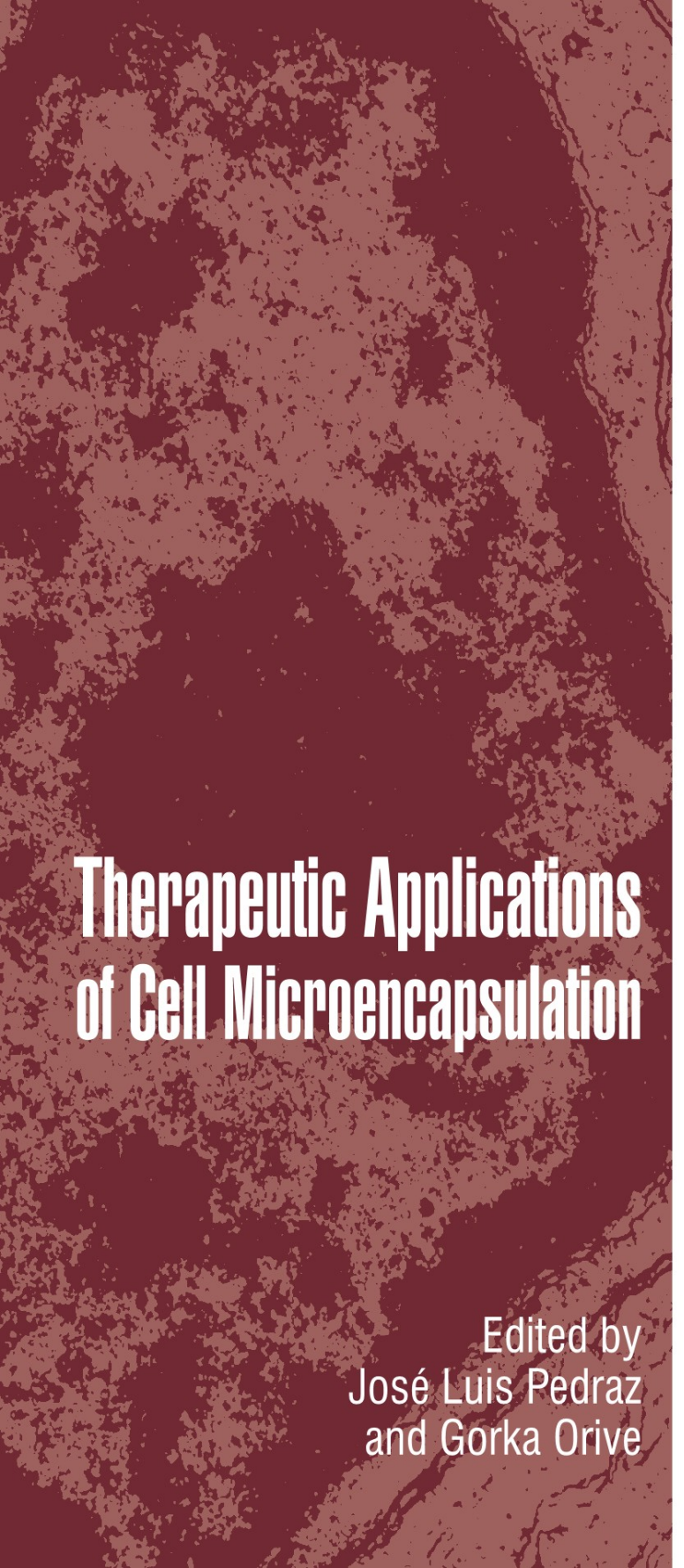

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Volume 670

Therapeutic Applications of Cell Microencapsulation

Edited by
José Luis Pedraz
and Gorka Orive

Therapeutic Applications of Cell Microencapsulation

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DEDICATION

Gorka Orive dedicates the present book to his parents Araceli and Ramón, his wife Raquel and his brother Ibon.

José Luis Pedraz dedicates this book to his parents José Juan and Luisa, his wife Angela and his children Diana and Carlos.

FOREWORD

The advancement of science is ever more contingent upon the interaction of experts with different yet complementary sets of expertise. This is a natural consequence of the vast amount of scientific information being gathered every day that exceeds the ability of any one scientist to acquire. As an illustration of the frantic pace of scientific discovery, no single molecular biologist was able to keep up with all the available knowledge in the field by the 1980s, a mere two decades after its inception. This challenge is even more acute in the case of scientific fields at the interface of different and seemingly distant areas of study. Amidst these, the field of cell encapsulation brings together an array of diverse disciplines such as molecular biology and biopolymers, gene therapy and inorganic membranes, stem cell biology and physicochemistry, immunology and nanotechnology. Clearly, such range of topics is too broad for any individual scientist to cover with sufficient depth. The field is experiencing such a remarkable rate of growth that regular updates are necessary to keep abreast of the current knowledge. This book is aimed at providing the reader with a detailed and up-to-date account of the state-of-the-art in the field of cell encapsulation.

