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Inorganic Nanoporous Membranes for Immunoisolated Cell-Based Drug Delivery

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Abstract

Materials advances enabled by nanotechnology have brought about promising approaches to improve the encapsulation mechanism for immunoisolated cell-based drug delivery. Cell-based drug delivery is a promising treatment for many diseases but has thus far achieved only limited clinical success. Treatment of insulin dependent diabetes mellitus (IDDM) by transplantation of pancreatic β -cells represents the most anticipated application of cell-based drug delivery technology. This review outlines the challenges involved with maintaining transplanted cell viability and discusses how inorganic nanoporous membranes may be useful in achieving clinical success.

Introduction

Cell-based drug delivery has been proposed as a treatment for diseases characterized by cell degeneration including Parkinson's disease,^{1,2} testicular dysfunction and hypogonadal disorders³ and liver failure.⁴ However, the driving force behind cell-based drug delivery research has been to improve the treatment of insulin dependent diabetes mellitus (IDDM). IDDM is characterized by the loss of pancreatic β -cell function which normally regulates the blood-glucose concentration by the secretion of insulin. Without functional β -cells, chronic hyperglycemia can lead to complications including retinopathy, neuropathy, nephropathy and death. Healthy β -cells secrete insulin in quantities that are highly sensitive to the blood-glucose level and successful IDDM treatment requires the same sensitivity to avoid debilitating events.

The first major advancement in treating IDDM occurred in 1922 with the first successful clinical trial using insulin.⁵ Unfortunately, while insulin-replacement therapy has saved countless lives, 82 years later in 2004 diabetes remained one of the most deadly diseases, ranking 6th in the United States.⁶ The most common insulin-replacement therapy requires frequent blood-glucose measurement through finger pricks as well as multiple insulin injections per day. The most advanced insulin-replacement therapy is approaching its ultimate goal of a closed-loop artificial pancreas, consisting of an artificial glucose sensor

coupled to an insulin delivery pump.⁷ So far the development has fallen short of its goals for two reasons. First, a fully implantable long-term insulin pump has not yet achieved clinical success, requiring the user to wear an external pump. Second, development of a long-term artificial glucose sensor remains elusive in part because of protein adsorption causing measurement drift, thus requiring frequent sensor calibration through finger pricking. As a result, while the current technologies offer remarkable advances for insulin-replacement therapy when used appropriately, proper treatment requires constant user attention. Lastly, even if glucose-sensing technologies improve, the algorithms with which the sensor communicates information to the pump to modulate insulin delivery kinetics represents only an approximation of blood-glucose regulation in healthy patients. Several companies continue to research towards a closed-loop artificial pancreas, including Medtronic Minimed Inc. and Roche Diagnostic's Disetronic.

Cell-Based Drug Delivery

An alternate approach to the replacement of insulin in treating IDDM is to transplant functional pancreatic β -cells either alone or as part of the Islets of Langerhans. The transplanted cells will sense extracellular glucose levels and secrete insulin accordingly, improving upon free drug delivery by eliminating the need for patient compliance and by enabling a more physiological regulation of glucose levels. While possessing greater therapeutic potential, cell-based drug delivery will not become widely accepted until its efficacy equals or surpasses that of insulin replacement therapy while offering decreased patient complications. Despite the promised benefits of cell-based drug delivery, however, sufficient transplant viability has not yet been achieved.

One challenge involved with cell-based drug delivery is immune-mediated destruction of the transplanted cells. The immune system can destroy transplanted cells through a variety of mechanisms. The most severe modality characterized by transplant rejection within minutes, called hyperacute rejection, has not frequently occurred with islet transplants in rodent models.⁸ The most common islet transplant rejection modality is a delayed antibody response for which the dominant mechanisms differ between allotransplants and xenotransplants. For allotransplants, antibody binding usually occurs with antigens presented on major histocompatibility complex (MHC) class I molecules on the surface of a cell. The MHC complex varies among a species more than the attached expressed peptides. As a result, peptides shed from an allogenic cell are unlikely to be recognized by antigen-presenting cells (APC's) for activation of the indirect presentation pathway. On the other hand, antibodies will recognize the variation in the MHC complex for activation of the direct presentation pathway. Xenotransplants express peptides that differ from those of the host and can be more potent activators of the indirect presentation pathway, resulting in B-cell activation and the production of secreted forms of antibodies that can target the transplanted cells. As a result, allotransplants in general are thought to be sufficiently protected by avoiding direct cell-cell contact whereas xenotransplants require the isolation of antibodies as well. It should be noted that allotransplants can also elicit the indirect antigen presentation pathway leading to destruction, although to a lesser extent than that from xenotransplants and therefore antibody isolation will likely result in improved viability of allotransplants as well.

An additional rejection modality for islet transplants is the production of macrophage-activating factors when under stress.⁹ Islet transplant viability has been correlated with the release of monocyte chemoattractant protein-1 (MCP-1) and tissue factor (TF).¹⁰ These cytokines are associated with macrophage recruitment and activation. Upon activation, macrophages release inflammatory cytokines including tumor necrosis factor- α (TNF- α) and interleukin 1- β (IL-1 β), which are implicated in β -cell death.⁹ Interestingly, one study suggests that bovine islets are less susceptible to human cytokines than they are to bovine cytokines, suggesting that xenogenic cells might be better able to survive a cytokine response than allogenic human cells.¹¹ Therefore, an important consideration in islet transplantation is providing an environment which limits the production of macrophage-activating factors.

The only chance of avoiding the above immune responses without immunosuppression or immunoisolation is to transplant cells that are genetically identical to the patient. For Type I diabetics, these are the cells that have degenerated and are therefore not available as autografts. Furthermore, Type I diabetes is thought to have an autoimmune etiology and therefore even the transplantation of pancreatic β -cells that are genetically identical to the recipient will be subject to the same degeneration that originally caused the patient's disease. Recently in Brazil, one study demonstrated the potential of autologous bone marrow-derived pancreatic stem cell transplantation following immune ablation.¹² Unfortunately, this study applied only to patients between ages 14–31 that were diagnosed with Type I diabetes within 6 weeks prior to treatment. Furthermore, patient hospitalization and isolation was required because of the temporarily weakened immune system caused by ablation. While a small subset of Type I diabetics may benefit from this treatment, more research is needed to determine whether it can be applied to a larger patient population.

