

Advances and Barriers in Mammalian Cell Encapsulation for Treatment of Diabetes

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Abstract: Mammalian cell encapsulation is under investigation for the treatment of a wide variety of diseases, since it allows for transplantation of endocrine cells in the absence of undesired immunosuppression. The technology is based on the principle that transplanted tissue is protected for the host immune system by an artificial membrane. In spite of the simplicity of the concept, progress in the field of immuno-isolation has been hampered. During the past two decades, three major approaches of encapsulation have been studied. These include (i) intravascular macrocapsules, which are anastomosed to the vascular system as AV shunt, (ii) extravascular macrocapsules, which are mostly diffusion chambers transplanted at different sites, and (iii) extravascular microcapsules transplanted in the peritoneal cavity. The advantages and pitfalls of the three approaches are discussed and compared in view of applicability in clinical islet transplantation. At present, microcapsules, due to their spatial characteristics, offer better diffusion capacity than macrocapsules. During the past five years, important advances have been made in the knowledge of the characteristics and requirements capsules have to meet in order to provide optimal biocompatibility and survival of the enveloped tissue. Novel insight shows that islet-cells themselves and not the capsule materials should be held responsible for loss of a significant portion of the immuno-isolated islet cells and, thus, failure of the grafts on the long term. New approaches in which newly discovered inflammatory responses are silenced bring the technology of transplantation of immuno-isolated cells close to clinical application.

Key Words: microencapsulation, macroencapsulation, islets, diabetes, alginate, immunoisolation, insulin, vascularization.

INTRODUCTION

Administration of insulin for the treatment of insulin-dependent diabetes is still associated with serious complications. Intensified insulin treatment has been shown to delay the onset and to reduce the progression of diabetic complications [1], but it requires multiple daily injections, frequent monitoring, dosage adaptations and, thus, patient compliance. Also, it is associated with life-threatening episodes of severe hypoglycemia and with hypoglycemia unawareness. This is the main rationale for many groups to design therapies to provide the diabetic patient with an endogenous insulin source that regulates blood glucose on a natural, minute-to-minute basis. Basically, there are two options, transplantation of the whole pancreas and transplantation of only the islets of Langerhans.

Transplantation of the whole pancreas is already a well-established mode of treatment with a worldwide experience of more than 15, 000 cases [2, 3]. Results have substantially improved during the past two decades and present patient and one-year graft survival rates almost equal to those of routine kidney transplantation (respectively 98% and 85%). A successful pancreas transplant provides almost normal glucose homeostasis, but it requires life-long immunosuppressive

medication and is associated with major surgery and high morbidity. Since it is still unclear whether the benefits of a pancreas transplant over continued insulin treatment outweighs the disadvantages, most transplant centers still restrict themselves to combined pancreas and kidney transplantation in diabetic patients with end-stage renal failure [2, 4].

Islet transplantation, in contrast to pancreas transplantation, requires no major surgery. Recent improvements in the technology are the administration of non-glucocorticoid immunosuppression (sirolimus, tacrolimus, daclizumab), which is associated with one-year graft survival of 100% of the transplanted diabetic patients [5]. These advances have led to a tremendous growth in the number of research groups aiming on human islet transplantation. Unfortunately, not all these groups have achieved the same level of success as the Edmonton group, which is usually attributed to different degrees in 'experiences' in efficacious isolation of functional islets and to application of different patient groups. However, in spite of the inability of many groups to fully reproduce the Edmonton results, the general impression is that recent advances of the Edmonton group have brought islet-transplantation close to wide-spread clinical application.

Another pertinent advantage of islet transplantation over whole pancreas transplantation is that islet transplantation without immunosuppression might be achieved by methodologies such as immuno-isolation. Immuno-isolation is a technology in which islet-cells are enveloped in semipermeable membranes that are impermeable for the hostile effect of the host

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immune system but are permeable for nutrients, glucose, and insulin.

In spite of the simplicity of the concept and the urgent need of such an approach as immuno-isolation, the progress in the field during the past decades could not meet with the high expectations. As a consequence, the field is not only suffering from scientific but also from many political impediments to success. A major political impediment during the past two decades is the high expectations, which have been a burden on the shoulders of researchers. Many of them were forced to perform transplantation studies far before the technology had reached the state of such an approach. As a consequence, many groups did not go further than demonstrating the principle applicability of the technology in different animal models rather than focusing on true issues bringing progress to the field. These true issues include a step-wise analysis of the chemical-physical properties the capsules have to meet in order to be accepted by the recipient. This type of research does not gain much popularity and it has taken almost 25 years before research groups started to focus on the characteristics of the capsules.

In the studies to demonstrate the proof of principle of islet immuno-isolation, normalization of blood glucose was obtained in both chemically induced and autoimmune diabetic animal models [6-16]. These studies used huge amounts of islets or other non-clinical applicable modifications in the technology, which falsely gave the impression that the technology is close to clinical application. It also minimized the perceived importance of conducting research on what appeared to be technical details.

Approximately 10 years ago, some institutes including the groups of the authors decided to start a true scientific step-wise approach to identify the factors determining the success and failure of immuno-isolated grafts. This included both *in vivo*, *ex vivo* and chemical analysis of the grafts. We did not hesitate to publish negative results as well, in order to convince the scientific community that the approach requires adaptations and in-depth analysis in order to become a successful mode of therapy for diabetes. As a consequence, during recent years, important advances have been made in the basic knowledge of immuno-isolation. This will be discussed in the present paper in view of future clinical applications.

APPROACHES OF ENCAPSULATION

The concept of immuno-isolation envelopes tissues in immuno-protective membranes in order to prevent graft rejection. The introduction of this concept dates back to 1933. Bisceglie *et al.* [17] have replaced the endogenous pancreas by insulin producing tissue encapsulated in a semipermeable but immuno-protective membrane to study the effects of the absence of vascularization on the survival of tissues. Bisceglie *et al.* [17] did not recognize the principle applicability of the approach for treatment of disease. It took until 1943 before Algire [18] recognized that graft failure could be delayed by encapsulating allo- and xenogenic tissues before transplantation. His group was the first to illustrate the importance of biocompatibility when they found that graft failure was always accompanied by cellular overgrowth of the membranes. In the past two decades, immuno-isolation has grown to a mature research field and is under study for the treatment of a wide

variety of diseases, including parathyroid cells [19, 20], hemophilia B [21], anemia [22], dwarfism [23], kidney [24] and liver failure [25], pituitary [26] and central nervous system insufficiencies [27], and diabetes mellitus [28].

Two major designs of encapsulation can be distinguished: intravascular devices and extravascular devices (Fig. (1)). Also there are categories of geometry: tissue can be enveloped in macrocapsules and in microcapsules. The macrocapsules contain groups of islets enveloped together in one immuno-isolating membrane that can be implanted as extravascular and intravascular device. With micro-encapsulation, the islets are individually enveloped by their own capsule. These two approaches will be discussed in the next section.

INTRAVASCULAR DEVICES

For application in the treatment of diabetes, the intravascular devices have a number of theoretical advantages over the extravascular approach. The islets in the devices are in close contact with the blood stream, which implies a fast exchange of glucose and insulin and, therefore, a tight control of euglycemia. The intravascular device is usually composed of a microporous tube with blood flow through its lumen and with a housing on its outside containing the implanted tissue [29, 30]. The device is implanted by vascular anastomoses to the blood streams of the host. The most intensively studied intravascular device is the modified diffusion chamber of Chick *et al.* [31]. It is technically advanced and has been tested extensively in small [32] as well as in large animals [32, 33]. The original device was composed of a number of small diameter artificial capillaries contained by one large diameter tube. The artificial capillaries were composed of fibers of polyacrylonitrile and polyvinylchloride copolymer (PAN-PVC) similar to those used in extravascular devices [34, 35]. This PAN-PVC ultrafiltration capillary design [26] has a lumen loaded with islets between the outside of the artificial capillaries. The design permits for close contact between the islets and blood, separated only by the microporous walls of the capillaries. These devices were found to induce normoglycemia in diabetic rats [32], dogs [33] and monkeys [32] but required systemic anticoagulation. The duration of this normoglycemia was usually restricted to several hours and successes of a somewhat longer duration were exceptional. Clotting of the blood in the lumen of these small diameters artificial capillaries proved to be a major obstacle, in spite of anticoagulant medication in massive doses. This thrombus formation was an early sign of insufficient biocompatibility, and has led to the use of tubular membranes with larger diameters in the hope of minimizing or eliminating clot formation in the absence of systemic anticoagulation.