At the core of this technology, there is an interaction of physicochemical and biological elements forming three distinct layers of complexity. First, the chemistry of the biopolymer dictates the degree of protein adsorption, vascularization, toxicity and biocompatibility of the microcapsules. Advances in biopolymer science are providing solutions to overcome existing challenges and to improve microcapsules as delivery vehicles. Second, the choice of cells, and more precisely the plethora of metabolites they secrete, and how they interact with the polymer and the host, are key in determining the immune response elicited by the host to implanted microcapsules. Adequate cell viability is crucial to achieve long-term therapeutic delivery. Finally, most microcapsule applications are aimed at delivering a given therapeutic product that is missing or deficient in the host, and thus it is recognized as foreign by the immune system. In response to this insult, the host often generates a vigorous immune response that can seriously compromise the therapeutic delivery. This immune response is antigen-dependent, it is not necessarily identical for all transgenes, and thus must be considered independently for every medical application. Adding to this complexity, changes in one of the above-mentioned layers may also affect the other layers. As an

example, the choice of encapsulated cells may determine whether there is an immune response against the secretable transgene. Similarly, the purity of the polymer may influence the viability of the encapsulated cells. Therefore, it is imperative to consider and study the interaction of all three layers.

Type 1, or insulin dependent diabetes, was one of the first main applications of cell encapsulation. However, the initial promise of a treatment for this disease that imposes such a tremendous burden on both patients and health care system has not yet fully materialized. Despite these shortcomings, recent findings have provided us with a more detailed characterization of the immune responses against encapsulated islets, and in the process, new possibilities for more effective treatments are opening. Beyond diabetes, applications of cell encapsulation have been expanded to multiple human disorders. The initial exploration of intrathecal implantation of encapsulated cells into the CNS, have been followed by additional studies aimed at developing potential applications to very serious medical conditions, such as Parkinson, Alzheimer's, and stroke. Similarly, there are efforts to exploit cell encapsulation as a treatment for cancer, blood diseases and metabolic disorders. The maturity of the field is reflected in the various clinical trials that have explored the application of cell microencapsulation in medicine.

Recent developments in cell encapsulation have improved the outlook of this technology as a viable treatment for medical diseases. The knowledge resulting from the clinical trials, together with additional research on the basic physicochemical and biological characteristics of cell encapsulation will ultimately determine its feasibility.

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PREFACE

The main objective of this book has been to analyze in depth and discuss the different aspects related to the design and elaboration of cell-enclosing microcapsules, even the regulatory features and clinical trials under development. These improvements will lead to progression in this therapeutic approach which may become one day closer to a realistic proposal for clinical application.

The advances in drug delivery technology have enabled a new era of drug discovery and development. In this regard, cell microencapsulation is a technology that opens new venues and possibilities to the administration of new active principles and may be the key to solve several issues related to the correct administration of new therapeutic agents to finally success in the clinical setting.

The editors believe that this technology may have important applicability not only in the field of drug delivery (to treat diseases such as cancer, neurodegenerative disorders, metabolic diseases etc.) but also in cellular therapy and tissue regeneration among others.

Due to the organization of the chapters, this book can be read at different levels and readers may analyze from basic aspects of encapsulation, biomaterials, clinical applications and regulatory and industrial issues of this technology. This book is useful for both graduate and PhD students in the pharmaceutical, engineering and biomedical fields. Nevertheless, we hope that this book is also useful to expert researchers who may find information and new ideas about the cell microencapsulation area.

The general aspects of the technology are analyzed in Chapter 1. The main issues related to the use and selection of biomaterials employed in this type of technology are discussed in Chapter 2. The importance of microcapsule size and the possibility of reducing it through different approaches are discussed in Chapter 3.

In order to succeed in the clinical setting, it is of great importance to take into consideration all the regulatory aspects that may determine the therapeutic applicability of this technology. These issues are discussed in Chapter 4.

In the following chapters the broad spectrum of pathologies that may be treated using cell microencapsulation technology are discussed. Chapter 5 presents the advances achieved in the treatment of diabetes using microencapsulated islets of

Langerhans. The use of microencapsulated cells for the treatment of diseases related to the haematopoyetic system are discussed in Chapter 6. Chapter 7 describes the latest advances achieved in the field of regenerative medicine and cell therapy using cell microencapsulation technology, which is nowadays gaining attention and being considered a very relevant and promising strategy for the area. Other pathologies discussed in the book (Chapter 8) deal with alterations and degenerative processes in the central nervous system and the approaches currently under study for the treatment of cancer (Chapter 9).

Chapters 10 and 11 examine the advances achieved in the field of inorganic nanoporous membranes and the aspects that will determine the future of this type of technology. Finally, Chapter 12 examines a very important issue related to the commercial applicability of cell microencapsulation.

Last but not least the editors would like to greatly thank all the participating authors for their dedicated contribution and help, which have been crucial to make the publication of this book possible.

