pancreatic islet implants is dependent on many factors: 1) pancreatic islet viability that varies considerably due to donor-associated variations and to differences in the efficacy of the enzyme-driven isolation process; 2) the alginate composition [43,73,74,77-81]; 3) the polymer characterization, including composition, molecular weight and purity [82-86]; 4) permselectivity; 5) the mechanical stability and the surface characteristics of the capsules [87]; 6) the biocompatibility, as well as 7) the transplantation site.

(**Table 1**).
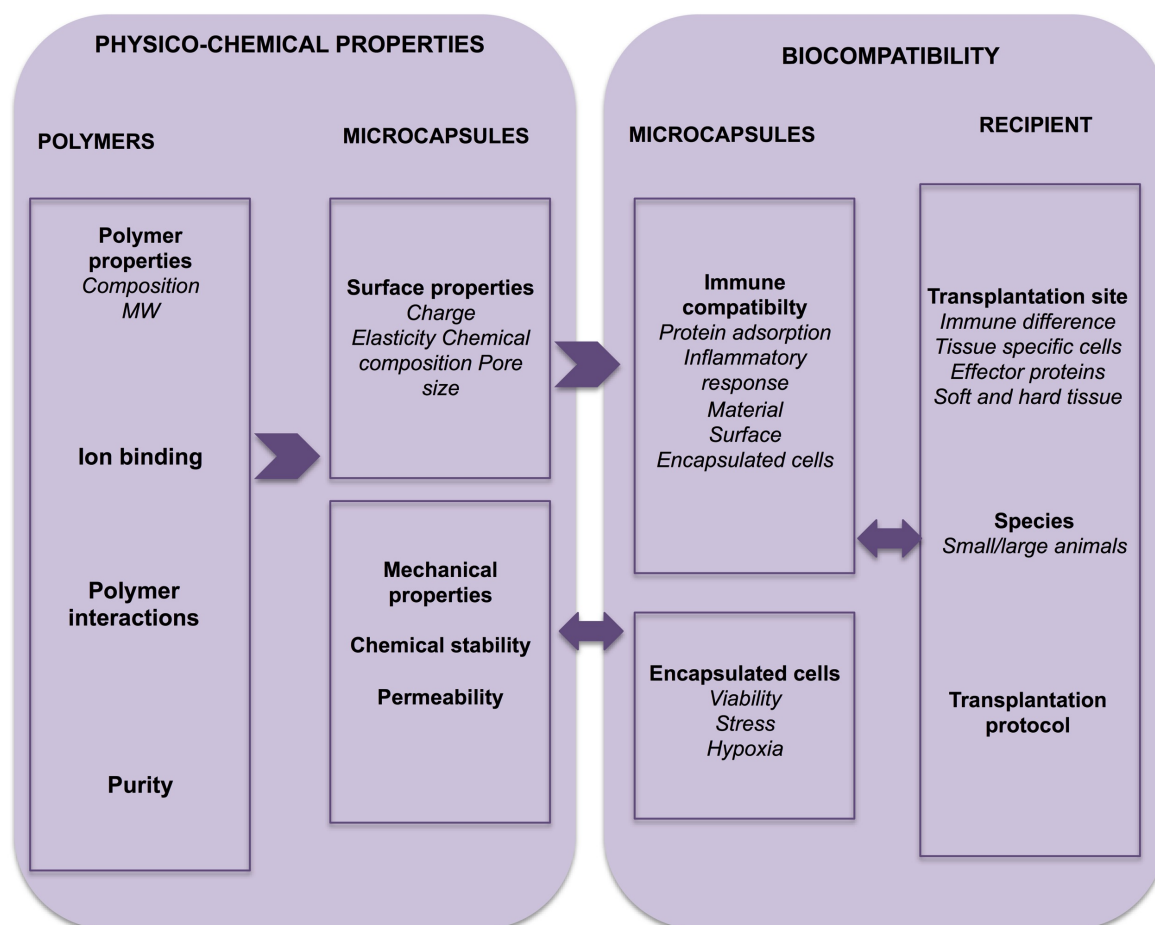
Table 1. Factors that control microcapsule features: choice of polymers and their physico-chemical properties can determine the capsule properties (surface properties, mechanical properties, chemical stability and permeability). Depending on these properties, capsule biocompatibility can be influenced, as well as viability and functionality of the encapsulated islets. *In vivo*, other factors, i.e. the transplantation site and the animal species, have to be taken in account for determining transplant success.

Likely, it is difficult to find an ideal alginate that fulfills the multitude of requirements as an encapsulation matrix for islet cells. However, alginate gel beads should ideally be characterized by: 1) high mechanical and chemical stability; 2) controllable swelling properties; 3) low content of toxic, pyrogenic and immunogenic contaminants and 4) defined pore size and a narrow pore size distribution.

2.5.1. Biocompatibility

The biocompatibility of the capsule material depends on many factors, such as chemical composition, surface charge, porosity, surface roughness, implant site and shape [88]. Lack of biocompatibility results in a host reaction to the biomaterials that takes to build-up a fibrotic capsule, which interferes with adequate nutrition of the encapsulated cells, and ultimately leads to necrosis of the enclosed cells. The implantation of a biomaterial always leads to an inflammatory response, which starts with the adsorption of cell adhesion proteins (such as vitronectin, laminin, fribronectin), of immunoglobulins, of complement components, of growth factors and of other tissue fluid proteins on the surface of the capsules. Macrophages can interact with the proteins that have been adsorbed by the implanted biomaterial through their cell receptors. Then, macrophages adhere to the surface of the biomaterials and they produce inflammatory cytokines, such as interleukin 1beta

(IL-1β), tumor necrosis factor alpha (TNF−α), and transforming growth factor beta (TGF-β), which further activate macrophages and fibroblasts [82,88,89]. The activation of macrophages and fibroblasts also leads to a cellular overgrowth on the capsule (thick fibrotic layer) that is detrimental to encapsulated islets, impairing nutrient diffusion to the islets. Further, the fibroblasts accumulating on capsules compete with islets for the oxygen and nutrient supplies, leading to their shortage. Another factor involved in the foreign body response is the complement system (C3). The activation of the complement might contribute to enhancing the immune response towards the encapsulated cells, stimulating the host immune cells in the graft vicinity to produce large quantities of inflammatory cytokines, which are able to travel through the capsule and damage the enclosed cells. The complement system is usually activated by chemical characteristics of the capsule surface, as well as the capsule pore sizes [88,90-92]

Although alginate meets the requirements as additives in food and pharmaceuticals, it contains various amount of other biological compounds such as endotoxins, proteins, complex polysaccharides and polyphenols which might be harmful to sensitive cells, or have impact on the biocompatibility of the capsules [93]. In the recent years, there has been much focus on the necessity of purification of the alginate material with regard to biocompatibility concerns, as some authors have claimed that alginate purity is the main factor determining the biocompatibility [50,94]. Several procedures for purifying alginates are found in the literature, and ultrapure grades of alginates approved for implantation purposes are available commercially [95]. However, evidence of overgrowth on capsules of highly purified alginate [30] suggests that there probably are additional factors influencing biocompatibility. It is controversially under discussion whether alginate with low or high content of G is advantageous, or if the molar mass has an impact on alginate

biocompatibility. However, due to the low cytokine response, alginates with a guluronic acid content higher than 50% has been recommended for transplantation purposes [96,97].

2.5.2. Permselectivty

Permeability is a major factor determining the functionality of alginate capsules, mostly for cell transplantation applications. Maintenance of cell viability and function requires both protection against the host immune system and sufficient supply of nutrients and oxygen. At the same time, the necessity of almost unrestricted diffusion of catabolic products like insulin out of the capsule should be met. Preventing immune cells from entering the capsule membrane is a relatively easy challenge to meet. However, these cells can secrete low molecular weight molecules that may be deleterious to cells. The challenges of keeping these outside the capsule membrane are more serious, since many of these components are smaller or similar in size to essential cell nutrients or products [98]. Hence, it is a difficult, if not impossible task to design a capsule meeting all these permeability requirements. Nevertheless, certain molecules of the immune system, like cytokines, immunoglobulins and elements of the complement system should ideally be completely, or at least partly, prevented from entering the capsule membrane to avoid or minimize the host reaction to the implant.

The permselectivity of the capsules depends on the balance obtained between the mass transport (which determines the supply of nutrients and release of therapeutics and metabolites) and the molecular weight cutoff (MWCO) of the membrane [99]. MWCO values (which determine the upper size limit of solute transport across the membrane) have been widely studied as a parameter used to

characterize membrane permeability in cell encapsulation devices and have been cited within the range of 50 to 150 kDa [47,100]. However, it is very difficult to determine permeselectivity starting form MWCO values. Firstly because macromolecules, such as alginate, usually exhibit a range of molecular weight values, rather than defined molecular weights. Secondly because diffusing solutes of identical molecular weights can vary dramatically in size, shape and relative charge, which often affect their diffusion behavior [101].

Alginate seems lacking proper immunoprotective properties [102] and to be permeable to immunoglobulin G (IgG, 150 kDa) and complement [71]. The high porosity of the alginate network has promoted the development of coating techniques. Formation of polyanion-polycation membranes with polypeptides or chitosan [53,54] has been used to prevent diffusion of antibodies through the capsule membrane, and the capsules can be made impermeable to TNF (51 kDa) using higher concentrations or exposure times in PLL [103]. Molecules as small as insulin can be retained after using poly-(D-lysine) as polycation [103]. However, the alginate coating has to provide not only a desired permselectivity, but also biocompatibility. Unfortunately, even with standardized protocol for coating, such as with PLL, issues related to biocompatibility have not been solved and a strong inflammatory response is still presented [104]. Others articles have shown that alginate/PLO capsules induce fibrosis event to a higher degree compared to alginate/PLL capsules [105].

2.5.3. Mechanical Strength

Microcapsules would be expected to last for a significant period of time in vivo. To meet this target, the capsule membranes have to demonstrate optimum

mechanical strength including stiffness (resistance to deformation) and toughness (resistance to fracture), and to withstand forces of compression and shear stresses imposed at the implantation site [95,106].

Failure of conventional alginate microcapsules after transplantation has been associated with their poor stability. Alginate capsules tend to swell and dissolve at physiological conditions. The swelling is caused by the gel network sensitivity towards chelating compounds such as phosphate, citrate and lactate, or non-gelling cations such as sodium and magnesium. Swelling and dissolution of the gel network represents a potential serious problem in immunoisolation systems. First of all, swelling leads to increased porosity and the loss of control of pore size. Secondly, disruption of the gel results in exposure of the transplanted cells [107,108]. Additionally, shear forces associated with the implantation procedure and the transplant site mechanical environment may further damage the microcapsules [109].

The mechanical stability of capsules is determined by different factors, such as the capsule composition (alginate type, alginate concentration, and the type of applied gelling cations) [107], the capsule geometry and the transplant site. By using UP-MVG alginate as capsule composition, it is possible to form stronger gels than high M-group alginates, because the Ca^{2+} cations bind with greater affinity to G-blocks than to M-blocks or MG-blocks. Despite such consensus, conflicting results have been published on the stability of alginate microcapsules in culture [96,110,111]. Additionally, there are no standard protocols for testing the overall mechanical properties of the alginate capsules.

A polyanion-polycation complex membrane, as already said, can stabilize against swelling both by increasing the elastic forces and by partly de-charging the

polymer network [112]. For small capsules where the surface to volume ratio is high this de-charging can be so effective that the capsules collapse [113]. Increasing concentration and exposure time to PLL and decreasing molecular weight of the polypeptide have been shown to increase the stability of capsules. In addition, PLL binds to a higher extent to high-M alginate than to high-G alginate, and higher concentration of polymer at the surface enhances the binding of polycations to alginate beads [56,114,115].

Another approach for stabilizing alginate gels is by covalent crosslinking. Various techniques have been applied, including direct crosslinking of the carboxyl groups [116] or covalent grafting of alginate with synthetic polymers [117]. A combination of covalent and ionic crosslinking has also been proposed [118] as well as covalent crosslinking of the PLL to the alginate [119]. A drawback using some of these methods has been the lack of selectivity characterizing chemical modifications that generally occur both on G and M residues, hampering the ability of the modified alginate to form instantaneous calcium gels.

2.5.4. Size

The size of the capsule is one of the major factor affecting not only cell viability, but also the in vivo graft outcome **(Figure 4)**. Capsules should be small to allow rapid diffusion of nutrients and the graft volume should be kept to a minimum. Indeed, it should be noted that the volume of a capsule is a function of the radius to the power of three, and therefore if the diameter of the capsule is reduced, the volume will be decreased to an eighth.
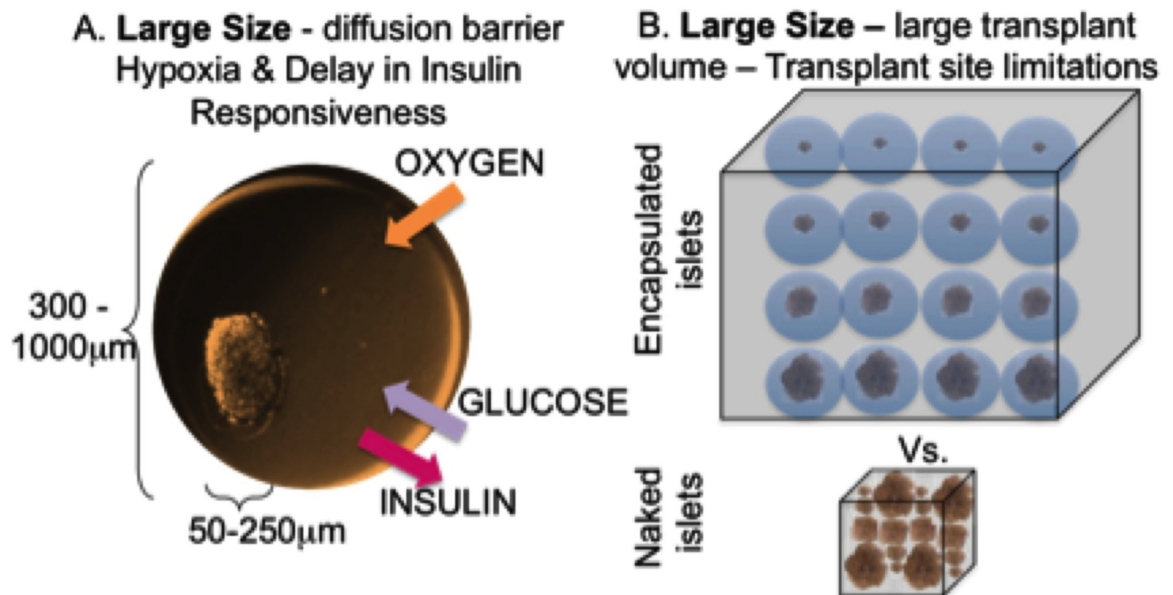
Figure 4. Schematic of limitations associated with traditional alginate microencapsulation. A) Diffusion limitations imposed by large capsule size (600-1000μm) that results in core hypoxia, central necrosis and delayed insulin secretion in response to glucose; B) Large volumes of encapsulated cells that do not allow implantation in sites that might be more favorable to islet cell engraftment but that can accommodate only small volumes.

Since islets have variable sizes (50 to 400μm in diameter) and microcapsules need to be large enough to include even the largest islets, traditional microcapsules range in size from 600 to 1000 μm; most of the volume is actually islet-free and biologically non-functional material. Transport through the capsule is mainly regulated by passive diffusion. Such large amounts of islet-free bulk capsule material are a barrier for diffusion of critical solutes, leading to core hypoxia and necrosis [120-122]. More importantly, large diffusion barriers will hamper the transport of glucose and insulin, leading to a delay in glucose sensing and insulin responsiveness of the encapsulated islets [123-125].

Large diffusion barriers resulting from the large size of traditional capsules may help explain why traditional islet encapsulation can fail in maintaining glucose homeostasis in patients transplanted with microencapsulated islets [73,74,125,126].

Moreover, the large size of traditional capsules increases the volume of transplantation materials up to 100 times over the volume of naked islets [48]. The volume of the graft is a critical in transplantation if we consider that to reverse diabetes in patients, up to one million islets need to be ideally transplanted, which would amount to volumes in the hundreds of milliliters [127]. Such large volumes can be transplanted only within the peritoneal cavity. It is easily accessible and thus the surgical risk is minimized for implantation of capsules. However, the peritoneal cavity is a rather harsh environment, characterized by high level of mechanical stresses, compared to the other more commonly used sites for islet transplantation [78,125,128,129]. After IP transplant, capsules fall by gravity and aggregate in the lower abdomen. Additionally, the foreign body reaction to the capsule material causes the formation of a thick layer of fibrotic tissue. Both packing of the capsules and the fibrosis further impair transport of critical molecules [122]. For all these reasons, to reverse diabetes with an IP transplant a high numbers of islets are required, leading to problem of tissue poor availability. It is clear that minimizing the islet dose per patient would make islet transplantation available to a larger number of patients.

A reduced capsule size therefore would also allow for a better nutrient supply to cells, and offers the advantages of an exponential decrease of the total implant volume.

Nowadays reducing the size of the capsule is possible using a high voltage electrostatic bead generator, by changing the voltage, needle diameter, distance between the needle and gelling solution or pump flow rate.

2.5.5. Implantation Sites for Encapsulated Islets

Since encouraging results were observed after free islet infusion in the portal vein, encapsulated islet transplantation was initially considered for the liver. However, due to the increasing graft volume after islet encapsulation, only a limited number of implantation sites can be considered for transplantation (**Table 2**). The peritoneal cavity has been used most often for encapsulated islet transplantation in clinical [122] and preclinical [130-132] studies in large models. It offers, as already discussed, the advantage a large space suitable for the implantation of large volumes of tissue. However it is poorly vascularized, and offers low oxygen levels [133]. Moreover, this site is highly proinflammatory, and implants are difficult to retrieve. The subcutaneous space is another good candidate site for transplantation of encapsulated islets because of a minimally invasive procedure performed under local anesthesia and easy graft removal without damage to other organs. It is disadvantaged by poor blood supply and the superficial location of the transplant, which is associated with a risk of mechanical stress and damage to the graft.

SITE	ADVANTAGES	DISADVANTAGES	COMPATIBILITY FOR MICROCAPSULES
Portal vein	Minimally invasive Physiologic insulin delivery	Early loss of islets Surgical complications Inability to biopsy or retrieval	For capsules< 150µm
Spleen	Physiologic insulin delivery Highly vascularized	Limited space Inability to biopsy or retrieval	For capsules< 150µm
Pancreas	Physiologic insulin delivery Highly vascularized	Surgical complications Limited space	Impossible
IP	Large space	Inflammatory site No direct vascular access Risk of hypoxia	Possible for small and large capsules
Kidney capsule	Highly vascularized	Limitation of space	For capsules< 150µm
Intramuscular space	Highly vascularized Accessibility	Extensive fibrosis Limited space	For capsules< 150µm
Subcutaneous space	Proximity to vascularization Easy to retrieve	Lack of early vascularization	Possible for small and large capsules

Table 2. Possible sites for microencapsulated islet transplantation.

An alternative site for islet transplants in humans is the omentum. Several properties support the choice of the omentum as an islet transplant site [134,135]. In surgery, the omentum is since long used for its wound-healing abilities [136]; technically, the anatomically structure of the omentum offers an advantage, as it allows for pouch formation or implantation between the two sheets [137]; this can facilitate implantation of larger cell volumes, including transplant devices, or implants consisting of cell mixtures. Additionally, its blood flow provides hepatic portal vein delivery, which approaches a physiologic route for released insulin [138]. Its high vascular density and angiogenic capacity may lead to a better revascularization and engraftment than in other sites [139]. Unlike humans, the mouse omentum is just a small piece of poorly vascularized tissue, making it almost impossible to successfully implant islet grafts. The EFP is a well-

vascularized and thin veil of tissue connected to the epididymis, with many properties similar to that of the human omentum. They are both fatty tissues located in the intraperitoneal cavity, have a reasonable size, and are well vascularized. Epididymal fat pad may serve as a useful site for islet implantation because it is easily accessible, the grafts may be easily re-moved without damage to other organs [140].

Poor islet revascularization after transplantation is one of the major impediments to long- term islet engraftment and function [141]. Native islets in the pancreas are highly vascularized by fenestrated endothelium throughout the islet core and receive 15–20% of pancreatic blood supply while comprising only 1–2% of the total mass [142,143]. This high degree of vascularization is rarely recapitulated in transplanted islets.

It is important to remember that cells in the capsules after transplantation cannot get fully revascularized, and the lack of direct vascular access limits exchange of glucose and insulin and the exchange of nutrients and metabolic waste. Such diffusion limitations are worsened when encapsulated islets are implanted in sites that do not get revascularized.

Attempts have been made to augment islet vascularization by gene or protein delivery in animal models [42,144], but many of these techniques are difficult to translate due to complex or insufficient protein delivery strategies and raise serious safety concerns associated with exogenous gene expression. Co-delivery of progenitor or endothelial cells has also been shown to augment islet vascularization [145-147].

Engineering the transplantation site is another strategy developed for increasing islet vascularization, as it will be discussed in the specific aim of this work.

2.5.6. Immunological response against encapsulated islets

Immune responses against the microcapsules is very complicated than the only biocompatibility, and composed of different separate immunological responses.

The host reaction against microcapsules can be classified into three groups:

a) *Non-specific activation of the innate immune system by the surgical procedure* (**Figure 5**)
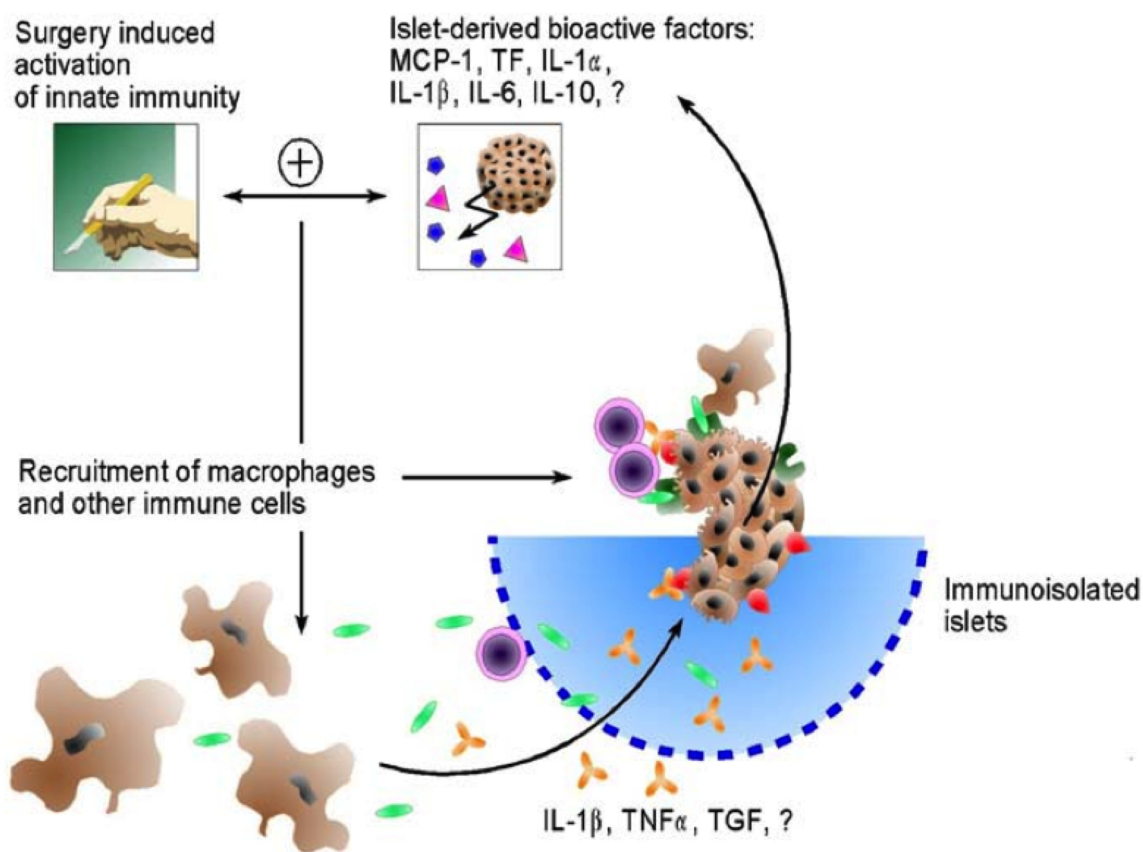
Figure 5. Schematic of surgery-induced activation of the immune system, which causes cytokine release and inflammatory cell activation [148].