The latter large lumen device is composed of a single, coiled, and tubular membrane with an internal diameter of 5-6 mm. The membrane is somewhat modified but still composed of PAN-PVC with a nominal molecular weight cutoff of 50 kDa. This membrane was found to be rather successful, since these devices implanted as high flow arteriovenous fistulas could remain patent for periods of seven weeks in the absence of systemic anticoagulant therapy [36]. This success is in part explained by the high flow rates through the device, which prevents adhesion of cells to the membranes or collection of those cells in the immediate vicinity [34].

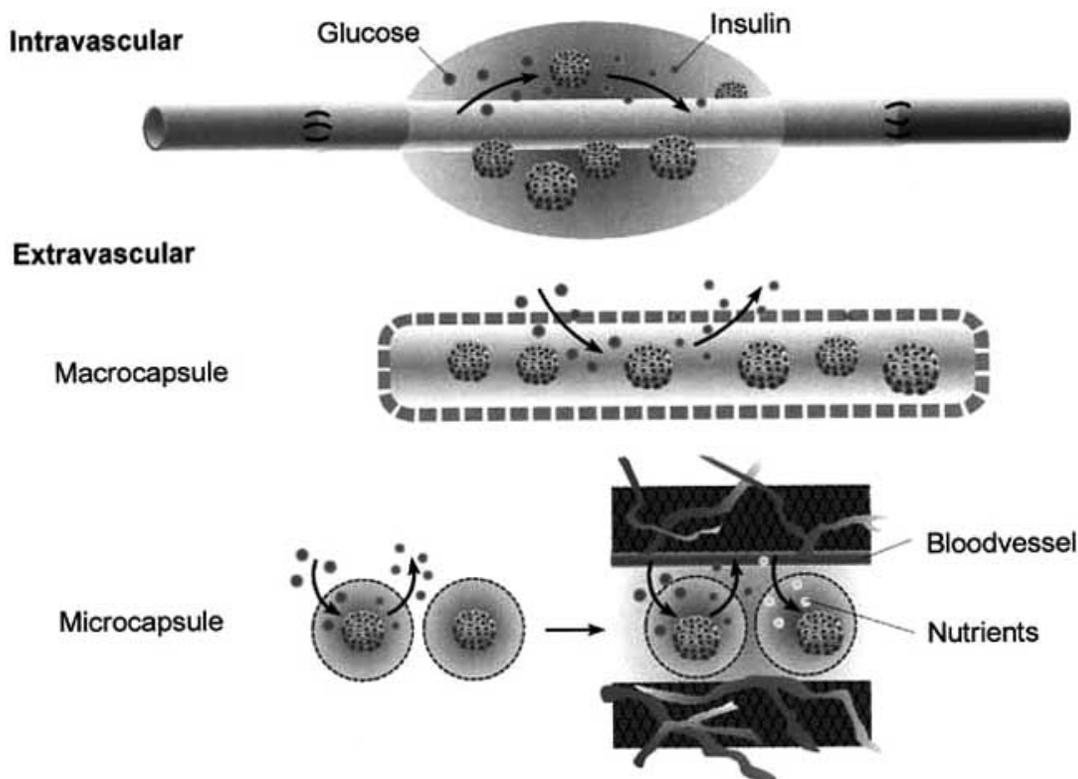


Fig. (1). Immuno-isolation approaches. In the intravascular device, islets are enclosed in a chamber surrounding a selectively permeable membrane. The device is implanted as a shunt in the vascular system. In the extravascular approach, islets are immunoisolated within membrane-diffusion chambers or enveloped in microcapsules and implanted, without direct vascular connection in the peritoneal cavity or subcutaneous site preferably near blood vessels.

Allo- and xenogenic islets in the high flow devices were successfully transplanted to diabetic dogs [37-42] but the efforts to improve the blood-compatibility have probably interfered with the efficacy of the device as an implantation site for islets. This view is derived from the following observations. First, two devices per recipient instead of one were required to achieve adequate secretion capacity while maintaining the same numbers of islets per device [43]. Furthermore, it has not been possible to load the space between the membranes and the housing with an islet-tissue density higher than 5-10% of the volume [26], in spite of the fact that the large lumen is exposed to arterial blood with optimal concentrations of nutrients and oxygen. It is quite plausible that the high flow rates through the device, which are required to keep the device patent, do not allow sufficient exchange of glucose, insulin and nutrients to permit long term survival and adequate function of the islets.

There are also indications that the materials applied in this kind of devices are not only thrombogenic but also insufficiently compatible with long term functional survival of the islets. For example, the polytetrafluoroethylene (PTFE) as used for vascular anastomosis of the device has been shown to induce interleukin (IL)-1 production by macrophages [44], where cytokine is lethal for islets [45, 46]. It is quite plausible that IL-1 causes loss of high numbers of islets during the period between implantation and complete integration of the prosthesis since macrophages are usually the first cells to invade the implant [47-49]. This is another explanation for the fact

that so many islets divided over two devices are required for maintaining normoglycemia in dogs.

Although the intravascular devices have shown some degree of success, the problems mentioned above should be solved if clinical application is considered. Even then, the complications associated with any type of vascular prosthetic surgery remain a serious threat, such as thrombosis, either primary or secondary to intimal hyperplasia at the venous anastomosis, defects of the device, or infection. This is a major drawback for wide application in large numbers of diabetic patients, since any alternative to conventional insulin treatment should preferably carry no additional risk.

EXTRAVASCULAR DEVICES

During the past decade, many groups have abandoned the intravascular systems and moved to the extravascular approach. The concept of extravascular devices does not require vascular anastomoses, since it is based on the principle of diffusion chambers [50]. The extravascular devices can be implanted with minimal surgery in different sites and are not associated with major risks such as thromboses. Extravascular devices can be categorized into two different types of devices, *i.e.* the extravascular macrocapsules and extravascular microcapsules.

The extravascular macrocapsules can be implanted with minimal surgery in different sites such as the peritoneal cavity [51-54], the subcutaneous site [12, 55-60], or the renal capsule [61]. The geometry of macrocapsules may be planar in the form

of a flat, circular double layer or tube-like as a so-called hollow fiber [50]. The extravascular macrocapsules can be readily retrieved.

Due to the advantages over other systems, macrocapsules have been intensively studied with application of many different biomaterials (Table 1). The tube geometry is usually preferred over the planar membranes for their higher degree of biocompatibility [62]. Most studies on tube-shaped fibers use fibers of polyacrylonitrile and polyvinylchloride copolymer (PAN-PVC) [34, 35]. They have been produced with a smooth or fenestrated outer layer. The design with the smooth outer skin proved to be the most successful since it provokes much less fibrosis than the rough fenestrated surface which allows host tissue to grow into the spongy matrix. Many modifications of this concept have been proposed in order to further improve the biocompatibility. One of those was the coating of the membranes with poly-ethylene-oxide to reduce protein adsorption [63]. Biocompatibility problems with extravascular macrocapsules are usually deleterious only to the function of the encapsulated tissue and have no or only minimal risk for the recipient. These biocompatibility problems are usually related to toxicity and activation of non-specific foreign body reactions resulting in fibrotic overgrowth with subsequent necrosis of the encapsulated tissue.