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CONTENTS

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Chapter 1

Highlights and Trends in Cell Encapsulation

Gorka Orive* and José Luis Pedraz*

Abstract

The exciting developments in the field of drug delivery have already had an enormous impact on medical technology, facilitating the administration of many drugs and improving the pharmacokinetics of many others. The past few years have also seen several firsts, including the design of novel tissue engineered approaches, intriguing advances in the fields of biomaterials and cell therapy and the improvements in the fabrication of more refined and tailored micro and nanocarriers for protein and drug delivery. The sinergy of some of these promising fields have fuelled the progress of cell encapsulation technology, a relatively old concept pioneered 60 years ago. The ability to combine cells and polymer scaffolds to create "living cell medicines" that provide long-term drug delivery has opened new doors in the use of allografts. In fact, transplanted cells may be isolated from the host's immune system by embedding them in a permeable device that controls the outward and inward diffusion of molecules and cells. As a result of this, the requirement for immunosuppresant drugs can be eliminated or at least reduced. At present, the burgeoning number of cutting edge discoveries is leading to the design of biomimetic and biodegradable microcarriers that can easily combined with stem cells. The latter will improve the protection and transport of the cells to the target injured tissue and then promote cell integration and consequently tissue repair or regeneration.

In the present reviews, we discussed the state of the art in the field of cell encapsulation technology. This book describes the most relevant aspects of the design and development of cell-loaded microcapsules. Some of the most interesting therapeutic applications of this technology are presented as are some of the limitations, future challenges and directions in the field.

Introduction

Exciting biotechnological approaches have been developed over the past two decades that have set the stage for tissue and organ replacement as well as for the continuous and controlled release of therapeutic agents to the host. Some of these advances can be summarized under the term "bioencapsulation". The latter involves the envelopment of tissues or biologically active substances in semipermeable membranes to protect the enclosed biological structures from potential hazardous processes.[1] The field of application of bioencapsulation is enormous. For example, in plant cell cultures,[2] bioencapsulation has been shown to be efficacious in mimicking the cell's natural environment. It improves the efficiency of production of different metabolites for industrial application. For fermentation[3] bioencapsulation is being applied for enlarging the cell density, aroma and capacity of the systems. In addition, bioencapsulation also has an outstanding and emerging application in

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medicine. It is, for example, used to protect biologically active substances or cells such as probiotica from the deleterious biological environment[4] and for delivery in specific sites such as the colon.[5]

A relatively large group of researchers apply bioencapsulation to drug delivery and the creation of bioartificial organs.[6] This approach, known in general as cell microencapsulation, involves the immobilization of therapeutic cells within polymer scaffolds usually surrounded by membranes that protect the cells against antibodies and cytotoxic cells of the host immune system.[7]

Cell microencapsulation represents one of these strategies which aim to overcome the present difficulties related to whole organ graft rejection and consequently the requirements for use of immunomodulatory protocols or immunosuppressive drugs. Additionally, it represents a new paradigm for local and systemic controlled release of drugs and growth factors. In the last few years, the principal applicability of the technology has been shown for the treatment of a wide variety of endocrine diseases, including anemia,[8] dwarfism,[9] hemophilia B,[10] kidney[11] and liver[12] failure, pituitary[13] and central nervous system insufficiencies[14] and diabetes mellitus.[15] More recently, microcapsules are also being used as new biodegradable scaffolds for stem cell proliferation and differentiation as well as in vivo administration.

The present book aims to provide an actualized and complete vision of cell microencapsulation technology for therapeutic purposes. The final objective is to gain knowledge on this technology from some of the most basic issues to clinical applications. Special attention will be paid to describing the most encouraging therapeutic approaches ranging from the treatment of anemia and diabetes to cancer and central nervous system (CNS) disorders. Last but not least, the latest data on encapsulation of stem cells, nanoporous micro- and macrosystems for cell encapsulation as well as regulatory considerations in the application of encapsulated cell therapies will be discussed.

Pivotal Issues for the Progress in the Field

Some of the main considerations for the progress of cell microencapsulation technology may include the selection of suitable cells for the intended therapeutic purposes, the optimization of the microencapsulation technology according to Good Manufacturing Practice (GMP), the selection of clinical grade biomaterials for microcapsule fabrication, the development of reproducible assays to measure capsule biocompatibility, permeability and mechanical stability and the improvement of biosafety considerations among others.

Initially, the elaboration of microcapsules for envelopment and protection of biologically active substances or cells starts with the selection of a suitable microencapsulation technology and encapsulation biomaterials. The majority of materials used in microcapsules are polymers, either naturally occurring or synthetic. A major pitfall in the field is the absence of guidelines for documentation of the characteristics of most of materials assayed. Therefore, it is mandatory that this documentation will be included since it is now widely accepted that the characteristics of the polymer is a dominant factor in determining the capsule properties.