The activation of the innate immune system starts with the surgery for encapsulated islet transplantation, which induces inflammation, ruptures of blood

vessels and the consequent release of cytokines, triggering the foreign body response towards the graft. Circulating and tissue-specific macrophages and granulocytes can engulf components of the foreign body. In the same time, lymphocytes can also be recruited in the vicinity of the implant.

 b) *Foreign body response against the capsules and the PAMPs contaminated material* (**Figure 6**)

It is well known that when preparing alginate-based capsules, the material has to lack any component that might provoke an innate immune system. For this reason, it is pivotal to apply alginate, pure from protein, endotoxins, and polyphenols [30]. When they are presented on the capsule surface, they can provoke strong a deleterious response, interfering with the exchange of nutrients and with the necrosis of the islets. Endotoxins contained in are, in fact, considered pathogen-associated molecular pattern molecules (PAMPs), which can promote activation of the host immune system with recruitment of immune cells after implantation.
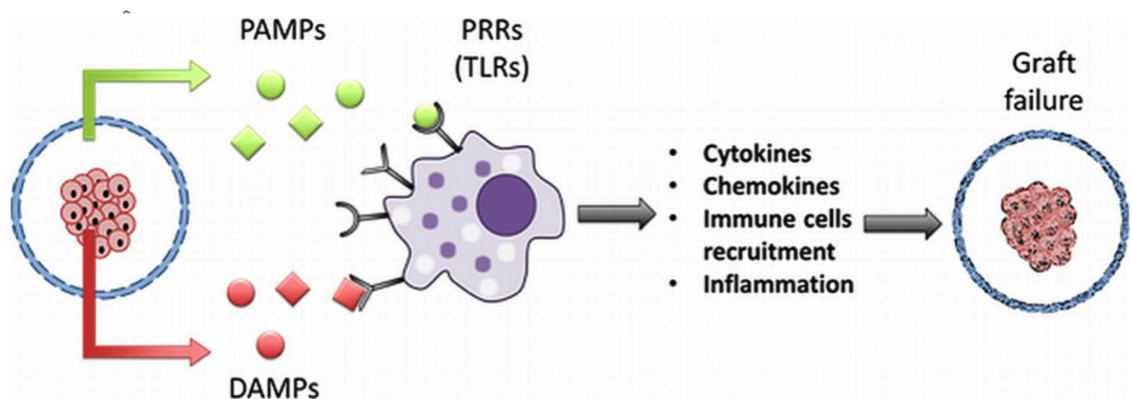
Figure 6. Schematic of the cytokine release caused by PAMPs in alginate binding PRRs, leading to the graft failure. Stressed islets can also release PRRs binding DAMPs and leading to the activation of both innate and adaptive immune system in the host [149,150].

Cells of the innate immune system react against the capsules, through a mechanism mediated by pattern recognition receptors (PRRs), such as the Toll-like receptors (TLRs), also engaged in the activation of innate and adaptive immunity [150,151]. The activation of this mechanism leads to the release of cytokines and chemokines [45,152], causing cell death and the graft failure. PRRs not only recognize PAMPs, but also intracellular component derived from dying cells in the capsules, called damage associated molecular patterns (DAMPs) that also play a crucial role in the response against the capsules and the encapsulated islet grafts [153].

c) Response provoked by the bioactive factors and allo- and xenogenic epitope release from encapsulated tissue.

This reaction is less acute and more difficult to prevent. It results in overgrowth of macrophages and lymphocytes on a small portion (~10%) of the capsules [154] and in a humoral immune response against the encapsulated tissue. Recently, it has been demonstrated that the islets in the capsules reinforce this reaction, being able to produce bioactive factors such as macrophage-colony-promoting factor 1 (MCP-1), interleukin-6 (IL-6), and nitric oxide (NO) [15], which activate inflammatory cells. This activation of inflammatory cells results in the production of cytokines, free radicals and NOs able to freely diffusion through the membrane.

2.5.7. Recipients for islet transplantation

The Non Obese Diabetic mouse (NOD) that spontaneously develops autoimmune diabetes is the only preclinical model of T1D. Similarly to clinical islet transplantation, transplantation of pancreatic islets from fully MHC-mismatched murine donors into the spontaneously diabetic NOD mouse

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exposes the islets to recurrence of autoimmunity, in addition to allogeneic islet graft rejection [155]. During the last decade, many groups have evaluated alginate capsules in preclinical islet transplantation models in the NOD mouse with different outcomes. In allotransplantation, microcapsules do not have to completely prevent diffusion of antibodies and cytokines to efficiently protect encapsulated islets. In fact, Ba-microcapsules transplanted IP in an NOD mouse showed to have a molecular weight cut-off of 600 kDa [64], allowing immunoglobulin G (IgG), the smallest of the immunoglobulins, which have a molecular weight of 140 kDa, and harmful cytokines, which have a molecular weight smaller than 100kDa, to diffuse through the capsule. However these Ba-alginate capsules allowed prolonged survival of allogeneic transplanted islets for more than 350 days. Thus, the protection provided by these alginate capsules is effective for a model of auto- and allotransplantation. When xenografts were encapsulated within this type of capsule the same long-term survival time could not be achieved, confirming the different requirements for capsule properties to prevent xenorejection [61,156].

Allotranslantation can be also performed into a nonautoimmune model of diabetes (chemically induced diabetes in strains that do not develop spontaneously this disease). The induction is made by streptozotocin (STZ). STZ is a glucosamine-nitrosourea compound that explains specific toxicity for β cells [157].

The NOD SCID mouse contains a homozygous, spontaneous mutation (Prkd^{scid}), which produces an immunodeficiency characterized by an absence of functional T and B cells, and defective NK cell function. It has been demonstrated that the in vivo NOD SCID assay is a good surrogate marker for islet potency and function in humans. Thus, the assay can be used to screen pancreatic islet suitability for transplantation [158].

2.6. Poly (ethylene glycol)

Peg is a polyether composed of repeating ethylene glycol units. Peg is produced by the interaction between ethylene oxide and water, ethylene glycol, or ethylene glycol oligomers.

It is material used for the encapsulation of a broad range of cell types, such as pancreatic islets [159-161], chondrocytes [124], osteoblasts [162], and mesenchymal stem cells [163].

Peg hydrogels have some advantages over other synthetic molecules that form hydrogels. They have high water content and a short diffusion time scale. Furthermore, Peg molecules can be easily coupled to functional peptides and mimic aspects of the extracellular matrix to support the survival and function of encapsulated cells. Another advantage is the low protein adsorption on Peg surfaces [164-166].

Peg has the ability to increase the durability and mechanical properties, and decrease permeability [167,168] of the capsules, when used as coating material.

Unfortunately, *in vivo* biocompatibility studies are still in progress and subjected to debates.

2.7. Specific aims of this thesis

A. Alginate Microencapsulation

Reduce size

B. Hybrid Microencapsulation

Increase stability (strategy #1)
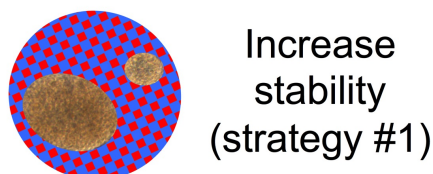
C. Double Microencapsulation

Increase stability (strategy #2)

Figure 7. Schematic of specific aims of the project, to increase stability of reduced sized alginate microcrocapsules.

Does decreasing size of alginate capsules improve function of enclosed cells?

As widely discussed above, large capsules cause impairment of transport of critical nutrients, oxygen, glucose and insulin to the encapsulated cells, leading to core hypoxia and death of encapsulated islets and delays in insulin secretion in response to glucose. Further, large capsules cause large implant volumes and limits transplant sites to sites that are less conductive to islet engraftment (more favorable sites can accommodate only small volumes). To understand whether failure of traditional alginate capsules is due to size we showed that capsule size is critical for determining the outcome of transplantation of encapsulated islets. In

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order to accomplish that we 1) minimized the size of traditional alginate capsules, and 2) determined whether decreasing size of traditional capsules affects outcome of islet transplantation. First we adopted conventional alginate microencapsulation and produced microcapsules with the traditional technology based on electrostatic droplet generators. Then we have optimized conventional alginate microencapsulation by minimizing capsule size (450-550µm in diameter vs. 600-1000µm of traditional capsules). This allowed for microencapsulated islet transplant into different sites rather than the peritoneal cavity. An alternative site is the omental pouch, which can be well represented (being too small in mice) by EFP that is well vascularized with good arterial supply, portal drainage and can accommodate large volumes. Reducing the capsules dimensions, we could transplant the encapsulated islets in the EFP site, improving outcome of islet transplantation with minimal islet dosages (750IEQ). To ameliorate outcome of islet transplantation in the EFP site and further promote islet engraftment and long-term function we have engineered the EFP site with pro-angiogenic hydrogels, to promoting islet engraftment and long-term function (as stabilization of blood glucose to normal values: < 200mg/dL). Pro-angiogenic hydrogels are fibrin matrices rendered pro-angiogenic by the incorporation of minimal doses of vascular endothelial growth factor-A165 and platelet-derived growth factor-BB, complexed to a fibrin-bound integrin-binding fibronectin domain.

We confirmed that the engineered EFP site allows for extended release of pro-angiogenic factors and for their synergistic signaling with extracellular matrix-binding domains in the post-transplant period.

Does improving stability of alginate capsules by addition of synthetic polyethylene glycol (Peg), creating hybrid capsules and double coatings, improve function of enclosed cells?

Since the mechanical stability of alginate capsules is determined by different factors, including the capsule composition [107], we decided to combine alginate with polyethylene glycol (Peg), because of the positive results that our group has obtained with Peg-based conformal encapsulation [48]. We have extensively characterized the conformal coating technology with Peg functionalized with maleimide groups (Peg-Mal) and crosslinked with dithiolthreitol (DTT) as hydrogel coating on islets from different sources (mice, rat, pig, non-human primates, and humans). We found that Peg-Mal has a well-defined permselectivity (MWCO between 250 and 500 kDa), and it does not affect viability and function, as assessed by both static glucose stimulated insulin release (GSIR) and perifusion.

The aim of the present work was to evaluate the specific effect of capsule composition on the and *in vivo* performance of alginate-based microcapsules to identify the optimal parameters for clinical translation.

The work was based on modulating the properties of the alginate microcapsules in order to increase the resistance of the capsules to swelling and osmotic stress, and to control the capsule permselectivity. We 1) generated materials and methods to reinforce traditional alginate, and 2) determined whether reinforcing alginate capsules affect outcome of islet transplantation.

To increase stability and mechanical properties of alginate, we have designed novel materials and technologies to reinforce alginate with Peg-Mal.

To reinforce MVG with Peg-Mal we have undertaken two different approaches: 1. we have fabricated <u>hybrid microcapsules</u> by mixing MVG with Peg-Mal and forming beads with the electrostatic droplet generator;

2. we have fabricated <u>double microcapsules</u> by coating MVG capsules with Peg-Mal through a custom optimized emulsification process.

We believe that such technologies, when combined with biocompatibility and the availability of novel sites of transplantation will improve immunoprotection of alginate encapsulated cells.

3. MATERIALS AND METHODS

3.1. Encapsulation materials

Ultra pure medium viscosity (>200 mPas) sodium alginate where 60% of the monomer units are guluronate (UP-MVG alginate, Novamatrix) was dissolved overnight in Hank's Balanced Salt Solution buffer without calcium and magnesium ions (HBSS w/o Ca^{2+},Mg^{2+}, Gibco) to a working concentration of 1.2% w/v. 50mM calcium chloride ($CaCl_2$) gelation solution was prepared by dissolving 5,55 mg $CaCl_2$, 2,09 mg 3-(N-Morpholino)propanesulfonic acid, 4-Morpholinepropanesulfonic acid (MOPS), 25,5 mg D-mannitol and 0,25ml Tween in 1L Milli-Q H_2O (Sigma-Aldrich). The final osmolarity of the prepared solution was 300mOsm, iso-osmotic with cells. For MicroMix capsule fabrication (1.2% UP-MVG-5% Peg-Mal), a solution of 5% (w/v) Polyethylene Glycol (Peg), 75% functionalized with maleimide groups (Peg-Mal, 10kDa, 8-arms, Jenkem Technology) was obtained by dissolving 50mg of Peg-Mal in 1 ml of 1.2% UP-MVG solution. For fabrication of double-coated capsules, Peg-Mal was dissolved in HBSS with calcium and magnesium ions (HBSS with Ca^{2+},Mg^{2+}) at 5% w/v concentration. The cross linking solution for the MicroMix capsules was prepared by dissolving 2.31mg of Dithiothreitol (DTT, OmniPur, Calbiochem) in 1 ml HBSS w/ Ca^{2+},Mg^{2+}, in order to obtain a 4:1 molar ratio of DTT (two reactive groups) to Peg (eight reactive groups): 1X DTT solution. The cross-linking solution for the double-coated capsules was prepared by dissolving 205 mg DTT in 333µl Dimethyl sulfoxide (DMSO, Sigma).

3.2. Islet isolation and culture

Male BALB/C Mice (Jackson Laboratories) and Lewis Rats (Harlan Laboratories) were housed in virus AND antibody–free rooms in micro isolated cages and exposed to a 12-h light/dark cycle with ad libitum access to autoclaved food and water and they were used as islet donors at 10-12 weeks of age for mice and 250-280g OF weight for rats. Animal studies were performed under protocols reviewed and approved by the University of Miami Institutional Animal Care and Use Committee (protocol 13-042). Islets were isolated by liberase (Roche) digestion followed by purification on Euroficoll density gradients (Mediatech), as described elsewhere [169]. Isolated pancreatic islets were cultured at 37 °C in standard 95%b RA/5% CO_2 incubators in 10cm petri dishes. CMRL (Mediatech) supplemented with 10% fetal bovine serum, 2mM glutamine (Gibco), 25mM HEPES buffer (Gibco) and 1% Penicillin/Streptomycin (Gibco) was utilized as islet culture media for both mouse and rat islets.

Nonhuman primate baboon (NHP; The Mannheimer Foundation, Inc., Homestead, FL, USA) islets were isolated using previously described methods [170]. NHP islets were cultured in CMRL 1066 (Mediatech) (supplemented with 10% fetal bovine serum (FBS; Sigma), 1% penicillin–streptomycin (Sigma), and 1% L-glutamine (Sigma).

3.3. Alginate based microcapsules: fabrication of cell-free capsules for optimization of encapsulation parameters

Microcapsules were formed using the electrostatic droplet generation method. This previously described method is based on the use of electrostatic forces to disrupt a liquid filament at the nozzle tip and form a charged stream of small droplets [171]. Droplet formation is a complex function of several parameters such as applied

electrostatic potential, needle diameter, electrode distance (needle-CaCl$_2$ solution) and geometry, flow rate of polymer solution, surface tension, density, and viscosity [108]. When cells are introduced within the polymer solution, both the polymer properties and the extrusion process are affected, requiring optimization.

The Nisco Electrostatic Droplet Generator machine was utilized to obtain alginate microcaspules. Voltage, flow rates and cell-loading density were optimized in order to minimize capsule size without compromising capsule integrity. 1.2% UP-MVG microcapsules and 1.2% UP-MVG-5% Peg-Mal MicroMix capsules were formed by extrusion of the polymer solution through a blunt stainless steel needle using a syringe pump (Harvard Apparatus, Holliston, Massachusetts) and a 3 mL plastic syringe (BD). Three needle diameters were evaluated: 0.17, 0.4, and 0.6 mm (internal diameter). The electric field was created between the needle (positive electrode-cathode) and the CaCl$_2$ hardening solution (ground). The potential difference was controlled by a high-voltage power supply and was varied between 8.8 to 10 kV range. Distances of 2 to 4cm between the needle tip and the hardening solution were tested, while the flow rate of polymer solution ranged from 5 to 50 μl/min.

The experimental set-up is presented in **Figure 8**.
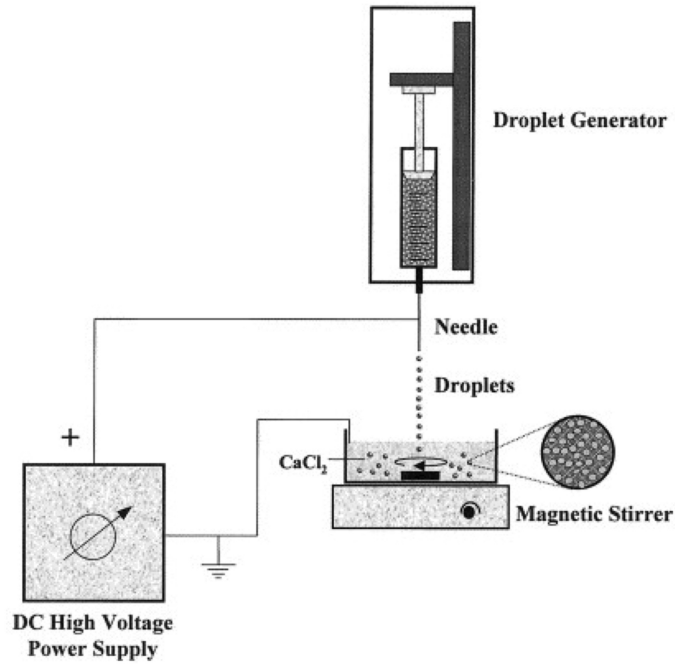
Figure 8. Schematic of electrostatic droplet generator

After extrusion, the 1.2% UP-MVG formed capsules were allowed to harden in the CaCl$_2$ bath for 10 minutes. Next, the fully polymerized microcapsules were washed 3 times in HBSS w/ Ca^{2+},Mg^{2+}, whereas the 1.2% UP-MVG-5% Peg-Mal MicroMix polymerized were washed only once in HBSS w/ Ca^{2+},Mg^{2+}, followed by the addition of 1 ml of DTT 1X for 1 minute to crosslink the Peg-Mal. MicroMix capsules were then washed three times in HBSS w/ Ca^{2+},Mg^2. A sample of 30 microcapsules from each cell-free run was taken (n=3) and the capsule diameters were measured within ±10 µm using a light microscope (LEICA DMIL, Germany) with a graded reticule. The average capsule diameter and standard deviations were calculated from the measured data.

3.4. Encapsulation of pancreatic islets

For encapsulation, isolated pancreatic islets were suspended in 100 µl of 1.2% UP-MVG alginate or in 1.2% UP-MVG mixed with 5% Peg-Mal (MicroMIx). Three islet loading densities were evaluated: 30,000, 15,000 and 5000 IEQ/ml.

The islet suspension was drawn up into the 0.4mm diameter needle at 100 μl/min by syringe pump and then extruded with the electrostatic droplet generator at a 10µl/min flow rate and 8.8kV voltage. After gelating the microcapsules and MicroMix capsules for 10 minutes in the calcium chloride bath, the formed cell-containing capsules were washed with the same steps detailed for the empty capsules.

3.5. Double coating of microcapsules by the emulsion technique

For the double-coated capsules, two different protocols were implemented.

Initially, 100µl of UP-MVG microcapsules were suspended in 1ml of 5% Peg-Mal (water phase) and double coated using an emulsion technique. A bath of light mineral oil (Sigma Aldrich) containing 5% Span80 (Sigma Aldrich) (oil phase) was stirred for 2 minutes at 350 rpm to guarantee that the surfactant was mixed with the mineral oil. The suspension of UP-MVG capsules and 5%Peg-Mal was added drop-by-drop to the center of the oil phase bath while the oil phase was continually stirred for 5 minutes. Then, 10X DTT in DMSO was added to drive Peg-Mal polymerization, and the stirring speed was increased to 450 rpm. Next, the double-coated capsules were allowed to crosslink for 15 minutes, under stirring, and then the gelated double-coated capsules were extensively washed with HBSS w/ Ca^{2+}, Mg^{2+} (**Figure 9**) By filtering the resulting double-coated and secondary beads suspension through a 70 µm filter, a large part of the secondary beads of 5% Pegmal were successfully eliminated.
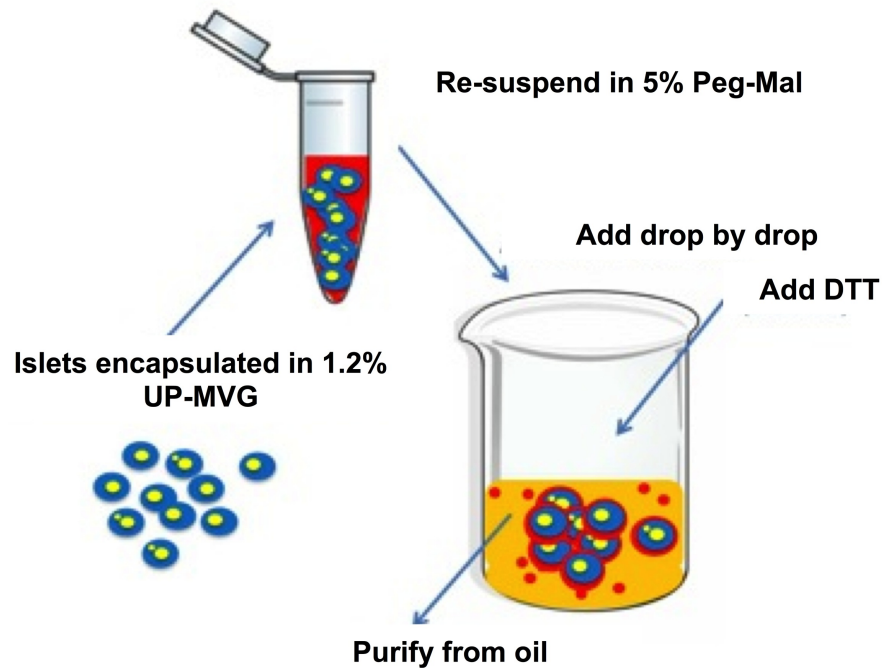
Figure 9. Schematic of the emulsion process: UP-MVG capsules formed running 100 μl of alginate were suspended in 1ml 5% Peg-Mal (water phase) and double coated using an emulsion technique. A bath of mineral oil (Sigma Aldrich) and 5%Span80 (Sigma Aldrich) (oil phase) was used. The suspension of UP-MVG capsules and 5%Peg-Mal was added drop-by-drop in the center of the oil phase bath and stirred for 5 minutes. Then 10X DTT in DMSO was added for Peg-Mal crosslinking and the stirring speed increased.

In order to increase the efficiency of purification of double-coated capsules from secondary Peg-Mal beads, a second optimized protocol was implemented. The initial stirring speed was first increased from 350 rpm to 400rpm. Then, the UP-MVG capsule-5%Peg-Mal suspension was added and stirred for 4 minutes before further increasing the stirring speed to 500 rpm. 1 minute after, DTT 10X was added. The capsules were then washed with the same protocol described for the preliminary studies. By utilizing this second purification procedure, there was no need to filter the double-coated capsules, because all of the secondary beads were eliminated during the washing procedure.

3.6. Peg labeling of MicroMix and Double coated capsules

Fluorescent labeling of microcapsules was achieved through a multi-step procedure. Fifty MicroMix and double-coated capsules were incubated in 1ml of 2% (v/v) Albumin (BSA, Sigma-Aldrich) solution in HBSS w/ Ca^{2+}, Mg^{2+} supplemented with 10% fetal bovine serum (FBS) and 4 drops of Streptavidin/Biotin Blocking solution (Vectors Lab). Beads were incubated for 30 minutes at room temperature. Next, the primary anti-Peg biotinylated antibody (AbCam) was diluted 1:200 in 1ml of 2% BSA in HBSS, supplemented with 4 drops of Streptavidin/Biotin Blocking solution and incubated with the alginate microcapsules for 2 hours. The capsules were then washed in HBSS twice and subsequently the capsules were incubated in AlexaFluor 488 streptavidin solution (1:500, Molecular Probes) for 1 hour, protected from light. Finally, the capsules were washed with HBSS. In the case of alginate-based capsules containing cells, nuclei were then counterstained with 4',6-diamidino-2-phenylindole (1:2000 Hoechst,Sigma)

3.7. Evaluation of mechanical properties of 1.2% UP-MVG microcapsules

3.7.1.Induction of osmotic stress

Osmotic stress was applied to microcapsules using a modification of a previously described procedure [172]. Aliquots of alginate microcapsules were placed in 10mL of double-distilled H_2O and incubated at 37°C for 2h. The capsules were then washed with normal saline and stained with 0.5% (w/v) trypan blue. The number of damaged capsules was assessed using an inverted light.

3.7.2. Induction of mechanical stress using the "agitation in presence of beads" method

Mechanical stress was applied using a modification of previously reported bead agitation procedures [173]. 1.2% UP-MVG capsules were placed into flasks containing approximately 6.5 g of 3 mm-diameter inert glass beads (VWR Scientfic Products Corporation) and 30mL of normal saline. Capsules were subjected to mechanical stress for 48 h by shaking at approximately 300 RPM using a Lab Line Orbital Shaker. The number of damaged capsules was determined by visual analysis and by handpicking under an inverted light microscope.

3.8. *In vitro* cytotoxicity assay

NIT-1 cells (insulin producing insulinoma cell line derived from non-obese diabetic mice (NOD), ATCC) were used for evaluating the cytotoxicity of the materials used for encapsulation. NIT-1 cells have become useful tool in cell biology studies, because they have been shown to retain the functional attributes of normal islets, with the advantages that they are easily available, stable and continuously proliferate in culture. 75,000 NIT-1 cells/well were seeded in a 96-well-plate and cultured in DMEM (Gibco), supplemented with 10% heat-inactivated FBS, 0.02% BSA, 0.15mM HEPES, and 100U/ml Penicillin/Streptomycin (Gibco) at 37 °C and 5% CO_2.

1.2% UP-MVG gel, crosslinked with 50mM $CaCl_2$, 5% Peg-Mal precursor, and 5% Peg-Mal gel, crosslinked with DTT were evaluated for their toxicity on NIT-1 cells. Additionally, the cytotoxicity of 1X DTT, alone, was evaluated. 50 µL/well of each gel precursor was placed in a 96-well-plate. For UP-MVG gels, $CaCl_2$ was added to the wells for 10 minutes to allow gelation to occur. Then several washes with

HBSS were performed. For 5% Pegmal gels, 10X DTT (1:10) was added to the wells for 15 minutes to allow gelation to occur (1X final concentration). Then several washes with HBSS were performed.

After manufacture, the materials were transferred to the wells containing the NIT-1 cells and co-cultured for 24 hours. The relevant controls utilized were incubation of the materials alone, the NIT-1 cells alone, and fully lysed NIT-1 cells.

