Chapter 1

Historical Perspectives and Current Challenges in Cell Microencapsulation

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Abstract

The principle of immunoisolation of cells is based on encapsulation of cells in immunoprotective but semipermeable membranes that protect cells from hazardous effects of the host immune system but allows ingress of nutrients and outgress of therapeutic molecules. The technology was introduced in 1933 but has only received its deserved attention for its therapeutic application for three decades now.

In the past decade important advances have been made in creating capsules that provoke minimal or no inflammatory responses. There are however new emerging challenges. These challenges relate to optimal nutrition and oxygen supply as well as standardization and documentation of capsule properties.

It is concluded that the proof of principle of applicability of encapsulated grafts for treatment of human disease has been demonstrated and merits optimism about its clinical potential. Further innovation requires a much more systematic approach in identifying crucial properties of capsules and cellular grafts to allow sound interpretations of the results.

Key words Encapsulation, Natural polymers, Synthetic polymers, Insulin, Alginate, Biocompatibility, Biotolerability

1 Introduction

Encapsulation involves the protection of living cells from hazardous effects of the host immune system by enveloping the cells in an immunoprotective membrane. The technology has received much attention by the scientific community in the past three decades but its introduction dates back to as far as 1933. At that time Bisceglie et al. [1] studied the effect of absence of vascularization on tumor cells by encapsulating the cells in immunoisolating membranes and transplanted them in the abdominal cavity of pigs. As membrane Bisceglie applied amnion tissue-sheets and demonstrated prolonged cell survival of the enveloped cells in the immunoprotective membranes [1]. Unfortunately, Bisceglie did not recognize the potential of his findings for the treatment of disease. It took until 1950, when Algire et al. [2] introduced the concept of immunoisolation by creating diffusion chambers for implantation purposes to cure

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disease by therapeutic cells. Algire also recognized as the first the importance of application of biocompatible materials and the need for constant, predictable properties of those materials for therapeutic application [2]. Since that time encapsulation devices have been produced in different conformations [3], with application of many different types of biomaterials [4] and has been applied for the treatment of many diseases that require a minute-to-minute regulation of metabolites such as in hemophilia B [5], anemia [6], dwarfism [7], kidney [8] and liver failure [9], pituitary disorders [10], central nervous system insufficiency [11], and diabetes mellitus [12].

2 Macrocapsules and Microcapsules

In the past three decades encapsulation of living cells has been applied in two families of geometries, i.e., macrocapsules and microcapsules. In macrocapsules, cells are packed in relatively large diffusion chambers. These chambers have semipermeable properties. The semipermeable macrocapsules have been produced in the form of hollow fibers, flat sheets, and disks [13]. Macrocapsules can be implanted as intravascular or extravascular devices [14]. When applied as intravascular device, a connection is made with the blood circulation. Usually the cells are seeded in polymeric capillaries and connected by anastomosis to the circulation. The advantage of this approach is the fast exchange of metabolites, nutrients, and oxygen [15]. A major disadvantage of this system is that thrombosis may occur after implantation. Up to now lifelong anticoagulation therapy has been a prerequisite with intravascular devices. This risk of thrombosis makes most intravascular approaches an unacceptable alternative for conventional treatment [16]. The side effects of implantation of the devices are simply too severe.

Because of the need of anticoagulation therapy most group nowadays focus on extravascular devices. In this approach therapeutic cells are encapsulated in semipermeable membranes and implanted without direct vascular connection. Exchange of therapeutic molecules, metabolites, and oxygen between the enveloped cells and the surrounding tissue depends on free diffusion over the membranes. An advantage of the system is that it does not need major surgery and can, in most cases, be easily replaced in case of failure of the graft. The numerous reports demonstrating successful application of extravascular devices in experimental animals and humans [17–20] illustrate the principle applicability of the approach.