Improving the technological properties of the capsules in terms of stability, permeability and biocompatibility is critical to ensure the future clinical success of the technology as it is to determine the optimal site for capsule implantation in each therapeutic situation. Reducing the diameter of cell-enclosing capsules may induce beneficial effects including better exchange between the enclosed cells and the ambient environment, higher mechanical stability and improved biocompatibility. Designing subsieve-size capsules is an interesting approach to address these issues and a particularly exciting alternative for drug delivery in the CNS.

Much effort is being devoted to improve the ability to monitor the implanted cell-loaded devices. Once microcapsules are transplanted, the only way to assess their functional state is through invasive recovery surgery. An interesting approach to overcome this situation has been recently proposed using alginate-based radiopaque microcapsules containing either barium sulfate or bismuth sulfate which could be monitored by X-ray.[16]

Another important issue corresponds to the regulatory aspects related with the clinical implementation of the technology. Recent legislation requires that investigational medicinal products, for instance gene therapy and cell-based therapies, should be produced according to GMP rules.

In addition, major efforts are needed to standardize the different microcapsule characterization assays, avoiding the multiple 'in-house' procedures currently available.

Therapeutic Applications of Cell Encapsulation Technology

The wide range of therapeutic applications of cell microencapsulation technology includes: (i) treatment of classical mendelian disorders, (ii) cancer treatment, (iii) Central Nervous System (CNS) diseases, (iv) artificial organs and (v) others. This book provides a comprehensive description of each of these therapeutic approaches.

In one of the chapters advances made in the field of diabetes treatment during the last two decades are presented. These include intravascular macrocapsules anastomosed to the vascular system as an AV shunt, the extravascular macrocapsules, which are mostly diffusion chambers transplanted at different sites and especially intravascular microcapsules. The advantages and pitfalls of the three approaches are discussed and compared with regard to clinical islet transplantation.

Another chapter presents the use of encapsulated genetically modified cells as an alternative approach for long-term drug delivery in different disorders. Taking erythropoietin as a model, the concept of living drug factories is presented in different animal models.

The use of cell-loaded systems for the treatment of CNS diseases is also highlighted. Microencapsulated cells can be implanted into the damaged brain area favouring the local, targeted and long-term release of drugs or proteins. Many studies have demonstrated the preclinical feasibility of encapsulation as a means of delivering factors to the CNS including the use of primary chromaffin cells for pain, PC12 cells for Parkinson's disease, genetically-engineered cells secreting trophic factors for Parkinson's (PD), Huntington's (HD), Alzheimer's disease (AD) and ALS. In general, these studies demonstrate that encapsulated cells can be protected and remain viable for extended periods of time to produce significant neuroprotective and behavioral benefits.

The use of encapsulated cells also has potential in the treatment of a variety of solid tumours, particularly those of unmet medical needs. The advantage to the use of encapsulated cells for the treatment of cancers is that therapeutic molecules can be locally delivered in a sustained manner from implanted cells since the cells are enclosed in microcapsules and are thus protected from host immune rejection. One of the chapters will summarise preclinical and clinical data from some of the more promising strategies involving encapsulated cells to treat tumours.

Another important application of capsules is as scaffolds for maintaining hESC pluripotency for extended periods of time via encapsulation in alginate hydrogels. As it has been demonstrated, human embryonic stem cells are capable of self-renewal and have an unlimited capacity to differentiate. The culture of hESCs, however, is hindered by high maintenance being a long, fragmented and labour-intensive process. As has been recently demonstrated, hESCs encapsulated in calcium alginate hydrogels remain viable in basic maintenance medium for a period of up to 260 days, indicating that it is possible to maintain hESCs in an undifferentiated state, without passaging or embryoid body formation and without animal contamination.[17] In addition, this technology can be further developed for generating tissue engineered structures. The integrated bioprocess may utilize stem cell encapsulation in alginate hydrogels and culture in rotating cell culture bioreactors. The process is amenable to control, automation and is scalable, providing an excellent platform to create different tissues such as bone.[18] This and other advances will be reviewed in one of the book chapters.

Therefore, one important consideration when designing the encapsulation systems is to define clearly their durability. For example, if the elaboration of an artificial organ or living drug delivery system is intended, highly stable and low-rate biodegradable capsules should be fabricated. On the other hand, capsules with a controlled biodegradation rate will be prepared if the administration and implantation of stem cells is desired.

Last but not least, the potential of inorganic nanoporous membranes for cell immobilization is presented in another book chapter. These types of membranes possess pore size distributions much tighter than that of polymer membranes, providing a better chance at appropriately balancing the requirements for immunoisolation and nutrient availability. Moreover, they can achieve smaller

and more accurate thicknesses, offering in the case of diabetes treatment improved blood-glucose control by decreasing the delay with which insulin regulates the blood-glucose level.

Conclusion

In summary, this book summarizes recent progress in the field of cell microencapsulation technology for therapeutic purposes and outlines what is needed to bring this technology closer to clinical application.

