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Review

Alginate-based microcapsules for immunoisolation of pancreatic islets

Paul de Vos[a,*], Marijke M. Faas[a], Berit Strand[b], Ricardo Calafiore[c]

[a]*Department of Pathology and Laboratory Medicine, Division of Medical Biology, University Hospital of Groningen, Hanzeplein 1, 9700 RB Groningen, The Netherlands*
[b]*Department of Biotechnology, Norwegian University of Science and Technology, Sem Sealand vei 6/8, 7034 Trondheim Norway*
[c]*Department of Internal Medicine, University of Perugia, Perugia, Italy*

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Abstract

Transplantation of microencapsulated cells is proposed as a therapy 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 foreign cells are protected from the host immune system by an artificial membrane. In spite of the simplicity of the concept, progress in the field of immunoisolation has been hampered for many years due to biocompatibility issues. During the last 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 not only the capsules material but also the enveloped cells should be hold responsible for loss of a significant portion of the immunoisolated cells and, thus, failure of the grafts on the long term. Microcapsules without cells can be produced as such that they remain free of any significant foreign body response for prolonged periods of time in both experimental animals and humans. New approaches in which newly discovered inflammatory responses are silenced bring the technology of transplantation of immunoisolated cells close to clinical application.

Keywords: Microencapsulation; Macroencapsulation; Islets; Alginate; Immunoisolation; Vascularization

Contents

1. Introduction

Grafting of therapeutic cells for treatment of human disorders such as hormone or protein deficiencies is not yet clinically applied on a large scale due to the necessity to use life-long immunosuppression for preventing rejection.

*Corresponding author. Tel.: +31 50 3611045; fax: +31 50 3619911.
E-mail address: p.de.vos@med.umcg.nl (P. de Vos).

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The necessity to apply immunosuppression can be bypassed by immunoisolating hormone- or protein-secreting cells in semipermeable membranes to protect donor-cells against antibodies and cytotoxic cells of the host immune system. This immunoisolation by encapsulation not only allows for successful transplantation of cells in the absence of immunosuppression [1–3] but also for transplantation of cells from nonhuman origin, i.e. xenografts, which could be a mean of overcoming the obstacle of limited supply of donor tissue [4,5]. The principle applicability of the technology has been shown for the treatment of a wide variety of endocrine diseases, including anemia [6], dwarfism [7], Hemophilia B [8], kidney [9] and liver [10] failure, pituitary [11] and central nervous system insufficiencies [12], and diabetes mellitus [1].

Microencapsulation of cells or tissues in alginate-based capsules, as originally described by Lim and Sun [1], is the most commonly applied procedure for immunoisolation. During recent years, important advances have been made with this technology. The first allotransplantations in humans with encapsulated parathyroid cells and pancreatic islets have been successfully performed [13–15]. Since most of the scientific research in the field of microencapsulation has been done with pancreatic islets in hydrogels, and since this is also the field of research of the authors, this review will mainly focus on the accomplishments with microencapsulated pancreatic islets in hydrogels.

In spite of the simplicity of the concept of microencapsulation and the urgent need for alternatives to immunosuppressives in transplantation, the progress in the field during the past decades could not meet with the high expectations. A casual factor in this has been insufficient knowledge of the microcapsule structure and properties in relation to its biocompatibility. Therefore, a number of groups including ours have performed a step-wise examination of the microcapsules properties and its concomitant biocompatibility. This has included in vivo, ex vivo, and chemical analysis of the capsules and grafts. Quite often this has led to design and application of new concepts. As a consequence, during recent years, important advances have been made in the basic knowledge of immunoisolation and the factors determining success and failure. This will be discussed in the present paper in view of clinical application. One of the authors is currently leading clinical trials with microencapsulated pancreatic islets for the treatment of type I diabetics (Fig. 1).

2. Biocompatibility issues in encapsulation

Biocompatibility is usually defined as the ability of a biomaterial to perform with an appropriate host response in a 'specific application' [16]. 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 bioartificial systems such as the immunoisolation technology. 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'. Both issues will be discussed in the present review.

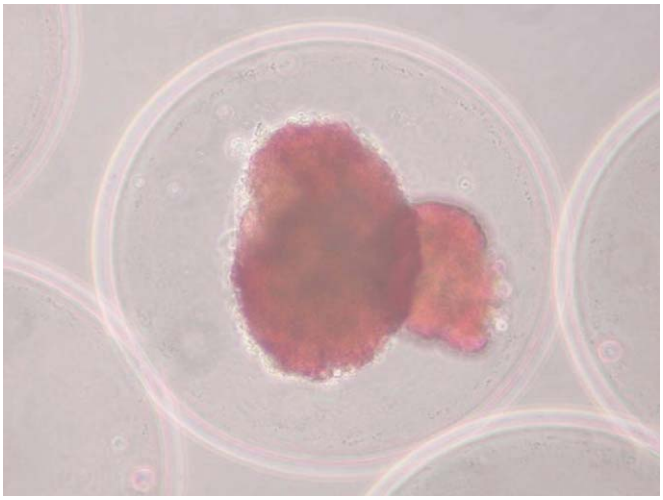

Fig. 1. A human pancreatic islet encapsulated in an alginate-based capsule before implantation in a type-I diabetic patient. The capsule contains two islets after dithizone staining.

Both intravascular and extravascular immunoisolation devices have been studied for application in Diabetics. In general, extravascular devices are beneficial because it requires not more than minor surgery with minimal risk for the patients.

Microcapsules have been the most intensively studied extravascular device because of the spherical shape and small size that offers an optimal surface to volume ratio and an optimal diffusion capacity when compared to the larger macrocapsules. Other advantages are that microcapsules cannot be easily disrupted, are mechanically stable, and do not require complex or expensive manufacturing procedures. Microcapsules can be produced from different materials and are being applied as planar beads or as coated, multilayered systems as will be outlined in the following section.