Immunosuppressed Cell Transplantation

One approach to providing protection for islet transplants has been to chronically administer immunosuppressive medication. In this pursuit, whole organ pancreas transplantations are possible with immunosuppression. The complications are deemed worthwhile only for patients that are already undergoing transplantation of a life-sustaining organ such as a kidney. These complications include susceptibility to infection, decreased capability of healing wounds properly and increased risk for developing lymphoma.^{13,14} Until recently, isolated islet transplantations had been much less successful than whole pancreas transplantations, with only 8% of patients maintaining insulin independence for up to one year in all procedures between 1990 and 1998.¹⁵ More recently, isolated allogenic islet transplantation was validated using a medication regimen outlined in the Edmonton protocol that resulted in 7 of 7 patients who remained insulin independent one year after transplant.¹⁶ However, a 5 year study of the same therapy resulted in only 10% of patients who remained insulin-independent.¹⁷ Additionally, this therapy requires human donor pancreatic islets of which the supply is limited.¹⁸ Currently, efforts are underway to differentiate pancreatic β -cells from human stem cell lines that could ultimately increase the supply for allogenic transplantations.¹⁹ Unfortunately, insulin-independence for the patient has not been achieved through immunosuppressed xenogenic islet transplantation for which the current supply is much greater.²⁰

Some methods have been developed to potentially reduce or eliminate the need for immunosuppressive medication during islet transplantation. In vitro culture prior to transplantation has demonstrated decreased immune rejection.²¹ Additionally, non-immunosuppressed xenotransplantation of embryonic pig tissue has demonstrated promise in treating diabetic rats.^{22–24} However, no success has been reported in larger animals, although research is underway to better understand the immune response of primates to fetal xenogenic transplants.²⁵ As a result, immunosuppressed cell-based drug delivery and strategies to avoid immune rejection have not yet provided a treatment option that can be widely administered.

Immunoisolated Cell-Based Drug Delivery

Origins

A solution to increasing the viability of allo- and xenotransplanted cells without the complications of immunosuppressive therapy is their encapsulation in an immunoisolating semipermeable membrane. The membrane serves to impede contact with antibodies, complement and cells, but allow transport of insulin, glucose, nutrients and waste products. The relatively smaller size of insulin, glucose and nutrients compared with antibodies, complement and cells, has inspired the development of immunoisolated cell-based drug delivery; a cell secretes insulin when stimulated by extracellular glucose but is protected from immune-mediated death by a semipermeable membrane.

One of the first attempts resembling immunoisolated cell-based drug delivery for diabetes treatment occurred in 1933 through xenotransplantation of human insulinoma tissue using membranous bags into rats.²⁶ However, the field of immunoisolated transplantation became more formally established in the early 1950's through a series of experiments that examined the survival rates of allotransplanted tissue into an extravascular space with and without a cell-impermeable encapsulating membrane.^{27–30} These experiments demonstrated prolonged survival of transplanted tissue when immune cell contact was prevented. The nonvascularized transplanted tissue, while receiving fewer nutrients, survived longer due to the lack of contact with the immune cells, preventing the direct antigen presentation pathway that leads to immune-mediated destruction.

The treatment of IDDM by immunoisolated cell transplantation was made possible only after the β -cell containing Islets of Langerhans were isolated in 1965.³¹ Several immunoisolated transplantation methods were subsequently developed, including intravascular chambers, microcapsules and extravascular chambers (Table 1).^{32–34} Each of these will be addressed in the following sections.

Intravascular Chambers

Motivation—Intravascular chamber development was motivated by the need for transplanted cells to regulate the blood-glucose level in a timely manner. These chambers directly access the blood, being separated only by a semipermeable membrane. Such an approach offers an advantage over both extravascular chambers and microcapsules, which are also implanted in an extravascular space, often in the peritoneal cavity. Glucose from the blood must first diffuse through the mesothelium that lines the peritoneal cavity in order to

access the cells. As a result, the cells receive blood-glucose information that is delayed. This delay is exacerbated in humans because of the greater thickness of human mesothelium compared with that of animals. For example, human mesothelium is 4–5 times thicker than that of a rat.³⁵ If the delay is significant in duration, the patient will experience peaks and valleys of blood glucose concentrations that will increase the chance of debilitating events. Therefore, the intravascular chamber approach avoids the increased delay and for this reason is a promising approach for immunoisolated cell-based drug delivery.

Development—The development of intravascular transplantation chambers began with the development of methods to culture cells on artificial capillaries by Knazek and Chick.^{36,37} Sun, Tze and Orsetti subsequently demonstrated some success in rats using Amicon (polyvinyl chloride-acrylic copolymer) membranes.^{38–40} These membranes comprise an artificial capillary that is attached to the animal's vascular system. The cells surround the semipermeable capillary which protects them from contacting the immune cells flowing through the blood. Glucose and other nutrients diffuse across the membrane, directly stimulating the cells to secrete insulin, which quickly disperses throughout the body to regulate the metabolism of glucose. More on intravascular transplantation chamber has been reviewed elsewhere.³⁴

Commercialization—The intravascular chamber approach at one time inspired several companies to further develop the technology. One example, BioHybrid Technologies, founded in 1985, developed an intravascular transplantation chamber with limited success in transplanting allogenic islets into pancreatectomized dogs.⁴¹ Unfortunately, commercial development of this approach was halted for reasons discussed below.

Failure Modes—The intravascular approach was abandoned due to the inability to control blood coagulation issues. This problem has not yet been overcome and these authors know of no current development in intravascular transplantation chamber technology. Perhaps as materials science advances or our ability to control biological processes improves and coagulation can be prevented, intravascular chamber transplantation for diabetes treatment will be revisited. However, even if coagulation can be controlled, the complications involved with implantation of an intravascular device are more dangerous than those involved with the implantation of an extravascular device.