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Chapter 2

Biomaterials in Cell Microencapsulation

Edorta Santos, Jon Zarate, Gorka Orive,* Rosa Mª Hernández and José Luis Pedraz

Abstract

The field of cell encapsulation is advancing rapidly. This cell-based technology permits the local and long-term delivery of a desired therapeutic product reducing or even avoiding the need of immunosuppressant drugs. The choice of a suitable material preserving the viability and functionality of enclosed cells becomes fundamental if a therapeutic aim is intended. Alginate, which is by far the most frequently used biomaterial in the field of cell microencapsulation, has been demonstrated to be probably the best polymer for this purpose due to its biocompatibility, easy manipulation, gel forming capacity and in vivo performance.

Introduction

Biomedicine improvements have permitted more precise and early diagnoses, less invasive and quicker procedures and fewer and shorter stay hospitals visits. Indeed, millions of patients worldwide have benefited from innovative biomaterial-based products, such as controlled drug delivery devices, joint replacement and dental implants, endoluminal stents, pacemakers, artificial hearts, contact lenses, surgical adhesives and antiadhesives, vascular grafts and contrast agents for imaging.[1]

The word biomaterial is defined in a general dictionary as a material compatible with an organism that can be used to make implants, prostheses and surgical instruments. The development of biomaterials range from older as metals, alloys and polyester to more challenging hybrid materials that associate inert and living material created by tissue engineering (i.e., cell cultures). At present, due to the increased accessibility of knowledge and modern technology combined with advances in understanding disease and tissue regeneration, biomedical engineers have more opportunities for the development of innovations based on biomaterials and interface them with different cell types including adult and embryonic stem cells. Thus, biomaterials for specific medical applications, such as targeted micro and nanodrug delivery vehicles,[2] materials that respond to physiologically regulated[3] or external stimuli,[4] high-throughput polymers for nonviral gene delivery,[5] biodegradable shape-memory materials for minimally invasive surgery and advanced materials for tissue engineering, could now be rapidly created.[6]

The development of biomaterials used for different biomedical applications is a long-term process of multidisciplinary research activities, which is subject to different regulatory requirements. In the European Union (EU) and the United States (US) general nature requirements such as current Good Manufacturing Practice (cGMP), current Good Tissue Practice (cGTP), several EU directives and quality management systems are applied. In addition to general nature requirements specific standards for testing of material properties are required; among others, United States or European Pharmacopoeia (USP, EP), standards by the International

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Organization for Standardization (ISO), Association for the Advancement of Medical Instrumentation (AAMI), American Society for Testing of Materials (ASTM), National Institute of Standards and Technology (NIST), etc.[7] In conclusion, the most important criteria for material selection are quality and safety of the final product. Thus, general and specific regulations of legal authorities should be followed.

In this context, cell encapsulation has gained a great interest in the field of drug and cell delivery. Accordingly, one of the key issues to solve when it comes time to face the design of a microencapsulation approach is the choice of a suitable material. The success of the implant lies among other properties in both the stability and biocompatibility of devices and these parameters are directly linked with the employed polymers. Furthermore, preserving the viability and functionality of enclosed cells becomes fundamental if a therapeutic aim is intended.

Hydrogels provide several characteristics that make appealing their use. Due to the hydrophilic nature of these materials they represent the most biocompatible features as compared with other biomaterial form.[8] In addition, hydrogels may mimic extracellular matrices and provides a number of advantages for microencapsulation.[9-12] Some naturally derived and synthetic polymers used in cell encapsulation include the alginate,[13] chitosan,[14] agarose,[15] hyaluronic acid,[16] poly(ethylene glycol) (PEG),[17] poly(hydroxyethyl)methacrylate (HEMA)[18] and copolymers of acrylonitrile (AN69).[19] Naturally occurring biomaterials usually demonstrate a better biocompatibility, whereas synthetic ones have a more reproducible composition.[20]

Alginate

Nature and Composition

Alginate is by far the most frequently used biomaterial in the field of cell microencapsulation. This is principally due to its capacity to form excellent gels in very mild conditions as well as its great biocompatibility shown in vivo. Alginate allows for a complete processing of capsules under physiological conditions. Thus, the encapsulation can be performed in aseptic environment at room temperature, at physiological pH and using isotonic solutions.[8] This results in an easier manageability of the whole process, a higher viability of enclosed cells and a lower risk of releasing harmful products in vivo derived of the use of toxic components during capsule elaboration. Furthermore, matrices obtained from this biomaterial show good mechanical properties and high porosity, making alginate an excellent material for bioencapsulation purposes.