3. Biocompatibility issues related to the materials applied

Prevention of cellular overgrowth of microcapsules is considered to be a crucial factor in biocompatibility of microcapsules. For some applications of biomaterials, such as implantation of artificial joints, growth of host cells and coverage of the implant with host-cells is considered as a benefit and a process that promotes the functional performance of the implant. For microcapsules, however, the growth of host cells on the capsule surface is considered to have negative effects because of reduced diffusion of oxygen and nutrients to the encapsulated graft resulting in necrosis of the enveloped cells. In addition, the cells on the

capsule surface are found to be mainly inflammatory cells secreting cytokines and chemokines that may have a negative effect on graft function.

In the past decade many groups have studied the applicability of hydrogels for extravascular encapsulation. 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 [17,18]. The most commonly applied materials for microencapsulation are alginate [1], chitosan [19], agarose [20], poly(hydroxyethylmetacrylate-methyl methacrylate) (HEMA-MMA) [21], copolymers of acrylonitrile (AN69) [22], and polyethylene glycol (PEG) [23].

Alginate provides some major advantages over the other systems. First it has been found, repeatedly, not to interfere with cellular function of the islets [24–26]. Alginate is one of the few materials that allows for processing of the capsules at physiological conditions. The encapsulation can be done at room or body temperature, at physiological pH, and in isotonic solutions. Also it has been shown that alginate capsules can provide a microenvironment which facilitates functional survival of islets. It has been demonstrated by several groups that islets can more readily and more adequately survive when being enveloped in alginate-capsules before long-term tissue culture [27,28]. A plausible explanation for this phenomenon is that the three-dimensional matrix provides a growth support for the islets and also prevents clumping and fusion of the free islets which can interfere with availability of nutrients and oxygen for the islet cells in the core of the clumps. A last, but certainly not the least, advantage of the alginate-based capsules is that they have been shown to be stable for years in small and large animals and also in men [14].

The microencapsulation 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, such as calcium or barium. Calcium beads are ussually coated with a polycation to produce an immunoprotective membrane while barium beads are applied by some groups as a immunoprotective system as such. Both calcium and barium require a specific alginate composition to adequately connect alginate molecules. Alginate molecules are linear block co-polymers of β-D-mannuronic (M) and α-L-guluronic acids (G) with a variation in composition and sequential arrangements (Fig. 2). Up to now, it was assumed that the G-blocks are the only molecules in alginate that bind divalent ions cooperatively and are, therefore, the main structural feature contributing to gel formation. Recent findings, however, show that not only G-blocks but also blocks of alternating M and G (MG-blocks) can form cross links with calcium. Hence, calcium junctions of GG–GG, MG–GG and MG–MG must be hold responsible for gel-formation [29].

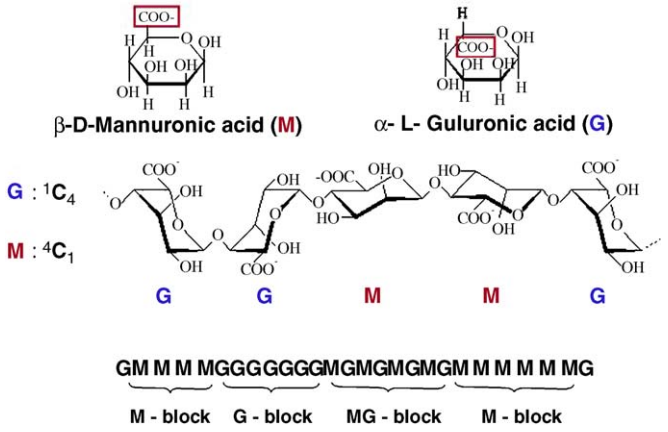

Fig. 2. The structure of alginate. Alginate molecules are linear block co-polymers of β-D-mannuronic (M) and α-L-guluronic acids (G) with a variation in composition and sequential arrangements.

Other cross-linking ions as an alternative for calcium have been used as well, in particular barium. Barium is preferred since it provides stronger gels [30] and since it may allow for transplantation of capsules in the absence of a polycation layer [31]. As barium is known to be toxic, concerns have been raised to using this ion as cross-linking agent. Studies of leakage of barium from alginate microcapsules of high-G material have shown, however, that when using low concentrations and intensive rinsing of barium beads, there is no barium leakage [32]. Barium forms stronger cross links with alginate which results in stronger gels than with calcium [30]. Notably, this only holds for specific types of alginates since recent studies showed that the effect of barium is only observed for alginates with a high-G content (more than 60% G). The enforcement of stability by replacing calcium for barium is absent for alginates enriched with M (less than 40% G) [33].

The composition and sequential structure of alginate is of great importance for its function as encapsulation material. Alginate is mostly isolated from seaweed and the composition varies widely depending on the source [34]. A lack of information from the manufacturer regarding composition of the alginate (i.e. G–M content, G–M ratios, and molecule length) is a major problem in the field of encapsulation as it is then not clear to researchers what type of polymer they are working with. In general, alginates with a high content of G have shown to form stable gels with a high permeability when compared to alginate microcapsules of high-M material [35]. When applying an outer coating of polycation (e.g. poly-L-lysine (PLL)), intermediate-G or low-G alginates have been shown to form more stable junctions than high-G alginates [32,36]. Thus, the adequacy of the type of alginate for a specific application depends on the type of cation and on the presence or absence of a polycation on the outside of the capsule.