In the past decade, many groups have studied the applicability of hydrogels for extravascular macroencapsulation. Hydrogels provide a number of features which are advantageous for the biocompatibility of the membranes. Firstly, as a consequence of the hydrophilic nature of the material, there is almost no interfacial tension with surrounding fluids and tissues which minimizes the protein adsorption and cell adhesion. Furthermore, the soft and pliable features of the gel reduce the mechanical or frictional irritations to surrounding tissue [64, 65].

The materials applied for the hydrogels are polyamide [66, 67], alginate [68-70], agarose [54, 71], 2-hydroxyethyl methacrylate (HEMA) [72, 73] and a copolymer of acrylonitrile and sodium-methallyl sulfonate, AN69 [74]. Some supporting results have been shown with the hydrogel membrane AN69, which induced only minimal fibrosis in the peritoneal cavity

of rats [75, 76]. Kessler *et al.* [75, 76] have introduced corona discharge to obtain a membrane with a more hydrophobic surface in order to facilitate diffusion of insulin. Fewer molecules adhered to the surface of such membranes, which improved not only the permeability for insulin but also its long term biocompatibility. One year after implantation of empty capsules in rats, only few macrophages were found on the membrane's surface. Surprisingly, up to now, there are no *in vivo* data available on the efficacy of the membranes in facilitating survival of encapsulated pancreatic islets. It seems that many groups have abandoned AN69 and have focused their research efforts on membranes prepared of polyvinylalcohol (PVA), which have been shown to allow for long-term survival of islet-tissue [77, 78].

Recently, it has been reported that a commercial available macrocapsule, *i.e.* TheraCyt, allows for survival of pancreatic islets in cynomolgus monkeys for a period up to 8 weeks [79]. Also, unconfirmed reports on meetings and in the US-press mention successful treatment of type I diabetic patients with macroencapsulated porcine islets. A 3-cm stainless-steel mesh capsule containing a removable Teflon cylinder was inserted into the abdominal cavity of each patient. Two months later, after a collagen membrane had formed around the capsule, the cylinder was removed and approximately one million pig islets were injected into the tube. The mixture of cells consisted of islet cells and testicular Sertoli cells taken from neonatal pigs. Sertoli cells are thought to have a special ability to subdue immune system T cells, which normally fight against the presence of anything foreign. The researchers gave the cells to 12 children with type 1 diabetes between the ages of 11 and 17 and did not administer any immunosuppressive drugs to protect the cells from being rejected. Six of the 12 patients had functioning grafts, and subsequent to receiving additional transplanted islets at 20 weeks, they had demonstrated improvements in their islet function. According to the researchers, the pig cells did not elicit the expected immune system response in the patients, and retransplantation of islets failed to stimulate a secondary response against the pig cells as well. One child remained insulin-independent one year after the transplant. Another was insulin-independent for six months and required 75 percent less insulin than before the procedure.

Table 1. Membrane Types Applied for Macroencapsulation of Pancreatic Islets

Membrane Geometry Proposed by	Geometry	Proposed by
nitro-cellulose acetate	Planar	Algire [18]
polycarbonate	Planar	Strautz [204]
2-hydroxyethyl methacrylate (HEMA)	Planar	Klomp [72]
polyacrylonitrile and polyvinylchloride	Tubular	Archer [51]
cellulose	Tubular	Zekorn [205]
polyamide	Tubular	L'Hommeau [66]
acrylonitrile and sodium-methallyl sulfonate (AN69)	Tubular	Kessler [74]
alginate-based	Tubular	Lanza [9]
polyvinylalcohol (PVA)	Tubular	Qi [77]
smart-membranes	Tubular	Kurian [80]

A novel, innovative approach in the field of macroencapsulation is the introduction of so-called "smart" membranes [67, 80]. These smart-membranes are composed of tricomponent amphiphilic membranes containing poly(ethylene glycol) (PEG), polydimethylsiloxane (PDMS) and poly(pentamethylcyclopentasiloxane) (PD(5)). It has been shown that these membranes in air are enriched by the hydrophobic components, PDMS and PD(5), while in water, the surfaces are rich in the hydrophilic PEG. This illustrates the versatile properties of the membranes under different circumstances. It has been shown that these versatile properties facilitate diffusion of essential nutrients such as oxygen and of therapeutic hormones such as insulin. Also it has been shown that the membranes allow for long term survival of encapsulated islet tissue.

All the above mentioned studies demonstrate the principle clinical applicability of the technology of extravascular macroencapsulation technology. However, the same studies also show that the technique is not yet ready for clinical application since graft survival is usually limited to several months. Critical issues such as the requirements the capsules have to meet in order to be biocompatible and the requirements the devices have to meet in order to allow for long-term survival of pancreatic islets remain subject of not more than a few studies. A detrimental factor in the geometry is the large distance that glucose and insulin have to diffuse in order to create a response. Although many groups have shown an improvement in glucose-disposal, the presence of a clear and adequate elevation in serum-insulin levels upon elevation in glucose levels remains subject of debate. The same geometrical problem interferes with adequate nutrition of the encapsulated tissue. Many groups have shown a slow but progressive development of necrosis in the islet tissue in the core of the macrocapsules with graft failure as a consequence [65, 81, 82]. Studies on mass transfer of oxygen have shown that in the extravascular spaces such as subcutaneously and intraperitoneally, the oxygen tension is much lower than in the systemic circulation [26, 83-89]. The membrane is another barrier to overcome, which limits the availability of oxygen for the tissue [83, 84, 89]. Similar calculations can be made for other, essential nutrients such as free-fatty acids, vitamins and free-radical scavengers. Many groups therefore consider vascularization in the vicinity of the macrocapsules after implantation, an obligatory event for long-term survival of the grafts. Approaches to achieve vascularization are inclusion or coating of the devices with angiogenic growth factors such as vascular endothelial growth factor or modification of the capsule surface in order to allow ingrowth of endothelial cells [65, 90-94].

An underlight issue in the field of extravascular macroencapsulation is the consequence of the immunological responses for survival of islet tissue. The macrocapsules predictably initiate many inflammatory reactions after implantation. These reactions can be categorized into at least three types. The first is the foreign body response against the capsules. Studies to this reaction mainly focus on modifications of the membrane to reduce the response rather than on identifying the cells and immunology behind the response [34, 35, 66, 78, 95-97]. As a consequence, there is still insufficient insight in the pathogenesis of the foreign body response against macrocapsules and it is impossible to decide whether the responses are caused by mechanical frictions *in vivo* or by chemical factors.

The other type of response is provoked by the enveloped tissue, which releases allogenic or xenogenic epitopes. It has been shown that this induces the formation of encapsulated tissue specific antibodies [41, 53, 98]. Most groups do not consider the formation of antibodies to be deleterious for the tissue since the capsules should adequately protect the tissue.

The last but rather newly identified type of response is the deleterious component of the vascularization process, which nowadays is considered to be mandatory for adequate function of macroencapsulated islets. This vascularization of a membrane is preceded by an inflammation episode which involves recruitment of many deleterious inflammatory cells in the vicinity of the capsules and with the formation of an extracellular matrix to facilitate ingrowth of endothelial cells [65, 99, 100]. The latter episode is not only associated with the presence of many deleterious cytokines and bioactive molecules but also with a period of ischemia. It is mandatory to study which factors macrocapsules should protect in both the immediate and late transplant period. It is predictable that the current generation of devices insufficiently protect the tissue for the factors involved in the immune responses.