Two types of extravascular devices are distinguished, i.e., the aforementioned macrocapsules and microcapsules. Conceptionally, encapsulation in macrocapsules is the simplest approach. Groups of cells are mixed within an immobilizing matrix and brought into one, large membrane and are subsequently implanted. Successful

application has been reported [21–28] but the system has a major drawback. All macrocapsules have compared to smaller spherical microcapsules a relatively large surface-to-volume ratio. This implies that high amounts of therapeutic molecules, nutrients, and oxygen are required to build an effective diffusion gradient for ingress and outgress of molecules. This interferes with an adequate response to changes in metabolites in the recipients and also delays supply of nutrients to the cells. Another issue is that the cell density in macrocapsules cannot be high. The cells compete for nutrients such as oxygen and will stop functioning or will become even necrotic when the density is too high [29–31]. As a consequence the seeding density should be quite low to guarantee adequate nutrition [14]. Within most applications, the cell density should not exceed 5–10 % of the volume fraction [15]. This suggests that if large numbers of cells are required to cure disease [15], either an artificial supply of nutrients should be incorporated in the design or several or very large devices must be implanted. In the past 5 years much attention has been given by the scientific community to means to enhance nutrition [23, 31–40] in the hope to be able to create smaller devices.

3 Insufficient Supply of Nutrients is Achilles Heel of Encapsulation

A transplantation area in which immunoisolation by encapsulation has a pertinent position is in research towards transplantation of pancreatic islets for the treatment of diabetes without immunosuppression. In this area most of the work on improving nutrition has been performed. Pancreatic islets are obtained from rare cadaveric donors and are also very sensitive for low oxygen tensions [41, 42]. Devices with optimal supply of nutrients are therefore an absolute requirement for application of pancreatic islets.

Research efforts to improve nutrition of immunoisolated islets started more than two decades ago by including different fenestrated membranes and angiogenic compound [43] in devices to enhance vascularization of the surface [32–34, 44, 45]. This however could never prevent the development of necrotic zones and loss of functional capacity of the grafts [44, 45]. Pancreatic islets need relatively high oxygen tensions for producing insulin and for regulation of glucose levels. The extravascular oxygen tensions around the macrocapsules in vivo of not more than 40 mmHg or lower are not sufficient to maintain optimal function and viability of the grafts [4, 46]. This has inspired a number of researchers to develop means to enhance the oxygen tensions in vivo. This has been done by inclusion of oxygen-generating chemicals in the capsules [47, 48], by inclusion of oxygen generating organisms [39], or by external supplementation of oxygen by means of an oxygen pump [31, 38, 40, 49].

The latter, the supply of oxygen by means of a pump, requires some further consideration as a recent series of experiments has shown that islets can function for a long time and keep on producing insulin in several animal models and even in a human in the absence of application of immunosuppression when oxygen is supplied in sufficient amounts [40, 49]. In this concept islets are surrounded by a polymeric shell and fed with oxygen by means of a manually operated oxygen pump. Supraphysiological amounts of oxygen are being pumped into the device inducing a gradient of oxygen in the islet graft [40, 49]. Islets proofed to perform quite well in this concept. They produce c-peptide in response to a glucose load in a human recipient [49] and histological examination confirmed the viability and absence of significant necrosis in the islets. This illustrates that the relative unfavorable surface to volume ratio of macrocapsules can be overcome by enhancing supply of nutrients.

The other promising concept that has been subject of intensive studies in the past three decades is the application of microencapsulation. Microcapsules are not associated with surface-to-volume ratio issues and are therefore preferred by some groups [4, 50]. Microcapsules have a size smaller than 700 μm and envelop individual clusters of cells or islets. The spherical geometry allows fast exchange of therapeutic molecules and nutrients between the surroundings and the encapsulated cells. Pancreatic islets in capsules have been shown to closely mimic the release of insulin and glucose of free, unencapsulated pancreatic islets [51, 52]. Manyissues related to biocompatibility have been studied with application of microcapsules and are discussed in the following sections. Also, many new polymers, immunoprotective membranes, and strategies to enhance longevity of grafts have been designed in the context of microcapsules.