Alginates are unbranched anionic polysaccharides extracted from brown algae (*Phaeophyta*) constituting about 20-40% of the total dry weight of these seaweeds. Chemically they are composed of linear block copolymers of β-D-mannuronic (M) and α-L-guluronic acids (G) where the composition and sequential arrangement of the two residues depends on the source from which they have been isolated. These monomers form structures of homopolymeric (GG and MM) or heteropolymeric regions (GM or MG) along the chains that associate with divalent cations to create the hydrogels. The most known model of linkage widely reported in the literature is the so-called "egg box",[21,22] in which G residues create a cavity that constitute the binding site to form the junction of the chains. Until now, the capacity to bind with the divalent cations was related exclusively to guluronate molecules, hence, assuming that it was the main responsible of gel formation and mechanical properties. However, recent studies have reported similar rules to the mixed junction given by blocks of alternating M and G (MG-blocks).[23] Moreover, Mørch et al showed that Ba^{2+} can also bind to M chains.[24,25] Therefore, gel-formation of alginate is governed by a greater complexity that was initially thought.

Biocompatibility and Purification of Alginates

Biocompatibility has been defined as the ability of a biomaterial to perform with an appropriate host response in a "specific application".[26] In our case, the interaction between the biomaterial and the encapsulated cells should also be considered.[9] Both aspects must be taken into account if a long-term survival of the cell loaded implant is intended.[27] The consequences of insufficient biocompatibility usually resulted in graft failure. Anyway, even though the employed material

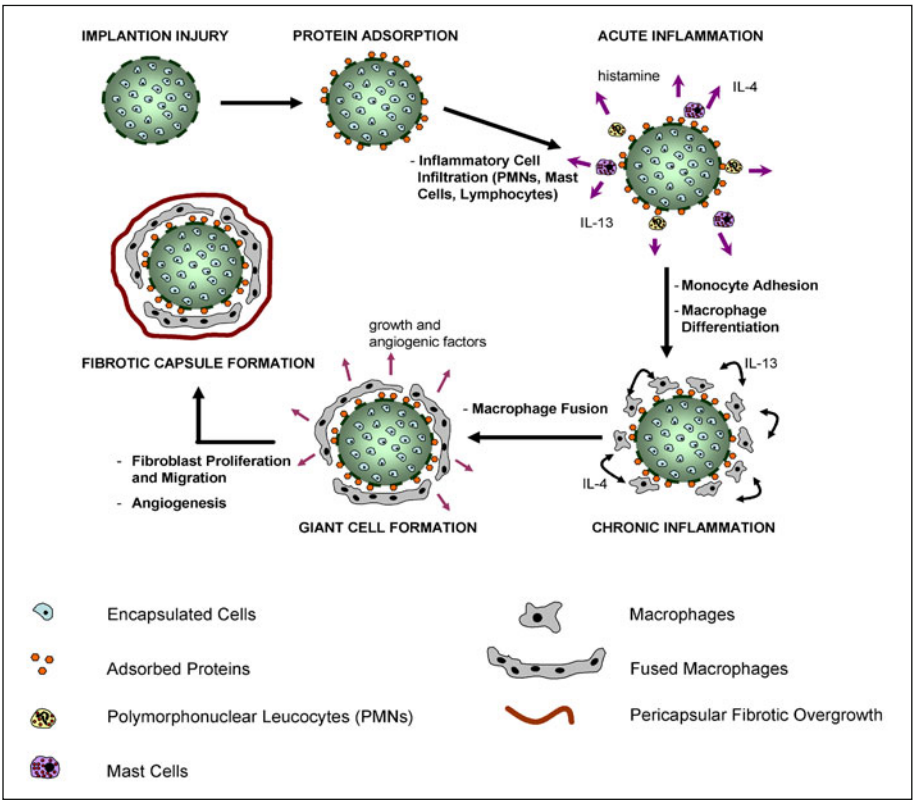

Figure 1. Diagram of the process of acute and chronic inflammatory responses in the foreign body reaction against implanted biomaterials.

presents an optimal biocompatibility, to achieve a successful implantation, the possible foreign body reaction produced by the surgical procedure should also be considered (Fig. 1).

As reported by Anderson et al,[28] surgical procedure triggers the immune response by the adsorption of proteins onto the biomaterial surface. The injury induced in vascularized connective tissue prompts thrombus formation, activating platelets and coagulation, complement, fibrinolytic and kinin-generating systems.[29] Due to that, it is induced the recruitment of neutrophiles, basophiles, mast cells and macrophages. The immune response acts through two main ways against foreign materials. (1) It develops a cellular overgrowth (mostly by macrophages and fibroblasts) that surrounds the microcapsules reducing diffusion of oxygen and nutrients within the matrix and leading to the necrosis of the entrapped cells.[30] (2) The immune cells attached to the implant secrete cytokines such as interleukin-1 (IL-1) that diffuse through the capsule and may have deleterious effects on the graft function.[9,31] Together with those processes, other inflammatory cytokines as they are tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), interleukin-4 (IL-4), interleukin-13 (IL-13) and histamine are also released, at the same time as fibrinogen adsorption is given.[32-35]

It has been observed that although the inflammation does not last more than two weeks, a few number of macrophages and fibroblasts remain attached to the 2-10% of the capsule surface.[9,36] In this respect, while some groups consider that the losing 2-10% of the surface area does not affect the functionality of the implant,[37-41] posterior data suggest that it is completely necessary to avoid capsule-surrounding overgrowth in order to maintain graft functionality.[9]