After 24 hours, the Multitox Fluor-Multiplex Cytotoxicity assay (Promega) to assess the ratio of live to dead cells was performed. The assay simultaneously measures two protease activities. The live-cell protease activity is indicative of cell viability because it is specific to intact viable cells and is measured using a fluorogenic, cell-permeant peptide substrate (glycyl-phenylalanyl- aminofluorocoumarin; GF-AFC). The substrate enters intact cells where it is cleaved by the live-cell protease activity to generate a fluorescent signal proportional to the number of living cells. This live-cell protease becomes inactive upon loss of cell membrane integrity and leakage into the surrounding culture medium. A second, fluorogenic, cell-impermeant peptide substrate (bis- alanyl-alanyl-phenylalanyl-rhodamine 110; bis-AAF-R110) was used to measure dead-cell protease activity, which is released from cells that have lost membrane integrity. Because bis-AAF-R110 is not cell-permeant, intact, viable cells generate no signals from this substrate. The live- and dead-cell proteases produce different products, AFC and R110, which have different excitation and emission spectra, allowing them to be detected simultaneously.

We added AFC and R110 1:1 directly in the 96-wells containing cells and materials and we incubated the plate for 1 hour. Then to detect viable cells the fluorescent signal was measured and quantified at ~400nm (excitation) and

~505nm (emission) for live cells and ~485nm and 520nm for dead cells.

3.9. Encapsulated pancreatic islets: assay

3.9.1 Static glucose-stimulated insulin release (GSIR)

Glucose-stimulated insulin release (GSIR) was performed to assess function of encapsulated islets and compare it to uncoated control islets (naked controls). A modified Krebs buffer containing 26mM sodium bicarbonate, 25mM HEPES and 0.2% w/v bovine serum albumin and either 2.2mM (low) glucose or 16.7mM (high) glucose was prepared and pre-conditioned at 37ºC and 5% CO_2. Approximately 5g of Sephadex G-10 (molecular weight cutoff <700kDa, GE Healthcare) was added to a 50mL beaker containing 20mL of HBSS w/o Ca^{2+}, Mg^{2+} (GIBCO) and boiled for 30 minutes to allow beads to swell and for disinfection. Naked and encapsulated islet aliquots (100 IEQ) were suspended in 0,5 ml of media and loaded between two layers of Sephadex within 10mL micro-chromatography columns (BioRad). Following bead loading, the bottom seals of each columns were removed and 4mL of low glucose buffer solution were added to each column to pack the beads and assure that flow was unimpeded through each column. Filled columns were then incubated in a standard 5% CO_2 37°C incubator for 1 hour. This step was followed by sequential incubations for 1 h each at 37°, in low, high and low glucose solutions. At the end of each incubation period, 1mL of column elute was collected by adding 1mL of Krebs low glucose buffer to each column. Insulin concentrations in eluted samples were assessed by ELISA (Mercodia). Glucose stimulated insulin release (GSIR) was represented as absolute values of insulin concentration in supernatants for each incubation step (low glucose 1, high glucose, and low glucose 2) and as stimulation index (the

ratio of insulin released after exposure to high glucose over the insulin released in basal low glucose 1 condition: GSIR Index).

3.9.2 Live Dead staining and confocal imaging

Naked and encapsulated islets were stained with calcein-AM and ethidium bromide (Live Dead cell viability kit, Molecular Probes), and imaged with a Leica SP5 inverted confocal microscope for viability assessment. Z-scans of up to 200µm volumes were performed (Slice thickness 5µm).

3.9.3 Dynamic glucose-stimulated insulin release (Perifusion)

Perifusion is a procedure that quantitatively and dynamically measures the insulin secreted by islets that are subjected to a continuous flow of stimulants [174]. The experimental conditions during islet perifusion are adjusted to simulate the islet "physiological" environment within the pancreas.

a) Materials for preparing C-10 solution and Glucose Solutions

C-10 solution was used as the base solution to prepare the various islet stimulation solutions. The C-10 solution is used to mimic the extracellular milieu experienced by the islet cells in their natural state (i.e. pH value, cations & osmotic effects).

1L of C-10 was prepared by mixing 800ml of deionized water with 125mM Sodium Chloride (NaCl, Sigma), 5,9mM Potassium Chloride (KCl, Fluka), 2,56mM Calcium Chloride ($CaCl_2$, Sigma), 1,2mM Magnesium Chloride (MgCl, Fluka), 25mM HEPES (Gibco), and 1mg/ml Bovine Serum Albumin (BSA, OmniPur). Using a magnetic stirrer, the solution was stirred at room temperature, and sterile filtered after adjusting pH to 7.4. 3mM glucose solution, 11mM glucose solution, and 25mM KCl solutions were prepared   in C-10 solution through the addition of the

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proper w/v amount of either D-Glucose or Potassium Chloride.

N=2 columns were implemented for each condition: 1) unencapsulated Lewis rat islets, 2) Lewis rat islets encapsulated in 1.2%UP-MVG microcapsules, 3) Lewis rat islets encapsulated in double-coated capsules. 100 unencapsulated or encapsulated IEQ were loaded in each column.

b) *Perifusion Procedure*

A perifusion machine (BioRep technologies, Miami FL) was used to subject islets to sequential stimulation of insulin secretion using, in order, the solutions, below, dispensed at a constant flow rate of 100µl/minute. Perifusion chamber (8 chambers) eluates were collected in a 96 well plate format with each column in the plate representing a single time point of collection for each group.

3mM glucose solution: 5 minutes

11mM glucose solution: 10 minutes

3mM glucose solution: 15 minutes

25mM KCl: 5 minutes

3mM glucose solution: 10 minutes

After the final solution exposure, the 96 well collecting plates were stored at -20°C for future immunoassays. The naked and the encapsulated islets in the perifusion columns were collected and stored for later DNA quantification.

3.10. DNA quantification

For all experiments where DNA normalization was utilized, the DNA extraction and quantification was performed using the DNAeasy Blood & Tissue Kit (Quiagen), following the manufacturer's protocol.

3.11. Mice transplant

3.11.1. Diabetes Induction

Diabetes was induced by a single intravenous injection of streptozotocin (200 mg/kg; Sigma-Aldrich, St Louis, Mo). Mice that showed blood glucose > 250mg/dL for a minimum of three days were considered diabetic.

3.11.2. Epididymal fat pad (EFP) transplantation

For EFP transplantation of either naked or alginate-based capsules, a small cutaneous incision and a small muscular incision were performed on the abdomen of recipient mice under general anesthesia (isoflurane), The EFP was then gently exposed and flattened. For unencapsulated (naked) islets, at the time of islet transplantation, 750 IEQ were collected with a Hamilton syringe and transferred to the surface of the EFP. 20µl of engineered fibrin gel were then pipetted on the EFP to cover the islets with gelation occurring in situ. The EFP was then wrapped and fibrin glue was used to seal the created 'EFP pocket'. The wrapped islet-containing EFP was gently placed back in the abdominal cavity of the mouse and the muscle and the skin were sutured. Alginate-based microcapsules were placed on the EFP with a spatula, followed by procedure detailed for naked islets. The engineered gels were comprised of a fibronectin (FN) fragment displaying the integrin-binding domain (FN 9-10), the growth factor (GF)-binding domain (FN 12-14) and the fibrin binding substrate: factor XIIIa. The FN fragment is covalently cross-linked into a fibrin matrix during the natural polymerization process of fibrin. Human VEGF-A165, human PDGF-BB and APROTININ were also incorporated within the gel to allow binding of the GFs to the FN12-14 sequence and to allow 1. controlled local release of GFs that are dependent on fibrin degradation; and 2. synergistic signaling of integrin and GF receptor [175]. Graft function was

monitored by measuring non-fasting blood glucose values using portable glucometers (OneTouch Ultra 2; LifeScan) **(Figure 10)**.
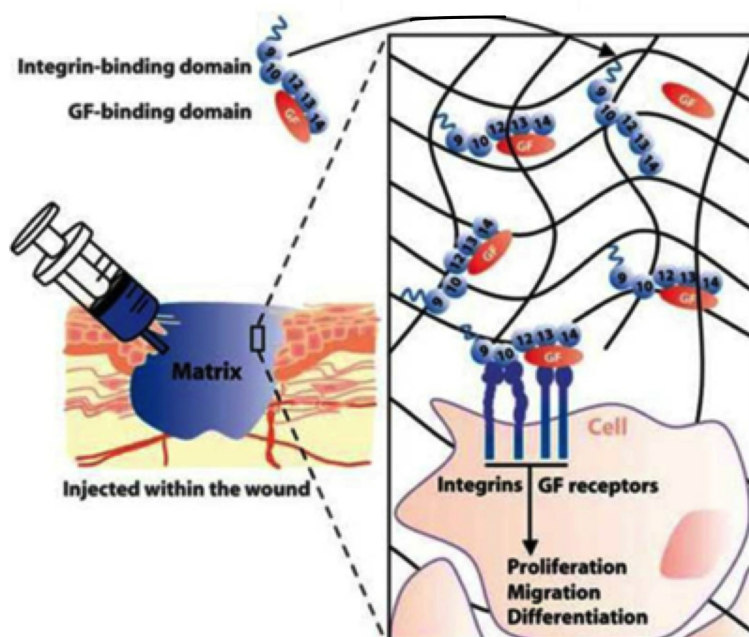
Figure 10. Fibronectin fragment is engineered to display 1) Integrin-binding domain (FN 9-10); 2) GF-binding domain (FN 12-14); 3) Fibrin binding sequence factor XIIIa. The fragment is covalently cross-linked into a fibrin matrix during the natural polymerization process of fibrin factor XIIIa. The engineered matrix allows sequestration of GFs [175].

Reversal of diabetes was considered when mice displayed at least three consecutive blood glucose readings < 250mg/dL after islet transplantation. Graft rejection was considered when mice displayed at least three consecutive blood glucose readings > 250mg/dL after reversal of diabetes following islet transplantation. To confirm graft function and exclude pancreas regeneration in mice that reversed diabetes after transplantation, EFP grafts were removed at 100-110 days after transplantation and mice were monitored to confirm return to hyperglycemia. The grafts were fixed in 10% buffered formalin.

3.11.3. Intraperitoneal (IP) transplantation

For intraperitoneal transplantation of unocoated islets and alginate-based capsules a small incision in the skin followed by an incision along the linea alba of the peritoneum were performed. Uncoated islets and alginate based caspules were suspended in HBSS containing calcium and magnesium, aspirated with a 1ml pipette and injected into the peritoneal cavity in a volume of approximately 0.2 ml. Muscle and skin were then sutured.

3.11.4. Intraperitoneal (IP) lavage

After sacrificing the mice by cervical dislocation, capsules were retrieved by opening the peritoneal cavity and washing out the capsules with HBSS w/ Ca^{2+}. Mg^{2+}. The capsules were fixed in 10% neutral buffered formalin and transferred to Ethanol 80% after overnight fixation. Typically, a low retrieval rate of capsules is indicative of low biocompatibility, because capsules that trigger higher fibrotic responses adhere to the peritoneal cavity preventing their retrieval through consecutive washes.

3.11.5. Islet transplantation in spontaneoulsy diabetic NOD mice

Female non-obese diabetic (NOD) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice that developed diabetes between 12 and 36 weeks of age received an insulin pellet (Linbit, COMPANY) to maintain glycemic control until the time of transplantation. Naked islets (1K IEQ, n=2) and alginate-based microcapsules (1K IEQ, n=3) were transplanted in the peritoneal cavity as indicated in the previous section of the Materials and Methods.

3.11.6. Islet transplantation in NOD SCID mice

Animals were rendered diabetic via a single intravenous administration of 200mg/kg of Streptozotocin (Sigma-Aldrich, St. Louis, MO). Non-fasting blood glucose was assessed by glucometer (Elite, Bayer; Tarrytown, NY or OneTouchUltra2, LifeScan, Milpitas, CA) and mice with sustained hyperglycemia (> 300 mg/dl) were designated for islet transplant. Islet grafts (1K IEQ or 2K IEQ) were transplanted under the kidney capsule (n=13 per group), as described elsewhere [169] or into the EFP (n=1for 2K IEQ or n=3 for 1K IEQ).

After transplantation, non-fasting blood glucose values were assessed daily for the first 2 weeks and then 3 times a week thereafter.

3.11.7. Biocompatibility test

Biocompatibility tests were performed by transplanting alginate based capsules in the EFP of C57BL/6 mice (n=6). 2 mice were sacrificed at each time point (t=7,14 and 21 days). Explanted grafts were fixed in 10% buffered formalin.

3.12. Graft Histology and Imaging

Formalin-fixed grafts were embedded in paraffin, sectioned at 5μm thickness and stained with standard Haematoxylin and Eosin (H&E) and Masson's Trichrome. Images were acquired with a Leica DMIRB light microscope and processed with the Leica Application Suite software.

3.13. Immunofluorescence staining

Formalin-fixed grafts were embedded in paraffin, sectioned at 5μm thickness, and stained with primary antibodies against glucagon (Biogenex; 1:250 dilution), insulin

(Dako; 1:250), CD3 (Cell Marque; 1: 250), CD45R-B220 (eBioscience; 1: 200), MAC2 (Cedarlane; 1:100), and CD31 (AbCam; 1:20). Secondary antibodies were purchased from Molecular Probes, Invitrogen and used at a dilution of 1:200. Nuclear counterstaining was done with 49,6-diamidino-2-phenylindole (DAPI, 1:10,000) (Sigma-Aldrich). Images were captured using a Leica SP5 inverted confocal microscope and processed with ImageJ (National Institute of Health).

3.14. Statistics

Prism 5.0 for Macintosh software (Graphpad, San Diego, CA) was used for analysis. Unless otherwise noted, data are presented as mean ± SD. Statistical comparisons were based on Student's t-test or analysis of variance (ANOVA) with Tukey post-hoc test for pairwise comparisons. A confidence level of 95% was considered significant. Actuarial survival curves and log-rank test were used to compare diabetes reversal amongst experimental groups. The statistical significance of differences among more than two groups for GSIR and cytotoxicity assay was analyzed by post-hoc Dunn's multiple comparison test after analysis of variance (ANOVA). P values <0.05 were considered significant.

4. RESULTS

4.1. Aim 1: Optimizing the traditional alginate microcapsules for clinical translation

4.1.1.Minimizing UP-MVG capsule diameter to minimize transport barriers and allow transplantation in islet-friendly sites

Our goal for this sub-aim was to generate minimal volume ultrapure alginate microcapsules by electrostatic droplet generator. As we discussed in the introduction, the size of traditional microcapsules (600-1000µm) causes central hypoxia in encapsulated islets and delays in insulin secretion in response to glucose, as a result of mass transfer limitation imposed by the capsule polymer. Further the size of traditional microcapsules obliges to transplant microencapsulated islets in the peritoneal cavity, which is the only site that can accommodate such large volumes (60-120ml in a human recipient). Such large volumes of microcapsules cannot be transplanted in sites that are more conducive to islet survival, like the omental pouch in humans and large animals or the EFP site in mice. Here we aimed at minimizing the diameters of microcapsules and thereby, mass transfer limitations to allow for transplantation of microencapsulated islets in the EFP in mice. To enhance biocompatibility and reduce host inflammatory reactions to the capsule material we chose alginate from a source that guarantees low endotoxin contamination and high purity.

As design parameters for microcapsule production we aimed at generating 1. a regular smooth, spherical geometry, 2. homogeneous and reproducible size distribution, 3. a bead diameter of 400-600µm, and 4. absence of irregular microcapsules. We first optimized the alginate concentration and the parameters of the electrostatic droplet generator (flow rate, voltage, and needle diameter) in

order to produce cell-free alginate microcapsules that conformed to optimal design parameters. Minimal size microcapsules can be obtained using a potential difference between 2 and 4 kV/cm (kV/height between needle tip and $CaCl_2$), independently of the needle size [176]. For this reason we set the voltage at 8.8 kV and the distance between the $CaCl_2$ bath and the electrode at 4 cm (giving a potential difference of 2.2 KV/cm, which is within the 2-4 kV/cm range) for the first optimization studies. Keeping the voltage and the bath-electrode distance fixed (fixed potential difference), we evaluate the effects of modifying the needle diameters and the flow rates of polymer extrusion on the average diameter of cell-free microcapsules. Keeping the potential difference (8.8 kV) and the flow rate (10μl/min) constant, we found that by decreasing the needle diameter from 0.6 mm to 0.17 mm, we could decrease the average capsule diameter from 749±35 μm to 279±29 μm **(Table 3A)**. Considering that pancreatic islets have a diameter between 50 and 350μm, we chose 400μm as the smaller needle diameter for use with islets preventing blockage of the needle and excessive shear stress. By varying the alginate solution flow rate, while keeping the potential difference (8.8 kV) and the needle diameter (400μm) constant, we found that by decreasing the flow rate from 50 to 10μl/min, we could decrease the average capsule diameter from 651±12 μm to 526±48 μm **(Table 3B)**. For flow rates lower than this value, the diameter distribution was wider and therefore less desirable (data not shown). For this reason we chose 10 μl/min as flow rate for further studies because it produced optimal capsules with uniform diameters in a reasonable time frame to maintain cell viability. Additionally, we evaluated the effects of changing the electrostatic potential difference by modifying the voltage and the distance between the $CaCl_2$ bath and the electrode, keeping the flow rate (10μl/min) and the needle diameter (400μm) constant. We found that increasing the voltage to 9-

10kV didn't change the capsules diameter, even when we decreased the distance between the bath and the electrode. Using 10kV as potential, it was not possible to decrease the distance between the bath and the electrode to 2 cm, because at such short distance, a spark between the bath surface and the needle tip was generated **(Table 3C)**.

A	Needle Diameter (mm)	Bead Average Diameter (µm)	Bead Diameter SD (µm)
	0,17	279	28,80
	0,4	500	16,95
	0,6	749	35,42

B	Alginate flow rate (µl/min)	Bead average diameter (µm)	Bead Diameter SD (µm)
	5	526	48,27
	10	502	8,36
	25	621	15,57
	50	651	12,44

C	Voltage (kV)	Electrode-Bath Distance (cm)	Voltage/cm (kV/cm)	Bead Average Diameter (µm)	Bead Diameter SD (µm)
	8,8	4	2,2	501,25	10,30
	8,8	2	4,4	519,25	9,77
	9	4	2,25	519,5	26,03
	9	2	4,5	542,5	28,72
	10	4	2,5	535,5	23,40
	10	2	5	-	-

Table 3. Determining the effect of needle diameter (A), alginate flow rate (B), and potential difference (C). In (A) the flow rate is 10 µl/min, electrode-bath distance is 4cm, and the voltage is 8,8kV. In (B) the needle diameter is 0,4µm, the voltage is 8,8kV, and the electrode-bath distance is 4cm. In (C) the needle diameter is 0,4µm and the flow rate is 10 µl/min.

Next, we optimized the concentration of the MVG alginate for our application. We found that the optimal concentration was 1.2% (w/v) MVG. Concentrations of alginate higher than 1.2% (range between 1.5% and 2%) resulted in the formation of larger microcapsules, whereas concentrations below 1% led to the formation of partially broken beads, likely due to mechanical stress as they are extruded into the gelation bath (data not shown). Spherical uniformity contributes to the biological and transport properties of alginate beads. For this reason, we aimed at generating spherical beads as a non-spherical geometry leads to non-uniform diffusion and aggravates foreign body/inflammatory responses to the bead material, resulting in the failure of microencapsulated islet graft.

From the optimization studies of cell-free microcapsules presented above we concluded that an integrated evaluation of the effects of different parameters for fabrication of alginate microcapsules with the electrostatic droplet generator allowed for the design of cell specific custom alginate capsules. From such optimization studies we concluded that 1) a 1,2% UP-MVG concentration, 2) an 8,8 kV potential difference, 3) a 10 μl/min alginate flow rate, 4) a 400 μm needle diameter, 5) a 4 cm distance between the needle tip and the $CaCl_2$ bath were the optimal parameters for obtaining microcapsules with small diameter (range: 450-500µm) and homogenous dimensional distribution (**Figure 11**).
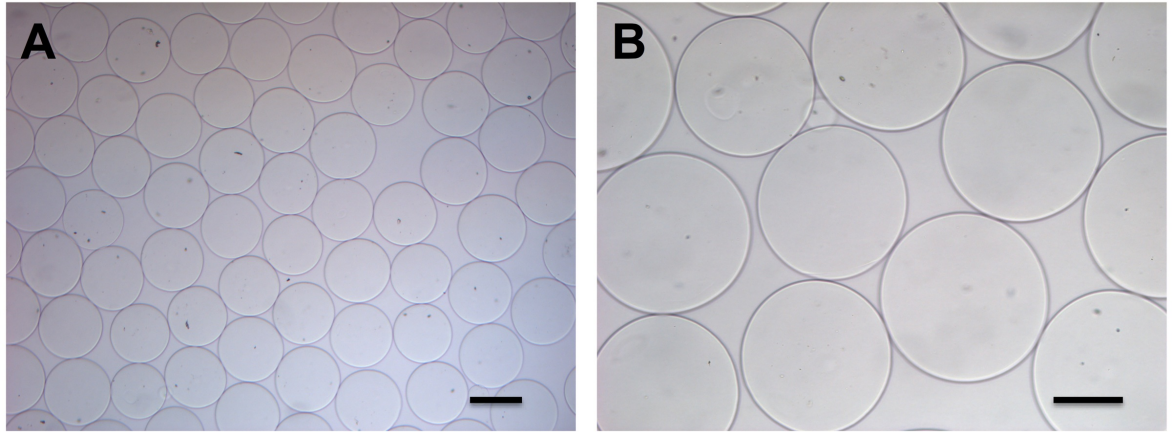
Figure 11. Optimized parameters allow fabrication of ultrapure alginate microcapsules with smaller diameter (average 500µm) and homogenous dimensional distribution (STDEV ±1,01). Scale bar (A)= 500 µm. Scale bar (B)= 200 µm.

Decreasing the diameter of conventional alginate capsules allows for their transplantation into sites that are potentially more suited to islet survival, but that cannot accommodate larger volume capsules, such as the EFP site. By transplanting diffusion-optimized minimal volume capsules in sites that are more favorable to islet engraftment and log-term function, we could potentially reverse hyperglycemia in diabetic patients diabetes with only a marginal mass of encapsulated islets. This is particularly appealing for islet transplantation because of the shortage of islet supply (currently isolated from cadaveric pancreases).

4.1.2. Evaluating the viability and functionality of pancreatic islets encapsulated within optimized alginate microcapsules

Viability and functional activity of encapsulated pancreatic islets of Langerhans, is critical in evaluating encapsulation strategies. For this purpose, we first evaluated the optimal cell loading density of optimized 1.2%UP-MVG alginate microcapsules. We sought to reduce the number of beads with partially protruding islets (this happens when cell density is too high), reducing the percentage of cell-free empty capsules (this happens when the density is too low) and to avoid core hypoxia

(this occurs when cell density is too high). Three different cell densities have been tested for encapsulation of pancreatic islets from Lewis rats: 500 **(Figure 12A)**, 1500 **(Figure 12C)** and 3000 IEQ **(Figure 12E)**, suspended in a volume of 100µl UP-MVG (final islet density: 5k, 15k, and 30k IEQ/ml). The 5k IEQ/ml density led to the highest percentage of empty capsules, while the 30k IEQ/ml density resulted in multiple islets per capsule. Multiple islets within the same capsule resulted in a massive oxygen and nutrients deficiencies, as expected as the typical loading density is around 1.5% (%islet volume/polymer volume) [80], while for 30k IEQ/ ml the density is 5,3%. The resulting nutrient and oxygen competitions between islets leads to central hypoxia and to necrotic core **(Figure 12F).** Live Dead staining and confocal imaging of capsules generated with 15k IEQ/ml **(Figure 12D)** islet density demonstrated higher cell viability than 30k IEQ/ml **(Figure 12F)**.
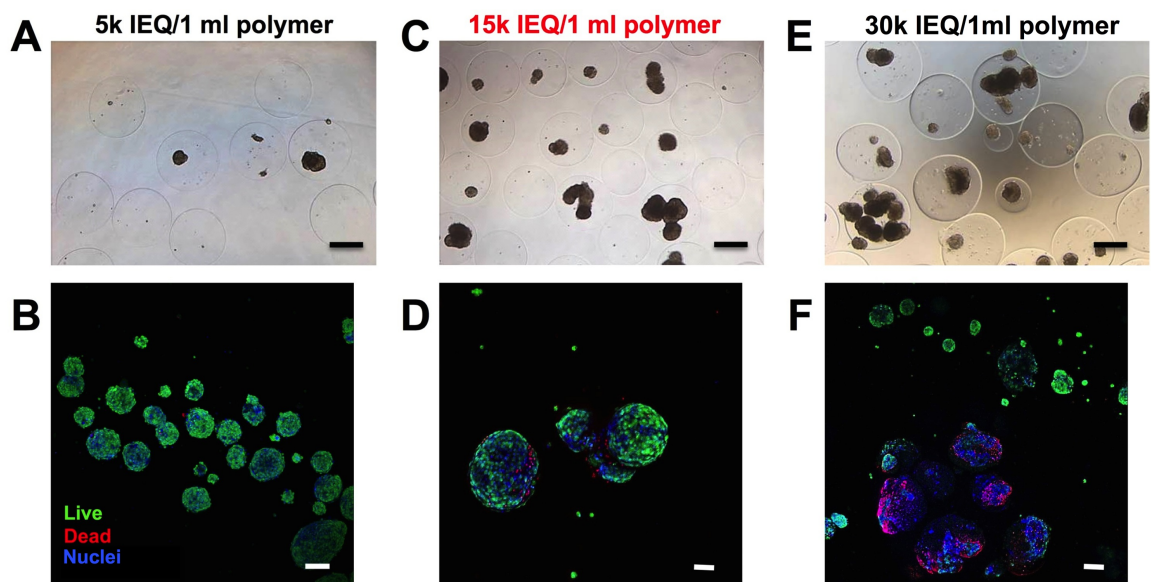
Figure 12. Optimization of loading cell density with pancreatic Islets from Lewis rats. Phase contrast images (A,C,E) and confocal images (B,D,F) of Live (green) Dead (red) Nuclei (blue) staining as viability assessment of islets encapsulated in 1.2% UP-MVG alginate microcapsules at 5k (A,B), 15k (C,D), and 30k (E,F) IEQ/ml densities. Phase contrast images scale bar= 500µm. Confocal images scale bar= 100µm.