Microcapsules

Motivation—Nutrient availability is another factor that determines the viability of cellular implants over time. For this reason, one design consideration in the early development of islet transplantation chambers were insulin and glucose diffusion across the membranes.⁴² In order to optimize these diffusion rates, the surface area to volume ratio should be maximized. As a result, researchers began transplanting cells encapsulated in semipermeable microcapsules.^{43,44} Furthermore, microcapsule implantation can occur through injection, offering a less invasive procedure than the surgery required for transplantation chamber implantation.

Development—Cell microencapsulation was first mentioned by Chang in 1964.⁴⁵ However, it was not until 1980 that Lim and Sun applied microcapsules to diabetes treatment, demonstrating prolonged isograft islet survival when microencapsulated in alginate-polylysine-polyethyleneimine microcapsules.⁴⁶ Initially, microencapsulated islet transplantation delayed the return to hyperglycemia compared with the transplantation of unencapsulated islets by only 10 days and failed due to a lack of biocompatibility of the microcapsule itself. The microcapsule material was improved in 1984 by O'shea and Sun who removed the polyethyleneimine component and designed alginate to be the outer layer of the microcapsule.⁴⁷ The improved material demonstrated significant improvement and in one of the five animals the microencapsulated islets remained viable for 365 days, when the experiment ended. An additional advantage of the new microcapsules was increased microcapsule strength. Efforts to further improve biocompatibility of alginate microcapsules involved decreasing the impurities and increasing the guluronic acid to mannuronic acid ratio.^{48,49} Other researchers questioned the reproducibility of alginate-polylysine microcapsules and explored either their coating with a polyethylene glycol hydrogel or manufacturing the microcapsules from a different material altogether such as a polyacrylate^{50,51} or silica.⁵² In an optimization effort, Wang et al evaluated over 1,000 combinations of polyanions and polycations with regards to suitability for cell encapsulation.⁵³ The result was a polyelectrolyte complexation process using 5 different polymers enabling independent control over capsule size, wall thickness, mechanical strength and permeability. For further information, microencapsulation technology has been extensively reviewed elsewhere.^{54,55}

Commercialization—The advances in microencapsulation technology have brought this approach to the forefront of islet transplantation therapy. Recent progress has resulted in several ongoing clinical trials. Dr. Calafiore led a study at the University of Perugia with two patients in 2006 receiving alginate-polylysine-polyornithine encapsulated islets.⁵⁶ Also, Novocell, Inc. recently presented interim data on a Phase I/II clinical trial using a photopolymerizable polyethylene glycol microcapsule.⁵⁷ In both cases, evidence existed that the islets were not rejected by the immune response throughout the duration of the trial. However, neither study resulted in insulin independence for the patient. It is important to note that although in 1994 Dr. Soon-Shiong was able to achieve insulin independence in a patient using alginate microencapsulated islets after 9 months, the patient was taking immunosuppressive therapy as well.⁵⁸ The work from Dr. Soon-Shiong's experiments is being pursued commercially by ReNeuron (previously Amcyte). In early 2007, Living Cell Technology (previously Diatranz) began their second clinical trial with a successful implant of neo-natal porcine islets encapsulated in alginate. Recently, interim data from Living Cell Technology indicates that one of two patients was successfully weaned off of insulin one month after transplantation, while the other was able to reduce exogenous insulin by 40%.⁵⁹ For how long the insulin independence will last is uncertain. Living Cell Technology's first trial was halted due to a ban on xenotransplantation issued by New Zealand in 1997 which has recently been repealed. Lastly, MicroIslet Inc. and Progenitor Cell Therapy are also working towards developing alginate-based microcapsules for diabetes treatment.⁶⁰ Clearly, the microencapsulation approach of immunoisolated cell-based drug delivery is flourishing.

Failure Modes—Despite significant activity, microencapsulation technology still has not achieved clinical success. Several experiments point to some key factors that may be playing a role in transplant failure. Originally, the lack of biocompatibility of the membranes was associated with cellular overgrowth of the capsule, particularly when the islets are not completely encapsulated and the resulting nutrient deficiency was blamed on transplant failure.⁶¹ However, improved materials and encapsulation techniques have enabled microcapsule implants that lack significant cellular overgrowth (<10% of the microcapsules).⁹ One study that analyzed the cause of failure in the absence of overgrowth suggested that the failure was likely due to nutrient deficiency throughout the encapsulated cluster of cells, as illustrated by necrosis of the cells furthest away from a nutrient source.⁶² However, a more recent study demonstrated that insulin secretion is also significantly reduced when the microcapsules are in a solution of activated macrophages compared to a solution without macrophages with identical nutrient availability.⁹ Cytokines secreted by activated macrophages such as IL-1 β (17.5 kD) and TNF- α (17 kD) have been implicated in transplant rejection.^{63,64} These cytokines are similar in size to insulin (5.6 kD). Therefore, any membrane that impedes diffusion of these and other cytokines will likely also affect nutrient and insulin diffusion. It is important, therefore, to ensure that the environment surrounding the transplanted cells minimizes the production of macrophage activating factors.

While microencapsulation technology is approaching human clinical success, there remain many disadvantages inherent with this approach. Microcapsule manufacturing processes have resulted in pore sizes with relatively broad distributions.⁶⁵ Even if cytokine-mediated cell death is limited, a broad pore size distribution presents a potentially insurmountable challenge in the attempt to isolate antibodies, complement and immune cells while allowing sufficient nutrient and insulin diffusion. An optimal membrane will completely isolate the encapsulated cells from the relevant antibodies and complement (IgG, IgM and C₁Q). Transport inhibition of such molecules is particularly necessary for xenotransplants because of increased indirect antigen presentation.⁸ Additionally, microcapsule walls are susceptible to having embedded islets enabling a portion of the islet that is not protected by the membrane to stimulate an immune response.⁶⁶ Although this limitation can be overcome, doing so typically requires a larger diameter microcapsule or a double layer, increasing the blood-glucose diffusion time.⁶⁷ Efforts are underway to create ultrathin microcapsule walls without any exposed portion of the islet, but in vivo success has not yet been demonstrated.⁶⁸

A further disadvantage of microcapsules is their difficulty in simultaneously achieving biocompatibility, immunoisolation and a suitable environment that minimizes stress on the islets. To date, the design of microcapsules has focused on biocompatibility as well as achieving immunoisolation while allowing sufficient nutrient availability. However, the design that optimizes these parameters may compromise the environment surrounding the cells and negatively impact cell behavior. In addition to biocompatibility, nutrient availability and immune protection, pancreatic β -cell behavior is also highly dependent on the surrounding matrix environment.⁶⁹ Therefore, the inability to independently control cell

environment from membrane permeability will continue to present challenges for achieving therapeutic success of microencapsulated cells.