On the contrary, when materials of poor biocompatibility are employed the inflammatory response continue turning more and more severe. As the implant is bigger than the adhered macrophages, they fuse each other to form multinucleated foreign body giant cells.[42] The final step of this process is the formation of collagenous fibrosis, which isolates the implant from the host inducing an irreversible graft failure.[43]

Despite its natural source several impurities have been described in alginate samples that compromise the biocompatibility of the implant. Fucoidan, derived from cell walls of brown algae, results highly mitogenic.[25] Moreover, raw alginates extracted from seaweeds contains polyphenols, proteins and endotoxins.[44] Polyphenols resulted toxic to the cells whereas endotoxins stimulate the immune system severely. Zimmermann et al have reported the great importance of obtaining the alginates from stipes of brown algae harvested freshly from the sea,[25] since those extracted from algae washed ashore present a high pollution and suffered degradation long before collection.

Hence, the purification of alginate is essential if its use for implant is pretended. Up to now, literature has described a number of in-house methods to carry out the purification of this biomaterial.[45-48] Dusseault et al[49] compared and evaluated three of the most employed purification methods including the method reported by De Vos,[50] the one of Prokop[51] and the one of Klöck.[52] However, in-house methods present the additional hurdle that the same purification procedure followed by various laboratories may provide different results.[32,53] Thus, industrially purified alginates may result a suitable option for implanting, not only for presenting the lower grade of impurities, but also for the standardization of the procedure and reproducibility of the batches. In fact, in relation with the controversy formed around the alginates of different M and G content, it has been shown that purity of employed alginates, rather than their chemical composition, is perhaps the main cause influencing capsule biocompatibility (Fig. 2).[54]

Some other authors have reported additional strategies to improve the biocompatibility of the cell-loaded microparticles, such as the administration of temporary immunosuppressants[55-57] or prevention of protein adsorption and cellular adhesion to the biomaterial surface in order to avoid foreign body response.[8]

It is important to emphasize that parameters such as purity standards established by health regulations should be defined for the clinical application of alginate.[49] For instance, endotoxin levels

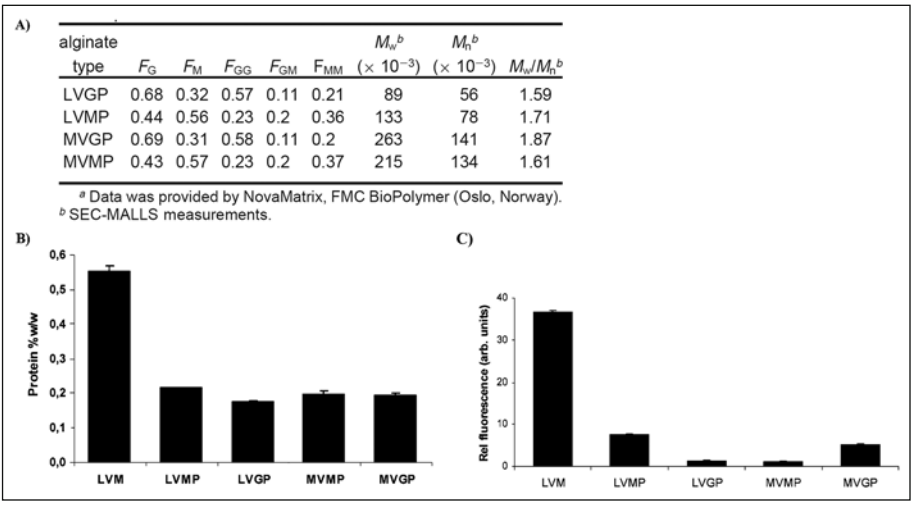

Figure 2. Evaluation of alginates with different compositions, purities and viscosities. A) Chemical sequences obtained by ^{1}H NMR spectra and SEC-MALLS measurements. B) Protein and C) polyphenol content of alginates. Reproduced with permission from Orive G, et al. Biomacromolecules.[44] © 2005 American Chemical Society.

must be kept below 100 EU/g for in vivo studies.[9,50,58] The microenvironment and ecologic of the growth place from which are harvested brown algaes are key factors in order to obtain reproducible batches of clinical grade alginate.[59] Indeed, a complete characterization by high-resolution specification of the growth area, as well as environmental and preparation data is mandatory.[25]

Besides all these aspects, other issues regarding the biocompatibility of the microcapsules are related to the physical properties of the devices. The elaboration of microcapsules with low surface roughness, lack of protuberant cells, reduced diameter and uniform size are critical issues to enhance the biocompatibility of the cell-loaded device. Another important factor that influences in the graft success is the choice of the employed polycation for the capsule coating. Indeed, this matter deserves a separate section and will be discussed later.