A more thoroughly studied system is the microencapsulation of islets in which islets are enveloped by their own individual capsule. Several arguments favor microcapsules over macrocapsules. Their spherical shape offers better diffusion capacity because of a better surface/volume ratio. Microcapsules cannot be easily disrupted, they are mechanically stable, they do not require complex or expensive manufacturing procedure, and they can be implanted into the patient by a simple injection procedure.

Reportedly, the clinical experience is still restricted to one recipient [101], but several research groups are concentrating on transplantation of microencapsulated islets in the experimental setting [11, 58, 59, 102-123].

EXTRAVASCULAR MICROCAPSULES

Due to the flexible and pliable characteristics, microcapsules are mostly produced from hydrogels. In Table 2 we have summarized a number of commonly applied materials for cell-microencapsulation. The authors of this paper have like others [11, 118, 123-126] concentrated on alginate-based capsules. This was done out of belief that a step-wise analysis of factors determining success or failure will deliver more information about the requirements a system has to meet than an approach of trial and error in which new materials are applied with some advantages but mostly with more 'new' obstacles. Alginate provides some major advantages over other systems. First it has been found, repeatedly, not to interfere with cellular function and alginate-based capsules have been shown to be stable for years in small and large animals and also in men [101]. The technique is based on entrapment of individual islets in an alginate droplet, which is transformed into a rigid bead by gelification in a divalent cation solution, mostly rich in Ca^{2+} . Alginate-molecules are composed of mannuronic (M) and guluronic acids (G). In the first step of the microencapsulation process (*i.e.* the gelification), the alginate-molecules are connected by Ca^{2+} through binding of consecutive blocks of G-molecules on each of both molecules.

Table 2. Membrane Types Applied for Microencapsulation of Pancreatic Islets

Main component of the capsule	Source	Initially proposed by
Alginate	Alga	Lim and Sun [28]
Chitosan	Alga	Zielinski [206]
Agarose	Alga	Iwata [116]
Poly(hydroxyethylmetacrylate-methyl methacrylate)(HEMA-MMA)	Synthetic	Dawson [207]
Copolymers of acrylonitrile (AN69)	Synthetic	Kessler [74]
Polyethyleneglycol (PEG)	Synthetic	Cruise [104]

Alginate-based capsules can be found as coated and non-coated beads. The most commonly and extensively studied non-coated alginate-beads are the Barium-crosslinked alginate beads. This methodology was developed by the Würzburg group [107, 124, 127-132] that found that the stability of alginate-beads increased by replacing calcium for barium as crosslinking agent. During the last decade, the Würzburg group has studied this method mostly in xenotransplantation models, with mixed success rates. More successful with this technology was the Boston-group who reported normalization of blood glucose for one year in the non-obese-diabetic (NOD) mouse, an auto-immune model of diabetes, using allogenic instead of xenogenic islets embedded in barium-beads [11]. This study and more recent studies [133] from the same laboratory provide additional support to a few basic concepts, as well as more insight on the potential for application of alginate-based capsules. First, the authors have showed that, in allotransplantation instead of in xenotransplantation, microcapsules do not have to completely prevent diffusion of antibodies and cytokines to efficiently protect encapsulated islets. The Barium-beads microcapsules used for this study had a molecular weight cut-off of 600 kD [133], whereas IgG immunoglobulins have a molecular weight of 140 kD and the molecular weight of potentially harmful cytokines range from 17.5 (IL-1) to 51 kD (TNF- α). The other important observation in this study is that the protection provided by these non-coated alginate beads is effective in auto-immune diabetes and that encapsulated islets may survive for periods longer than a year [11]. Since the life span of a Beta cell is approximately three months [134], the study suggests that regeneration of islet cells occurs in capsules. Unfortunately, the Boston-group could not achieve the same success rates when rats instead of mice were applied [133]. The authors suggest that microcapsules are submitted to a higher mechanical stress in rats than in the mouse peritoneum. The authors suggest to reinforce the mechanical stability of barium alginate beads in order to allow testing in larger animals than mice.

Coated alginate beads have a broader potential application than barium-beads because the coating induces an increase in mechanical stability and a restriction in permeability, which makes xenotransplantation of islets a feasible option. The most commonly used alginate-based capsules are formed by the alginate-polylysine system. After gelification of the beads in calcium, the beads are coated with a polylysine membrane by suspending the beads in a poly-L-lysine (PLL) solution. During this step, PLL binds to mixed sequences of G and M in the alginate molecules [135, 136]. This induces the forma-

tion of complexes at the capsule surface surrounded by super-helically orientated polysaccharide chains [136]. The presence of these complexes decreases the porosity of the membrane. By varying the molecular weight and the concentration of the polylysine, and the incubation time, one can modulate the porosity of the capsule membrane [114, 137-142]. Usually, five to ten minutes incubation in 0.1% polylysine with a molecular weight of 22 kDa is sufficient to form an immunoprotective membrane. However, it should be emphasized that the binding of polylysine does not only depend upon the incubation time and the molecular weight of the polylysine but also on the type and concentration of alginate [141, 143] as well as on the temperature of an incubation [140, 141, 143]. In a final step, to provide biocompatibility, the capsules are suspended in a solution of alginate or other negatively charged molecules [140, 143] to neutralize positively charged polylysine residues still present at the capsule surface.

Another very important aspect for preparing successful alginate-PLL capsules, which requires a lot of experience in the microencapsulation process, is a smooth and mechanically stable microcapsule. In our laboratories, we have observed that high rather than low viscosity alginates produce smooth beads with no obvious tails or strains. Moreover, we have found that after implantation, alginates with low-G concentrations had a tendency to swell with subsequent breakage of the PLL membrane followed by cellular overgrowth of the capsules. Therefore, for our studies we have chosen the alginate with an intermediate G-concentration.

The stability of capsules is determined by stability of a membrane and stability of an alginate core. The stability of membrane can be controlled by the PLL-step since shorter incubation time, lower PLL concentrations, and lower PLL molecular weight than described above, were associated with an increase of the number of capsules with broken membranes [64, 114, 144-147]. Additionally, incubation at 4°C instead of room temperature led to less stable microcapsules. Finally, we have not applied EGTA or citrate [148] to liquify the inner core of the capsule. The reason for this modification of the original Lim and Sun method [28] was that we observed many capsules losing their integrity during the treatment.

BIOCOMPATIBILITY ISSUES IN MICROENCAPSULATION

For many years researchers have considered insufficient biocompatibility to be a major threat for clinical application of microcapsules. Failure of microencapsulated islet grafts was

usually interpreted to be the consequence of insufficient biocompatibility of the materials applied, which induces a non-specific foreign body reaction against the microcapsules and results in progressive fibrotic overgrowth of the capsules (Fig. (2)). This overgrowth interferes with adequate nutrition of the islets and consequently causes islet cell death. During the past decade, many improvements have been introduced in the encapsulation technology, which have solved the vast majority of biocompatibility problems. It was shown that pure alginate rather than commercially available crude alginates should be applied for encapsulation [65]. Crude alginate was shown to be associated with overgrowth of the capsules by inflammatory cells (mostly macrophages and fibroblasts) with necrosis of the enveloped therapeutic cells as a consequence.

Another important factor is the type of alginate applied. As aforementioned, alginates are composed of guluronic acid (G) and mannuronic acid (M) and can be obtained with varying G/M-content. Theoretically high-G alginates are preferred over alginates with a lower-G content since high-G capsules are more durable [149-153] and associated with less protruding islet cells [114, 146] than the other types of alginates. *In vivo*, however, it was found that high-G alginates are associated with much more inflammatory reactions than intermediate-G alginates. This is due to different binding properties of polylysine to high-G and intermediate-G alginates [149].

When inadequately bound to alginate, polylysine can be a strong initiator of fibrosis. This was shown by others [154] and by us [149] when comparing the biocompatibility of high-G alginate-polylysine capsules and high-G alginate beads in the absence of polylysine.