Alginate is considered a biocompatible material for both the cells in the microenvironment and for the cells inside the capsules as it generally does not interfere with cellular function. Also, since alginates are negatively charged, the attachment of cells is limited due to the negative charge also on the cell surface. However, soluble alginates with a high content of M (>90% M) have been shown to stimulate monocytes in vitro through CD14 and toll-like receptors (TLR)-2 and TLR4 [37]. Additionally, antibodies to high-M material have been identified in transplanted mice [38]. As calcium and barium do not form cross-links with M-blocks, high-M material predictably leaks out of the capsules [39]. However, this can be prevented by washing the beads, which reduces the final leakage and the content of immune stimulating high-M alginate of the capsules to a minimum. This procedure increases the biocompatibility of alginate-based capsules but is unfortunately not fully recognized and applied by all groups in the field.

Crude alginate from seaweed contains polyphenols, proteins, and endotoxins [40]. Polyphenols are known to be toxic to cells while endotoxins are potent stimulators of the immune system. Polyphenols are also responsible for ORD-catalyzed depolymerization of alginates and a subsequent loss of viscosity [41]. Therefore, purification of alginate is required before application as an implantation material. Many purification methods have been published [3,40,42–44]. It is now also possible to buy ultrapure alginates with endotoxin levels of less than 100 EU/g (NovaMatix, Drammen, Norway). It has been shown that purification of alginates improves biocompatibility of alginate-based microcapsules [3,45]. 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. This reaction can be deleted by application of purified alginates. The vast majority of groups nowadays apply pure alginates with low content of endotoxins and lacking immunogenic effects. When alginates are implanted as barium–alginate beads the majority of groups never observe any tissue response illustrating the optimal biocompatibility of the alginate [3,13,17,45–54]. It should be mentioned, however, that purification of alginates requires insight in the chemistry and rationally for performing specific procedures. Unfortunately, the same purification procedure may give different results in various laboratories. This was recently illustrated in a paper of Robitaille et al. and Tam et al. [55,56] showing that some laboratories have difficulties in setting up purification procedures and to achieve the same efficacy as the original laboratories that designed the technology [57]. It should be mentioned that another factor may have influenced their results. Robitaille et al. and Tam et al. [55,56] did not thorough characterize the alginate after the purification, which means that both alginate composition and molecular weight and molecular weight distribution was lacking in the data. These factors may have had a large influence on the observed lack of biocompatibility as well.

It should be mentioned that most groups in the field-test the alginate samples routinely on endotoxin content before application. The efficacy of the purification can be tested by measuring the endotoxin content. Alginates above an endotoxin content of 100 EU/g will never be applied for in vivo studies [3,17,47,58]. If one is not able to obtain this efficacy of purification of alginate, it is advisable to either obtain pure preparations from other laboratories or to apply commercially purified preparations in order to prevent that purity issues are interfering with the success of the capsules.

Alginate-based microcapsules have been applied for immunoisolation as coated and non-coated beads. The coated systems are subjected to a coating step with a polycation such as PLL. The most commonly and extensively studied non-coated alginate beads are the barium-cross-linked alginate microbeads. This methodology was developed by the Würzburg group [42,52,59–63] that found that the stability of alginate beads increased by replacing calcium for barium as cross-linking agent. During the last decade, the Würzburg group has studied uncoated alginate beads with mixed success rates. More successful with this technology was the Boston-group who reported normalization of blood glucose for 1 year in the non-obese-diabetic (NOD) mouse, an auto-immune model of diabetes, using allogenic islets embedded in barium beads [54]. This study and more recent studies [64] 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 [64], whereas Immunoglobulin G (IgG), the smallest of the immunoglobulins, has a molecular weigh of 140 kD and the molecular weigh of potentially harmful cytokines range from 17.5 (IL-1β) to 51 kD (TNF-α). It must be noted, however, that microcapsule permeability is dependent on the three-dimensional size (e.g Radii of gyration) as well as the charge of both the molecule of interest and the polymer network in addition to the pore size and pore size distribution. It might therefore be that the beads have protected against small bioactive molecules which on their basis of molecular weight should have been able to enter the beads.

Another important observations 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 [54]. Since the life span of a β cell is approximately 3 months [65], the study suggests that regeneration of islet-cells occurs in capsules. Unfortunately, the Boston-group could not achieve the same long-term survival times

when rats instead of mice or xenografts were applied [4,64,66].

Alginate beads coated with a polycation may have a broader potential application than barium-beads because the coating induces an increase in mechanical stability and a further restriction in permeability which e.g. makes xenotransplantation of islets a feasible option and provides mechanical stability features required for application in large mammals and men. The most commonly used alginate-based capsules are formed by the alginate-PLL system, but also other polycations such as polyethyleneimine, poly-L-ornithine (PLO), poly-D-lysine, chitosan and polymethylene-*co*-guanidine have been used. After gelification of the beads in calcium, the beads are coated with the polycation membrane by suspending the beads in polycation solutions such as PLL. During this step, polycations bind to alginate molecules [67,68] and induces the formation of complexes at the capsule surface [32,68]. The presence of these complexes decreases the porosity of the membrane [69–72].

Soluble and noncomplexed PLL as such is an inflammatory molecule and responsible for fibrotic overgrowth when not adequately bound to alginate [73–76]. We have shown that soluble PLL induces cytokine production in monocytes and can cause cellular necrosis [75]. Soluble alginate reduces the effect of PLL toxicity. This is also observed in vivo where it was found that high-G alginates are associated with a stronger inflammatory reactions than intermediate-G alginates when PLL was applied as the polycation [76]. This illustrates the importance of understanding and the design of approaches to allow optimal complexation of PLL with the alginate network.