4 Biocompatibility and Biotolerability

A subject that after three decades of research is still subject of intensive debate is the biocompatibility of encapsulation devices. Biocompatibility is a complex, difficult concept with an even more complicated definition. Biocompatibility is usually defined as “the ability of a biomaterial to perform with an appropriate host response in a specific application.” This definition was formulated at a time of emerging application of fully artificial organs, such as artificial hips and knees [53]. These fully artificial constructs induce innate immune responses resulting in fibrosis around the devices and integration of the artificial materials in the surrounding tissue. The immune responses and integration of the fully artificial devices in tissue is desired and therefore defined as an “appropriate host response”. For bioartificial organs, such as encapsulated cells,

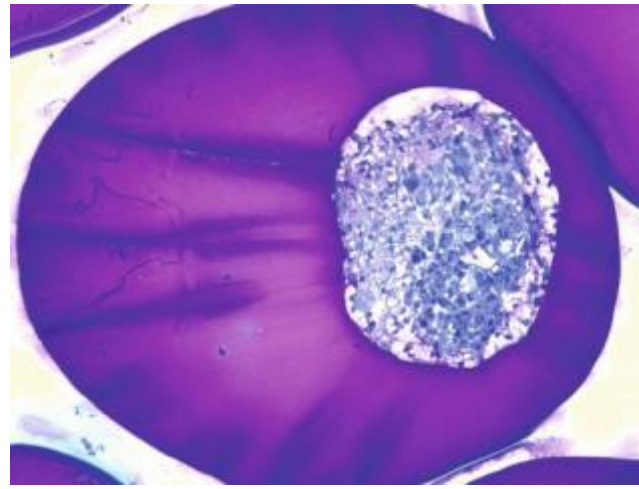

Fig. 1 Preferably no or minimal inflammatory responses should be elicited by encapsulate cells. This requires full control of critical capsules parameters. The proof of principle: an encapsulated pig pancreatic islet 4 weeks after xenotransplantation in a streptozotocin diabetic C57/b6 mice. GMA-embedded capsules. Original magnification ×40. Note absence of cellular adhesion

defining the appropriate host response is more complicated as inflammatory responses are associated with diffusion of harmful cytokines into the capsules with death of the cells as a consequence [50]. Preferably no or at least minimal cellular overgrowth should be provoked by the capsules to ensure free diffusion of nutrients and oxygen and to guarantee exchange of therapeutic molecules (Fig. 1). Because of the complexity of the biocompatibility issues the field has adopted a new term and definition for describing tissue responses against capsules. This term is "biotolerability" [50] and defined as "the ability of a material to reside in the body for long periods of time with only low degrees of inflammatory reactions" [54]. This definition also covers another important requirement which involves the "friendliness" or compatibility of the biomaterial with the encapsulated cells. Cells should grow and function in the polymer network as adequately as in their natural environment [55, 56].

Biotolerability of immunoisolating devices is defined by a complex combination of factors. A significant amount of research efforts has been spend in the past two decades on creating pure polymers that contain no or minimal amounts of endotoxin. It has been shown by many that even minimal contaminations with endotoxins can induce severe inflammatory responses with fibrosis of the capsules as a consequence [57–61]. Alginate is the best-studied encapsulation material and still the most commonly applied polymer in encapsulation research because of the friendly circumstances by which is can be applied to envelope cells and because of its

relative high biotolerability. Almost 20 years ago the essence of applying purified alginates has been reported [62]. In the years after, commercial purified alginates were being brought to the market. The alginates were without exception low in the endotoxin lipopolysaccharide (LPS) but still provoked responses in the hands of some groups. This has stimulated research on the identification of factors determining the presence or absence of immune responses against capsules. It was found that capsules with a too high surface roughness provoke strong innate responses [28, 63, 64] with cell adhesion in mice and rats as a consequence. For that reason we usually keep the surface roughness below 10 nM to avoid these type responses [55]. However, even with low surface roughnesses responses may occur when pro-inflammatory chemical groups are exposed. Notorious are for example polyamide groups that are applied to reduce the permeability of the membranes to provide immunoprotection. Poly-L-lysine and poly-L-ornithine are often applied for these purposes but are highly proinflammatory when not in the correct conformation [65–69]. Poly-L-lysine has to be forced into a superhelical core with alginate and beta-sheets to prevent responses [65, 68]. Seemingly minor changes in the encapsulation procedure such as changes in temperature can have a profound effect on the surface chemistry and induce strong inflammatory responses.