Extravascular Chambers

Motivation—Meanwhile, membranes manufactured from materials that cannot be formed into microcapsules have continued to advance. These membranes can be incorporated into a transplantation chamber such as those used by the early researchers in this field^{27–30,70,71} (See Fig. 2). Additionally, the design of the matrix environment surrounding the cells is independent from the design of the membranes, allowing for greater design flexibility. A further advantage of the extravascular transplantation chamber is that it is more easily retrievable than both intravascular chambers and microcapsules after implantation.

Development—The extravascular chamber method developed by Algire, Weaver and Prehn, discussed earlier, in the 1950's for transplanting tissues was a natural starting point from which researchers could develop an extravascular chamber for immunoisolated islet transplantation.^{28–30} During the 1970s, Millipore Corporation produced a commercially available extravascular transplantation chamber using the Algire approach.³⁴ These membranes typically had pore sizes on the order of 450 nm, a size sufficiently small to prevent direct cell-cell contact and therefore promising for allotransplants. Studies by Algire and colleagues demonstrated improved cell viability when encapsulated in these membranes.^{27,70,71} Although many of the initial experiments involved syngeneic cells, transplant failure occurred nonetheless due to fibroblastic overgrowth of the graft and chamber, highlighting the importance of biocompatibility of the chamber to transplant success.³⁴ Significant advances have been made since these early experiments and they have been reviewed extensively elsewhere.^{34,72,73}

Commercialization—In the 1980s and 1990s, extravascular chamber technology became sufficiently advanced that many companies were funded for commercialization purposes. BetaGene partnered with Gore Hybrid Technologies to create a transplantation chamber for xenogenic immortalized pancreatic β -cells that Dr. Newgard, one of the founders, believed would possess better transplant viability. Baxter Healthcare developed a device for xenogenic immortalized pancreatic β -cells with some success in NOD mice.⁷⁴ Encelle Inc., recently acquired by Pioneer Surgical Technology, produced a biocompatible transplantation chamber to be implanted intramuscularly.⁷⁵ Cytotherapeutics Inc. created a similar transplantation chamber but for the application of Parkinson's treatment using immortalized neurosecretory cells that secrete dopamine and other factors. iMedd, Inc. investigated the use of silicon nanoporous membranes, which will be discussed in more detail later, for cell-based drug delivery based upon studies from the Desai laboratory (Fig. 1).^{76–78} Cerco Medical (previously Islet Sheet Medical) is currently developing a transplantation chamber in the geometry of a sheet of islets surrounded by an alginate membrane.⁷⁹ Despite all of this activity, as far as these authors are aware, current clinical trials are not underway for cell-based drug delivery using transplantation chambers.

Failure Modes—Despite having the longest development history, extravascular transplantation chambers have not yet achieved clinical success. In the past it has been

suggested that host fibroblastic response, poor graft oxygenation and poor graft nutrition hindered the effectiveness of this immunoisolation approach.³⁴ However, current extravascular chambers can incorporate materials with improved biocompatibility and diffusion characteristics that may overcome these challenges, as discussed below. The remaining failure mode for extravascular chambers that cannot be overcome is the diffusion delay of glucose and insulin between the transplanted cells and the bloodstream. Further evaluation is required to determine whether this is an insurmountable obstacle preventing clinical success. This evaluation is ongoing for microencapsulated cells, where the diffusion delay of glucose and insulin is similar to that associated with extravascular transplantation chambers. Therefore, if microencapsulated cells demonstrate clinical success, the failure mode associated with the delay of glucose and insulin diffusion between the transplanted cells and the bloodstream should not prevent extravascular transplantation chambers from also achieving clinical success.

Inorganic Nanoporous Membranes

Material advances inspired by the semiconductor, electronics, sensor and solar power applications have brought about the development of inorganic nanoporous membranes that have demonstrated promise for therapeutic applications such as cell-based drug delivery. Currently, inorganic nanoporous membranes that are useful for cell encapsulation can be manufactured from silicon, aluminum and titanium. The nature of these membranes makes them useful only for extravascular transplantation chambers. Transplantation chambers compare favorably with microcapsules because of: (1) the ability to independently control the cell matrix environment and the membrane parameters, enabling the design of an environment more likely to achieve proper cell behavior and (2) the ability to avoid the risk of incomplete cell protection by loading the cell-matrix after the membrane has been fabricated. Additionally, inorganic nanoporous membranes compare favorably with membranes traditionally used for transplantation chambers as well as microcapsules because of: (1) the tighter pore size distribution of inorganic nanoporous membranes and (2) the decreased diffusion time and variability associated with a thinner and more precisely controllable membrane thickness. The membranes traditionally used for extravascular transplantation chambers as well as microcapsules have been polymer membranes and will be referred to from now on as such (Table 2).

Silicon Nanoporous Membranes

Silicon nanoporous membranes are the most extensively studied of the inorganic nanoporous membranes.⁸⁰ The processes for altering the surface of a silicon wafer are well understood as a result of integrated circuit development for computer chips. This precise control has enabled the fabrication of a nanoporous membrane with incredible precision that has proven useful for cell-based drug delivery.

Preparation—Silicon nanoporous membranes are prepared initially from silicon wafers. A comprehensive outline of the history and development of the silicon membrane was previously reviewed by Leoni.⁸¹ Presented here is the most current manufacturing strategy, also previously described (Fig. 2).^{82,83} First, a support ridge structure is photo-

lithographically etched to provide mechanical support to the final structure.⁸⁰ A low-stress silicon nitride layer is deposited over the top surface of the wafer. The membrane structure will be formed on top of the silicon nitride, which will serve as an etch-stop for future processes. This etch-stop layer is very thin and small in comparison to the depth between support ridges. A polysilicon film, henceforth referred to as the base layer, is deposited on top of the silicon nitride layer, filling the remaining space between support ridges. The thickness of the base layer will determine the overall thickness of the nanoporous membrane.