We concluded that 15k IEQ/ml was the optimal cell density to allow entrapment of 1 to 2 islets within UP-MVG capsules, minimizing the percentage of cell-free beads and maximizing viability of encapsulated islets.

We performed glucose-stimulated insulin release (GSIR) of microencapsulated murine islets to evaluate whether optimized microencapsulation of islets (15k IEQ/ml) affected the viability and functionality. We found that the encapsulation, even at higher than typical density (3% vs. 1.5%) does not compromised the insulin secretory response to glucose stimulation (**Figure 13A)**. There was not statistical difference between the index values of islets within microcapsules relative to the naked islets control (by post-hoc Dunn's test, P: nonsignificant) (**Figure 13B**). Since there is intrinsic variability among different batches of pancreatic islets (in terms of dimensions, purity, viability and glucose response) for each experiment we performed we have normalized the results of the microencapsulated islets to the results of their naked controls**.**
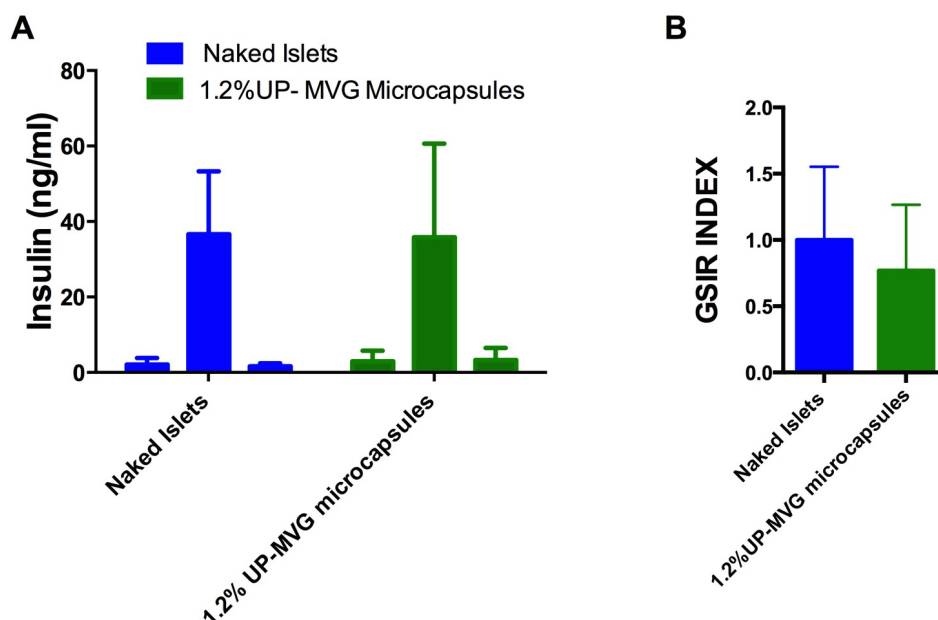
Figure 13. GSIR assessment: comparison between naked murine pancreatic islets and 1.2% UP-MVG microcapsules. (A) Absolute values of insulin concentration in supernatants for each incubation step (low glucose 1, high glucose, and low glucose 2).

(B) Stimulation index (the ratio of insulin released after exposure to high glucose over the insulin released in basal low glucose 1 condition).

We concluded that, by using an electrostatic droplet generator, we could reduce the diameter of standard alginate microcapsules without impairing the viability and the functionality of encapsulated pancreatic islets. Pancreatic islets viability and functionality are a critical requirement to increase the likelihood of successful *in vivo* transplantation. Transplanting cells that are suffering from oxidative stresses, causing the release of inflammatory cytokines increases the host response and the likelihood of the graft failure.

4.1.3. Determining whether optimized microcapsule allow engraftment and long-term function of islets in the autoimmune murine model of diabetes (NOD mouse) in the peritoneal cavity

As already discussed in the introduction, different issues can lead to failure of alginate microcapsules grafts. Among those issues are: 1) the transplantation site, 2) material impurities, which triggers inflammatory response, 3) inadequate mass transfer resultant from capsule dimension, and resulting in necrosis of the islets, and 4) inadequate permselectivity.

Our aim is to determine whether we could prevent graft rejection caused by a combination of allo- and auto-immune responses, utilizing our microcapsules.

We compared engraftment and long-term function of 1000 IEQ BALB/c transplanted into the intraperitoneal cavity of recipient NOD mice either encapsulated in 1.2% UP-MVG or non-encapsulated (naked) islets. The naked islet control is necessary to assure islet quality, as lack of diabetes reversal after islet transplantation (diabetes reversal should happen within the first 10 days after

islet transplantation of full islet mass doses in mice) would indicate primary non-function due islet isolation. Also the naked islet control is necessary to confirm that in absence of immunoisolation provided by the microcapsules, naked islets are rejected within 30 days in presence of allorejection, and within 10 days in presence of both auto and allo-rejection.

In our study we found that 0/2 mice that received 1000 IEQ naked IP restored euglycemia after islet transplantation **(Figure 14A,B).** On the other hand, 2/3 mice that received 1000 IEQ IP within 1.2% UP-MVG microcapsules (average diameter 498±2,06) restored euglycemia in 7±3 days (median reversal time: 10 days vs. undefined for naked controls) after transplantation suggesting proper engraftment of encapsulated islets **(Figure 14A,B).** The median survival time of those microencapsulated islet grafts that reversed hyperglycemia after transplantation was 53 days **(Figure 14C).**
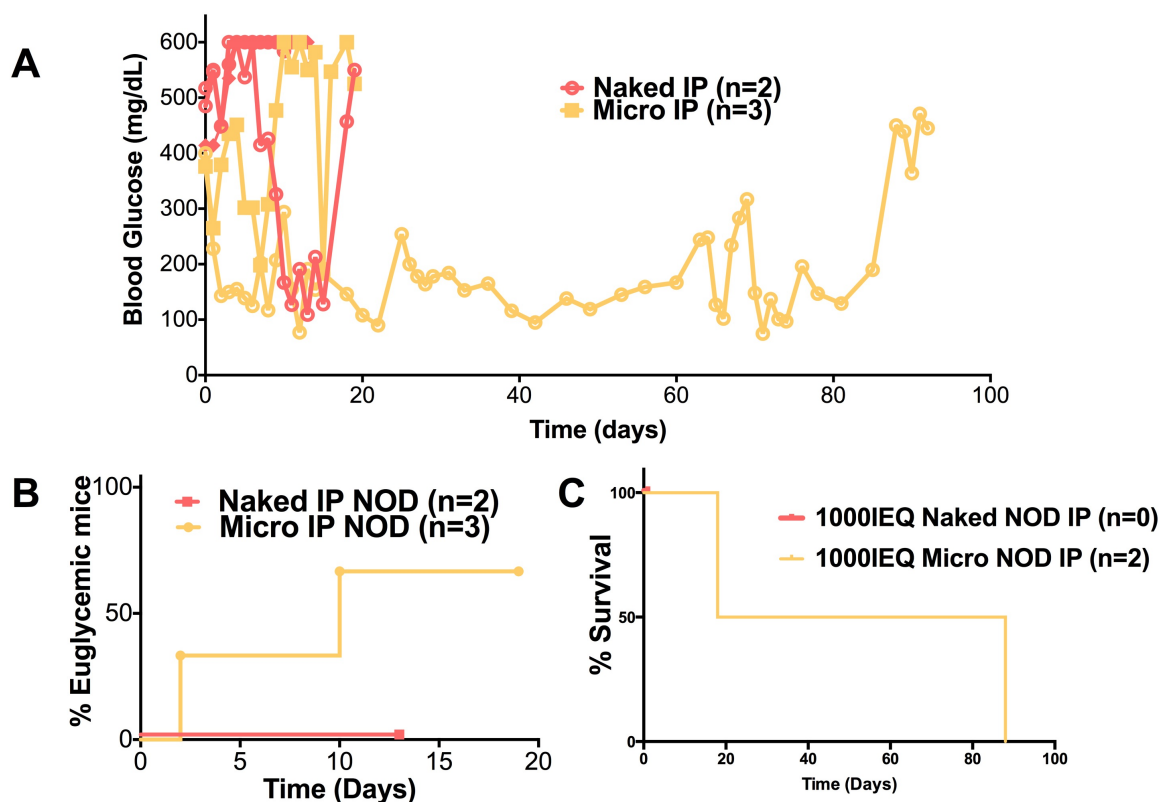
Figure 14. Transplantation of 1000 IEQ BALB/c islets encapsulated in optimized 1.2% UP-MVG microcapsules in allogeneic and spontaneously diabetic NOD mice. (A) Blood glucose of NOD mice transplanted with 1000 IEQ 1.2% UP-MVG microencapsulated islets from fully MHC-mismatched BALB/c mice intraperitoneally without any immunosuppression and compared to naked non- encapsulated islets. (B-C) Percentage of NOD mice that reversed hyperglyemia (B) and maintained graft function (C) after islet transplantation.

At graft rejection (90 days after transplantation), we performed an IP lavage to retrieve the capsules. We could efficiently retrieve the majority of the microcapsules – i.e. the capsules were floating in the peritoneal cavity without signs of adherences – suggesting lack of high inflammatory responses to the material or cells. Retrieved microcapsules showed preservation of their spherical shape, without any breakages **(Figure 15A,B,C).**
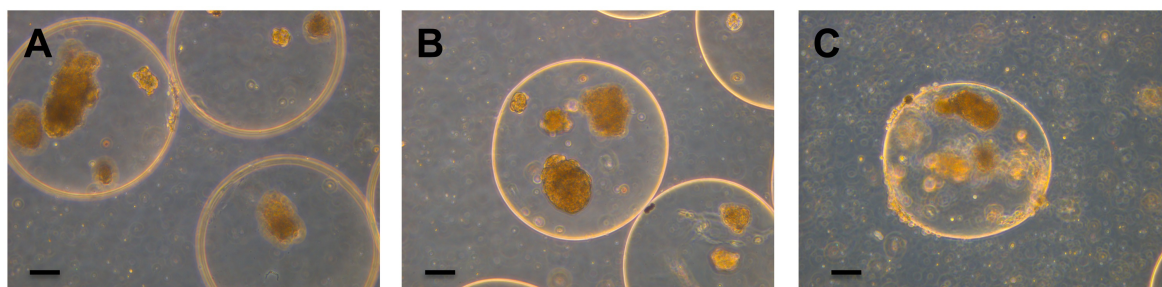
Figure 15. Phase contrast representative images of 1.2% UP-MVG capsules retrieved through intraperitoneal lavage from NOD mice that reversed diabetes and maintained euglycemia for 90 days after transplantation of fully MHC-mismatched islets from BALB/c mice. (A,B,C) Representative images of the majority of transplanted capsules that did not show either cell accumulation or deposition of fibrotic tissue around the capsules; Scale bars: 100μm.

These preliminary studies suggest that the optimized UP-MVG capsules are immunoprotective and preserve islet viability and function, even in the most challenging preclinical model of allogeneic IP transplantation into the autoimmune NOD mouse.

4.1.4. Determining whether improving the islet transplant site can improve engraftment and long-term function of encapsulated islets

By minimizing the size of the microcapsule geometry we were able to transplant microencapsulated islets in sites that are volume restricted and more conducive to islet survival. We chose the EFP site because it is highly vascularized, it can be wrapped to contain the graft within a pocket, for easy monitoring and retrieval, and it resembles the human omentum (that we are currently exploring as novel transplant site in a phase I/II clinical trial) more closely than the murine omentum. To promote rapid revascularization of the islet graft in the EFP site we have previously engineered the EFP site with pro-angiogenic hydrogels as described in the Introduction (manuscript submitted to Biotechnology and Bioeneigneering).

We transplanted 750 IEQ naked vs. 1.2% UP-MVG microencapsulated islets from BALB/c mice in the engineered EFP site or intraperitoneally without the engineered fibrin gels) of fully MHC-mismatched C57BL/6 mice rendered diabetic by chemical treatment (as described in the Methods). We found that as little as 750 IEQ naked islets reversed diabetes within 5 days (median reversal time: 1 day) and maintained euglycemia when islets were transplanted in the engineered EFP site, but not in the IP site (median reversal time: undefined, P=0.0003 when compared to naked islets in the EFP site), confirming that the engineered EFP site is more islet-friendly (it allows diabetes reversal with marginal masses of islets) than the peritoneal cavity **(Figure 16A,B)**. Despite prompt reversal of hyperglycemia, naked islets were rejected within 30 days (median survival time: 18 days) (**Figure 16C**). On the other hand, 750 IEQ islets enclosed in 1.2% UP-MVG microcapsules and implanted in the engineered EFP site reversed hyperglycemia in 1 day (median reversal time: 1 day, P=0.09 when compared to naked islets in

the EFP site and P=0.006 when compared to microencapsulated islets in the IP site) and maintained euglycemia for more than 100 days (median survival time: undefined, P=0.005 when compared to naked islets in the EFP site) in absence of immunosuppression (**Figure 16A,C**). When implanted in the peritoneal cavity, the same number of encapsulated islets reversed diabetes within 7 days (median reversal time: 4 days, P=0.0003 when compared to naked islets in the IP site), but we observed more fluctuations in the fasting blood glucose relative to the capsules implanted in the engineered EFP site. Further, when implanted in the peritoneal cavity, microencapsulated grafts showed a trend towards decreased survival, in terms of mice maintaining euglycemia for 100days, relative to microencapsulated islets transplanted in the EFP site, but not statistically different (median survival time: undefined, P=0.08).
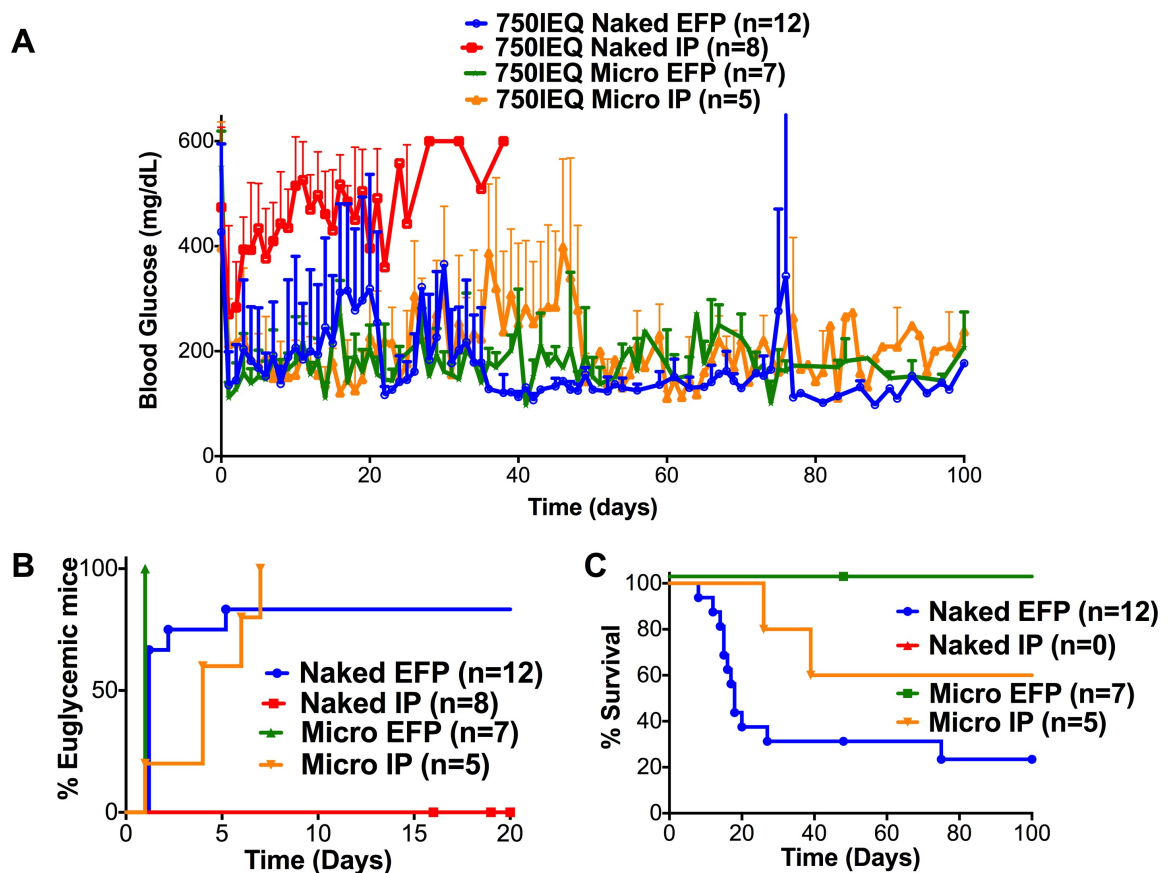
Figure 16. Transplantation of pancreatic islets encapsulated in optimized microcapsules in the IP and in the engineered EFP sites. (A) Blood glucose of C57BL/6 mice rendered diabetic by STZ treatment and transplanted with 750 IEQ naked or encapsulated (1.2% UP-MVG optimized microcapsules) islets from fully MHC-mismatched BALB/c mice in the EFP or IP sites without any immunosuppression; (B) Percentage of mice that reversed diabetes after transplantation of naked vs. encapsulated islets in the EFP vs. IP sites; (C) Percentage of naked vs. encapsulated islets that survived allorejection after transplantation in the EF vs. IP sites.

 We concluded that encapsulation of pancreatic islets in optimized UP-MVG microcapsules allows engraftment and long-term function of marginal masses of islets in the peritoneal cavity – i.e. the same number of islets fail to reverse diabetes when they are transplanted non-encapsulated.

Further, transplantation of islets in optimized UP-MVG microcapsules in the engineered EFP site, improves islet engraftment (as percentage of mice that reversed diabetes and as time required for diabetes reversal after islet transplantation) and long-term function (as stabilization of blood glucose to normal values: < 250mg/dL for more than 100 days after transplant). This is more than likely the result of improved vascular access in the EFP making the site more conducive to islet survival.

Of note, the transplantation of microencapsulated islets in the more supportive EFP site was only possible due to the capsule size/volume reduction afforded by the optimization of the electrostatic droplet generator.

4.1.5. Determining whether EFP transplant of optimized alginate capsules into NOD SCID mice improve engraftment and long-term function of non-human primate islets

750 1.2% UP-MVG encapsulated baboon (NHP) IEQ were transplanted in the engineered EFP site of chemically diabetic NOD-SCID mice and compared to 1000 and to 2000 IEQ naked islets transplanted in the engineered EFP site or in the renal subcapsular space (KD). We utilized the EFP site as a preclinical site to assess clinical translation and compared the results to the standard KD site utilized for evaluating the potency of clinical preparations of human islets. We found that as little as 750 IEQ microencapsulated islets reversed diabetes in 100% (n=3) of the mice within 10 days (median reversal time: 9 days) and maintained euglycemia when islets were transplanted in the engineered EFP site. Conversely, diabetes was reversed in only in 25% of the mice (median reversal time: 36 days, P=0.003 when compared to 750 IEQ microencapsulated islets in the EFP site) **(Figure 17A,B)**, when 1000 IEQ or 2000 IEQ naked islets were transplanted in the EFP. When transplanted in the KD site, naked islets rapidly reversed diabetes suggesting that poor islet engraftment in the EFP site was not due to poor islet potency **(Figure 17B)**.
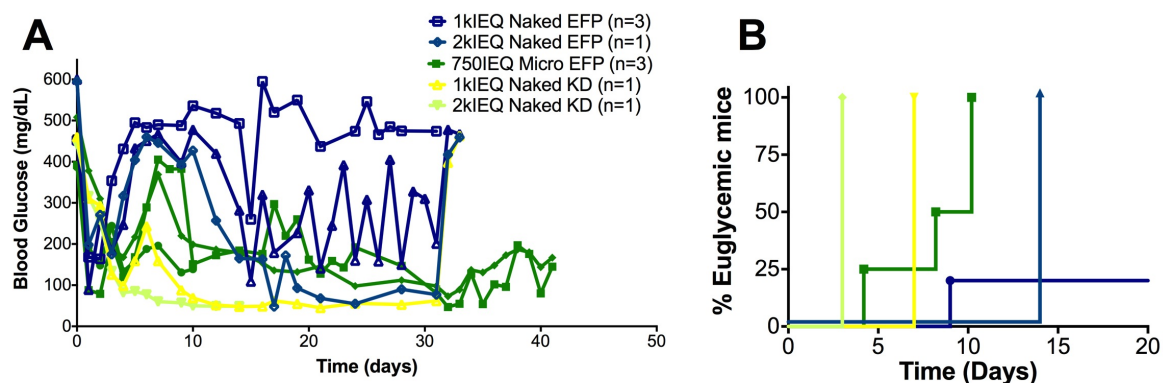
Figure 17. Transplantation of pancreatic islets from Baboon non-human primates (NHP) encapsulated in optimized microcapsules in the engineered EFP of chemically diabetic

NOD-SCID mice and compared to the renal subcapsular space (KD). (A) Blood glucose of NOD-SCID mice rendered diabetic by STZ treatment and transplanted with 750 IEQ encapsulated (1.2% UP-MVG optimized microcapsules, green) or 1000 (blue) – 2000 (black) IEQ naked islets from NHPs in the EFP site and compare to naked islets in the KD capsule at two different doses: 1000 IEQ (yellow) and 2000 IEQ (light green); (B) Percentage of mice that reversed diabetes after transplantation of naked vs. encapsulated islets

We concluded that a combination of minimizing transport barrier, utilizing ultrapure alginate and improving the islet transplant site in a clinically relevant site improves engraftment and long-term function of non-human primate islets for clinical translation.

4.2. Aim 2: Determining the effect of key capsule parameters on the in vivo performance (engraftment and long-term function) of encapsulated islets

4.2.1. Stability of 1.2% UP-MVG microcapsule

Since after implantation alginate capsules are expected to swell due to the exchange of Ca^{2+} with Na^+, which leads to destabilization of the gel network [149], we compared the swelling capacity of 1.2% UP-MVG microcapsules to 5% Peg-Mal capsules after exposure to osmotic pressure.

We found that UP-MVG capsules increased in size within minutes of incubation either in saline or in H_2O and completely disappeared. On the other hand, 5% Peg-Mal capsules presented no sign of swelling, neither after exposure to the hypotonic solution nor to normal saline. Our mechanical stress studies suggest that only Peg-Mal capsules resist osmotic stress **(Figure 18A,B)**.

To mimic the *in vivo* shear stress, additional studies were performed by incubating microcapsules in presence of large inert glass beads that were shake for 48 h. Only a negligible percentage of either 1.2% UP-MVG or 5% Peg-Mal broken

capsules was observed, suggesting that both capsule compositions were resistant to shear forces **(Figure 18C)**.
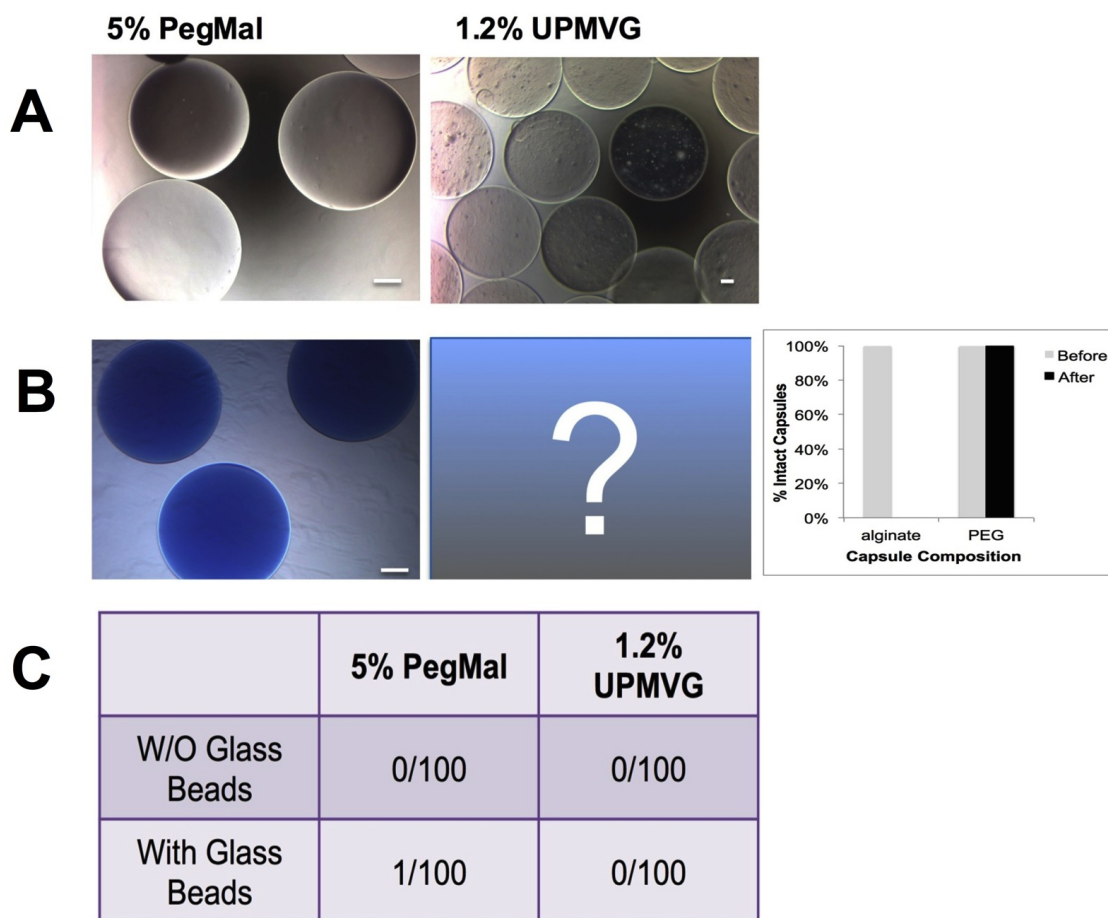
Figure18. Contrast phase images of 5% Peg-Mal caspules and 1.2% UP-MVG microcaspules. Images from osmotic stress test: (A) at t =0, immediately after the incubation in double-distilled H_2O. (B) at t=2 hours, double-distilled H_2O was replaced with normal saline and the capsules colored with 0.5% Trypan Blue to visualize breakages. Images from mechanical stress test: (C) 5% Peg-Mal and 1.2% UP-MVG showed good resistant to shear stress. Scale bar= 100 µm.