Besides poor control of the aforementioned physicochemical variations, also simple items in the production process can influence biotolerability. In the past years there is an emerging trend of introducing new technologies to produce encapsulated islets. Sophisticated emerging technologies such as microfluids and electrospinning [70] are being proposed as alternative for the more conventional droplet formation technologies [71]. What has been overlooked and is still insufficiently recognized in the field is that the new technologies produce capsules with unique and technology dependent variations in capsule surface properties. Even with application with exactly the same polymer, our group has observed differences in surface roughness and chemistry. This has an impact on biotolerability. Also, physicochemical variations between capsules were observed and even inhomogeneity on surface characteristics on a single capsule. This is in our opinion an important item in the reported variations in success of encapsulated islets.

Recently it was reported that also the size of the capsules matters [72]. It was reported that smaller capsules (500 μm) provoke a stronger response than larger capsules (1800 μm). Remarkably, this difference in response was never observed by others [73–76]. In fact we never see any responses against planar alginate beads as applied by the authors [77]. Variations in the production pro- cess might be held responsible for the finding that smaller and larger capsules provoke different responses in some groups [73]. The most likely explanation is however the following. The group

[72] does not apply purified alginates but a crude alginate that

based on an in vivo skin study was selected for provoking lesser tissue responses than other unpurified alginates [78, 79]. When unpurified alginates are applied, endotoxins will be exposed on the surface of the capsules. Due to the higher surface to volume ratio of smaller capsules more endotoxins will be present on the smaller capsules compared to the larger capsules with differences in responses as a consequence [46, 73, 80]. This phenomenon is known for many years and emphasizes the importance of controlling and documenting all known factors responsible for induction of inflammatory responses against capsules. Side-by-side comparison of data sets is only possible when all physicochemical variations, including endotoxin content, are known [50]. In our hands and that of others size does not matter, at least not in the size ranges that are conventionally applied.

5 Impurities Revisit

Although most groups nowadays apply homemade or commercially available purified alginates, unrecognized contaminations might still contribute to the enormous variations in reported immune responses against capsules [46, 73, 80].

LPS is the best-recognized endotoxin present in encapsulation polymers. LPS is an endotoxin and also referred to as pathogen associated molecular pattern (PAMP). LPS as PAMP binds to Toll-like receptor 4 and induces proinflammatory responses in a wide variety of immune cells [81–83]. There are however other PAMPs that are having at least the same immune stimulatory capacity as LPS. In a recent study our group found some of these strong immune stimuli in alginates and some other polymers applied for immunoisolating devices. In unpurified alginates lipoteichoic acids (LTA) and proteoglycans (PG) was found [80]. These PAMPs stimulate TLR-2 and were responsible for proinflammatory responses in vitro and in vivo [46, 73, 80, 84]. Removal of these contaminations was found to be cumbersome as all purification procedures up to now were focused on removal of LPS. Novel methodologies for removal had to be developed. Also it was observed that PAMPs could be reintroduced during purification procedures or during storage [80] by for example unsterile conditions or use of equipment or disposables that contained PAMPs.