Holes are then etched through the base layer but not through the nitride etch-stop layer. The geometry of the holes determines the shape of the pores. This geometry is defined by a thermally-grown oxide layer mask and etched using chlorine plasma. Another sacrificial thermally-grown oxide layer is formed, covering all silicon surfaces, but not the nitride etch-stop layer. The thickness of this sacrificial layer will determine the pore size. This oxide layer can be controlled to within 0.5 nm in thickness through thermal oxidation in dry oxygen, enabling pore sizes between 10 and 100 nm as well as tight pore size distributions.⁸³

The next step involves plugging the holes that were created in the base layer. In order for the plug material to become attached to the base material, anchor points are defined by selective etching of the oxide layer. Another polysilicon layer, henceforth referred to as the plug layer, is then deposited that fills the holes, attaching to the base layer at the anchor points. The surface is then planarized using chemical mechanical polishing to remove the over-filled plug layer until it exists only within the base layer, leaving a smooth surface with the sacrificial oxide exposed.

Subsequently, a nitride protective layer is deposited completely covering both sides of the wafer. This layer is impervious to KOH etching. Windows are etched through the nitride layer in the areas where membrane exposure is desired. Then, an 80°C KOH etch is performed that will remove the exposed silicon only as far as the nitride etch-stop layer. Finally, a HF etch removes the protective and etch-stop nitride layers as well as the sacrificial oxide layer. The finished product is a silicon nanoporous membrane with highly controllable pore channel widths (Fig. 3).⁸⁴

Advantages—The silicon nanoporous membrane has the potential to overcome all of the limitations associated with polymer membranes discussed above. Pore widths of 18 nm have demonstrated significant diffusion resistance to IgG while allowing relatively unrestricted diffusion of insulin and glucose.^{78,85} Furthermore, the highly controllable pore channel width to within 0.5 nm⁸⁶ results in a substantially tighter pore size distribution of approximately 5% compared with the 30% distributions that can be associated with polymer membranes.⁶⁵ It has been suggested that if only 1% of the pore sizes exceed the desired cut-off, sufficient quantities of antibodies, complement and cytokines will diffuse to cause immune-mediated death.⁴³ In order for less than 1% of the pores to exhibit sizes above the desired cut-off, a broader pore size distribution necessitates a smaller nominal pore size. However, a smaller nominal pore size will result in decreased diffusion of insulin, glucose and nutrients, leading to a greater chance of nutrient starvation and poor insulin secretion

kinetics. Additionally, 18 nm pore width membranes have demonstrated protection for islets when placed in a serum complement/antibody solution over a 2-week period as measured by improved glucose stimulated insulin secretion compared with unencapsulated islets.⁸⁷ Furthermore, in vivo studies have confirmed both short-term biocompatibility of the membranes and increased insulinoma cell viability.⁷⁷ All of these results support the potential that silicon nanoporous membranes have in providing adequate immunoisolation to encapsulated cells.

Silicon nanoporous membranes offer an additional advantage due to their small thickness of only a few microns. The diffusion of molecules through a membrane depends upon both the pore thickness and shape. Pore thickness impacts the diffusivity of all molecules equally. Pore shape, on the other hand, plays a significant role in altering diffusion in a size discriminatory manner. Ideally, the pore shape even at small thicknesses will completely block IgG yet allow unrestricted insulin and glucose flow. Therefore, the ability to manufacture silicon membranes to a thickness much smaller than that of polymer membranes, which are on the order of 100 μm thick, represents a significant advantage because of increased diffusivity of insulin and nutrients. As it turns out, a 6 μm thick, 18 nm pore width silicon nanoporous membrane has demonstrated favorable IgG diffusion characteristics.⁷⁸ For pore sizes that equally restrict IgG diffusion, the silicon membranes' reduced thickness will enable an increased diffusion of insulin and nutrients compared with thicker polymer membranes. Additionally, the thickness of a silicon membrane can be controlled more precisely than that of a polymer membrane. As a result, in addition to providing adequate immunoisolation to encapsulated cells, silicon nanoporous membranes can offer excellent transport characteristics of insulin and nutrients.

Disadvantages—The silicon nanoporous membrane possesses one disadvantage compared with polymer membranes as well as the alumina and titania membranes that will be discussed later. Currently, it is only feasible to manufacture silicon membranes with rectangular pores, whereby the width can be in the nanometer range but the length is limited by that which traditional etching methods allow. The width of the pore and not the length serves to restrict antibody and complement diffusion. When considering diffusion of a protein through a pore, however, the 3-dimensional conformation of the protein must be considered. IgG is a relatively flexible y-shaped molecule that can assume conformations that minimize width and extend length, allowing enhanced diffusion through a rectangular pore compared with a circular pore. This phenomena has been demonstrated by the restricted diffusion of IgG through an alumina nanoporous membrane with 75 nm diameter pores compared with a silicon nanoporous membrane with 49 nm wide pores.⁸⁸ The alumina membrane also restricted glucose diffusion more than the silicon membrane; this was likely due in part to the larger alumina membrane thickness. However, the difference in restricted glucose diffusion was less than that for IgG. Therefore, at least part of the decreased IgG diffusion was due to the circular nature of the pores in the alumina, suggesting that a circular pore can provide improved immunoisolation. While currently not easily available, technologies for creating circular nanopores in silicon may someday become commercially available by using more advanced lithographic techniques such as electron-beam or nano-

imprint lithography.⁸⁹ Until then, the silicon nanoporous membrane, while extensively studied and promising, possesses the disadvantage of containing rectangular-shaped pores.