New physicochemical technologies have come to the field to explain the observation that the biocompatibility and the adequacy of binding with poly-L-lysine vary with the G-content of the alginate. In order to provide more insight in the structure of alginate-PLL capsules, we have performed a physicochemical analysis of the capsules by applying X-ray photoelec-

tron spectroscopy [155-157]. This technique allows for identification of the chemical groups on the surface of the capsule on an atomic level. Up to now, the capsule was assumed to be composed of a core of Ca-alginate, which is enveloped by a membrane composed of two layers, *i.e.* an inner layer of alginate-PLL and an outer layer of Ca-alginate [28, 151, 158]. The data, which have led to this model, were almost exclusively obtained by studying the chemical interactions of PLL with solvated, non- Ca^{2+} bound and often individual components of alginate (*i.e.* G-acid and M-acid monomers) and not by studying the chemical structure of the capsules as such. In our studies on true capsules, we never observed Ca^{2+} in the membrane of the capsules, which has the following implications for the assumed structure of the capsules. First, the alginate-PLL layer is not composed of the combination of alginate-PLL and Ca^{2+} -alginate but of the alginate-PLL only. The absence of Ca^{2+} also implies that the outer Ca-alginate layer does not exist and, consequently, that the membrane is not composed of two but of one layer only. Finally, we found sodium in the membrane, which is bound by carboxyl groups on consecutive blocks of G- and M-molecules, which remain unbound between the complexes of PLL and mixed sequences of G and M.

We [159] combined attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR), X-ray photoelectron spectroscopy (XPS), with time-of-flight secondary ion mass spectrometry (ToF-SIMS) to define the alginate-PLL capsule membrane at the micrometric and nanometric scale. These three surface sensitive techniques have a dept of analysis in the micrometric range for ATR-FTIR, up to 100 Å for XPS and 1-2 nanometers for ToF-SIMS. These studies showed a relatively high amount of PLL near the microcapsule surface. ToF-SIMS analysis demonstrated that PLL was present at the outermost 1-2 nanometer of the membrane. This represents a dept of 1-2 atoms. In addition, ToF-SIMS imaging showed that PLL exposure at the true surface of capsules was not due to defects in alginate coating. Instead, PLL, alginate, and larger PLL-alginate complexes were all very uniformly distributed at

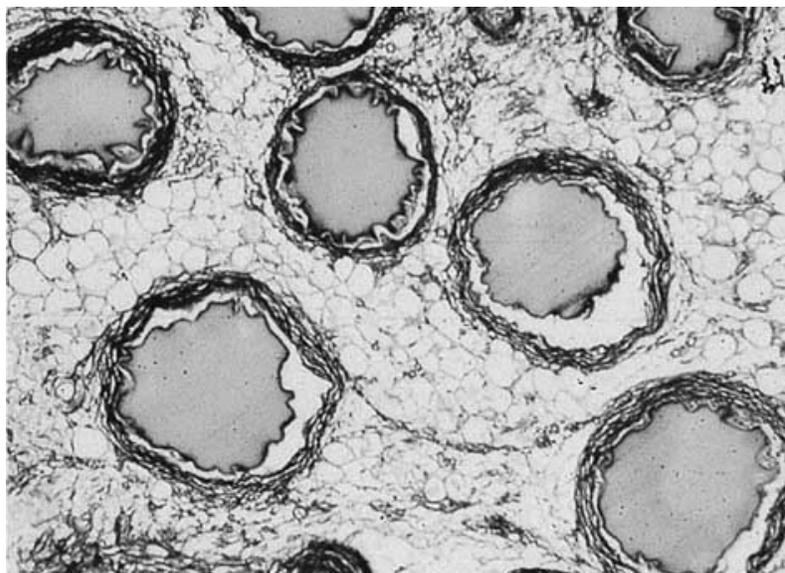


Fig. (2). The final result of a foreign body response against alginate-polylysine capsules. The capsule is overgrown by fibroblasts and connective tissue already at one month after implantation in the rat. (GMA-embedded section, syrius red staining, original magnification x 50).

the microcapsule surface. Fig. 3 shows the actual structure of alginate-PLL capsules.

These findings have serious implications for biocompatibility issues associated with microcapsules since it implies that the proinflammatory PLL is always on the surface of the capsules in direct contact with the inflammatory cells. Also, it shows that it is mandatory to include physicochemical technologies in the field. The present data suggest that, for optimal biocompatibility, we have to focus on understanding and improving the interaction of the inflammatory polycations with alginate rather than improving the second coating step with alginate. Alternatively, we can perform an additional envelopment step of the capsules to prevent direct contact between the polycation with the surrounding tissues in the implantation site [160].

In a recent study, we have applied all the current knowledge for the requirements of producing a biocompatible alginate-PLL capsule. These capsules were implanted in the peritoneal cavity of rats and retrieved two years later, *i.e.* the life-span of a rat, for studying the biocompatibility of the capsules. It was found that the vast majority of the capsules could be retrieved after this 2 years period. Of the retrieved capsules only a portion of 2-10% was overgrown with inflammatory cells while 90-98% of the alginate-PLL capsules were completely free of any inflammatory overgrowth [155]. This study shows

that it is feasible to produce fully biocompatible alginate-PLL capsules in spite of the inflammatory reactions individual components of the capsules can provoke.

LONGEVITY OF THE GRAFT

Unfortunately, the improvements in the capsule's chemical composition did not bring about the ultimate goal of encapsulated-cell research, *i.e.* predictable long-term survival of the grafts. Although, the overgrowth rate of capsules was reduced from 80-100% of the capsules to a portion of only 2-10%, the engrafted immunoisolated cell survival time was not permanent but increased from 3 to 6 months [161]. It has been argued that the microenvironment of the capsule itself interferes with long-term survival. Recent findings however show the opposite; *in vitro*, encapsulated islets show better survival rates than free islets [162]. In part, this can be explained by retention of hypertrophic autocrine and paracrine signal molecules in the microcapsule micro-environment that promotes islet survival.

Another important factor interfering with survival of encapsulated islets is the lack of direct vascular access due to the presence of the physical barrier of the capsule that interferes with direct vascularization. This lack of direct vascularization not only interferes with optimal nutrition of the immunoisolated graft but also with the functional performance of the