We concluded that Peg-Mal capsules are more resistant to mechanical stresses than UP-MVG capsules.

4.2.2. Physic-chemical stability

Based on previous results, which showed beneficial properties of Peg addition to alginate capsules to control permselctivcity and those obtained from the mechanical tests, we hypothesized that the addition of Peg-Mal hydrogels to alginate microcapsules could improve stability of the alginate microcapsules. By limiting the swelling of alginate capsules in presence of ion –rich physiological fluids in the *in vivo* setting, we hypothesize that we could increase the long-term immunoprotection.

4.2.3. Production of MicroMix and Double coated microcapsules

In order to fabricate hybrid 1.2% UP-MVG microcapsules reinforced with 5% Peg-Mal (**MicroMix**), we utilized the electrostatic droplet generator and the previously optimized fabrication parameters for 1.2% UP-MVG microcapsules. The addition of 5% (w/v) Peg-Mal had no effect on shape, diameter, or the homogeneity of the MicroMix capsules. We performed immunofluorescence staining of the MicroMix capsules with an anti-Peg antibody and we confirmed that Peg was uniformly distributed throughout the hybrid capsules (data not shown).

Double coating of 1.2% UP-MVG microcapsules with Peg-Mal (**Double**) was achieved through the design and optimization of a novel double emulsion method. First, we investigated the emulsion parameters that allow minimizing coating thickness while guaranteeing coating completeness. We found that the emulsion parameters that control coating properties were (i) the stirring speed (range between 400 rpm and 800 rpm) of the oil phase (emulsion external phase), (ii) the polymer (emulsion internal phase) volume, (iii) the number of UP-MVG beads re-suspended within the Peg-Mal polymer solution, and (iv) the percentage of surfactant in the oil phase (range between 2.5% and 20%). Initially, a total volume of 100µl of 1.2% UP-MVG microcapsules was re-suspended in 1 ml of 5% Peg-

Mal solution and then, this capsule suspension to the oil phase drop by drop, while constant stirring (as indicated in the methods). High stirring speeds (>600rpm) led to fracture of the alginate capsules, likely due to the high shear stress. Conversely, utilizing a speed below 400 rpm led to a non-uniform suspension of capsules in the oil phase. The utilized 1ml was the optimal volume of Peg-Mal that allowed us to obtain complete double coatings on all alginate microcapsules (**Figure 19C,D)**, while reducing the number of Peg-Mal secondary beads (not covering the alginate microcapsules) produced with the emulsion technique. The presence of these secondary beads can be deleterious for the in vivo performance of encapsulated cells, even within this novel double coating. Additionally detrimental, empty secondary beads increase the graft volume thereby potentially increasing mass transfer resistances and limiting the available sites. Most of the observed secondary beads were smaller than 70µm in diameter and could successfully be removed by filtration through a large pore (70µm) filter. Regarding surfactant percentage, the optimal percentage of surfactant was found to be 5%, as both a lower and the higher percentage led to abnormalities and lack of uniformity in coating shape **(Figure 19E)**. Despite obtaining a complete coating around the microcapsules, we could not achieve a uniform double coating around microcapsules, a uniform double coating thickness was never achieve either on different capsules or on the same capsule (**Figure 19A,B, and C)**. The high irregular thickness of the coating will have to be addressed in further studies, if in vivo applications are to be considered as the coating inconsistencies may result in poor biocompatibility, as described in the following sections.
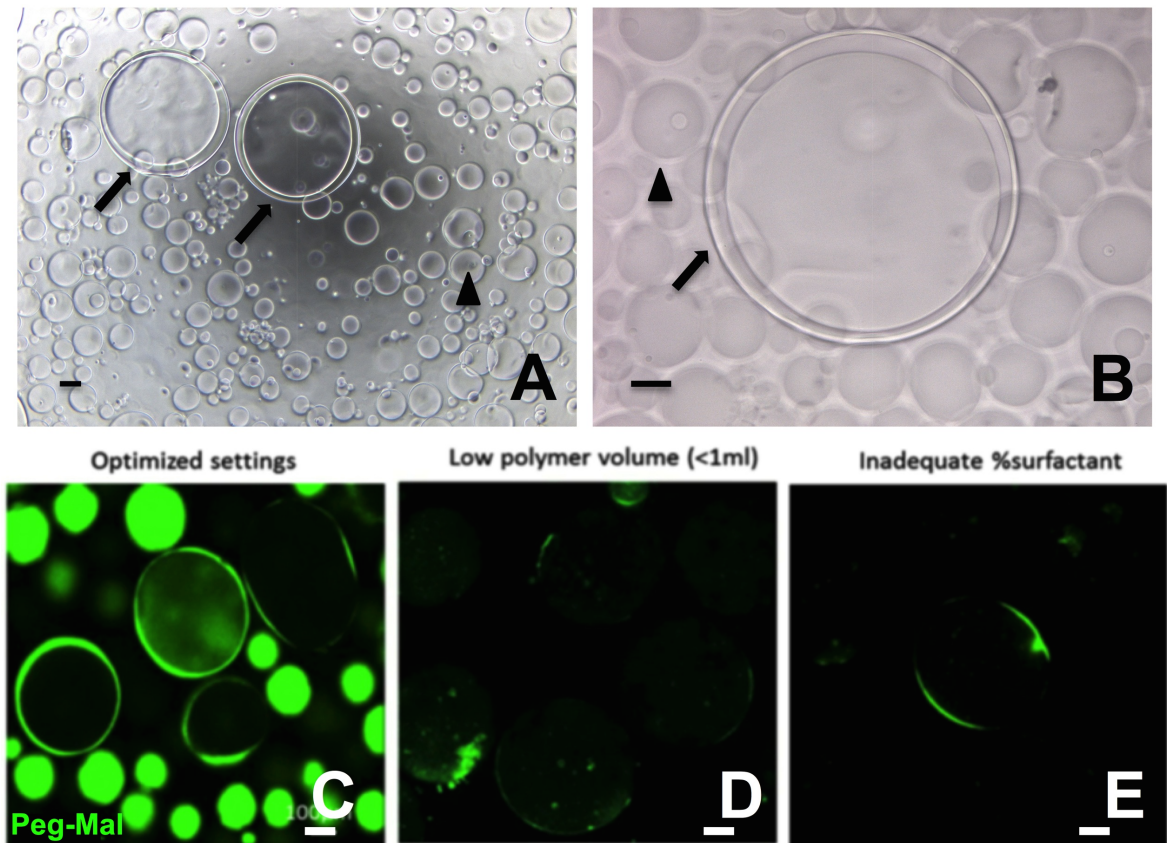
Figure 19. Optimization of the emulsion technique parameters to achieve double coatings of alginate microcapsules with Peg-Mal. (A) Phase contrast images of Double coated capsules (arrows) and Peg-Mal secondary beads (arrowheads) at low (A) and high (B) magnification. (C-E) Effects of varying the emulsion parameters on the Peg-Mal double coatings: optimized settings (C) are compared to low (insufficient) polymer volume (D) and low surfactant percentage (E), which led to uncompleted coating. Scale bars: 100 µm.

4.2.4. _In vitro_ viability and functionality of pancreatic islets enclosed in MicroMix and Double coated alginate capsules

After optimizing the hybrid and double coatings, the technologies were implemented on isolated rodent islets, generating both MicroMix **(Figure 20A,C)** and Double capsules **(Figure 20D,F, and G)**. Interestingly, islets both enclosed with Peg-alginate hybrid capsules (MicroMix) and in Double coated alginate microcapsules (Double) showed preserved viability, as assessed by confocal imaging of live/dead stained encapsulated cells **(Figure 20B and Figure 20E, respectively)**. The staining qualitatively demonstrated the lack of potentially

harmful coating materials and procedures.
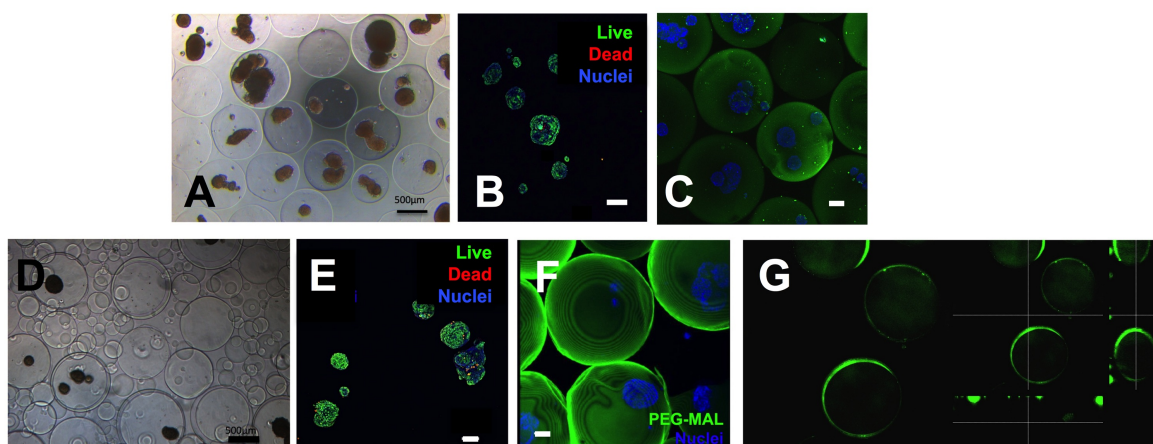
Figure 20. Coating completeness and viability of Peg-alginate MicroMix and Peg Double alginate capsules. MicroMix capsules: (A) phase contrast image, (B) Live (green) Dead (red) staining, and (C) anti-Peg staining. Double coated capsules: (D) phase contrast image, (E) Double coated capsules stained for Live (green) and Dead (red). Nuclei are counterstained with HOECHST (blue), and maximum projections (F) and Z-slice (G) of 200 μm thick z-stack confocal images of Double coated capsules stained with anti-Peg antibodies (green) and nuclear counterstaining (DAPI) (blue). Contrast phase image scale bars: 500 μm. Confocal image scale bars: 100 μm.

To evaluate the effects of MicroMix and Double coatings on the functionality of murine islets after coating, we performed GSIR studies. Both hybrid and double encapsulation procedure had no negative effect on the insulin secretory response to glucose stimulation (**Figure 21A,C**). There was not statistical difference between the index values of islets coated with the MicroMix technology **(Figure 21B)** or the double coating technology **(Figure 21D),** when compared to the naked islets control. For each experiment we have normalized the results of the encapsulated islets to the results of their naked controls (Figur) (by post-hoc Dunn's test, P: nonsignificant).
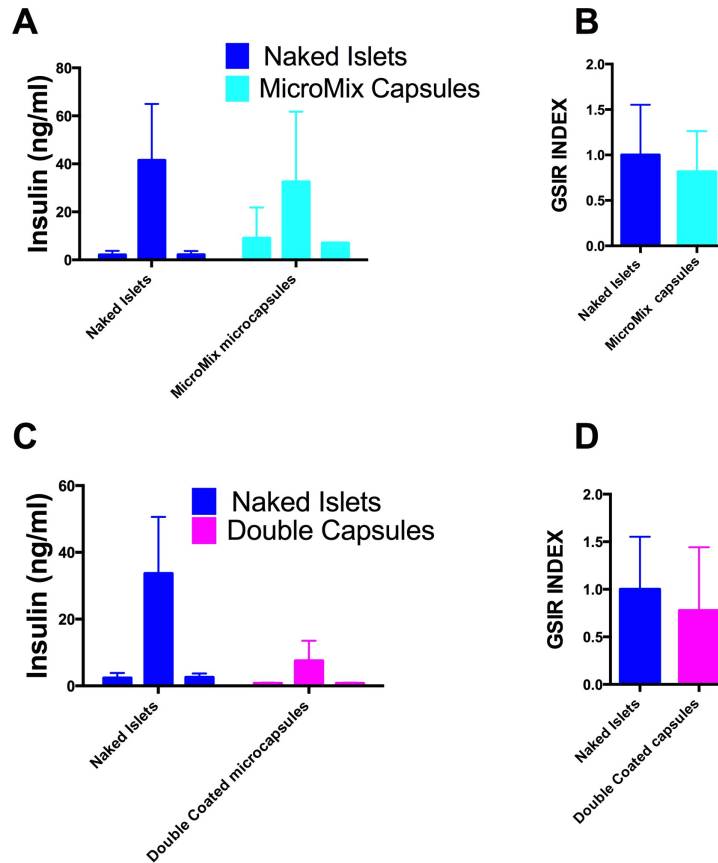
Figure 21. Assessment of function of murine islets enclosed in MicroMix. (A) Absolute values of insulin concentration in supernatants for each incubation step (low glucose 1, high glucose, and low glucose 2). (B) Stimulation index (the ratio of insulin released after exposure to high glucose over the insulin released in basal low glucose 1 condition). Assessment of function of murine islets enclosed in Double coated capsules. (C) Absolute values of insulin concentration. (D) Stimulation index.

We concluded that Peg-alginate hybrid (MicroMix) and Peg double encapsulation of alginate microcapsules (Double) procedures have no negative effects on cell viability and functionality. Finally, before assessing *in vivo* performance of MicroMix and Double capsules through transplantation, we performed cytotoxicity and in vivo biocompatibility assays to evaluate the effects of direct contact of the biomaterials with cultured cells and of host interactions to alginate/Peg-Mal capsule composition and geometries, respectively.

4.2.5. *In vitro* cytotoxicity and in vivo biocompatibility of alginate and Peg-alginate based capsules.

Cytotoxicity tests (n=3 independent experiments) were performed using cultures of a murine insulinoma cell line (NIT-1). Exposure to biomaterials and procedural solutions was performed for 24 hours. The statistical significance of differences was analyzed by Dunn's test, as described in Materials and Methods section. The results showed that exposure of NIT-1 cells to UP-MVG alginate gels for 24 hours had no adverse effect on cell viability relative to untreated control cells (P=non-significant), suggesting that UP-MVG has no toxicity, as described in literature [148]. The exposure of NIT-1 cells to 5% Peg-Mal precursor showed not significantly affect cell viability relative to cells both untreated and exposed to UP-MVG, although a viability decrease can be observed. Conversely, the presence of 5% Peg-Mal gel (crosslinked with DTT) and DTT alone in contact with NIT-1 for 24 hours significantly reduced cell viability (P<0.05) **(Figure 22)**. Despite the observed cytotoxicity results, we speculate that inclusion of 1.2% UP-MVG alginate in the Peg-Mal/DTT gels might ameliorate the deleterious effects of the DTT on the islets, thereby improving the chance of cell survival.
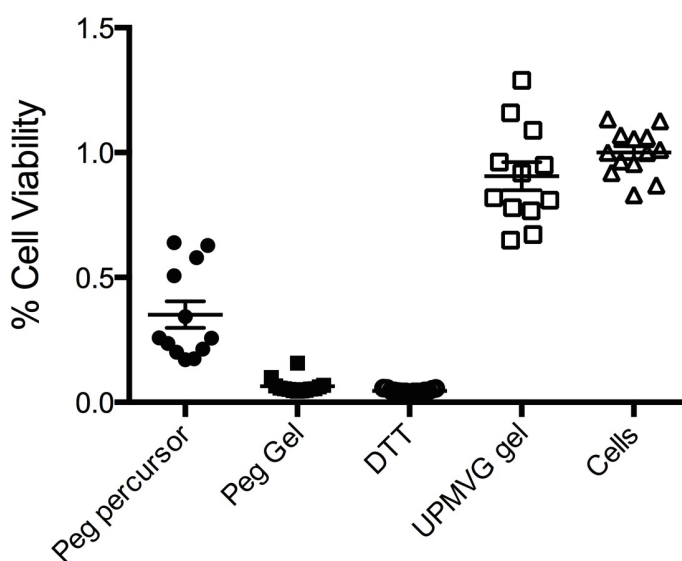
Figure 22. Cytotoxicity of UP-MVG, Peg-Mal gel, Peg-Mal gel precursors, and DTT crosslinker to NIT-1 after 24-hour exposure (MultiTox).

To assess the biocompatibility of the different capsule compositions H&E and Masson's trichrome staining was performed on explanted capsules at 7, 14 and 21 days post-implantation. Limited overgrowth was observed on the surface of both 1.2% UP-MVG and 5% Peg-Mal – 1.2% UP-MVG hybrid MicroMix capsules **(Figure 23A,C,E and Figure 24A,C)**. Collagen deposition, shown by the trichrome staining (**Figure 23B,D,F and Figure 24B,D)**, peaked at 14 days after implantation, suggestive of a characteristic fibroproliferative response with an influx of fibroblasts and the development of fibroconnective tissue. This was more prevalent for MicroMix capsules **(Figure 24B,D)**. Likely, the observed collagen deposition is due to the foreign material reaction, in which the critical cellular elements are always collagen-secreting fibroblasts and activated macrophages, releasing growth-regulating cytokines [82,90]. Same results are confirmed by fluorescence staining. CD3$^+$ lymphocyte recruitment was absent **(Figure 23G,I and 24E,G,I)**. The observed inflammatory cells for the MicroMix, mainly MAC2$^+$ macrophages, found on the capsule surface at day 7, gradually decreased with time **(Figure 24F,H,L)**, in line with the early inflammatory response to the biomaterials and transplants. Throughout the whole graft process, 1.2% UP-MVG **(Figure 23H, L)** showed a constantly lower level of macrophage infiltration than MicroMix capsules.
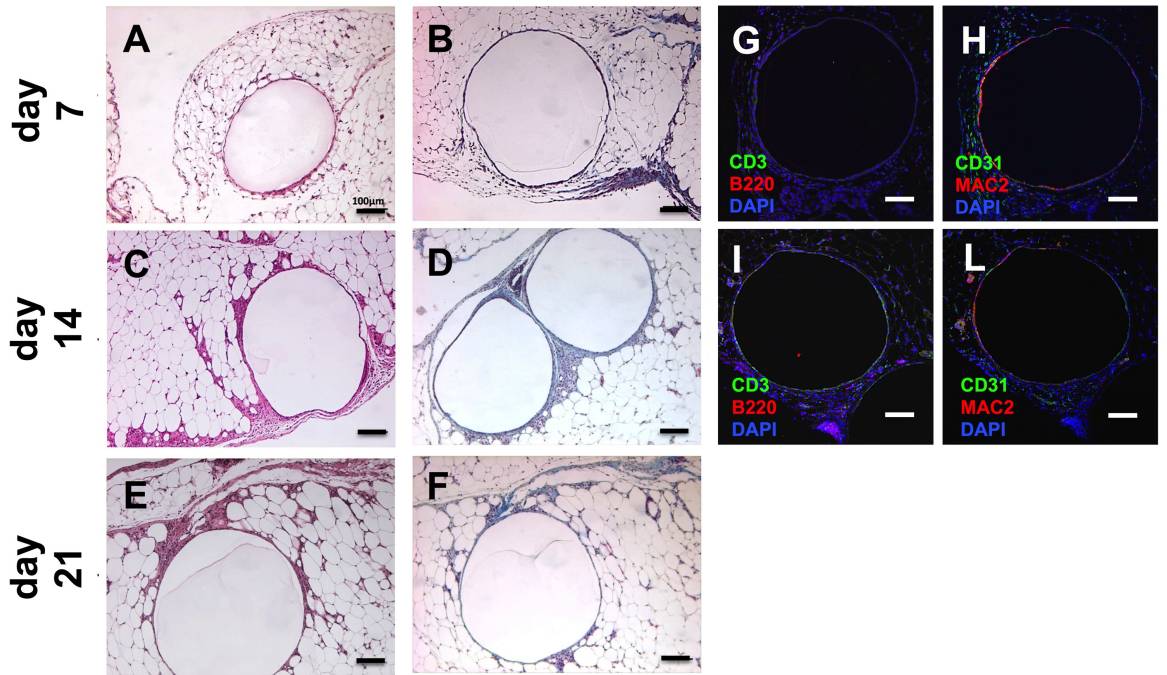
Figure 23. Empty 1.2% UP-MVG microcapsules were transplanted in the EFP of C57BL/6 mice to assess the biocompatibility of the capsule material 7, 14, and 21 days after implantation. H&E (A, C, E, respectively), Masson's Trichrome (B, D, F, respectively), and confocal images of T cells (CD3: green) and B cells (B220: red) (G, I), and of endothelial cells (CD31: green) and macrophages (MAC2: red) (H, L) are shown for each time point analyzed. Nuclei were counterstained with DAPI (blue). Representative images of n=3 mice are shown. Scale bars: 100 µm.
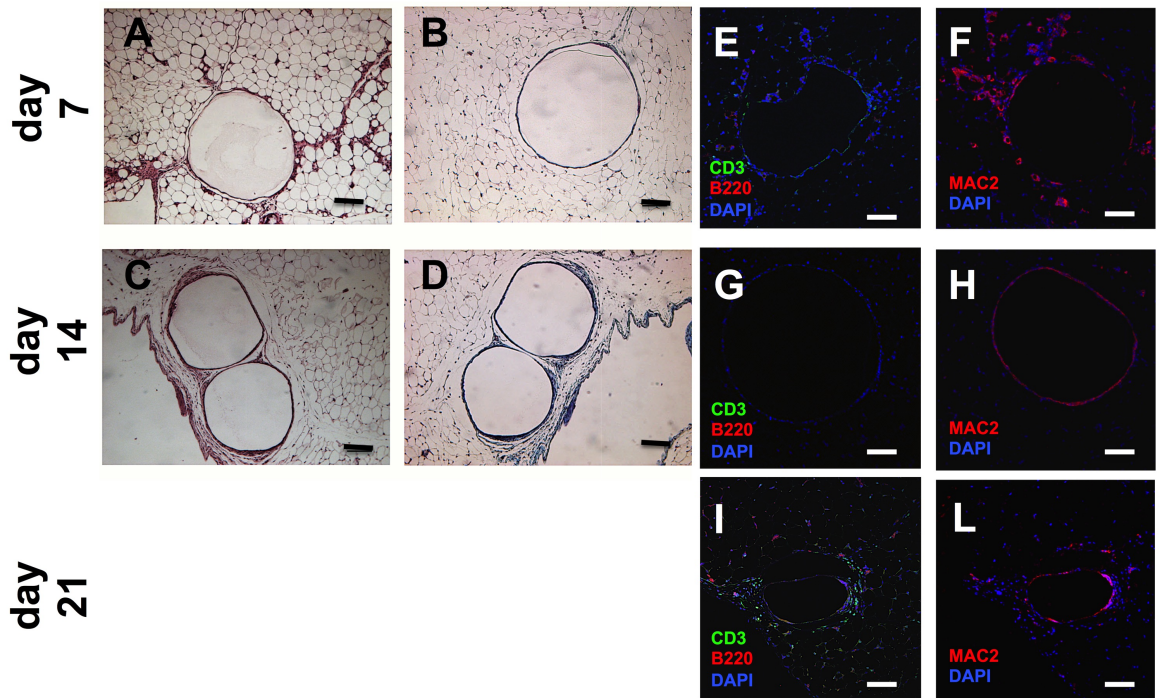
Figure 24. Empty 1.2% UP-MVG and 5% Peg MicroMix capsules were transplanted in the EFP of C57BL/6 mice to assess the biocompatibility of the capsule material 7, 14, and 21 days after implantation. H&E (A, C, respectively), Masson's Trichrome (B, D, respectively), and confocal images of T cells (CD3: green) and B cells (B220: red) (E, G, I), and of macrophages (MAC2: red) (F, H, L) are shown for each time point analyzed. Nuclei are counterstained with DAPI (blue). Representative images of n=3 mice are shown. Scale bars: 100 μm.