Alumina Nanoporous Membranes

Alumina nanoporous membranes, originally developed for electronics and sensor applications, take advantage of the self-organizational behavior of anodized alumina.⁹⁰ Soon after discovery of this phenomenon, a process resulting in straight nanoholes through a thin film of alumina was developed, resulting in the creation of a self-organized nanoporous alumina membrane.⁹¹ This technology was adapted to control molecular release through a nanoporous cylindrical alumina membrane embedded within an aluminum-manganese alloy capsule.⁹² The alumina membrane can also be formed on flat sheets of aluminum.⁹³ More recently, the alumina nanoporous membranes have demonstrated promise for cell encapsulation.⁸⁸

Preparation—Although nanoporous anodized alumina membrane fabrication depends on the application, a general process for fabrication to be incorporated into a cell encapsulation device is presented here, as adapted from previous reports (Fig. 4).^{88,92,93} First, an aluminum alloy ($\text{Al}_{98.6}\text{Mn}_{1.2}\text{Cu}_{0.12}$) is cleaned by sonication in acetone and deionized water and then dried with nitrogen. The next steps described are specific to a membrane formed in a cylindrical aluminum tube from the inside out. Although membranes can be created from the outside of an aluminum tube, they have demonstrated decreased mechanical strength.⁹⁴ Furthermore, when prepared from the inside, the membrane exists within a recess and is less susceptible to external damage. To achieve inner-wall membrane formation, the outside of the tube is protected by spin-coating a thin layer of polymer, typically ethyl acetate and butyl acetate (nail polish). Prior to polymer spinning, an oxalic acid anodization process produces a very thin oxide layer that allows for polymer adhesion.

After the polymer has been coated to the outside of the tube, the first anodization process involved in membrane formation occurs in 0.25 M oxalic acid using platinum as the cathode and the polymer-covered aluminum tube as the anode. This process yields a layer of alumina on the inside of the aluminum tube, where the surface is not protected by the polymer. Next, this layer of alumina is etched in a 4% (w/w) chromic acid and 8% (v/v) phosphoric acid mixture for 10 minutes at room temperature. The result is a uniform concave array of nucleation sites that is critical to achieving tight pore size distributions. The organization of nucleation sites depends on the voltage used during the first anodization step.

The second anodization step involved in membrane formation needs to occur at the same voltage as the first. The duration determines the membrane thickness and the voltage determines the pore diameter with each applied volt increasing pore diameter by 1.29 nm. The resulting alumina layer will serve as the nanoporous membrane.

In order to expose the nanoporous membrane to the outside of the tube, a window-area is created in the polymer film through the selective application of acetone and a cotton swab. A 10% NaOH solution can be poured for 15 minutes to completely remove the unwanted layer of alumina that is formed during the second anodization step. Parafilm or silicone plugs are capped on the tube ends to protect the inside of the tube from the subsequent

etching step. After a thorough rinse in DI water, the unprotected aluminum in the window is etched using a 10% (w/w) HCl and 0.1 M (CuCl₂) solution, exposing the transparent alumina membrane. Finally, a 10% (w/v) phosphoric acid solution for 1 ½ hours at room temperature removes the barrier oxide layer on the outside of the nanoporous alumina. After the parafilm or silicone plugs are removed, the result is an aluminum cylinder with a nanoporous alumina membrane window.

More recently, greater flexibility for nanoporous alumina configuration has been achieved by the use of a lithographically-produced photoresist polymer to replace the initial polymer coating.⁹⁵ Additionally, nanoporous alumina membranes have been fabricated on flat sheets.^{90,91,93,95,96} As a result, alumina nanoporous membranes can be easily fabricated in a variety of configurations that could be useful as a membrane for immunisolated cell-based drug delivery (Fig. 5).⁹⁵

Advantages—The alumina nanoporous membrane may overcome the limitations associated with polymer membranes discussed above, although it has not been as extensively evaluated as the silicon nanoporous membrane for this application. The pore size distribution within an alumina nanoporous membrane becomes tighter with decreasing pore diameters. A 46 nm pore created from a 40 V anodization process resulted in a 2.35 nm standard deviation, compared with a 5.48 nm standard deviation associated with a 50 V induced 58 nm pore.⁸⁸ Although these distributions are greater than those achievable with a silicon nanoporous membrane of the same pore width, they compare favorably with those of polymer membranes.

Additionally, the pore density of an alumina nanoporous membrane can exceed that for both polymer and silicon nanoporous membranes.⁸⁸ The ability to increase pore density offers a potential advantage in the design of a cell encapsulation device in the pursuit of balancing the requirements for immunisolation and nutrient availability. If the pore diameter sufficiently impedes antibody and complement diffusion, the larger pore density will increase the diffusion of insulin, glucose and nutrients more than it will increase the diffusion of antibodies and complement in a size specific manner. Additionally, alumina nanoporous membranes improve upon polymer membranes by offering greater control over membrane thickness.

Furthermore, the circular nature of the alumina membrane pores offers an advantage for inhibiting diffusion of the flexible IgG molecule. As a result, alumina nanoporous membranes have demonstrated greater diffusion resistance to IgG than silicon membranes.⁸⁸

Lastly, the studies evaluating the biocompatibility of alumina nanoporous membranes have been favorable. Alumina has demonstrated bio-inert characteristics in humans for certain applications, enabling its use in hip and knee replacements.⁹⁷ More recently, alumina nanoporous membranes have not caused fibroblast cytotoxicity nor complement activation in vitro. In vivo studies in the same report reveal that membrane-containing capsules are free from fibrous growth and membranes remain intact when implanted in the peritoneal cavity of rats for up to 4 weeks.⁹⁸ Tissue samples surrounding the implants do show signs of inflammation, but samples taken from tissue surrounding polyethylene glycol (PEG) coated

alumina nanoporous membrane capsules exhibited less severe signs of inflammation which receded after 4 weeks.^{93,99} These results suggest that the inflammation from PEG-coated capsules occurs from the surgery itself and not from the implanted capsule. In vivo studies with encapsulated cells have not yet been performed. In conclusion, the alumina nanoporous membrane offers many promising characteristics that can be applied to immunoisolated cell-based drug delivery.

Disadvantages—One limitation that the alumina nanoporous membrane has compared with the silicon nanoporous membrane is the thickness of the membrane. The alumina nanoporous membrane has been fabricated with thicknesses as small as 70 μm and although thinner membranes are possible, such modifications will negatively affect membrane strength. As discussed above, a thicker membrane results in delayed diffusion of glucose information to the cells and insulin secretion to the body. The relationship between having an increased pore density but a thicker membrane needs to be more thoroughly evaluated. The advantage of increased pore density could potentially reoperate any diffusion loss due to membrane thickness in comparison to silicon nanoporous membranes. Regarding biocompatibility, it is unclear in vivo whether alumina nanoporous membranes can be as stable as either polymer membranes or silicon nanoporous membranes. As a result, despite promising results thus far, further evaluation will be necessary to determine whether the alumina nanoporous membrane is the ideal choice for immunoisolated cell-based drug delivery.