A technology platform was developed to screen encapsulation polymers for PAMPs [80] (Fig. 2). This platform involves a cell-based series of experiments combined with ELISA approaches to identify the contaminant present in the polymers [80]. This platform has been applied to screen and determine the purity of commercially produced purified alginates that were in some cases recommended for human application. Without exception, all the commercially available alginates tested up to now contained significant amounts

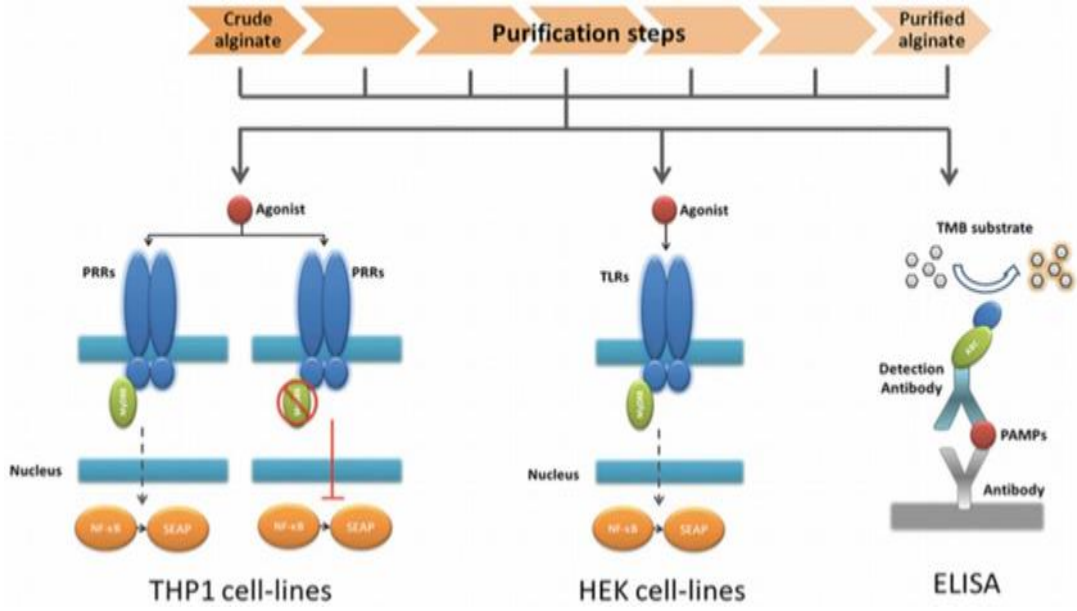

Fig. 2 Schematic presentation of the technology platform to identify the presence of pathogen associated molecular patterns (PAMPS) in polymers applicable for cell encapsulation as published in ref. [80]

of PAMPs and induced immune responses in immune cells. This is a serious issue impeding application and is not solved yet by the companies involved.

6 Strategies to Improve Tolerability

As outlined above it is far from simple to produce biotolerable capsules. After three decades of intensive research most factors interfering with biotolerability are identified but only a few groups have the skills and technology available to control, measure, and document the critical parameters for capsule formation [75]. Producing biotolerable capsules requires the application of a combination of physical and chemical approaches and an extreme control of the production process. It is laborious and requires experienced and trained technicians. Unfortunately the skills for producing capsules are underestimated. This is interfering with side-by-side comparison of data and is frustrating progress in the field [50]. Strategies to improve tolerability should therefore meet more prerequisites than just reduction of tissue responses. It should be simple and readily applicable in laboratories that lack physicochemical methodologies.

Many have been the efforts to change the surface properties of capsules by adding chemical groups to reduce the surface properties or to change coagulation and adhesion of proteins [64, 85–101]. Improvement of performance by reducing tissue responses

was reported but often the coating was not permanent or inter- fered with diffusion of essential molecules such as insulin. A rela- tive new emerging approach is the building of the so-called polymer brushes on surfaces of capsules. Normally molecules that bind to capsule surfaces bind in the so-called mushroom formats [102–107]. By increasing the grafting density more molecules will be bound on an identical surface area impeding with the formation of mushrooms. When a critical grafting density is accomplished the molecules will stretch and form the so-called polymer brush [106]. These brushes prevent adhesion of proteins, do not interfere in the desired length ranges with permeability [106, 107] and can bring improvement of biotolerability. This can even be accomplished in systems that are not produced according to the international standards [107].