New physicochemical technologies have come to the field to explain the observation that the biocompatibility and the adequacy of binding with PLL vary with the G-content of the alginate [32,36]. In order to provide more insight in the structure of alginate-PLL capsules the Groningen group has performed a physico-chemical analysis of the capsules by applying X-ray photoelectron spectroscopy (XPS) [47,77,78]. 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 calcium-alginate which is enveloped by a membrane composed of two layers, i.e. an inner layer of alginate-PLL and an outer layer of calcium-alginate [1,32,79]. The data, which have lead to this model, were almost exclusively obtained by studying the chemical interactions of PLL with solved, non-calcium bound and often individual components of alginate (i.e G and M monomers) and not by studying the chemical structure of the capsules as such. In our subsequent studies on true capsules, we combined Fourier transform infrared spectroscopy (FT-IR), [77] XPS [48], and confocal microscopy [80] to study the structure of the alginate-PLL capsule membrane. From confocal images and from electron microscopy pictures it can be seen that the PLL penetrates the alginate core, forming an alginate-PLL complex of about 30 μm, depending on the exposure time to PLL [32,80]. It was found that the capsules were not composed of a generally considered three layer system of alginate-polycation, and an outer alginate layer but only of an alginate-core surrounded by an alginate-polycation core. This was recently confirmed by Tam et al. [81] by applying ToF-SIMS imaging. 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 polycations such as PLL is always on the surface of the capsules in direct contact with the host-inflammatory cells in the vicinity. The present data suggest that, for optimal biocompatibility, we have to focus on understanding and improving the interaction of the inflammatory polycations with the core of alginate and not

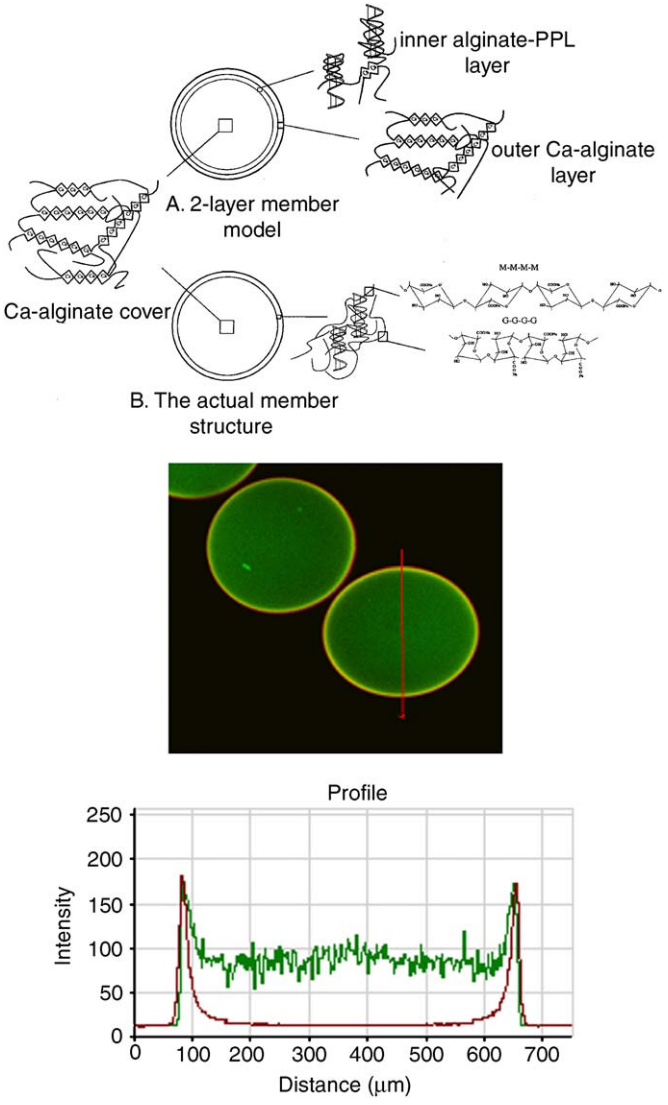

Fig. 3. A: The considered and the actual structure of alginate-PLL capsules. The capsule is not composed of three layers as generally assumed but of two layers. B: Alginate (green)-PLL (red) capsules visualized in the confocal microscope. The optical slice is through the equator of the capsule.

only on improving the second coating step with alginate or other polymers.

PLL binds to alginate by forming complexes with M-G sequences on the surface of the alginate beads [68]. To make these M–G sequences available for PLL binding, an incubation step in calcium-free medium of the calcium beads is required [17,82,83]. A recent FT-IR study by van Hoogmoedt et al. [77], showed that this step has different effects on intermediate-G and on high-G alginate calcium beads. The calcium-extraction step leads to extraction of more calcium from high-G calcium beads than from intermediate-G calcium beads and induces different conformations on the surface of intermediate-G and high-G beads. The most important observation is that high-G beads contain after calcium-free washing more intermolecular hydrogen bounds involved in intermolecular connections which are not available for PLL binding. In the subsequent, coating step with PLL, PLL diffuses into the beads and forms α-helixes, antiparallel sheets and random coil formation [77]. Due to the high number of available binding sites in intermediate-G alginate, the PLL is adequately bound. This was not observed on the high-G capsules where after the PLL binding much more incompletely bound PLL molecules was found than on intermediate-G capsules [47,48,77].

The uncomplexed polycations on the surface of the microcapsule are usually complexed in a final incubation with diluted alginate to reduce the attachment of host cells [73]. Dilute alginate solution of the same composition as the core alginate has mainly been used. It has been shown, however, that alginates with a lower molecular weight than the one used in the core shows a higher binding efficacy to PLL structure on the surface [32,84]. This has also been confirmed in vivo, where better coating of the PLL by using tailored alginate resulted in improvement in biocompatibility of the alginate-PLL-alginate microcapsules [85]. The success and efficacy of this alginate-coating step, however, is largely determined by the properties of the alginate in the core of the capsules [32,36,45].