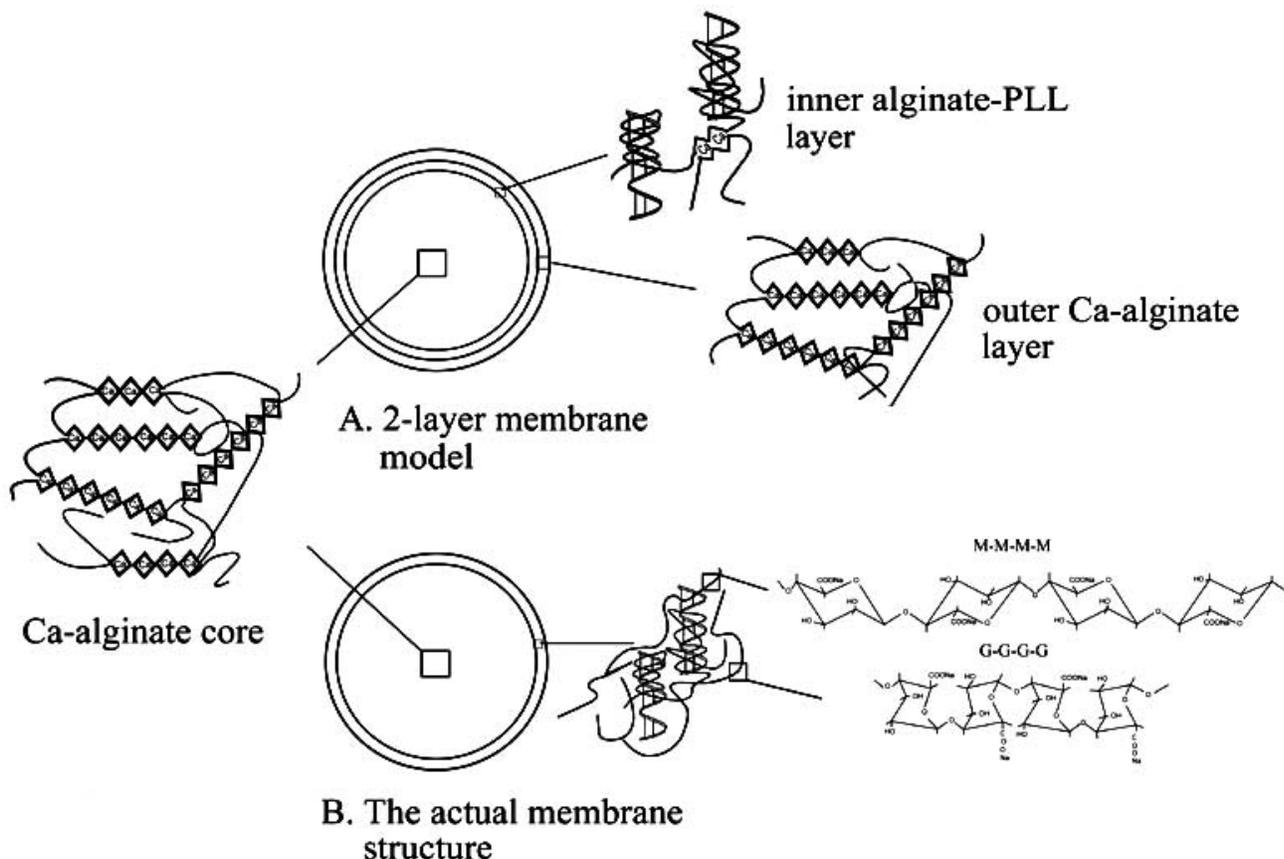


Fig. (3). The considered and the actual structure of alginate-PLL capsules. The capsule is not composed of three layers as generally assumed but of two layers.

grafts [65, 81, 82]. Apparently, it is obligatory for clinical application to find a site where encapsulated islets are in close contact with the blood stream. Unfortunately, it is difficult to find such a site since it should combine the capacity to bear a large graft volume in the immediate vicinity of blood vessels. The peritoneal cavity is the only site available able to carry a graft with the size of an encapsulated transplant but it does not have the required degree of vascularization. To allow transplantation in other sites, it is obligatory to reduce the capsule size.

In most tissues, the maximum diffusion distance for effective oxygen and nutrient transfer from capillary to cells is 200 μm . [163-166]. The absence of convection movement within a capsule induces a nutrient- gradient from the capsule surface to the center of the islet [167, 168]. A reduced capsule size therefore would allow for a better nutrient supply to cells, and offers the advantages of an exponential decrease of the total implant volume. Based on the principles set forth by Hommel *et al.* [7, 169], an electrostatic pulse generator was designed to produce alginate beads and capsules as small as 185 μm in diameter, which is fourfold smaller than the conventional 800 μm capsules [123]. These small capsules can be implanted in the intraperitoneally implanted solid support system for pancreatic islets [81], which was recently introduced. This site allows for implantation of high numbers of islets, which can readily be retrieved and, theoretically, can be designed as such that it is highly vascularized.

This solid support system was developed from expanded polytetrafluoroethylene (ePTFE) since ePTFE has been shown to be biocompatible and to become neovascularized after implantation in the peritoneal cavity. Initially, we applied the concept of Thompson *et al.* [170, 171] in which solid supports are coated with collagen type IV (*i.e.* the collagen type predominantly present in the basal membrane) and acidic-fibroblast growth factors (α -FGF) to facilitate the ingrowth of blood vessels [81, 170, 171].

This solid support was shown to be very efficacious since normoglycemia was induced in all diabetic rats with islet transplants in the solid supports while this was only the case in 40% of the diabetic rats transplanted with islets in the unmodified peritoneal cavity without the solid support [81].

In our subsequent studies on vascularization of solid supports, we have focused on vascular endothelial growth factor (VEGF)-165 [172-174], which is considered to have the greatest potential as angiogenic stimulus when compared to other angiogenic growth factors like acidic-fibroblast growth factor, basic-fibroblast growth factor, platelet derived growth factor, epidermal growth factor, transforming growth factor alpha and beta, interleukins (IL-8 and TNF) or prostaglandins. It has been shown in rats that islets show much better survival rates and function in these devices [65] but with encapsulated islets, the survival was still not permanent.

LONGEVITY AND ISLET-DERIVED BIOACTIVE FACTORS

At this point, it was obscure what was causing failure since it was generally assumed that the loss of 2-10% of capsules cannot explain the failure of the cells in the remaining 90-98% of the capsules [143, 175-179]. A recent series of experiments brought us new insight in the pathogenesis of encapsulated cell

failure: the transplanted cells and not the capsule's materials were the principle cause of failure. We found that encapsulated cells such as immunoisolated pancreatic islets under stress (by adding IL-1 and TNF-) can produce the cytokines MCP-1, MIP, nitric oxide (NO), and IL-6 which are well-known to contribute to recruitment and activation of inflammatory cells. In a subsequent experiment, we demonstrated that activated macrophages on the 2-10% of overgrown capsules do secrete the cytokines IL-1 and TNF- when they were co-cultured with islet-containing capsules and not with empty capsules [147, 180]. This process was accompanied with a gradual loss of function of the encapsulated tissue [180, 181]. These experiments showed that graft-derived cytokines diffuse out of the capsules and on their turn, activate the macrophages to secrete cytokines with a vicious circle of activation as a consequence (Fig. 4).

The initiation of this vicious circle of activation has to be sought for in the immediate period after transplantation, *i.e.* the tissue responses associated with implantation surgery. In a recent paper [157], we have shown that the very first step in the tissue response is not related to the implantation of the 'foreign' capsules but to the required surgical procedure for implantation (it was also observed in shams) [157, 182]. Although it is only minor surgery, the procedure is associated with tissue damage and release of bioactive proteins such as fibrinogen, thrombin, histamine, and fibronectin [183-185]. These factors have chemotactic effects on inflammatory cells and induce influx of high numbers of granulocytes, basophiles, mast-cells, macrophages to the peritoneal site in the first days after implantation [157].

Especially, the observation that mast-cells and macrophages are present in the first days after implantation is important since these cells are potent producers of the bioactive factors IL-1, TNF-, TGF-, and histamine, which further activate inflammatory cells in the vicinity of the foreign materials [184-188], and, more importantly, stimulate the cells in the capsules to produce graft-derived cytokines.

Within two weeks, basophile and granulocytes gradually disappear from the graft site while macrophages and some fibroblasts remain attached to the portion of 2-10% the capsules [157]. These attached macrophages remain activated and, therefore, contribute to the vicious and deleterious circle of activation. Thus, although we and others [143, 175-178] considered the loss of 2-10% of capsules of minor importance for the function of the remaining 90-98% of the graft, our data show the opposite and illustrate that it is mandatory to completely delete overgrowth of the capsules.