Polymer brushes need to be created from relative long molecules for optimal efficacy. They can be di-block polymers with a group that readily binds to the core capsule materials and an outside that is known to provide beneficial properties such as biotolerability. In our group we have selected a series of candidate molecules [108] applicable for encapsulation of cells and for improvement of biotolerability by relative simple and disturbance free procedures that can be performed in labs with minimal equipment. The hope is that these types of approaches will make encapsulation a more straightforward approach and facilitate biotolerability and application.

7 Biotolerability from the Inside Out

Much attention has been focused in the past decades on creating capsules that provoke minimal or no tissue responses but even if this has been accomplished the grafts are having a limited survival time [4, 50, 109]. The longevity of a graft is not only influenced by the events from the outside but also by the intracapsular environment that should support long-term survival of tissue. A too rigid intracapsular environment does contribute to cellular rearrangements with either formation of nonfunctional multinucleated giant cells or death cells as a consequence [55]. The process responsible for these rearrangements is called mechanotransduction [55, 110–115]. During mechanotransduction mechanical forces on cells are transformed into biochemical signals [55, 110– 115]. Cells respond by adjustments in cellular and extracellular structure. This results in modulation of cellular functions such as proliferation, differentiation, migration and apoptosis, and is harmful for cellular homeostasis. The molecular processes and sensors by which mechanotransduction in cells occurs are largely unknown, but recent studies suggest that integrins play a key role in mechanotransduction. With application of very rigid alginate matrixes to

enforce the mechanical resistance of capsules, we recently encountered mechanotransduction as a homeostasis interfering process [55]. Different types of cells lines were encapsulated in alginates of different composition. Specific alginates were found to interfere with cell survival in capsules while other types supported cell survival [55]. Alginates are composed of mannuronic acid (M) and guluronic acids (G). The guluronic acid components, that link constitutive molecules in an egg-box model, provide rigidity to the inner matrix and are therefore preferred by many for application in sites where high shear forces are to be expected such as in the brain [56]. Despite a long term history of application of high-G alginates, we found that this matrix induced mechanotransduction associated cell death in a few days after encapsulation. This mechanotransduction induced cell death was not observed in less rigid matrices or in inner capsules with an alginate of a different type. More research efforts are required to determine the circumstances under which mechanotransduction does or does not occur after encapsulation.

The presence of an extracellular matrix (ECM) may also be a requirement for long-term survival of encapsulated cells. The majority of polymers currently applied do not support interaction with the many integrins and other cell-regulatory anchoring receptors on cells [56]. Especially in those applications where cadaveric cells are being applied to restore organ function, an ECM may be a requirement. ECM provides a matrix for cells to grow on [116–119], it serves as depot or binding site for many growth factors, or is a scavenger for many deleterious molecules. A few groups have applied RGD in their concept of encapsulation [120, 121] and have reported long periods of grafts survival in small as well as large animal models. It remains subject of debate but application of ECM might be a mandatory step when long-term function of grafts is envisioned such as in transplantation of pancreatic islets.

The consequences of a nonoptimal intracapsular environment for longevity of an encapsulated cellular graft may be larger than considered up to now. Currently, loss of cells is assumed to be an issue that can easily be overcome by transplanting a larger cellular mass. This however might be an underestimation of the consequences of dying cells as they may contribute to inflammatory responses by releasing specific alarm molecules and induce immune responses leading to graft failure [122]. Loss of cells in capsules occurs via three processes as recently reported [122]. It involves autophagy, necrosis, necroptosis, and apoptosis [122]. All these cell processes are associated with release of intracellular compo- nents such as DNA, RNA, and HMGB1 that bind to specific recep- tors on immune cells. These specific receptors are the so-called pattern recognition receptors (PRRs). Examples of PRRs are Toll- like receptors (TLRs), NOD receptors, and C-type lectins [123– 127]. The DNA, RNA, and HMGB1 are also referred to as