The importance of an adequate alginate composition for the biocompatibility of alginate-polycation capsules was further substantiated in a recent study on zeta-potentials of capsules [86]. The zeta-potential is a measure for the electrical charge of the surface and a predictive value for the interfacial reactions between the biomaterial and the surrounding tissue [87–89]. When comparing the zeta-potential of capsules prepared of intermediate-G and high-G alginate-PLL capsules under a physiological pH value of 7, we found no differences in zeta-potentials and thus in electrical charge distributions. A difference in zeta-potential between the two capsule types only became apparent at a lower pH. On the first sight this does not seem to have any value for understanding biocompatibility issues. However, an event that is insufficiently realized is that the direct environment of the capsules changes directly after implantation. A pertinent change is a drop in pH as the consequence of a temporary inflammation process due to the mandatory surgery. Such a drop in pH can for instance induce changes in the charge density of the capsules and make the capsule more vulnerable for adhesion of proteins and cells. Capsules should be able to withstand these kinds of environmental changes. We found that high-G capsules showed statistical significantly more positive charges at lower pH than intermediate-G capsules which corresponds with the higher degree of biocompatibility of intermediate-G capsules.

The above-mentioned studies clearly show that it is mandatory to include physicochemical technologies in the field in order to clarify the true biocompatibility issues. Another important issue that has recently been described is the surface roughness of capsules. Bünger et al. [90] showed that alginate-PLL capsules provoke a strong tissue response in rats when capsules were implanted with a strong surface roughness as visualized by atomic force microscopy. This was plausibly caused by an inadequate interaction of the PLL molecules with the alginate at the surface of the capsules. The authors subsequently added polyacryl acid on the surface which profoundly decreased the surface roughness (Fig. 4) and almost completely abolished the observed tissue responses [90]. These studies not only illustrate the importance of the surface roughness on biocompatibility but also clearly show that the surface property requires further study since this may be a crucial area determining biocompatibility in vivo.

In a recent study, the Groningen group has applied all the current knowledge for the requirements of producing a biocompatible alginate-PLL capsule in a long-term biocompatibility study. The capsules were implanted in the peritoneal cavity of rats and retrieved 2 years later, i.e. the life span of a rat. 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 [47]. 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.

The above-mentioned studies have been employed with PLL as the cross-linking agent. Other characteristics and prerequisites apply when other types of polycations are applied. PLO is another successfully applied polycation in alginate-based capsules. It is preferred by the Perugia group because it is in their hands more chemically stable as compared to alginate-PLL capsules but also immunoselective in terms of nominal membrane's molecular weight cut-off and also biocompatible [91,92]. To make a homogeneous and biocompatible hydrogel, PLO needs to be ionically complexed with a mannuronic acid-enriched (70% M) alginate. Capsules prepared from this alginate are very resistant to mechanical burst and the only way to dissolve them is by exposure to strong bases. Long-term studies, where empty alginate PLO microcapsules were injected intraperitoneally in rodents, dogs, or pigs have

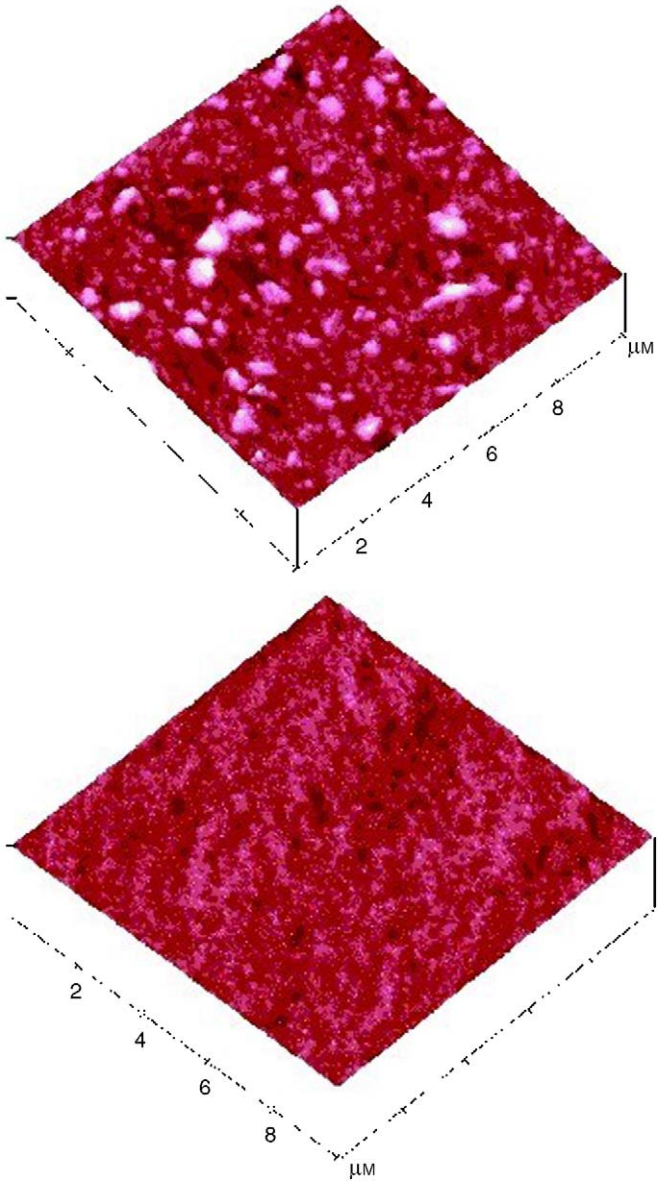

Fig. 4. A two-dimensional atomic force image ($10 \times 10\,\mu m$) of (A) conventional alginate-PLL capsules and (B) alginate-PLL-poly acrylic acid capsules. The RMS roughness of the films are (A) 7.3 and (B) 3.4 nm.

always resulted in retrieval of intact and overgrowth-free microcapsules up to one year post-implant.