A conceivable approach to overcome the problem of islet-derived cytokines is reduction of the capsule permeability. The diffusion of graft-derived and inflammatory cell-derived cytokines is a major threat for the longevity of the encapsulated grafts [147, 189]. The permeability of the capsules for cytokines has always been a subject of debate. Scepticists have always assumed that the membranes of capsules cannot adequately protect against deleterious cytokines with an approximate molecular weight of insulin or essential nutrients (5-15 kDa). Therefore, diffusion of cytokines into the capsules has always been the Achilles heel of immunoisolation. Combined efforts of the De Vos-group and that of Marchetti in Pisa have shown that this is not an insurmountable problem. It has been

shown that the final effect of cytokines is dependent on the combined presence of different cytokines and on the concentration of cytokines [82]. It was found *in vitro* that decreasing the

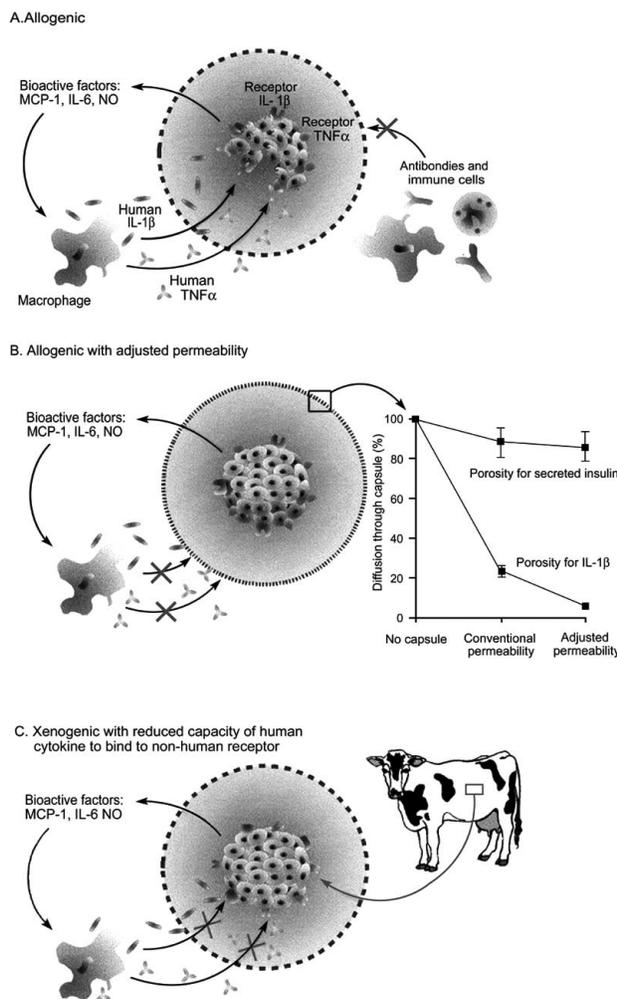


Fig. (4). The vicious circle of activation causing failure of 60% of the islets in the immediate period after transplantation. Islets release cytokines which act in concert with cytokines released by a surgery induced activation of the immune system on the recruitment and activation of inflammatory cells in the vicinity of the graft.

permeability by chemical modification of the capsules prevents diffusion into the capsules of large and multimeric cytokines such as TNF- α . Also, diffusion of small cytokines (e.g. IL-1 β) was reduced by changing the permeability of the membrane, which was unexpected as the molecular weight of small cytokines such as IL-1 β (17 kDa) was far below the molecular cut-off of the applied capsules (100 kDa) (Fig. 5). The mechanism is that the chemical modifications for decreasing the permeability increased the negative charges of the alginate in the capsules, which induces repulsion of the negative charges on the tested cytokines-molecules.

Also, we found evidence that cytokines may not interfere with islet function in case of xenografting of encapsulated islets in humans. We have observed that following exposure to

a combination of human cytotoxic cytokines, a marked decrease in functional survival and a high percentage of apoptotic cells could be found in human islets but not in bovine islets [190]. Preliminary data show that this is due, at least in part, to the fact that xenogenic islet cells are less capable to bind and to take up human cytokines. This implies that, at least in some combinations, even when capsules are applied which are permeable for cytokines, the function and survival of xenogenic islet-sources will be less affected.

CONSIDERATIONS FOR FURTHER RESEARCH

Present data suggest that we have to focus on at least two critical issues in order to reduce the drawback in viability of the encapsulated tissue as the consequence of inflammation in the immediate post-transplant period. The first is to completely delete overgrowth of the capsules. It is well known that the overgrowth of the 2-10% of the capsules is caused by individual imperfection on the capsules such as protruding cells [146] and polyaminoacid molecules with an inflammatory conformation [155, 156]. A conceivable approach is to cover the surface of the capsules with an additional layer of highly biocompatible alginate [144] or other materials [160] in order to completely mask the surface of the capsule for the inflammatory cells in the surroundings.

Another mandatory step is to delete the effects of the tissue responses associated with implantation surgery. This response is responsible for loss of 60% of the islets in an encapsulated graft [179] in spite of application of fully biocompatible capsules that remain completely free of inflammatory overgrowth. Recently, Büniger *et al.* [145] showed that suppression with dexamethasone of this surgery-induced activation of the immune system in the immediate period after implantation was successful in preventing responses against the capsules. It was found that a four weeks period of administration completely blocked the influx of inflammatory cells and cytokine release and, most importantly, that the response did not appear after ceasing the release of dexamethasone. Although this study demonstrates the efficacy of the approach of interfering with the innate immune response, we cannot apply this classical method of suppressing the innate immune response to islet-transplantation, since corticosteroids interfere with the functional survival of islet-tissue [191, 192] and therefore should be avoided. At present, we are testing new anti-inflammatory agents having a minimal or no effect on the survival of islets.

CONCLUDING REMARKS

The encapsulation-field has experienced more scientific progress in the past five years than in the 25 years before that in spite of reduced funding as the consequence of political impediments. It is now clear and accepted in the field that much more requirements have to be met in order to produce biocompatible capsules than originally described [8, 109, 193-200]. Also, it is now better understood that biocompatibility of immunisolating devices is much more than only prevention of tissue responses.

Biocompatibility is usually defined as the ability of a biomaterial to perform with an appropriate host response in a 'specific application' [201]. With fully artificial organs such as artificial hips, knees or middle ears, this definition is easy to interpret. It is, however, far from simple to interpret with