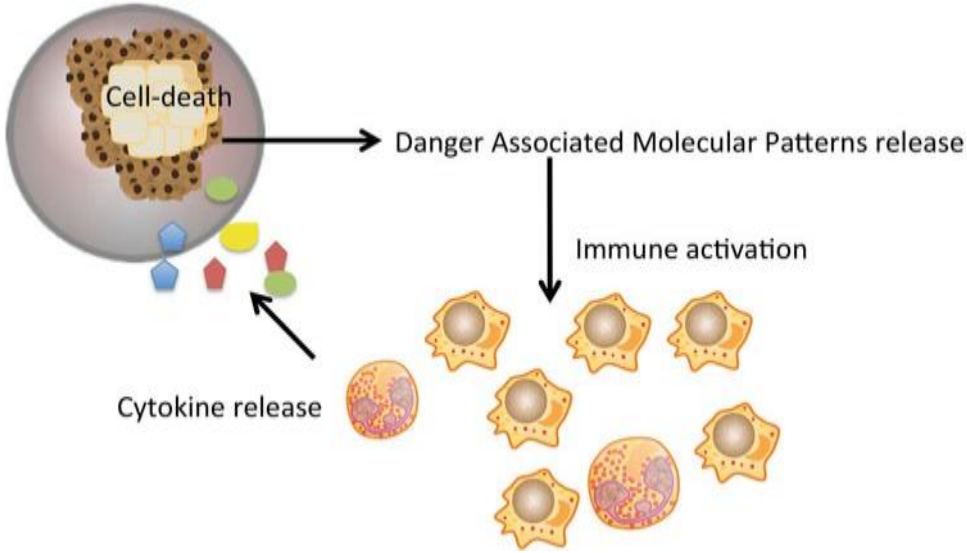

Fig. 3 Cell death does not only induce loss of the functional mass of cells in an encapsulated graft but also contributes to inflammatory responses. Dying cells release danger-associated molecular pattern molecules (DAMPs) which are potent activators of immune cells found in the transplantation site. After immune activation, cytokines are released that are small enough to pass the capsule membrane and induce more cell death

alarmins or Danger Associated Molecular Patterns (DAMPs) and bind or activate the PRRs and are very potent stimulators for immune cells. In recent studies it has been shown that encapsulated pancreatic islets do release DAMPs and activate immune cells in vitro [122] (Fig. 3). By applying a membrane that does not allow for entry of molecules smaller that 100 kDa some DAMPs can be retained but diffusion of DAMPs and associated immune activation cannot be completely blocked by the current generation of immunoprotective membranes [122]. We have indications that the quality of islet grafts and associated release of molecules such as DAMPs has a profound influence on the functional survival of islet grafts [122]. DAMP release can only be prevented by supplementation pharmaceuticals that stop cell death processes such as necroptosis [122] or by making the intracapsular environment a friendly environment that supports survival of cells.

8 Concluding Remarks and Future Considerations

Important advances have been made in the past decades with encapsulated cell therapy. Many new biomaterials and concepts have been introduced and many factors have been identified that determine the presence or absence of a tissue response against capsules. Clinical trials have started with some degree of success [49, 128].