Recently, the Perugia group extended these findings for the first time in clinical studies in nonimmunosuppressed humans [15]. In this instance, in order to start a phase-1, closed pilot clinical trial of microencapsulated islet grafts into nonimmunosuppressed patients with T1DM, specific issues have been extensively reviewed with the Italian Ministry of Health which finally released an ad hoc authorization to begin the study. In particular, alginate pharmacotoxicology has been carefully scrutinized. In fact, for human application, use of "clinical grade" alginate is mandatory [92]. Preliminary evidence of graft metabolic function coupled with host's immune unresponsiveness to the encapsulated islets is very encouraging, although the restricted procurement of cadaveric donor organs mandates that alternative islet cell sources, with special regard to neonatal pig islets [93], take over human donor tissue in the near future. Also, the life span of the cells in the capsules is a critical issue that requires further consideration since in most studies the longevity of the cells was of a limited duration in spite of adequate biocompatibility of the capsules.

4. The influence of the presence of cells in the capsules

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 is reduced to a minor portion of the capsules the survival time is not permanent but limited to periods up to a year [48].

A crucial factor in the limited longevity may be the lack of sufficient supply of nutrients and oxygen to the islets. The presence of the physical barrier of the capsule 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 grafts [18,94,95]. The principle success of improvement of blood supply for function and survival was shown by the Perugia group. To improve oxygen and nutrient supply to the enveloped islets, allogenic and xenogenic islets were enveloped within vascular prostheses directly anastomosed to blood vessels, in dogs and humans [96]. The islets showed optimal functional survival. However, while associated with no side effects, the procedure because of its intrinsic potential thrombogenicity, would have to face serious regulatory concerns.

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 to carry a graft with the size of an encapsulated transplant but it is not having 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 [97–100]. The absence of convection movement within a capsule induces a nutrient-gradient from the capsule surface to the center of the islet [101,102]. 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. Application of new droplet formation technologies such as an electrostatic pulse generator [103,104] has allowed for the production of alginate beads as small as 185 µm in diameter which is fourfold smaller than the conventional 800 µm

capsules [105]. There is however a drawback on decreasing the capsules size. With reduction of the capsule size the number of capsules containing partially protruding islets will proportionally increase [83,106]. This obviously will also increase the number of capsules affected by an inflammatory response. To reduce the number of cells one can decrease the number of islets per volume of alginate [107]. It has been shown that every capsule size is having an optimal islet density which has to be determined experimentally [107]. Usually this is associated with a slight increase in the number of empty capsules. This however has to be accepted in order to keep the number of protruding islets minimal.

The small capsules can be implanted in the intraperitoneally implanted solid support system for pancreatic islets [94] which was recently introduced. This site allows for implantation of high numbers of islets, which can readily be retrieved and can be engineered as such that it is highly vascularized. It has been shown in rats that islets show much better survival rates and function in these devices [18] but with encapsulated islets the survival was still not permanent. This illustrates the involvement of other factors than insufficient supply of nutrients in failure of microencapsulated islet grafts.

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 [58,108–112]. A recent series of experiments have brought new insight in the pathogenesis of encapsulated cell failure with biocompatible capsules: the transplanted cells and not the capsule's materials were the principle cause of failure. It has been shown that pancreatic islets secrete cytokines upon stress [113]. The Groningen group found that encapsulated cells such as immunoisolated pancreatic islets under stress (by adding IL-1β and TNF-α) 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 [114–116]. In a subsequent experiment it was demonstrated that activated macrophages on the 2–10% of overgrown capsules do secrete the cytokines IL-1β and TNF-α when they were co-cultured with islet-containing capsules and not with empty capsules [115,116]. This process was accompanied with a gradual loss of function of the encapsulated tissue [116,117]. 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. 5).

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 [78], the Groningen-group has 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) [55,78]. This is later confirmed by

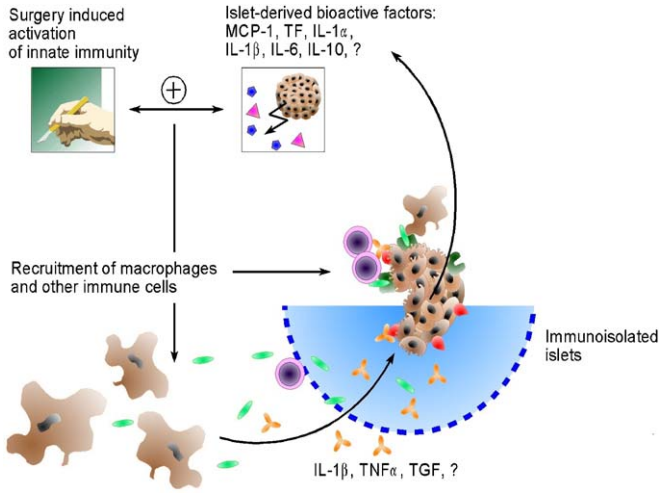

Fig. 5. The vicious circle of activation causing failure of 60% of the islets in the immediate period after transplantation. Islets release cytokines which act in concert with cytokines released by a surgery-induced activation of the immune system on the recruitment and activation of inflammatory cells in the vicinity of the graft.

others [55]. Although transplantation of encapsulated cells to the peritoneal cavity only requires minor surgery, the procedure is associated with tissue damage and release of bioactive proteins such as fibrinogen, thrombin, histamine, and fibronectin [118–120]. 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 [78].

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-1β, TNF-α, TGF-β, and histamine which further activate inflammatory cells in the vicinity of the foreign materials [119–123], and, more importantly, stimulate the cells in the capsules to produce graft-derived cytokines.

Within 2 weeks, basophiles and granulocytes gradually disappear from the graft site while macrophages and some fibroblasts remain attached to a portion of 2–10% of the capsules [78]. These attached macrophages remain activated and, therefore, contribute to the vicious and deleterious circle of activation. Thus, although we and others [108–112] 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 it is mandatory to completely delete overgrowth of the capsules.