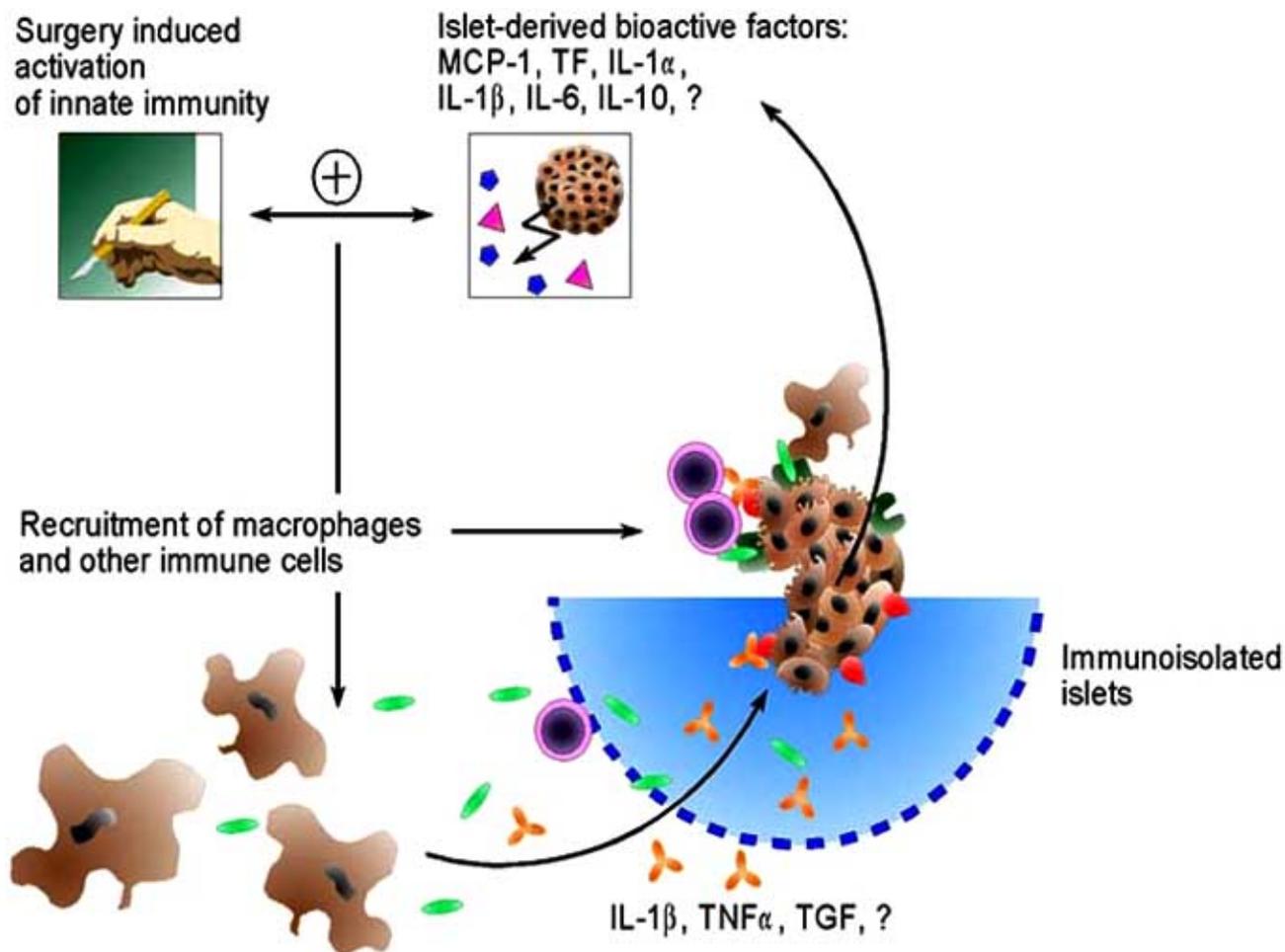


Fig. (5). Approaches to prevent deleterious effects of diffusion of cytotoxic cytokines and chemokines into the immunoisolating capsules after transplantation. (A). Islets produce cytokines that diffuse out of the capsules and activate inflammatory cells such as macrophages in the vicinity of the microencapsulated islets. The cytokines secreted by the macrophages diffuse into the capsules and induce massive cell death in the allogenic human islet cells. (B). By adjusting the porosity of the capsules for IL-1- and for secreted insulin, we found that the permeability can be lowered so that cytokines cannot pass the membrane, whereas the porosity of the capsules for insulin remains unaffected (graph show mean \pm sem of 5-7 experiments). (C). In case of xenotransplantation, we found that cytokines of human origin are less deleterious for islets of animal origin such as bovine islets, possibly due to reduced interaction at the receptor level.

bioartificial systems such as the immunoisolation technology. Until a few years ago, it was assumed with extravascular devices that a fully biocompatible system would be achieved with membranes which elicit no or not more than a minimal foreign body reaction, since overgrowth on the surface of the membrane interferes with optimal diffusion of nutrients and metabolites [109, 141, 202]. Now we have reached the state in which we can prevent overgrowth on the majority of 90-98% though we still observe limitations in functional survival in our 'specific application'. With intravascular devices, the overgrowth of the membrane is considered to be of minor importance. Here, a permanently patent tubular membrane without thrombotic or other adherent layers is required for optimal biocompatibility. Irrespective of the question whether the approach is intravascular or extravascular, the host response to the biomaterial was generally considered as the only and single response causing biocompatibility problems. However, with immunoisolating devices, there is not only an interaction between the biomaterial and the tissues of the exterior, host

environment but also between the biomaterial and the encapsulated donor tissue. Although this aspect is not covered by the current definition of biocompatibility, it should be considered a true biocompatibility issue since long-term survival of the tissue is required for this 'specific application'.

Factors not related to the capsules materials are of equal importance for the survival and longevity of encapsulated tissue. The surgery induced activation of the immune system in the immediate period after implantation is a rather unrecognized reaction with a profound, deleterious effect on encapsulated islets. This immediate response is not directly related to rejection or autoimmunity and requires more intensive studies in order to find means to interfere with the response. We feel this response should be blocked by temporary rather than permanent pharmaceutical intervention by release of anti-inflammatory products in the first two weeks after implantation, since it will be difficult to overcome this issue by modification of the capsule membrane.

An underlight issue is the fact that we require methodologies to produce capsules that can withstand the shear-forces capsules are exposed to in larger mammals. Recently, Dusseault *et al.* [203] developed a method to encapsulate living cells in membranes with covalent crosslinks between adjacent layers. Since the extreme conditions required to break covalent links are incompatible with cell survival, these capsules are virtually indestructible in the conditions found in the human living body. This technology should be combined with the approach for producing the smallest capsule possible. This is not only pertinent for allowing transplantation of encapsulated islets near blood vessel but also for allowing fast exchange of glucose and insulin, since encapsulated islets should closely follow the kinetics of insulin release of a free, nonencapsulated islet.

An obstacle in clinical application of encapsulated islets that is not directly related to the encapsulation technology is the absence of an adequate islet source. Irrespective of the species (*i.e.* rat or human), we require multiple donors for a single transplantation. It is mandatory to gain sufficient insight in which factors determine success or failure of an islet isolation procedure. For islet-encapsulation, it is of an additional importance that the islets are sufficiently 'clean', *i.e.* not contaminated with exocrine tissue since this holds the risk of protrusion of cells [114, 146] and death of the encapsulated islets as a consequence. Many groups focus their research efforts on generation of islet-cells from stem-cells for solving the obstacle of insufficient supply of islets and abandon the approach of improvement of islet-isolation from adult sources. It should be noted that this is not without risk since in spite of a decade of intensive research, there are no reports showing full mature insulin secretion profiles of islet cells produced from stem cells while the number of publications on improvement of adult islet isolation has decreased dramatically. It is plausible that the high expectations of the stem-cell technology will run into the same political impediments that the immunoisolation field has met during the past ten years, which will block scientific progress in the generation of sufficient islet sources.

Finally, we feel that transplantation of encapsulated cells will come to clinical application if two major issues will be solved. The first is transplantation of the encapsulated cells near blood vessels in order to allow optimal nutrition of the encapsulated tissue and control of glucose metabolism. This site should also allow for retrieval of the graft in case replacement is necessary. The second issue is the reduction of loss of islets in the immediate transplant period as the consequence of a surgery induced activation of the immune system. We propose administration of anti-inflammatory pharmaceuticals during and directly after transplantation to reduce the effects of the surgery induced activation and to implant the encapsulated islets in a prevascularized solid support system or any other highly vascularized site in which long-term function and optimal exchange of glucose and insulin are guaranteed.

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ABBREVIATIONS

PAN-PVC	= Polyacrylonitrile and polyvinylchloride copolymer
PTFE	= Polytetrafluoroethylene
IL	= Interleukin
HEMA	= 2-hydroxyethyl methacrylate
HEMA-MMA	= Poly(hydroxyethylmethacrylate-methyl methacrylate)
AN69	= Copolymers of acrylonitrile
PEG	= Polyethyleneglycol
PVA	= Polyvinylalcohol
PDMS	= Polydimethylsiloxane
D(5)	= Polypentamethylcyclopentasiloxane
PLL	= Poly-L-lysine
M	= Mannuronic acid
G	= Guluronic acids
NOD	= Non-obese-diabetic
ATR-FTIR	= Total reflectance Fourier transform infrared spectroscopy
XPS	= X-ray photoelectron spectroscopy
ToF-SIMS	= Time-of-flight secondary ion mass spectrometry
ePTFE	= Polytetrafluoroethylene
a-FGF	= Acidic-fibroblast growth factors
VEGF	= Vascular endothelial growth factor
NO	= Nitric oxide

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