While the addition of Peg to alginate throughout the capsule (MicroMix) did not appear to adversely affect the capsule biocompatibility, the capsules with a pure Peg external layer (Double) initiated a stronger host inflammatory response than to the MicroMix capsules **(Figure 25A,C,E)**. This observed inflammatory response was more frequently in response to pure Peg secondary beads formed during double encapsulation procedure. An early inflammatory cellular reaction was observed 7 days after transplantation. In addition, severe fibrosis surrounding both the Double capsules and the Peg secondary beads was observed at each time point **(Figure 25A-F)**. We hypothesize that the observed fibrotic capsule

overgrowth together with the macrophage recruitment **(Figure 25H,L,N)** to the capsules likely creates an unfavorable microenvironment for islet engraftment and long-term function. This type of response is associated with the release of inflammatory cytokines that are cytotoxic to the islets.
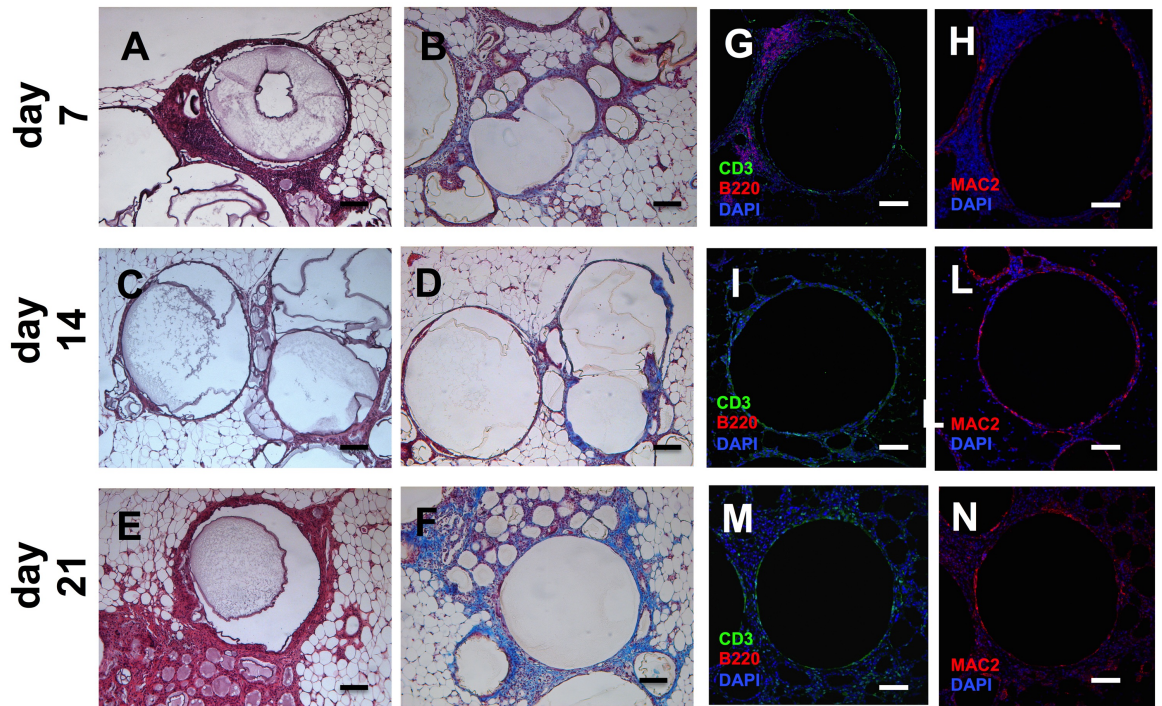
Figure 25. Empty Double coated capsules were transplanted in the EFP of C57BL/6 mice to assess the biocompatibility of the capsule material 7, 14, and 21 days after implantation. H&E images (A, C, E, respectively), Masson's Trichrome images (B, D, F, respectively), confocal images of T cells (CD3: green) and B cells (B220: red) (G, I, M), and of macrophages (MAC2: red) (H, L, N) are shown for each time point analyzed. Nuclei are counterstained with DAPI (blue). Representative images of n=3 mice are shown. Scale bars: 100 µm.

Our results suggest that despite similar Peg-alginate composition, the surface properties of MicroMix and Double capsules induce different host responses after implantation. The presence of Peg-Mal mixed with alginate throughout the capsule

(MicroMix) does not induce a pronounced inflammatory response. It is likely that the inflammatory response that we observed for Double capsules depends on the direct contact of the Peg-Mal double layer with the host tissue. Our hypothesis is supported by the inflammatory reaction that we observed in the tissue surrounding the pure Peg secondary beads. To address this issue and confirm our hypothesis that poor biocompatibility of Double capsules was associated with the amount of pure Peg in double coating and in secondary beads, we further optimized the emulsion technique to minimize secondary beads contamination and to reduce the thickness of the Peg-Mal outer layer.

In some cases capsules could not be retrieved from the EFP. Additionally, an absence of capsules in some histological sections of retrieved tissue was observed. This might be related to the fixation and the staining procedure, during which the capsules could swell, break, and detach from the glass slides. Currently the protocol for fixation and sectioning of alginate-based capsules is being optimized to better preserve the capsule graft.

4.2.6. *Re-optimization of the emulsion technique*

To further optimize the emulsion procedure, reduce the amount of Peg in double coatings and minimize secondary beads   rat islets were first encapsulated in 1.2% UP-MVG microcapsules, and then immediately double coated. Phase contrast evaluation of optimized Double capsules **(Figure 26A,B)** demonstrated a lack of Peg secondary capsules and a minimal double coating thickness. To confirm the presence and uniformity of the Peg double coatings on alginate microcapsules, the anti-Peg immunostaining was performed. A thin, uniform layer (15±2 µm) of Peg was found on 100% of the Double capsules **(Figure 26C,D,)**. By increasing the

emulsion stirring speed the size of the Peg secondary beads was minimized. N=3 rounds of post-emulsion purification served to eliminate any remaining secondary beads. To determine the effect of increased stirring speed on viability, Live Dead confocal imaging and GSIR functional assessment were performed on islets encapsulated within optimized Double capsules. No adverse effect was observed on either viability or functionality of these islets relative to naked controls, even 48 hours post-encapsulation. (**Figure 26E**)
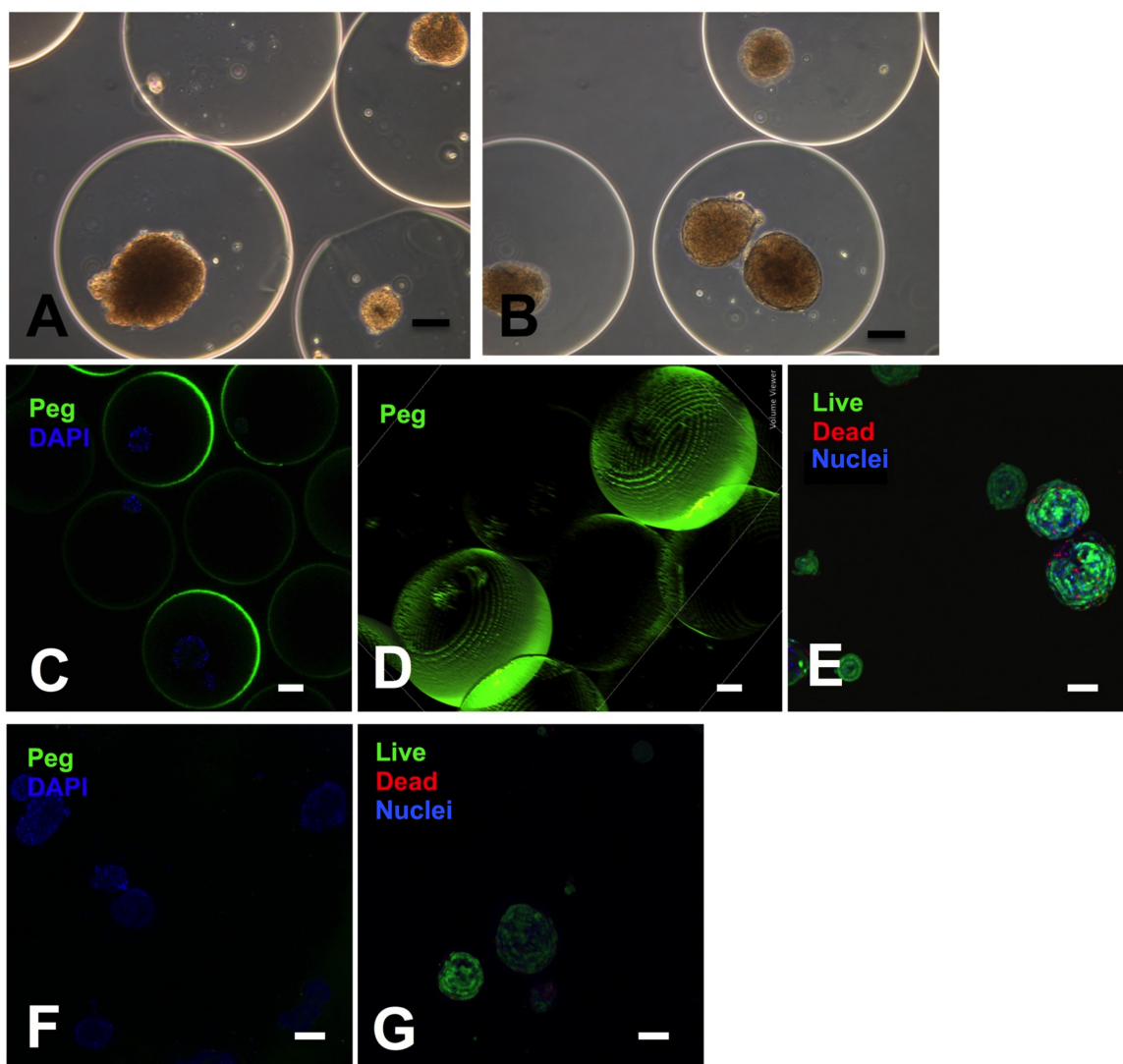
Figure 26. Coating completeness and viability of optimized alginate capsules double coated with Peg-Mal (Double capsules). (A,B) Phase contrast images of rapresentative Double capsules; confocal images of anti-Peg immunostaining (green): Z slice (C)

and 3D reconstruction of 200µm thick Z stacks are shown. (D) Live Dead staining of Double capsules 48 hours after double encapsulation. (E) Nuclei are counterstained with Hoechst (blue). Double capsules compared to uncoated alginate microcapsules: (F) and (G) confocal images of anti-Peg immunostained (green) and Live Dead staining of 1,2% UP-MVG uncoated microcapsules. Scale bars: 100 µm.

To exclude that the Peg double coating of rat islets either impaired or delayed insulin secretion in response to glucose the GSIR and the perifusion assay were performed compared to unencapsulated and alginate microcapsule controls. The. Double capsule had no effect on the timing of insulin secretion in response to glucose. There was not statistical difference between the index values of microcapsules or Double coated capsules, when compared to the naked islets control. For each experiment we have normalized the index of the encapsulated islets to the index of their naked controls (by post-hoc Dunn's test, P= nonsignificant) (**Figure 27 A,B**).
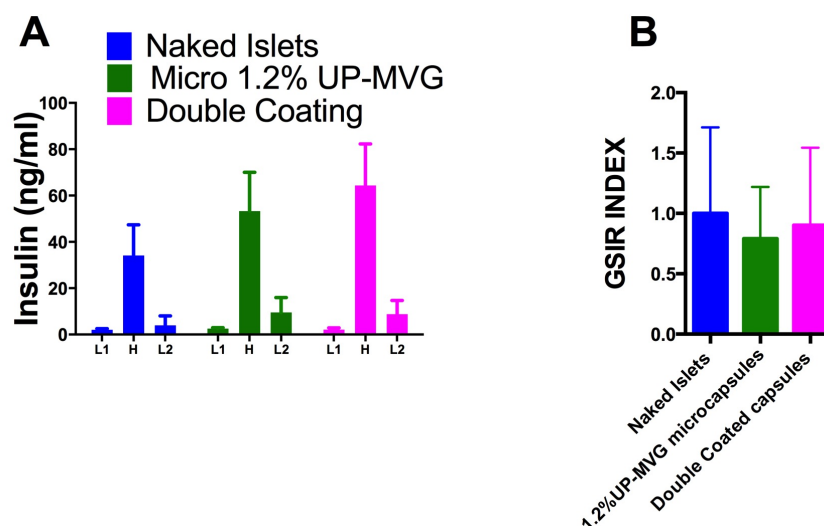
Figure 27. Assessment of function of naked rat islets vs. microcapsules vs. Double Coated capsules. (A) Absolute values of insulin concentration in supernatants for each incubation step (low glucose 1, high glucose, and low glucose 2). (B) Stimulation index normalized to naked islet index

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Additionally, when compared to both unencapsulated and microcapsule controls, islets within Double capsules showed comparable insulin secretory response to dynamic glucose challenge (perifusion assay, **Figure 28**). Encapsulated islet insulin secretion values were normalized to the LG secretion values of the naked islets.
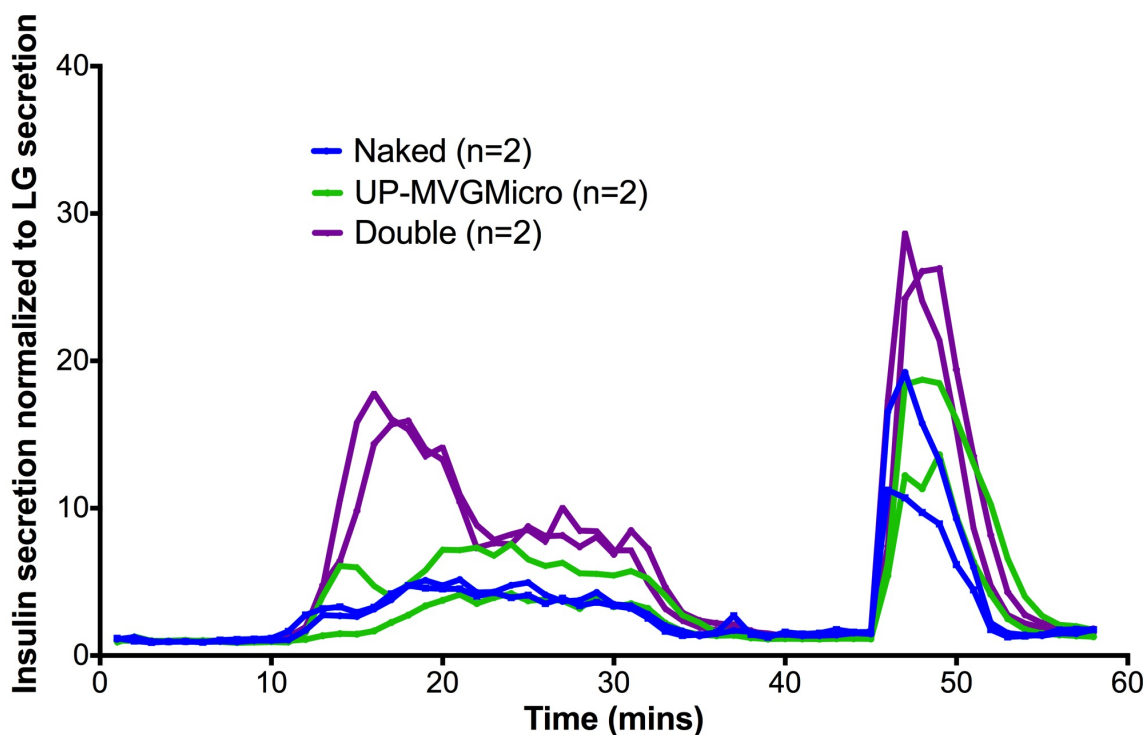
Figure 28. Assessment of insulin secretory response of naked rat islets vs. microcapsules vs Double Coated capsules.

4.2.7. *In vitro* comparison of viability and function of rodent islets after encapsulation in Micro vs. MicroMix vs. Double capsules.

Since the viability of pancreatic islets after encapsulation has been correlated with encapsulated islet graft survival, the effects on islet viability of the UP-MVG, UP-MVG/Peg-Mal hybrid and Peg-Mal Double coating encapsulation were carefully examined.

As schematized in **Table 4**, rat pancreatic islets were isolated on day 0, followed

by microencapsulation in 1.2% UP-MVG (Micro and Double) or 1.2% UP-MVG/5% Peg-Mal (MicroMix) 1 day after islet isolation.

	ISO day	ENCAPSULATION day	DOUBLE COAT day	ASSESSMENT (TX) day
TX #1	Day 0	Day 1	Day 1 or Day 2	Day 3
TX #2	Day 0	Day 1	Day 1 or Day 2	Day 4

TABLE 4. Timeline for evaluation of pancreatic islet viability and functionality after UP-MVG encapsulation, UP-MVG-Peg Mix encapsulation, and Double coating after UP-MVG encapsulation.

Further, the effects of performing the double coating with Peg on day 1 (right after the first alginate coating) or day 2 (24 hours after the first alginate coating) were compared.

Viability (through Live/Dead staining) and function (through GSIR) of encapsulated islets were assessed 1) at day 3: 48 hours after performing the Micro, MicroMix coatings and 24 or 48 hours after performing Double coating (day 3,**)**; 2) at day 4: 72 hours after performing the Micro, MicroMix coatings and 48 or 72 hours after performing Double coating (day 4**)**.

The goal of these studies was to determine the optimal time frame for the transplantation of maximally viable and functional encapsulated islets.

The encapsulation procedures used for both the alginate microcapsules and the MicroMix capsules had no adverse effect on islet cell viability after either 48 or 72 hours (day 3 and 4, respectively), as demonstrated by the Live Dead staining

(**Figure 29**).

Performing the double coating procedure at day 1 or day 2 post islet isolation (immediately or 24 hours after the alginate encapsulation) did not result in any detriment to islet viability, when assessment was performed at day 3 and day 4, 48 hours or 72 hours after double encapsulation (**Figure 29**). However, at earlier time points (24 hours after the double coating), cells viability was decreased, as indicated by a qualitative increase in the amount of core necrosis observed in L/D imaging. (**Figure 29**).
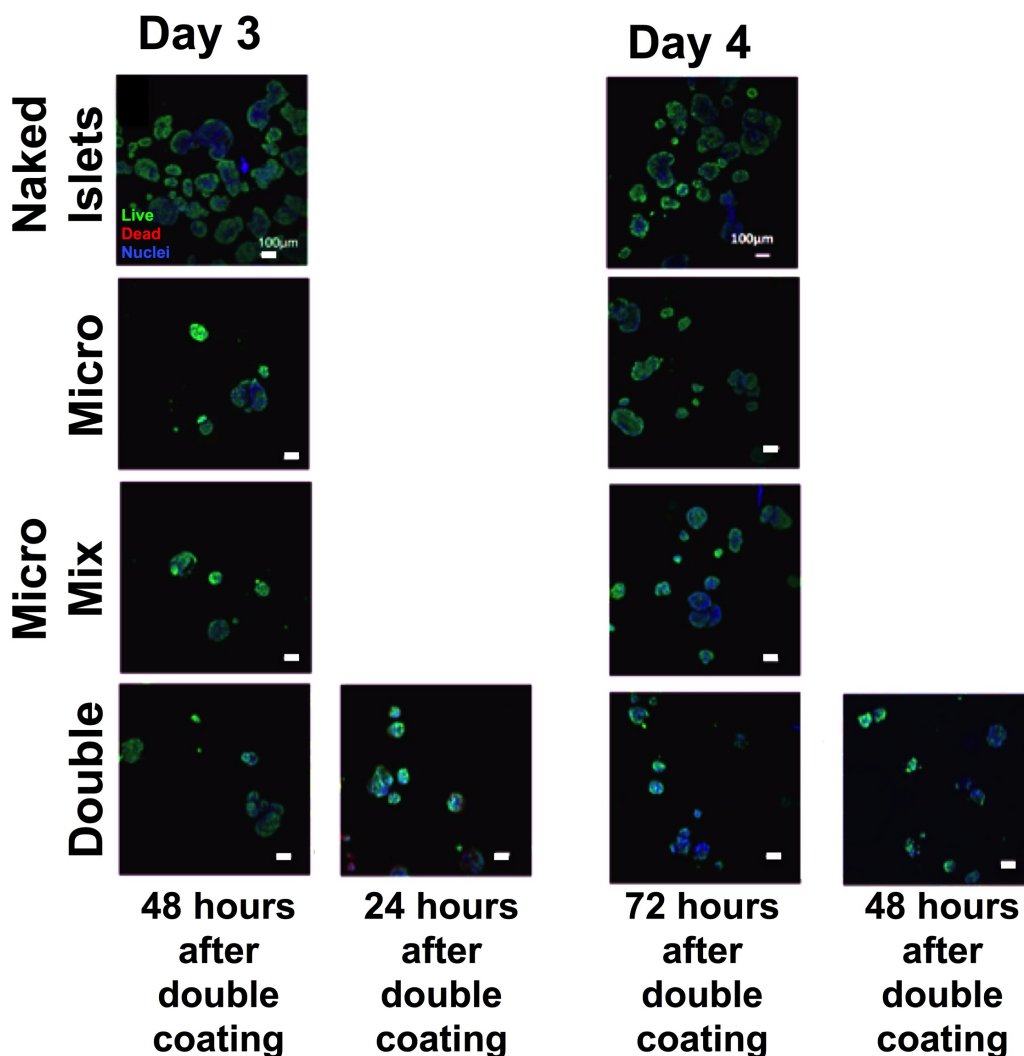
Figure 29. Assessment of Micro, MicroMix and Double capsules during culture after encapsulation and compared to naked islets. Confocal Live/Dead stained images of pancreatic islets at day 3 and 4 after isolation.

GSIR of islets encapsulated with the three utilized methods displayed no impaired function relative to naked islets, at all time points indicated in **Table 4**. For each time point (Day 3 and Day 4), there was not statistical difference (by post-hoc Dunn's test, P: nonsignificant) between the index values of microcapsules, MicroMIx or Double coated capsules, when compared to the naked islets control **(Figure 30)**. For each time point (D3 and D4) the index of the encapsulated islets was normalized to the index of their naked controls.
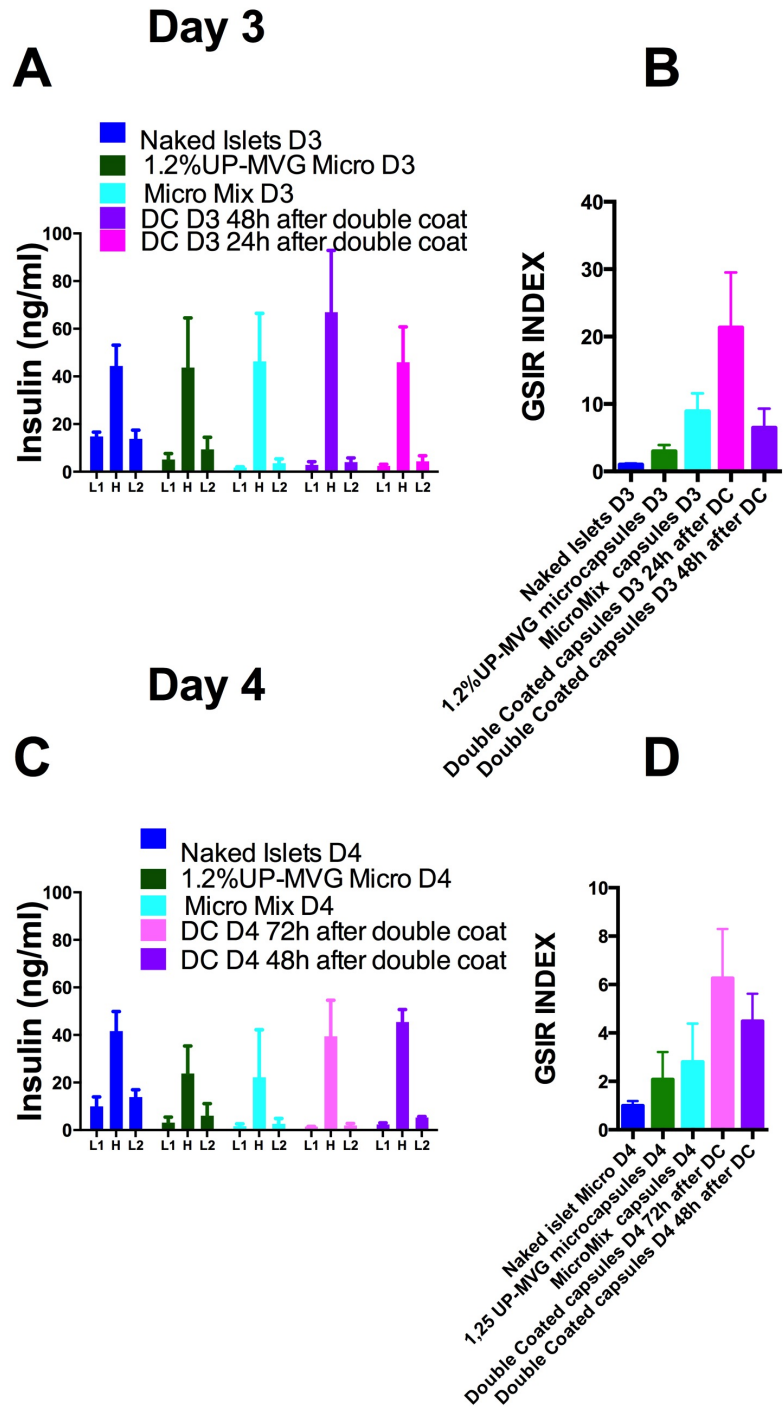
Figure 30. Assessment of Micro, MicroMix and Double capsules during culture after encapsulation and compared to naked islets. GSIR of pancreatic islets at day 3 (A,B) and day 4 (C,D) after isolation.