5. Immunological responses against encapsulated tissue

From the foregoing follows that it is more accepted that immune responses against the microcapsules prepared of 'foreign materials' is far more complicated than initially

assumed and composed of different separate immunological responses. The reactions against capsules can be categorized into at least four types. The first is a nonspecific activation of the innate immune system by the surgical procedure of transplantation. The second is the foreign body response against the capsule. The third type of response is provoked by the enveloped tissue which releases bioactive factors but also allogenic or xenogenic epitopes. This implies that the reaction of the host immune system towards the capsule and the encapsulated tissue is both through the innate and the adaptive lineages. The last identified type of response is the deleterious component of the vascularization process which only applies for capsule types which will be vascularized after implantation.

The activation of the innate immune system already starts with the mandatory surgery to implant the 'foreign material'. This mandatory surgery induces an inflammatory response due to rupture of bloodvessels which is associated with influx of inflammatory cells and release of bioactive factors such as cytokines and fibronectin. It depends on the material's properties whether this results in adsorption of proteins and subsequently cell adherences onto the surface. The second response, the foreign body response against the capsules can now start depending on the characteristics of the materials applied. The severity of this reaction may be species dependent. This has been shown e.g. in different strains of mice. The C57Bl/6 mouse provokes a significantly higher response to the encapsulation material than the Balb/c mouse [76].

Fibrosis which affects the whole graft and not only a portion of the capsules has become a rare phenomenon since the introduction of purified alginates. Recently, a detailed study on the tissue responses against alginate-PLL capsules has been published by de Vos et al. [78] and Robitaille et al. [55]. Robitaille et al. [55] observed a strong fibrogenic response with high concentrations of fibrogenic cytokines such as TGF-β which does not corroborate the results of de Vos et al. [78]. It should be noted, however, that the alginates applied in the Robitaille-study had a low purity degree and therefore a low biocompatibility. There is obviously a large difference in pathophysiology of the reaction between application of capsules with a high and a low degree of biocompatibility since the Groningen group using highly purified alginate never observes these responses. When applying capsule grafts in which the majority of the capsules remain free of overgrowth, we do not observe a fibrogenic response but a temporary increase in macrophages, granulocytes and cytokines characteristic for an activation of the innate immunity [78,90]. This response is usually extinguished within 2 weeks. Unfortunately, this response is still responsible for loss of a significant number of the encapsulated cells [58,78].

The effect of the response initiated by the enveloped tissue has gained much attention during recent years [57]. Immune cells such as circulating and tissue-specific macrophages and granulocytes can take up components of the foreign material or specific allogenic and/or xenogenic epitopes and initiate a specific immune response characterized by presence of lymphocytes in the vicinity of the materials. It has been shown that this induces the formation of encapsulated tissue-specific antibodies [124–126]. Most groups, however, do not consider the formation of antibodies to be deleterious for the tissue since the capsules should adequately protect the tissue. The severity of the response, however, may vary with the applied transplantation site. A recent study by Dufrane et al. [127] shows that xenogenic tissue in alginate microcapsules transplanted to the peritoneal cavity of mice provoked a higher response than capsules transplanted under the skin or under the kidney capsule.

Release of bioactive factors by the encapsulated tissue have recently been identified as a causal factor for the loss of 60% of the engrafted tissue in the 1st months after transplantation [58]. It has been shown that the diffusion of graft-derived and inflammatory cell-derived cytokines is a major threat for the longevity of the encapsulated grafts [115,128]. A conceivable approach to overcome the problem of islet-derived cytokines is reduction of the capsule permeability. However, 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 Groningen group and that of the Pisa-group have shown that this is not an insurmountable problem [26,95,129]. 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 [114–116,130]. It was found in vitro that decreasing the 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-1β) was reduced by changing the permeability of the membrane which was unexpected as the molecular weight of small cytokines such as IL-1β (17 kDa) was far below the molecular cut-off of the applied capsules (100 kDa) (Fig. 6). Finally, the deleterious effects may be decreased by changing the capsule size. The Perugia-group has shown that cytokine-induced damage to the microencapsulated islets is minor in "medium size" (400–500 μm) capsules and increases with smaller capsules [131]. This observation seems to suggest that microcapsules may perform better than conformal coatings in terms of immunoprotective capacity [131].

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 [132]. It has been shown that this is due to the fact that xenogenic islet cells are less