Titania Nanoporous Membranes

Titanium foil when anodized in certain conditions will cause the growth of an array of nanotubular titania structures from the surface.^{100–105} The commercial interests driving the developing of nanotubular titania have been for photovoltaics, sensing, water photolysis, molecular filtration and tissue engineering.¹⁰³ However, when the array of nanotubular titania is released from the substrate from which it is grown, a titania nanoporous membrane is produced that may prove useful for immunoisolated cell-based drug delivery.¹⁰⁶

Preparation—The nanotubular titania can be grown from a titanium foil in several ways. The formation of nanotubular titania described here is adapted from previous reports (Fig. 6).^{100–105,107} First, high purity titanium foil (99.97% or higher, thickness approximately 250 μm) is degreased by sonication in acetone, ethanol and DI water, followed by a DI water rinse and nitrogen drying. The growth of the nanotubular titania occurs with a subsequent potentiostatic anodization in a 2-cell electrode electrochemical cell connected to a dc power supply, using platinum foil as the counter electrode at room temperature. Methods of controlling nanotube diameter and length have recently been elucidated, although this research is still in its infancy and greater optimization will likely occur in the future.

The first successful nanotubular titania growth occurred through anodization of titanium foil a 0.5% (w/w) HF solution.¹⁰² Under these conditions, the nanotubular structure is formed at voltages greater than 10 V and less than 40 V. Nanotubes fabricated using this process have diameters ranging from 25–65 nm and thicknesses up to 500 nm.^{102,103} The first event in the anodization process occurs within 10 seconds when the titanium film is covered by a

compact oxide film of uneven height. At 30 seconds the oxide film begins to dissolve exposing a continuous nanoporous layer without the presence of any tubular structures. After 8 minutes of continued anodization, the oxide layer is completely removed, exposing discrete emerging nanotubular structures. It has been proposed that nanotubular structure formation occurs by the following mechanism: At sufficiently high anodization voltages, the electric field strength will mobilize titanium ions from the surface in between the pores and facilitate their migration to the oxide/solution interface, resulting in the growth of tubular structures from the titanium surface.¹⁰²

Techniques to increase the length of the titania nanotubes have been elucidated. The thickness of the membrane is determined by the equilibrium between the electrochemical formation and dissolution of titania.¹⁰⁰ By inducing localized acidification at the pore bottom the titania dissolution rate is adjusted, allowing greater control over titania length which allows for the fabrication of membrane thicknesses up to 7 μm .^{103,108} Furthermore, the use of non-aqueous organic polar electrolytes during anodization has enabled membrane thicknesses of up to 134 μm .¹⁰³ More recently, potentiostatic anodization of titanium foil yielded membrane thicknesses of 1000 μm .¹⁰⁶ With a relatively simple fabrication process allowing for significant design control over the characteristics, titania membranes may prove useful for immunoisolated cell-based drug delivery (Fig. 7).¹⁰³

Advantages—Nanoporous membranes fabricated from titanium offer a distinct advantage compared to all other membranes mentioned thus far mainly due to their widely accepted biocompatibility. Titanium has been approved by the FDA for use in many kinds of implants, including into the peritoneal cavity as exemplified by Medtronic's Isomed approval in 2000. Alumina has also been approved for some implant indications, such as the recently approved NOVATION™ Ceramic Articulation Hip System by Exactech, Inc. However, accepted implant sites for alumina do not include inside the peritoneal cavity, a promising implant location for a cell encapsulation device. While some of the polymer membranes as well as the alumina and silicon membranes currently appear biocompatible, the regulatory process associated with receiving approval for marketing those materials as biocompatible will likely be more rigorous than that for titanium.

Another distinct advantage that titania has over all other membranes discussed here is the proven ability to fabricate over a wide range of thicknesses. This design variability compares favorably with silicon nanoporous membranes which have thinner membranes as well as alumina nanoporous membranes which have thicker membranes. Control over this design variable will enable more flexibility in optimizing the diffusion requirements for immunoisolation and nutrient availability for cell encapsulation applications. It is important to note that adequate mechanical stability has not yet been evaluated for thin titania membranes. Nonetheless, if the titania membranes are patterned into a thicker titanium substrate, similar to the ridge support structure associated with thin silicon nanoporous membranes, it is feasible that titania nanoporous membranes can be made mechanically stable even at small thicknesses. Finally, for the same reasons discussed above regarding the alumina nanoporous membrane, the titania nanoporous membranes provides an advantage because of the circular nature of the pores and the increased achievable pore density. In

conclusion, the titania nanoporous membranes are an excellent choice for incorporation into cell-based drug delivery devices.

Disadvantages—The titania nanoporous membrane development is still in its infancy. Many qualities necessary for the successful application of titania nanoporous membranes to cell encapsulation have not yet been evaluated, such as durability in vivo, immunoisolation characteristics, compatibility with implanted cells and pore size distribution. It is premature to comment on the disadvantages of the titania membrane until further evaluation and fabrication optimization has been performed.