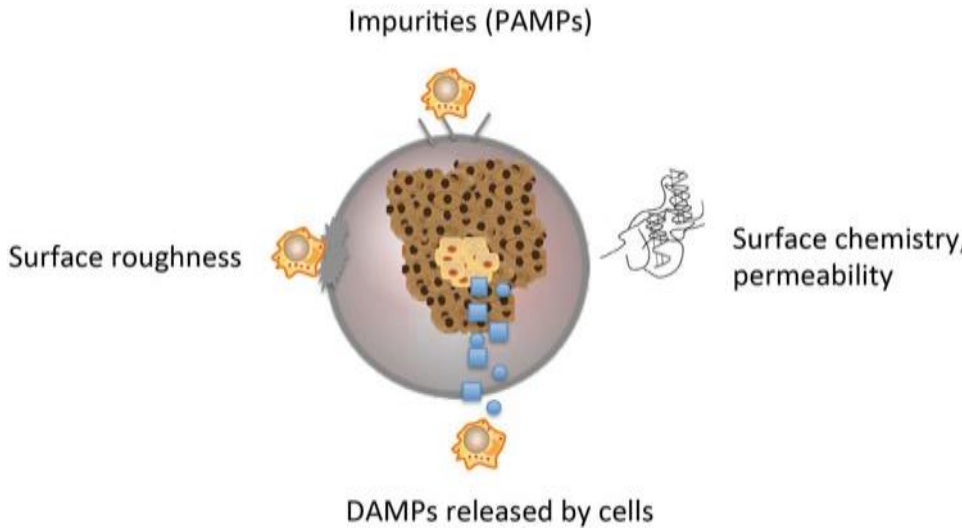

Fig. 4 Identified capsule properties that have a profound influence on the biotolerability of the encapsulated cellular graft. Minor changes in surface roughness, chemistry, presence of proinflammatory molecules such as pathogen associated molecular patterns may induce inflammatory cell adhesion and tissue responses against the capsules. Unfortunately only rarely these essential properties are reported on in transplantation studies

A major issue that is influencing the general opinion about applicability of the technology is the enormous lab-to-lab variation in success of the encapsulated cellular grafts [4, 50]. It is well known that the cause of this lab-to-lab variation is the poorly controlled physicochemical circumstances under which capsules are produced (Fig. 4). In a European consortium a stepwise analysis of capsule properties was performed to identify the minimum set of characteristics that has to be documented in order to reproduce the encapsulation procedure [75]. These were simple parameters such as polymer molecular weight and composition, permeability, mechanical strength, surface properties, and characteristics of the production process [75]. Unfortunately, until now it are only the former members of the European consortium that document and report on these essential parameters [50]. It is essential however for understanding the factors responsible for success and failure that the aforementioned critical parameters will be documented by the scientific community as a whole. Without it is currently impos- sible to do side-by-side comparisons of data-sets from different research groups.

A current regulatory trend in the USA is the suggestion that nonhuman primates are the ultimate models for human application of encapsulated islets grafts. As recently reviewed this might be a suggestion that interferes with progress [46]. Nonhuman primates have innate and adaptive immune responses that divert on essential parts from humans. Especially innate immune pathways that are involved in responses against capsules are different in nonhuman

primates. Responses that will be seen in nonhuman primates will be very different from that in humans [46].

It has repeatedly been shown that capsules do work in small species but that scaling up to larger animals and humans in cumbersome. In our experience changing species of encapsulated islet graft recipients means many adaptations in capsule properties such as mechanics, osmolarity, and permeability. Up to now these adaptations have not been documented and it is not known what the requirements are that capsules have to meet to work in large animals or humans [4, 50]. A systematic approach to identify the values is lacking up to now. A major challenge for the near future is to set the essential parameters for application in humans and to make the procedures more reproducible. This is not only a scientific challenge but also a regulatory challenge. Bioartificial organs are a relative new area of application and many regulatory issues are coming from other areas such as organ transplantation. Safety and functionality are associated with different items in the bioartificial organ field; for example, regulations about presence of endotoxins are set too high for the cell encapsulation field [75]. Also there is no regulation for types of endotoxins present in the capsular grafts. The aforementioned LTA and PG are highly inflammatory molecules and present in most polymers currently applied for encapsulation. Not only does their presence negatively impact biotolerability, it also may influence human health. Regulation might therefore facilitate progress.

The historical and more recent review of challenges hopefully illustrates two important critical recommendations. The first is that the proof-of-principle studies with encapsulated grafts demonstrate the principal applicability of the technology for treatment of human disease. The second is that the field needs a much more systematic approach in characterizing properties of capsules and cellular grafts to allow for sound interpretations of the results and further innovation.

Acknowledgements

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