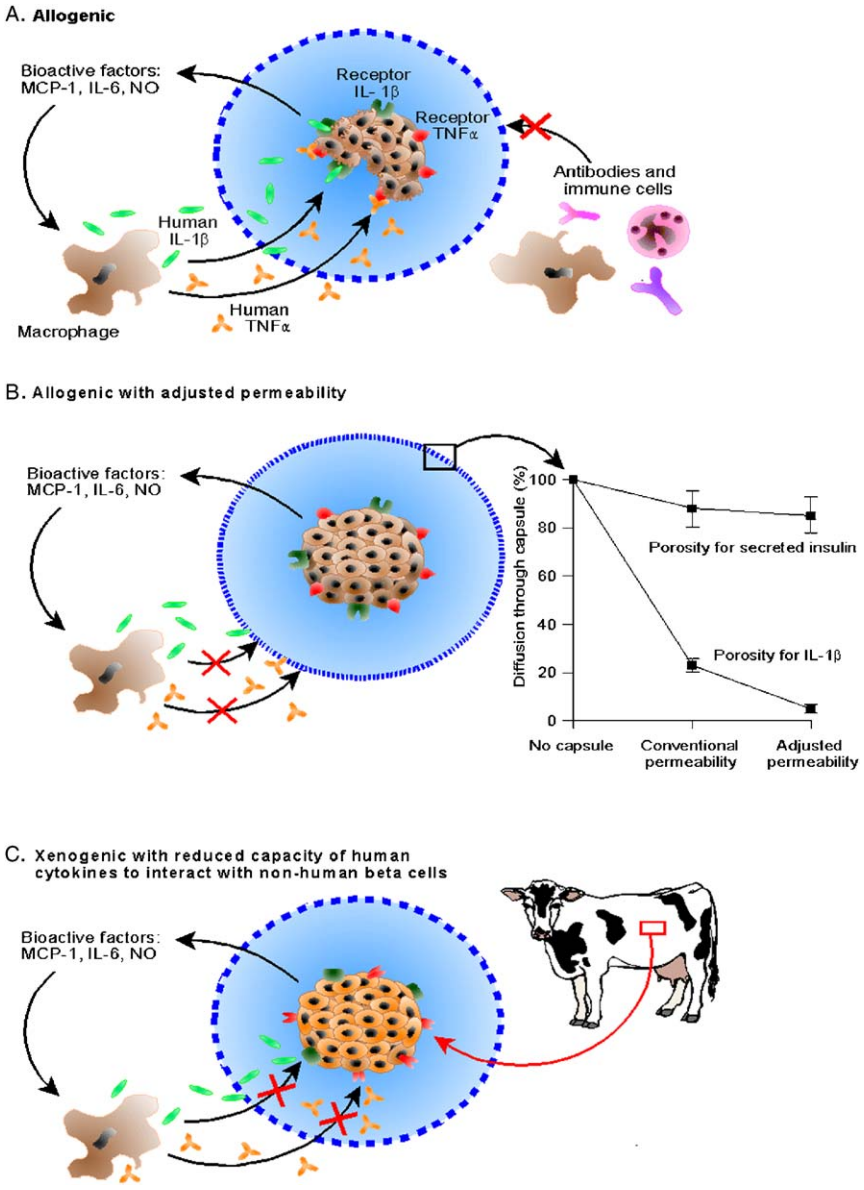

Fig. 6. Approaches to prevent deleterious effects of diffusion of cytotoxic cytokines and chemokines into the immunoisolating capsules after transplantation. (A) Islets produce cytokines that diffuse out of the capsules and activate inflammatory cells such as macrophages in the vicinity of the microencapsulated 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-1β 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.

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.

Some apply specific capsule materials to enforce vascularization of the capsule membranes in order to promote exchange of nutrients and therapeutic agents between the bloodstream and the encapsulated tissue [133–136]. This approach is for example being applied by Novocell in their phase I clinical trials with poly-ethylene-glycol capsules. 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 [18,137,138]. The latter episode is not only associated with the presence of many deleterious cytokines and bioactive molecules but also with a period of ischemia. On the basis of the above-mentioned studies it is advisable to apply prevascularized approaches [18,94] in order to reduce above-mentioned deleterious effects.

6. Metabolic control by a microencapsulated islet graft

Although the topic of the present review is biocompatibility of alginate-based microcapsules we do not want to neglect another very important prerequisite of a graft to be applied in diabetics. Any new therapy for the treatment of diabetes should provide a minute-to-minute regulation of the glucose levels in order to improve the quality of life of the patient and to avoid the side effect of the present exogenous insulin therapies. For this reason microencapsulated islets have been subject of many metabolic studies.

A favorable feature of microcapsules over other encapsulation systems is their spherical shape which offers better diffusion capacity because of a better surface/volume ratio. In vitro, insulin release from microencapsulated islets in capsules up to 800 μm have been shown to be similar if not identical to insulin release profiles from free, nonencapsulated islets [26]. The capsule as such is therefore not considered to have any influence on the insulin release kinetics. However, in vivo de Groningen group has repeatedly shown that there is a slight delay of uptake of insulin from encapsulated islets into the systemic circulation [94,117,139,140]. This is caused by the lack of direct vascular access due to the presence of the physical barrier of the capsule that interferes with direct vascularization [94,117,139,140]. When functioning grafts were tested by oral or intravenous glucose challenge, glucose tolerance was found to be rather adequate as illustrated by normal HBAc1 levels [3], and maximum glucose levels of only 8.3 mM but a rise in systemic insulin levels was never observed. We have further substantiated this observation experimentally by assessing portal and systemic insulin responses and glucose levels after gradual infusion of low amounts of insulin into the peritoneal cavity, thereby mimicking the gradual release of insulin from the capsules of intraperitoneal graft. We found that the dose-dependent rise of insulin and decrease of glucose levels with intraperitoneal insulin infusion were strongly delayed and reduced as well as prolonged in comparison to intraportal insulin infusion [117,139]. Obviously, the anatomy of the peritoneal cavity does not allow for instant transport of insulin to the systemic circulation.

In the subsequent experiments on function of intraperitoneally transplanted microencapsulated islets, we assessed C-peptide in the systemic circulation instead of insulin. C-peptide is released in equimolar concentrations with insulin, is not readily absorbed by the abdominal organs and does not undergo hepatic extraction. With this approach, we have found for the first time a glucose induced response from the encapsulated islets as evidenced by an increase of C-peptide in systemic circulation, when diabetic mice were subjected to meal challenge [141]. Surprisingly, glucose clearance was about the same as that of mice transplanted with free, nonencapsulated islets [141]. This can all be explained by the lack of direct vascular access in the peritoneal cavity. Apparently, also for optimal functional performance it is obligatory for clinical application to find a site where encapsulated islets are in close contact with the blood stream. The recently designed intraperitoneally implanted prevascularized solid support system for pancreatic islets [94] which has been discussed in a previous paragraph can serve as such a site.

7. Concluding remarks

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 [142–144]. Now that we have reached the state in which we can prevent overgrowth on the majority of 90–98%, we still observe limitations in functional graft survival when encapsulating pancreatic islets for the treatment of type-1 diabetes. From the studies addressing the identification of the casual factors for this failure of encapsulated cells, it became clear that the host response to the biomaterial is not the only and single response causing failure of the grafts.

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 pharmaceutical intervention, that is, we should prevent the release of anti-inflammatory products in the first 2 weeks after implantation, since it may be difficult to overcome this issue by modification of the capsule membrane.

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