Conclusion

The field of cell-based drug delivery has come a long way towards overcoming the challenges that have limited successful clinical treatments. Several challenges remain, however, including attaining a sufficiently available cell supply, means of maintaining cell viability for a therapeutically useful duration and minimizing the delay of glucose-stimulated insulin secretion. Immunosuppressed cell transplantation does not adequately overcome the cell supply issue and leaves the patient with undesirable complications. Immunoisolated cell transplantation via intravascular transplantation chambers has not overcome the coagulation issues associated with graft failure. Microencapsulated cell transplantation is the only immunoisolated cell-based drug delivery approach being evaluated in clinical trials. However, all microcapsules comprise a polymer membrane with inherent limitations including broad pore size distributions, thick membrane walls and interdependency of membrane and cell matrix design. Extravascular transplantation chambers, on the other hand, allow both for the independent design of the cell matrix and membrane as well as the incorporation of inorganic nanoporous membranes. Currently, inorganic nanoporous membranes can be fabricated from silicon, alumina and titania. Additionally, recent research has elucidated new inorganic nanoporous materials that could someday be investigated for use in an extravascular transplantation chamber.^{109,110} The inorganic nanoporous membranes possess pore size distributions much tighter than that of polymer membranes, providing a better chance at appropriately balancing the requirements for immunoisolation and nutrient availability. Inorganic nanoporous membranes also have displayed promising biocompatibility characteristics as well as allow for the cell matrix environment to be independently designed from the membrane. Additionally, the silicon and titania nanoporous membranes can comprise smaller and more accurate thicknesses, offering improved blood-glucose control by decreasing the delay with which insulin regulates the blood-glucose level. Therefore, the inorganic nanoporous membrane-enclosed extravascular transplantation chamber offers great promise for developing a widely-available treatment for insulin dependent diabetes mellitus.

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Figure 1.

Extravascular Transplantation Chamber. A device encloses a collection of cells with an immunoisolative membrane. Shown here is a cross-section of a device with cells in between two nanoporous membranes. Reprinted with from Leoni L, Desai TA. Micromachined biocapsules for cell-based sensing and delivery. *Adv Drug Deliv Rev* 2004; 56(2):211–29, with permission from Elsevier.

Figure 2.

Silicon Nanoporous Membrane Fabrication. A) Support ridges are fabricated from a silicon wafer using lithography; Silicon nitride etch-stop layer is deposited; Polysilicon base layer fills the remainder of the space between support ridges. B) Holes etched through the base layer define the geometry of the pores. C) Sacrificial oxide layer is thermally-grown which defines the width of the pores. D) Sacrificial oxide is selectively etched to reveal anchor points; Plug polysilicon layer is deposited. E) Surface is planarized until sacrificial oxide layer is exposed. F) Nitride protective layer is deposited covering all sides of the wafer; Windows are etched through nitride layer in areas where membrane exposure is desired. G) 80°C KOH etches exposed silicon up to silicon nitride etch-stop layer. (HF etch removes all nitride and sacrificial oxide layers—not shown). Reprinted with permission from Leoni L, Attiah, Darlene et al. Nanoporous platforms for cellular sensing and delivery. *Sensors* 2002; 2:111–120.

Figure 3. SEM Micrographs of Silicon Nanoporous Membrane. A) Top view detail. B) Side view detail. Reprinted with permission from Leoni L, Boiarski, Anthony et al. Characterization of nanoporous membranes for immunoisolation: Diffusion properties and tissue effects. *Biomedical Microdevices* 2002; 4(2):131–139.

Figure 4.

Alumina Nanoporous Membrane Fabrication. Reprinted with permission from Swan EE et al. Fabrication and evaluation of nanoporous alumina membranes for osteoblast culture. J Biomed Mater Res A 2005; 72(3):288–95.

Figure 5.
SEM Micrograph of Alumina Nanoporous Membrane. Reprinted with permission from
Swan EE et al. Fabrication and evaluation of nanoporous alumina membranes for osteoblast
culture. *J Biomed Mater Res A* 2005; 72(3):288–95.

Figure 6. Titania Nanoporous Membrane Fabrication. A) Oxide layer formation. B) Pit formation on the oxide layer. C) Growth of the pit into scallop-shaped pores. D) The metallic part between the pores undergoes oxidation and field-assisted dissolution. E) Fully developed nanotubes with a corresponding top view. Reprinted with permission from Mor GK, Varghese, Oommen K et al. Fabrication of tapered, conical-shaped titania nanotubes. *J Mater Res* 2003; 18(11).

Figure 7. FESEM Micrographs of Titania Nanoporous Membranes. A) Cross-section at lower magnification. B) Cross-section at higher magnification. C) Top-surface image. Reprinted with permission from Paulose M et al. Anodic growth of highly ordered TiO₂ nanotube arrays to 134 μm in length. *J of Phys Chem B* 2006; 110(33):16179–16184.

Table 1

Advantages and disadvantages of immunoisolation technologies

Immunoisolation Technology	Advantages		Disadvantages	
Intravascular chamber	1	Vascular access results in decreased diffusion time for glucose and insulin	1	Blood coagulation leads to transplant failure
	2	Independent design of cell matrix environment and membrane	2	Increased complications due to invasive surgery
			3	Currently limited to polymer membranes
Microcapsules	1	Improved nutrient availability depending on design	1	No vascular access results in increased diffusion time for insulin and nutrients
	2	Less invasive implantation procedure	2	Currently limited to polymer membranes
			3	Interdependent design of cell matrix environment and membrane
Extravascular chamber	1	Flexibility of membrane material (i.e., inorganic nanoporous membranes)	1	No vascular access results in increased diffusion time for insulin and nutrients
	2	Independent design of cell matrix environment and membrane	2	Limited diffusion depending on chamber design
	3	Less invasive implantation procedure compared with intravascular chambers		

Table 2

Advantages and disadvantages of polymer and inorganic nanoporous membranes

Membrane Material	Advantages	Disadvantages
Polymer	1 Circular pore geometry	1 Broad pore size distribution
	2 High pore density	2 Broad thickness distribution
	3 Biocompatible	3 Thick membrane
	4 Inexpensive	
Silicon	1 Tight pore size distribution	1 Low pore density
	2 Tight thickness distribution	2 Rectangular pores
	3 Proven fabrication of channel widths as small as 10 nm	3 Expensive
	4 Thin membrane	
	5 Biocompatible	
Alumina	1 Tight pore size distribution	1 Thick membrane
	2 Tight thickness distribution	2 Fabrication of pore diameters as small as 10 nm has not been proven
	3 Circular pore geometry	3 Biocompatibility unclear
	4 Inexpensive	
Titania	1 Tight pore size distribution	1 A thin membrane has not yet been proven to be adequately mechanically robust
	2 Tight thickness distribution	2 Fabrication of pore diameters as small as 10 nm has not been proven
	3 Circular pore geometry	
	4 Biocompatible	
	5 Material is FDA approved for implant into the peritoneal cavity	
	6 Inexpensive	