4.2.8 Determining the effect of capsule composition (microcapsules, MicroMix capsules and Double coated capsules) on *in vivo* performance of encapsulated islets.

Next, the effect of capsule composition on the performance of encapsulated islets: alginate microcapsules) vs. MicroMix capsules or Double coated was evaluated,.

In vivo performance was evaluated in fully MHC-mismatched allografts in mice in two transplant sites: the EFP and the IP sites.

a) EFP site

750 BALB/c IEQ were transplanted into the engineered EFP site of C57BL/6 recipients rendered diabetic by chemical treatment (STZ). All transplants were performed without immunosuppressive regimens. Hybrid MicroMix and Double coated capsules were compared to naked islets and those encapsulated in 1.2% UP-MVG microcapsules.

When implanted in the EFP site, islets within MicroMix capsules engrafted as well as naked islets (MicroMix median reversal time: 3 days vs. 1 day for naked islets, P=0.3). However, there was a significant difference in the engraftment time when compared to alginate microcapsules transplanted in the EFP (microcapsule reversal time: 1 day. P< 0.01) (**Figure 31A,B**). Islets within alginate microcapsules reversed diabetes in 7/7 mice that were transplanted, as islets enclosed in MicroMix in 6/7 mice. Hyperglycemic excursions were more prevalent in the MicroMix capsules when compared to naked and microencapsulated islets. Graft survival analysis performed on MicroMix capsules compared to microcapsules did not show a statistically significant difference (MicroMix median survival time: undefined vs. Micro median survival time: undefined, P=0.06). There was also an improved survival (P= 0.08) in MicroMix capsules when compared to

naked islets (median survival time undefined vs. 18 days) (**Figure 31C)**.

Islets within Double capsules displayed significantly inferior engraftment when compared to naked islets (P=0.02), microcapsules (P=0.001) and MicroMix capsules (P=0.03) (**Figure 31A,B).** Islets within double capsules reversed diabetes in only 3/8 mice that were transplanted. Islets within Double capsules poorly controlled fasting blood glucose of recipient animals when compared to naked and microencapsulated islets. Islets within Double capsules showed inferior survival compared to microcapsules (P<0.05). However, Double capsules displayed comparable survival to MicroMix (P>0.1) (**Figure 31C)**.

Figure 31. Effects of capsule composition on the *in vivo* performance of encapsulated islets in the engineered EFP site. 1.2% UP-MVG optimized microcapsules are compared to 1.2% UP-MVG-5% Peg-Mal hybrid MicroMix and to 1.2% UP-MVG double coated with 5% Peg-Mal hybrid Double capsules. Non-encapsulated islet controls are also shown. (A) Blood glucose of C57BL/6 mice rendered diabetic by STZ treatment and transplanted with 750 IEQ naked or encapsulated islets from fully MHC-mismatched BALB/c mice in the

EFP site without any immunosuppression; (B) Percentage of mice that reversed diabetes after transplantation of naked vs. encapsulated islets in the EFP site; (C) Percentage of naked vs. encapsulated islets that survived allorejection after transplantation in the EFP site.

b) IP site

750 IEQ BALB/c islets were transplanted into the IP site of C57BL/6 mice rendered diabetic by chemical treatment. All transplants were performed without immunosuppressive regimens. MicroMix and Double coated capsules were compared to naked islets and those encapsulated in 1.2% UP-MVG microcapsules.

When implanted in the IP site, islets within MicroMix capsules engrafted significantly faster than naked islets (MicroMix median reversal time: 2 days vs. undefined for naked islets, p < 0.01). When compared to microcapsules, MicroMix capsules displayed an increased rate of reversal (MicroMix median reversal time: 2 days vs. microcapsule median reversal time: 4 days. (P=0.007) (**Figure 32A,B**). Islets within microcapsules and MicroMix reversed diabetes in all the mice transplanted (5/5 and 3/3, respectively). The glycemic control afforded by both microcapsules and MicroMix transplanted in the IP site was qualitatively inferior to microcapsules transplanted in the EFP site, with more observed hyperglycemic excursions. Survival of islets within MicroMix capsules was comparable to islets within microcapsules (MicroMix median survival time: undefined vs. microcapsule median survival time: 73.5 days. P=0.5) **(Figure 32C)**.

Microcapsules (microcapsule median reversal time; 4 days. P=0.07) exhibited a trend towards increased IP engraftment compared to Double coated capsules (Double median reversal time: undefined), though the difference was not statistically significant. Islet within Double capsules showed a comparable engraftment to islets within MicroMix capsules (MicroMix median reversal time: 2

days. P=0.14). Islet containing Double capsules transplanted in the IP site displayed inferior control of blood glucose when compared to microcapsules transplanted in the EFP. Survival of islets within Double capsules was comparable to islets within microcapsules (P=0.14) and MicroMix (P=0.1) capsules, despite a smaller percentage of animals reversing diabetes (25%) **(Figure 32A,B, and C)**.

These studies confirmed that the IP site is sub-optimal for naked islets and all encapsulated islet formulations. The time to restore euglycemia in mice after transplantation of 750IEQ was between 1 and 7 days for 1.2% UP-MVG microcapsules. Furthermore, not all grafts survived allo-rejection for more than 100 days. Additionally, blood glucose levels fluctuated between 180mg/dl to 250mg/dl, suggesting that IP microcapsules do not exhibit tight control of fasting blood glucose levels, confirming previous results. MicroMix capsules reversed hyperglycemia in 100% of the mice in a mean reversal time of 2.5 ±1 days. Higher percentages of MicroMix mice survived allo-rejection for more than 100 days than those transplanted with microcapsules. This suggests that Peg reinforcement of alginate capsules via material mixing could improve the *in vivo* performance of encapsulated islets in the IP site, likely through improved capsule stability.

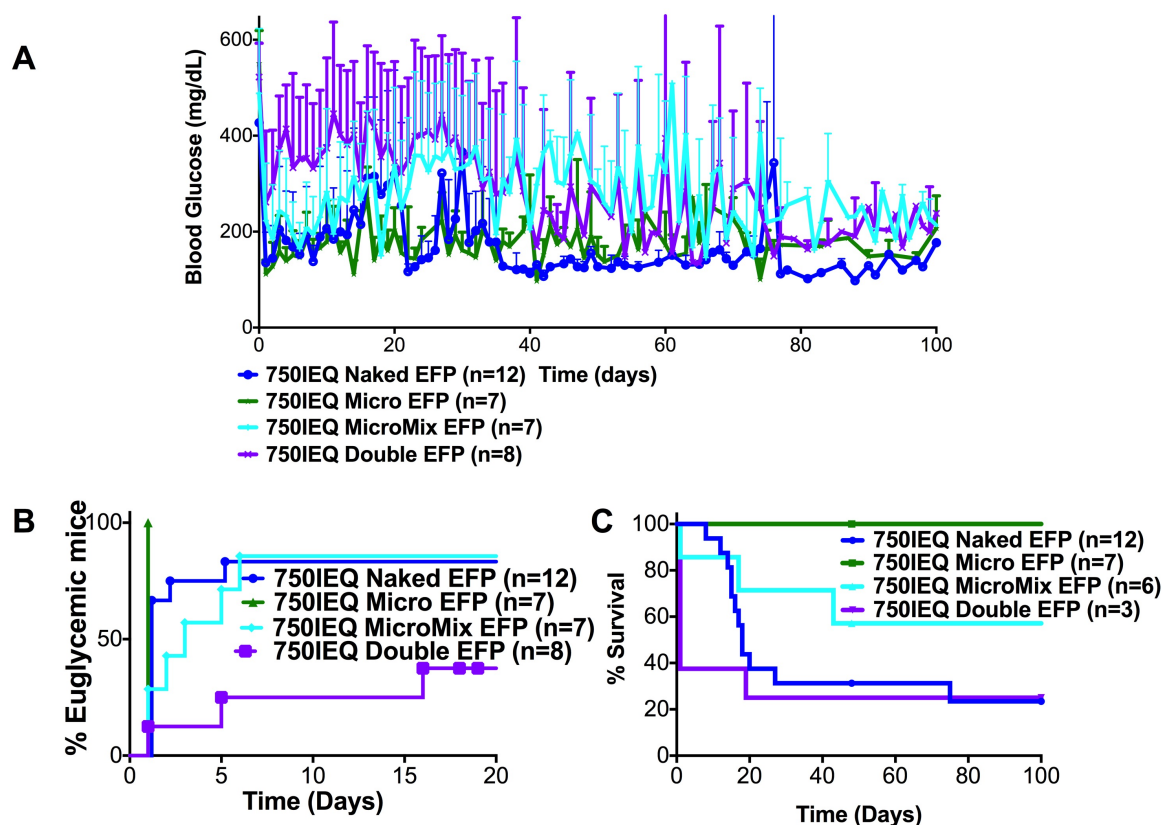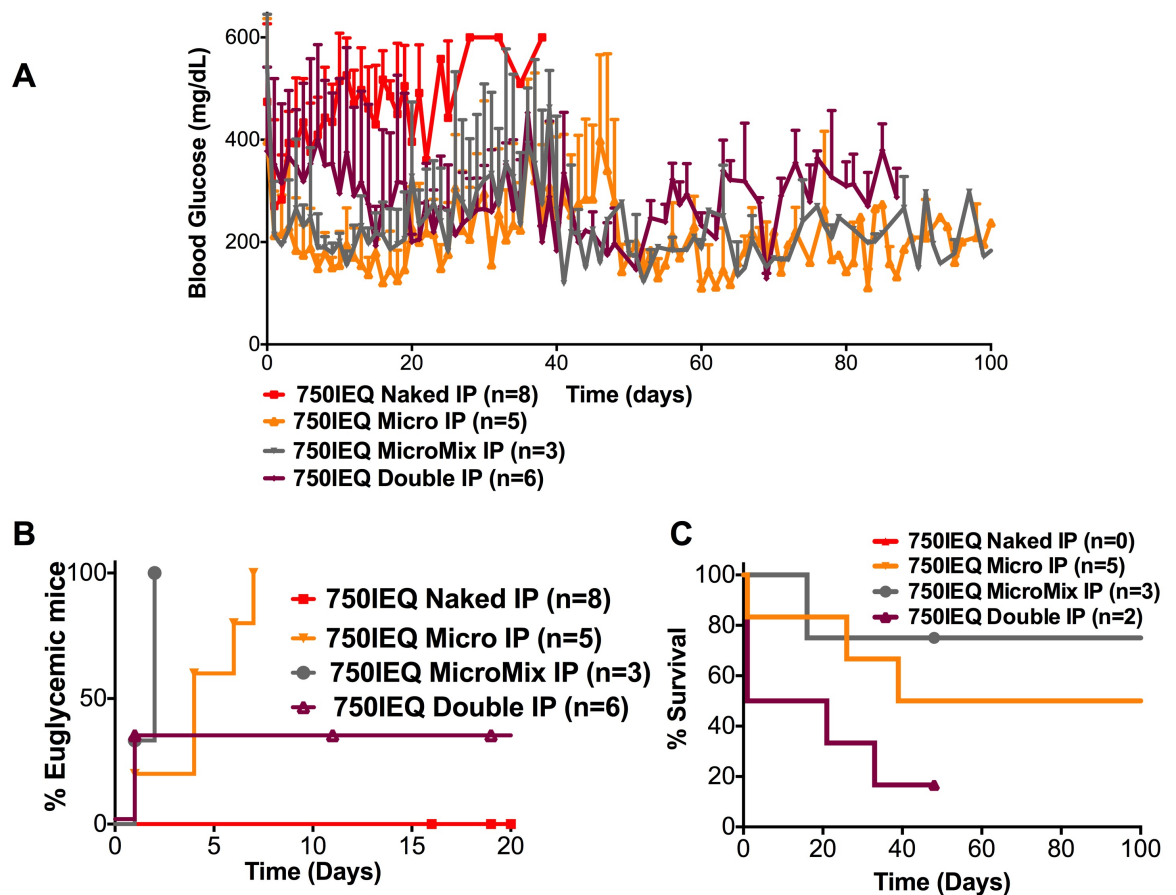**Figure 32**. Effects of capsule composition on the *in vivo* performance of encapsulated islets in the IP site. 1.2% UP-MVG optimized microcapsules are compared to 1.2% UP-MVG-5% Peg-Mal hybrid MicroMix and to 1.2% UP-MVG double coated with 5% Peg-Mal hybrid Double capsules. Non-encapsulated islet controls are also shown. (A) Blood glucose of C57BL/6 mice rendered diabetic by STZ treatment and transplanted with 750 IEQ naked or encapsulated islets from fully MHC-mismatched BALB/c mice in the IP site without any immunosuppression; (B) Percentage of mice that reversed diabetes after transplantation of naked vs. encapsulated islets in the IP site; (C) Percentage of naked vs. encapsulated islets that survived allorejection after transplantation in the IP site

4.2.9. Determining the effect of capsule transplant site (EFP vs. IP) on *in vivo* performance of encapsulated islets.

Our previous data confirmed that the transplant site is a key determinant of encapsulated islet *in vivo* performance. Further investigation was done to examine the effects of transplantation site (EFP vs. IP) considering one encapsulation method at a time. 750 IEQ BALB/c   islets were transplanted into the IP site of

C57BL/6 mice rendered diabetic by chemical treatment. All transplants were performed without immunosuppressive regimens

Engraftment of microencapsulated islets was higher in the engineered EFP site than in the IP site (median reversal time in the EFP site: 1 day vs. 4 days in the IP site, P<0.01). This suggests that the engineered EFP site is more conducive to islet engraftment **(Figure 33A)**. Survival of islets in microcapsules transplanted in the EFP site showed superiority relative to the IP site (P=0.04), suggesting that survival of islets within microcapsules is dependent on the transplantation site, independent of recipient allogeneic responses **(Figure 33A)**.

Engraftment of MicroMix encapsulated islets and glycemic control was qualitatively improved in the IP site compared to the engineered EFP site, though not statistically significant (median reversal time in the IP site: 2 days vs. 3 days in the EFP site, P=0.19). Unlike what was observed in microcapsules, survival of islets in MicroMix capsules transplanted in the IP and the EFP sites were comparable (P=0.62) (**Figure 33B**).

Engraftment of islets in Double capsules was comparable in the IP and the engineered EFP site (median reversal time in the IP site: undefined vs. undefined in the EFP site, P=0.99) **(Figure 33C).** Survival of islets in Double capsules transplanted in the EFP site was higher than in the IP site, though not statistically significant (P=0.9).
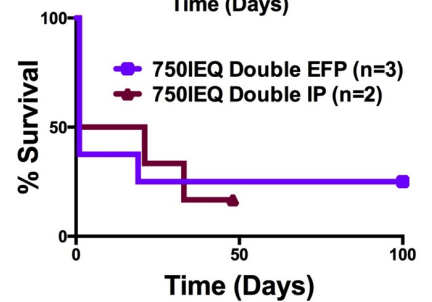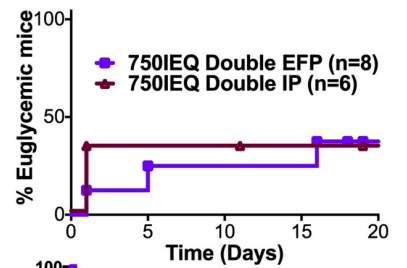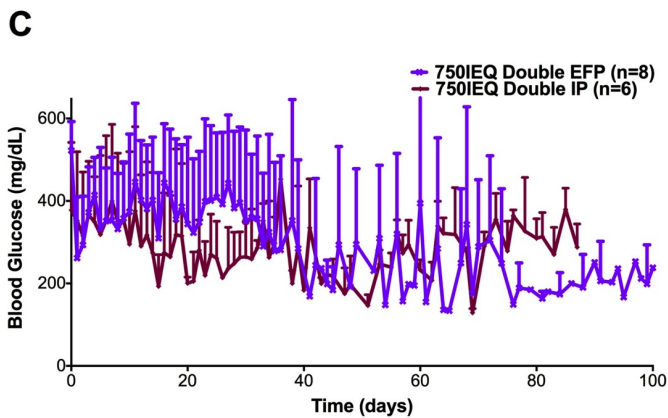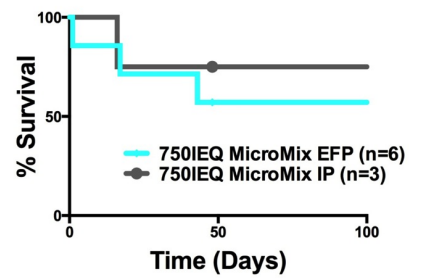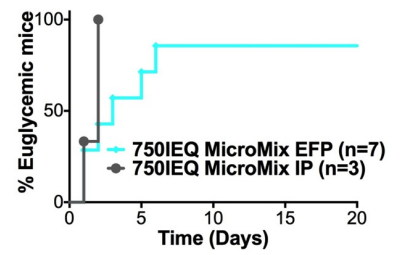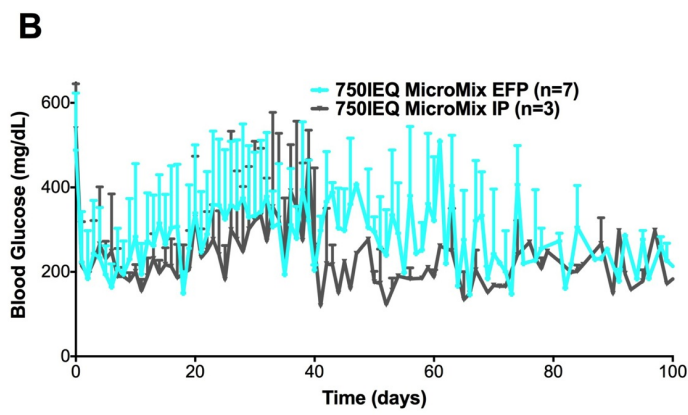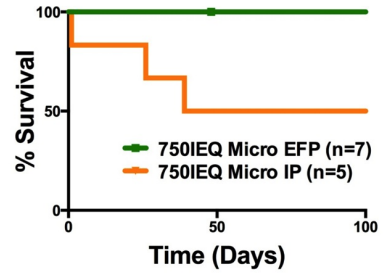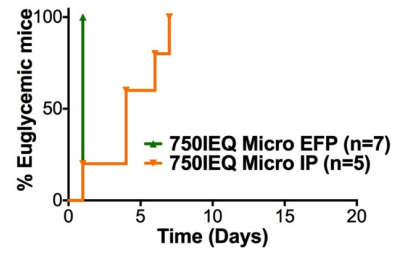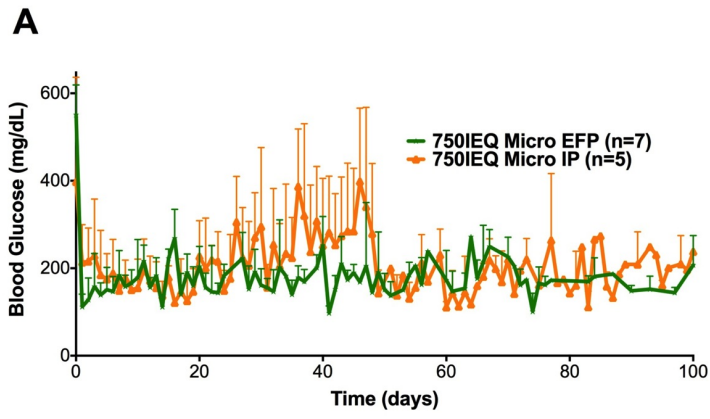
Figure 33. Effects of transplantation site (EFP vs. IP) on the *in vivo* performance of encapsulated islets. Blood glucose, percentage of mice that reversed diabetes, and percentage of encapsulated islets that survived allorejection after transplantation into C57BL/6 mice rendered diabetic by STZ treatment. (A) 750 IEQ islets from fully MHC-mismatched BALB/c mice encapsulated in 1,2% UP-MVG microcapsules. (B) 750 IEQ islets from fully MHC-mismatched BALB/c mice encapsulated in MicroMix capsules. (C) 750 IEQ islets from fully MHC-mismatched BALB/c mice encapsulated in Double coated microcapsules.

4.2.10. Correlating the histology of explanted grafts to the *in vivo* performance of islets encapsulated in Micro vs. MicroMix vs. Double capsules

Here, the *in vivo* function (long-term function) of fully MHC-mismatched allogeneic islets encapsulated (microcapsules, MicroMix or Double capsules) and transplanted (EFP or the IP site) in mice was investigated. Grafts were removed from the EFP site. Recipient animals were then monitored for the reversion to hyperglycemia insuring that post-transplant restoration of euglycemia was due to the function of the encapsulated grafts. Histological processing was then performed on the retrieved grafts, as described in the materials and methods. For islets transplanted in the IP site, the graft could not be retrieved (IP lavage) by survival surgery. After retrieval (100 days or upon rejection), EFP and IP grafts were fixed in formalin, embedded in paraffin, sectioned (5µm thick) and processed for H&E and immunofluorescence staining. Analyzing the H&E staining of grafts that functioned for more than 100 days after transplantation, immune cell infiltration was not observed in the majority of the Micro and MicroMix capsules. However, a few capsules, particularly those retrieved from the IP site, did present a thin surface layer of host cells (**Figure 34A,B**). Both Micro and MicroMix capsules were intact (no visible damage and/or fracture) and their spherical shape was preserved. The islets within the capsules retrieved from the EFP site had no evidence of degranulation, loss of integrity or central necrosis, indicating

maintenance of overall viability. Conversely, islets analyzed within the capsules retrieved from the IP site had some sign of central necrosis (**Figure 34A**).

The host inflammatory response to Double capsules retrieved from both the EFP and the IP sites was pronounced, unlike that observed in response to the Micro and MicroMix capsules, Double capsules permitted the intra-capsular penetration of immune cells, at least in the external part of the capsule, particularly evident in those retrieved from the peritoneal cavity. Additionally, Double capsules had compromised spherical shape and large portions of the capsule material appeared damaged. Islets within Double capsules, irrespective of retrieval site, displayed pronounced fragmentation and loss of pericapsular membrane, indicative of compromised viability (**Figure 34A**).

Immunofluorescent staining of the grafts explanted from the EFP site was also performed. Islets housed within microcapsules stained positive for both insulin and glucacon, confirming the presence of beta and alpha cells, respectively **(Figure 34B). The** immunoisolation of islets within microcapsules was confirmed by the lack of CD3$^+$ T cells, B220$^+$ B cells, and MAC2$^+$ macrophages within the capsule. Islets encapsulated in MicroMix microcapsules were also positive for insulin and glucagon, confirming cell viability and functionality (**Figure 34B**). The qualititative material and cell biocompatibility of microcapsules and their immunoisolating properties correlates well with the *in vivo* performance of microcapsules in the EFP site. Microcapsules and MicroMix capsules were immunoisolating, lacking infiltration of T cells, B cells and macrophages.

Unlike the microcapsules, MicroMix capsules were covered with one layer of macrophages, suggesting higher host reactivity to the capsule biomaterial than microcapsules, and correlating with their slower engraftment and lower survival than microcapsules implanted in the same site.

Islets encapsulated in Double capsules showed low insulin and glucagon staining, potentially in line with their sub-optimal function in the EFP site. The observed dysfunction of islets within the Double capsules could be attributed to an aggravated inflammatory response to the external layer of Peg-Mal, as observed in the cell-free material biocompatibility studies. Despite these observed inflammatory responses, reduced macrophage, and no T cell and B cell invasion of the capsules was observed.
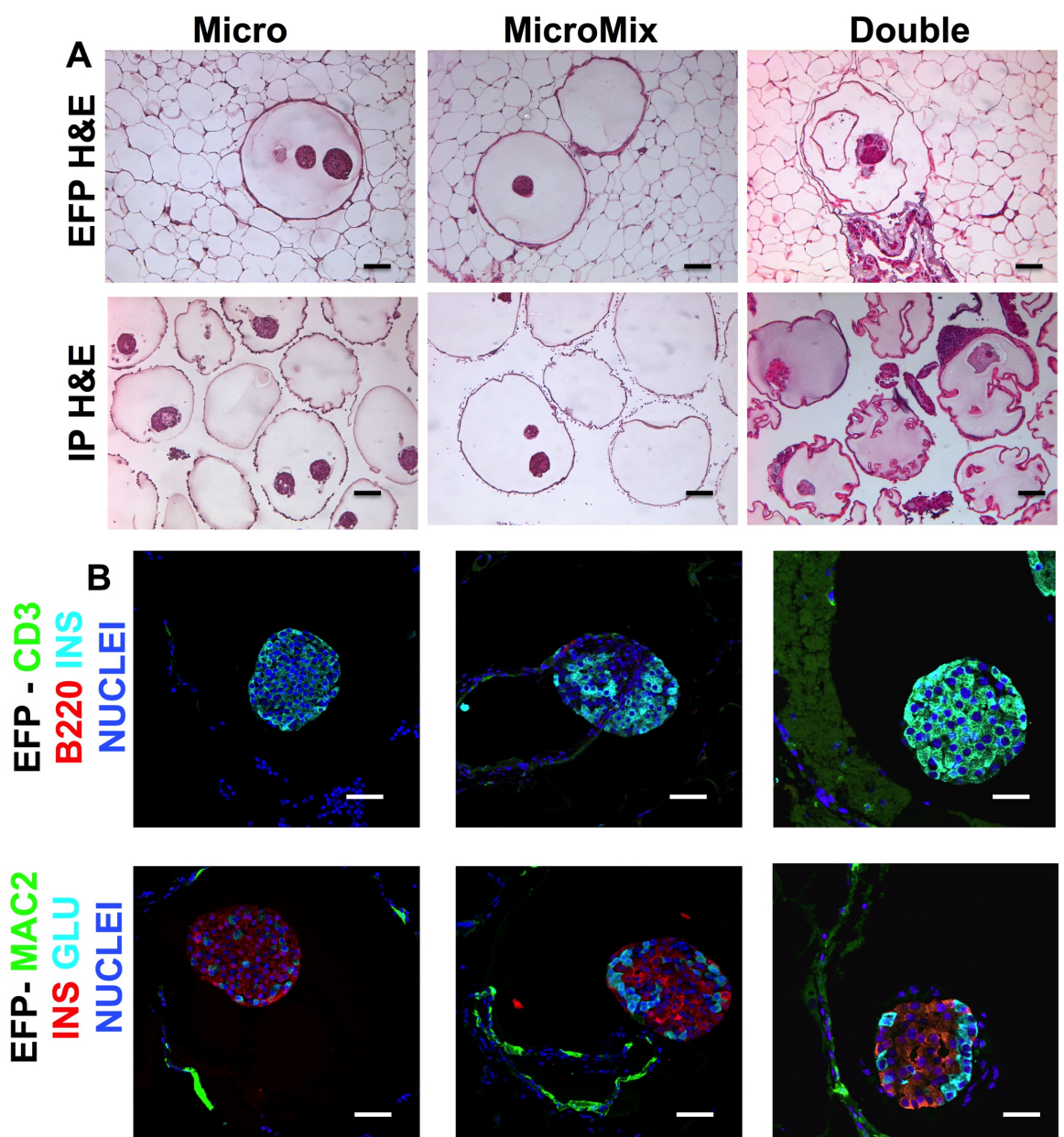
Figure 34. Characterization of fully MHC-mismatched islet allografts encapsulated in

Micro vs. MicroMix vs. Double capsules. (A) H&E staining of formalin-fixed paraffin-embedded EFP grafts (EFP) or capsules retrieved from the IP site through lavage. Micro and MicroMix reversed diabetes and maintained euglycemia both in the EFP and IP for more than 110 days after transplantation. EFP grafts for the Double maintained euglycemia for more than 110 days. IP grafts for Double capsules were retrieved 50 days after transplantation and were not functioning. (B) Confocal images of EFP grafts retrieved 110 days after implantation and immunofluorescently stained to identify T cells (CD3$^+$),B cells (B220$^+$), beta cells (INS$^+$), alpha cells (GLU$^+$), and macrophages (MAC2$^+$) Scale bars H&E images: 100 µm; Confocal images: 100µm.

Likely, graft failure of the Double capsules implanted in the EFP graft site was due to early inflammatory macrophage response to the biomaterial resulting in free radical damage to the islets, fibroblast recruitment and inhibition of oxygen and nutrient mass transfer. As these first grafts were explanted well beyond the period of early inflammatory response, Double coated EFP grafts rejected 21 days after transplantation were also retrieved and processed for staining (**Figure 35)**. Islets within Double capsules showed pronounced fragmentation with the loss of pericapsular membrane. Double capsules showed compromised shape and a pronounced host inflammatory response, extended in the proximity of the whole graft (**Figure 35 A,B)**. Confocal images showed MAC2$^+$ cells around the capsules and confirmed the absence of T and B cells (**Figure 35 C,D)**. Islets within Double capsules showed low insulin and glucagon (**Figure 35 C,D)**, in line with the compromised viability suggested by the H&E staining. These results may indicate that the early inflammatory response occurring for a short term after transplantation is the most critical for islet engraftment and survival. The principal immune players seem to be macrophages, which subsequently release bio-reactive agents (free radicals).

Further experiments will be required to elucidate the precise mechanisms of rejection. The observed cellular outgrowth from the biomaterials also needs to be

further investigated by immunostaining and flow cytometry for clarification.
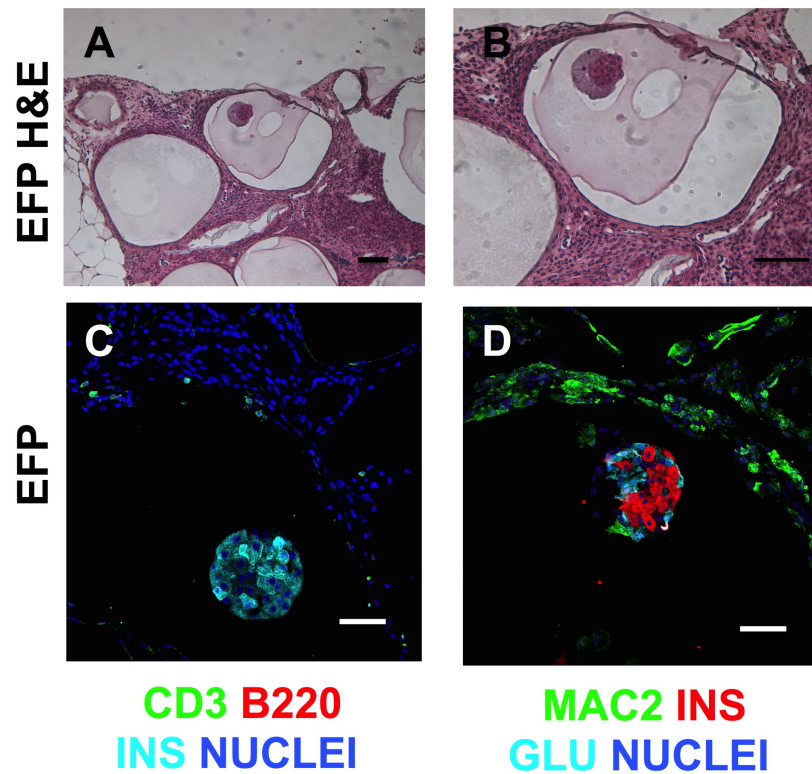
Figure 35. Characterization of fully MHC-mismatched Double coated encapsulated islet allograft. The graft was rejected 21 days after transplantation. (A) H&E staining of formalin-fixed paraffin-embedded EFP graft. (B) H&E staining of formalin-fixed paraffin-embedded EFP graft at higher magnification. (C), (D) Confocal images of EFP graft stained to identify T cells (CD3$^+$), B cells (B220$^+$), beta cells (INS$^+$), alpha cells (GLU$^+$), and macrophages (MAC2$^+$) Scale bars H&E images: 100 µm; Confocal images: 100µm.

5. DISCUSSION

Aim 1: Optimizing the traditional alginate microcapsules for clinical transplantation.

Encapsulation of a 200-µm islet in an 800-µm average diameter capsule is associated with a 64-fold increase in graft volume over naked islets, whereas graft volume increase is limited to 15.6 times when islets are enclosed within a 500-µm average diameter capsule. Reducing the average diameter from 800 µm to 500 µm is associated with a four-fold reduction of the total volume of the encapsulated graft. As a consequence of the large volume of islet grafts, islets encapsulated in 800-µm average diameter capsules can only be transplanted into the peritoneal cavity. Unfortunately, as discussed in the Introduction, the IP site is not ideal for islet transplantation because of nutrient mass transfer limitations and suboptimal oxygen supply [97]. Also, graft retrieval is difficult, which is a concern when sequential transplant procedures are required if a prior graft fails.

Therefore, the first aim of the present work was to develop a protocol to minimize the size of 1.2% UP-MVG microcapsules. Reduced alginate capsule diameter improves diffusion of nutrients and oxygen, pancreatic islet viability, and allows for transplantation in volume-limited alternative sites better suited for islet survival.

In our hands, the size of alginate capsules was consistently reduced to an average diameter of 500±1.01µm, without impairing their physical integrity and spherical shape. This allowed for an increased islet loading density of 15k IEQ/ml (~2 fold the standard recommended loading density of 8000/mL). This increased loading density would reduce the volume of microcapsules for transplant. Additionally, this higher loading density reduces the probability of unwanted empty capsules. There are, however, two drawbacks to reducing size and increasing cell concentration.

The first is the increased likelihood of necrotic cell core formation, due to the competition for oxygen and nutrients within a potentially overloaded capsule. The second is the presence of protruding islets [177] that could lead to an increased inflammatory response due to oddly shaped capsules or uncoated tissue. Choosing 15k IEQ/ml, we demonstrated that the encapsulated pancreatic islets maintain their viability and functionality, none exhibited islet protrusions exposed outside the capsule surface, and *in vivo* they did not showed evidences of necrotic core formation. This result is profoundly encouraging, considering the optimal loading density in the field of 8K/ml (~1.5% loading density) [177]. This was increased to nearly 3% loading density in our hands without impairing either pancreatic islet viability or capsule shape.

The use of reduced diameter 1.2% UP-MVG Microcapsules with high cell loading density could make a profound clinical impact by reducing transplant volume. Additionally, cytokine-induced damage to the microencapsulated islets has been shown to be minimal in ''reduced size'' (400–500 μm) capsules and increases with capsules smaller than this range [178,179]. This observation seems to confirm the high immunoprotective capacity of 1.2% UP-MVG microcapsules produced following our optimized protocol. In general, the developed alginate capsule intermediate range (400–500 μm) allows for a good balance between correct oxygen and nutrient diffusion (impaired for "bigger size"), and the decrease of cytokine-induced damage (increased for "smaller size").

The 1.2% UP-MVG capsules transplanted into NOD mice confirmed their immunoprotective properties, even in a preclinical model of autoimmunity and in a typically suboptimal site (IP).

In diabetic NOD mice, Barium gelled M-alginate capsules have been shown to

provide protection of xenogeneic adult porcine islet grafts for longer than 50 days in the absence of immunosuppression [180]. Further, these barium gelled M-alginate capsules have functioned as long as 466 days when transplanted IP into NOD mice with a continuous dual co-stimulatory blockade (Hong Cui transplantation 2009). However, in both cases the diameter of the capsules ranged between 850-880µm. Additionally, 9000 IEQ were transplanted in a volume of 0.8 ml (~2% v/v loading density). As intraportal transplantation of 20000 IEQ/kg is required for insulin independence in a human recipient, the only feasible site for 850-880µm encapsulated islet transplantation in a human would be the intraperitoneal cavity. A further risk is the use of barium, which is a known neurotoxin.

In our hands, we found that manufacturing microcapsules from UP-MVG alginate gelled in a calcium solution, we prevented several problems associated with poor M-group biocompatibility or and Ba^{2+} leakage/toxicity [107,177]. Even though several articles have presented the hypothesis that MVG calcium gelled microcapsules require a polycation coating to reduce the permeability and insure immunoprotection [110], our results showed the contrary in the NOD providing complete long-term immunoprotection, without an additional polycationic layer.

Our optimization of microcapsule composition, dimension, and islet loading density provided promising results for scale-up and further testing with complete protection from auto and allo-immune response, and excellent biocompatibility.

The only concern regarding NOD IP transplants was related to the site. The limits of the peritoneal cavity, in particular the poor vascularization, represent an important variable affecting the long term success/failure of the graft. Further experiments, will be performed to investigate alternate transplant sites, e.g. the engineered EFP, in the NOD mice, with a marginal mass of encapsulated

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islets.

In the second experiment we investigated the importance of the transplant site, considering the peritoneal cavity and the engineered EFP site. Encapsulated islets transplanted in the peritoneal cavity remain free-floating in the peritoneal fluid without direct vascular access. This adversely affects glucose-induced insulin release both in the sensing of glucose levels by the encapsulated islets and the circulatory uptake of the produced insulin. Glucose must first pass through the basement membranes of the capillaries in the peritoneal cavity. This process can take up to 5 min post-glycemic increase [181]. The released insulin then builds a gradient from islet to capsule surface before it is released via diffusion into the IP cavity and, further, into the systemic circulation [123]. The peritoneal cavity is also considered a harsh environment that can induce a pronounced inflammatory response and subsequently, overgrowth of fibrotic tissue around capsules [122,182]. The delay of insulin diffusion through the peritoneal membrane and possibly the cell overgrowth around capsules can explain: 1) the longer time observed in our studies to restore euglycemia, 2) the trend towards decreased survival (P<0.1) when compared to alginate capsules in the EFP), and 3) the presence of pronounced fasting glucose fluctuations.

Conversely, the combination of 1,2% UP-MVG Microcapsules and the engineered EFP site showed a significant performance improvement *in vivo*, in terms of engraftment and prolonged function

Likely, the performance of immunoprotective 1.2% UP-MVG microcapsules could be further improved through the engineering of islet-conducive site. The fibrin gel utilized in the studies increased the   vascularization with a long term beneficial

effect on islet engraftment and function. Additionally, the capsules wrapped within the EFP were likely subjected to less mechanical stress relative to the IP site.

The omentum is considered a favorable site for islet engraftment and survival in rodent and large animal models [134]. The engineered murine EFP site, as an omental pouch surrogate, has been used to assess the clinical relevance of the omentum as an alternate site for islet transplantation. As the gold standard for the assessment of islet quality and potency, islet transplantation is performed in an immune deficient murine model (NOD SCID or Athymic Nude).

For these reasons, NHP islets were transplanted in the renal subscapular space of NOD SCID mice rendered diabetic by chemical treatment. These served as potency controls for each transplanted islet preparation. Typically, the NHP islets reverse diabetes in a few days post transplant under the kidney capsule. If they are not able to reverse diabetes, it means that the islet potency is suboptimal. The results of the kidney capsule transplants showed that full mass (2000 IEQ) and marginal mass (1000 IEQ) NHP islets were fully functional, reversing diabetes rapidly at both does. In further study, 750 NHP IEQ encapsulated in 1,2% UP-MVG and transplanted in the engineered EFP were able to reverse diabetes and maintain euglycemia for the entire experiment period. Interestingly, full and marginal doses of naked islets transplanted in the engineered EFP displayed a significantly reduced functionality relative to the naked islets in the kidney and to the Microcapsules in EFP. This suggests that the EFP site is more conducive to islet engraftment when encapsulated but not when in direct contact with the epididymal tissue.

The combined effect of the rich   vascular supply and mechanical protection

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afforded by the engineered EFP and the immunoprotection provided by the optimized microcapsules is promising in the perspective of clinical translation.

Aim 2: Determining the effect of key capsule parameters on the in vivo performance (engraftment and long-term function) of encapsulated islets

The ability of alginate to resist changes in the *in vivo* microenvironment is a determining factor for long-term functional survival of encapsulated islets. Even though an alginate with high G-group concentration was utilized, resulting in stronger and more mechanically stable capsules [104], the capsules swelled and completely solubilized in 0.9% normal saline. Peg-Mal, on the other hand, is stable in saline solution, resisting breakage or size changes due to swelling. This suggests that a combined use of alginate and Peg-Mal might have a positive effect on the long-term performance of encapsulated islets, preventing *in vivo* instability and compromised immuno-protection due to swelling or breakage. In line with this hypothesis, some published studies have reported the use of Peg as double coating to restrict the pore size and simultaneously increase the mechanical properties and durability of alginate microcapsules [159-161]. However, Peg-Mal as material for encapsulation is suboptimal due to the following issues. First, the use of reactive thiol crosslinkerrs (DTT), known to induce direct cytotoxicity during culture and during the encapsulation procedure and second, the restricted capsule pore size has been shown to limit nutrient delivery to encapsulated cells. However, the issue of pore size/nutrient transport can be addressed by decreasing the capsule wall thickness [159,160].

The results from the direct exposure cytotoxicity assay confirmed the limitations related to the use of Peg.

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DTT/Thiol crosslinker cytotoxicity represents a significant obstacle to clinical implementation of Peg. Toxicity of Peg crosslinked with DTT is due to the well-established direct cytotoxic effect of DTT or, additionally, could be due to extended duration of exposure. In the study, leeching of the Peg from formed gels was observed (data not shown) and as the DTT is supplied in exact molar ratio to the functional groups of the Peg, we cannot exclude that this leakage could represent a loss of functional groups (Maleimide) during storage. This excess DTT can freely diffuse or remain entrapped inside the Peg-Mal gels, with a direct cytotoxic effect on the cells during the encapsulation procedure and during post-procedure culture. The observed, though non-significant, cytotoxicity of Peg-Mal gels needs to be taken into account because upon encapsulation, pancreatic islets remain in direct contact with the encapsulating material for the duration of graft survival. A potential advantage of both the MicroMix and Double hybrid encapsulation methods is that the islets are in direct contact with Peg-Mal to a lesser degree, likely preventing the cytotoxic effects observed with Peg-Mal gels crosslinked with DTT and DTT alone, as our viability and functionality results would indicate. The observed DTT cytotoxicity presents a formidable challenge because this cross-linker is one of a few that can be utilized for the formation of Peg-Mal gels. One possible solution is to decrease the amount of DTT used for Peg gelation or the time for gelation. Alternatively, other appropriate cross-linkers, such homo-functional linear or branched Pegs at different molecular weights could be investigated and screened. However, it is important to highlight that complete gelation is required to guarantee desirable mechanical properties and to avoid leakage of excess cross-linker from the capsules after polymerization. Presently, studies have demonstrated that only DTT meets these critical properties [48].

One possible factor contributing to the loss of islet viability and function after

transplantation is insufficient biocompatibility of the capsules. Alginate is generally considered a biocompatible material in both the transplant microenvironment and for the encapsulated cells. Whether the biocompatibility of alginates is dependent on the M/G ratio is debated. It has been shown that alginates containing more than 35% M-groups are potent stimulators of the immune system, due to the mannuronic acid oligomers capacity to provoke macrophage cytokine release [84], while alginates with high G-group concentration have been found to be less reactive. For all of the presented studies 1.2%UP-MVG alginate was utilized containing an endotoxin level of less than 100EU/g, to avoid the inflammatory response due to impurities or high M-group incompatibility.

The results from our *in vivo* biocompatibility test showed direct correlation between Peg-Mal amount and reduction of biocompatibility. The 1.2% UP-MVG Microcapsules had superior biocompatibility compared to both of the hybrid capsule formulations. The inflammatory response against the MicroMix was qualitatively comparable to the microcapsules except for a more evident collagen deposition at day 14. Conversely, Double coated capsule showed a marked inflammatory response, with cell migration, overgrowth and collagen deposition. In particular, this pronounced host response seemed amplified by the presence of empty/cell-free Peg-Mal double capsules in the EFP. One possible explanation is that high concentrations of secondary Peg-Mal beads could lead to exacerbated material response, macrophage recruitment and cytokine release resulting in fibrotic capsule overgrowth and the production of an unfavorable microenvironment for islet engraftment and long-term function.

After these initial studies were performed*, in vivo* studies were halted and two procedural optimizations were undertaken in an attempt improve the hybrid

encapsulated islet transplant results:

1) Re-optimization of the Double coated capsules

The re-optimization of the double coating emulsion procedure was effective at reducing the amount of Peg-Mal in the double coating and minimizing the presence of unwanted secondary beads. The new process had no negative effects on cell viability and functionality, as confirmed by the assessment (Live Dead, GSIR, and perifusion).

2) Screening of the optimal time frame for the transplantation

An important requirement for successful cell encapsulation is that the encapsulation procedure itself should not be harmful to the cells. Since pure alginate encapsulation is generally performed under physiological conditions and doesn't require the utilization of toxic crosslinkers or chemicals, a negative effect of the alginate microencapsulation procedure on islet viability is not expected [183]. However, it is widely accepted that pancreatic islet encapsulation affects not only islet viability and insulin secretion [184], but also induces cell stress, and the release of inflammatory cytokines from the encapsulated cells.

No statistically significant difference was observed on post-procedural islet viability and function between all the conditions, suggesting that none of the encapsulation procedures have negative effects on pancreatic islets viability and functionality. However, double coated islets at 24 hours post-procedure were more stressed than at the other screened time points, with evidence of decreased viability, as observed with Live Dead staining.

The ideal time frame for microcapsule and MicroMix encapsulation was found to be 24 hours after the pancreatic islet isolation, while for the double coating after

either 24 or 48 hours. For all procedural conditions the *in vivo* experiments were performed 96 hours after the isolation.

In this way, transplantation of sub-optimally viable and functional encapsulated islets was minimized. In this way, the variables affecting *in vivo* results are limited to the host immune response (cell-mediated and material- mediated) to either the materials or the encapsulated cells.

Transplantation of alginate encapsulated BALB/C islets into diabetic C57BL/6 utilizing the newly optimized methods led to confounding results. They again confirmed the superior performance of 1.2% UP-MVG microcapsules, particularly evident in the engineered EFP. Further results suggested that the presence of Peg-Mal in the MicroMix configuration improves the *in vivo* engraftment of alginate microcapsules in the IP site. However, they displayed only a trend towards increased rate survival compared to microcapsules transplanted IP. Additionally the survival of islets within the MicroMix did not present statistical difference relative to the survival of microencapsulated islets within the same site. Conversely, MicroMix capsules transplanted in the EFP showed a significantly reduced engraftment relative to microcapsules.

There are a few proposed reasons for the comparable performance of hybrid capsules in the IP site. The improved performance of MicroMix capsules in the IP site relative to the EFP might be due to their improved mechanical properties through the addition of Peg-Mal particularly resistance to osmotic stress imparting an added strength *in vivo*.

Double capsules displayed significantly inferior engraftment comparing to all the other conditions. Only 3/8 mice reversed in the EFP site. It is possible that there is a critical phase in the early inflammatory stage immediately post-transplantation. Short-term graft can be attributed to the host cells covering the capsules, and

resulting in cell death from induced hypoxia or starvation. Also, there is a potent cytokine/reactive species mediated mechanism, caused by macrophage recruitment. This mechanism may also explain why immune cells (macrophages; T or B cells) were not observed in the explanted graft. Grafts were explanted long after the early inflammatory phase had likely subsided and the cells had been destroyed by penetrating molecules (superoxides or other radicals). It is likely that islets surviving this acute phase may function long-term. This is evidenced by the fact that double coated islets transplanted IP showed an inferior, even though not statistically different engraftment, for example. Likely, in the IP site, the unfavorable characteristics of the site coupled with the poor biocompatibility of the Peg external layer might exacerbate the poor *in vivo* Double capsule islet performance. In the EFP, the direct tissue/material contact modulated host inflammatory response to Double capsules may negate the possible mechanical reinforcement benefits of the Peg-Mal.

Of note, for all the formulations in the IP site we observed a lack of blood glucose homeostasis. This could be due to the fact that the IP site has reduced oxygen tension due to low vascular density. Additionally, the fluid cavity does not easily allow for rapid revascularization of the islet graft nor is it conducive to engineering for the promotion of angiogenesis, as performed in the EFP. The lack of vascularization and the reduced oxygen tension in the IP site likely results in islet hypoxia and core necrosis, as previously described. As a consequence, graft failure and delay in physiological insulin response can occur. Moreover, for the alginate/Peg-Mal capsules, the presence of Peg/Mal reduces pore size and increases permselectivity,likely exacerbating mass transfer. It is not known, yet, if these changes increase the immunoprotective properties of the MicroMix and Double coated capsules without impairing the diffusion of other critical molecules.

Decreased permeability to nutrients and oxygen could be resultant from a Peg outer layer placed around the alginate. .

These results suggest that Peg-Mal as an outer coating for alginate microcapsules, rather than when mixed throughout the capsules (MicroMix), interfere with nutrient permeability properties and trigger a host immune reaction.

Unfortunately, even though we attributed the low biocompatibility of the Double coated capsules to the presence of the empty Peg beads, their presence only exacerbates the inflammatory response, as seen in the biocompatibility test. It is clear that even a small layer of Peg-Mal in direct contact with the host tissue can have negative effects on the transplant outcome. This result is in line with the observed cytotoxicity test results. Further experiments will be performed using a decreased amount of DTT (preventing leakage of excess cytotoxic crosslinker) or screening different crosslinkers.

We hypothesize that the MicroMix "configuration" shows a superior performance to the double capsules because it has intermediate properties between alginate and Peg-Mal, in terms of mechanical properties, permselectivity, and biocompatibility. As the Peg-Mal is homogeneously distributed inside the capsules, the amount of Peg-Mal in direct contact with the host tissue is reduced relative to the outer layer of the double capsules. Additionally, in the MicroMix encapsulation procedure, the exposure of the pancreatic islets to DTT is decreased relative to the Double coating procedure (1 minute vs. 15 minutes).

Further experiments will be performed to assess if the MicroMix islet capsules can successfully engraft in the fully immunecompetent/autoimmune NOD model, in the EFP and IP sites.

Future work will more throughly examine the mechanical properties of microcapsules relative to the MicroMix and Double capsules, to evaluate whether the addition of the Peg-Mal improves the mechanical stability of alginate capsules.

In order to understand whether engineering the EFP site with pro-angiogenic matrices contributed to the enhanced engraftment observed for microcapsules, future studies are planned in which engraftment of islets within microcapsules will be compared varying the amount and type of growth factors. Additonally, synergistic signaling between growth factor receptors and integrin receptors will be studied to determine if there is improved engraftment of encapsulated islets.

To determine if immunoisolation of microcapsules is affected by transplant site, and the site-specific host reactions, future transplants in the absence of allogeneic rejection (syngeneic transplants) will be performed. In particular, these studies will focus on innate immune components, such as early inflammatory response, in the absence of adaptive response. Additionally a third transplant site, such as the intramuscular or subcutaneous site will be evaluated.

Overall the main conclusion of our study is that encapsulated islet engraftment and long term functionality can be achieved through a synergy between all the capsule properties (material composition, dimension, permselectivity, biocompatibility), the site of transplantation and type of transplant. Only an accurate analysis of all the process critical parts, starting from the choice of the material to the transplant procedure, will lead to a widespread success in the field of pancreatic islet transplantation.

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