Properties: Stability, Permeability and Viscosity

The properties of the gels are governed by several parameters including the composition and the purification process of the alginate, the viscosity of pregelled solution, the cation employed for the linkage of the chains and the polycation used to coat the capsules. Additional factors such as pH, temperature, the presence of ion sequestering (as they are EDTA or citrate) or nonbindign ions (such as Na) in the cross-linking solution and encapsulation methodology should also be critically considered.

Mechanical stability is crucial for protecting transplanted cells and ensuring a sustained therapeutic efficacy during long periods of time. Indeed, the breakage of the capsules allows for the exposure of the entrapped cells to the immune system leading to the graft failure. In addition, it has been seen that the stiffness of the hydrogels may have influence on morphology and attachment of the cells, as well as on the regulation of several cellular activities such as proliferation, differentiation and apoptosis by focal contacts.[8,60-62]

A number of studies have demonstrated the great importance of the alginate composition and sequence for the properties of the gelled matrices. Features such as mechanical stability, capsule diameter, permeability and degradation rate are conditioned by this simple variable. Gelation process is based on the affinity of alginates towards divalent ions and the ability to bind these ions in a selective and cooperative manner.[63] Overall, alginates with high content of G result in more stable gels of greater permeability than those with high M.[64,65] That is mainly due to the fact that G residues, or more precisely, the length of the G-blocks are the principal responsible of the cross-linking with the great majority of usually employed divalent cations.[66] On the other hand, M residues are known to provide elasticity to the gel. In fact, it has been shown that the relative stiffness of the polymer chains increases as follows: MG < MM << GG.[67,68] As a result, gels with high G content which possess long G blocks and short elastic segments produce stiff open and static networks, while high M content alginates become more dynamic favouring more entangled networks due to their relative long elastic segments (Fig. 3).[63]

With the aim of obtaining tailored alginate gels Mørch et al reported the use of C-5 epimerases which converts M monomers to G in a postpolymerization step. This epimerization process increases the G content and changes the sequential arrangement of the chains, allowing for the alteration of both flexibility and stiffness of the polymer as desired.[67,69,70]

The rheological properties of the pregelled solution deserve especial attention, since they result of vital importance to maintain optimal cell viability. In the encapsulation process a mixture of cells and alginate solution is extruded through the electrostatic or air droplet device. In this step, mixing cells with high viscosity solutions may have deleterious effects for viability due to the shear stress that could damage cell membranes.[8,71]

Viscosity of the polymer solution prior to gel depends on the length of the chains, the concentration and the purity. The longer the chains, the higher the viscosity. Additionally, varying the concentration (w/w) of the polymer desired solutions can be easily obtained. In this way, low viscosity solutions lead to weak gels with poor stability and the viscosity required to form strong gels may be harmful for cells. Kong et al achieved decoupling this dependence of rheological/mechanical properties of hydrogels.[72] They employ a blend of low M_w and high M_w alginates to

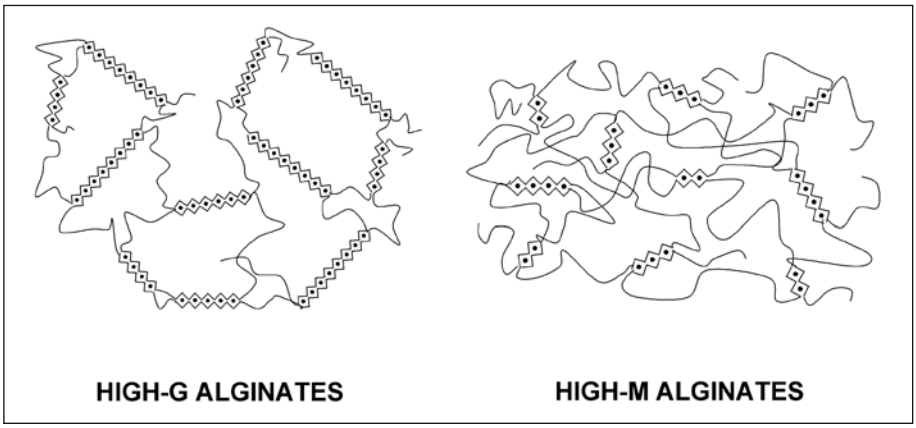

Figure 3. Model for network structure and porosity shown by hydrogels obtained from alginates of different composition.

obtain a bimodal molecular weight distribution and increase cell viability while producing gels of higher rigidity. Short chains provide stiffness to the gels with negligible contribution to the viscosity. On the other hand, long chains are responsible of maintaining the high strain at failure, since gels formed solely by low M_w alginates may result in too much fragile gels due to their rigidity. Thus, it is possible to increase the starting solution concentration without altering the viscosity of pregelling solution and additionally increase the mechanical stability of derived gels. This idea results very appealing for hydrogels, however, its application for cell encapsulation is still unclear.

Moreover, as mentioned above, several factors related with the purification process of the alginate should be taken into account. First of all, in the purification process there are certain steps, such as dialysis, that likely remove the smallest chains increasing the viscosity of the alginate. On the other hand, impurities of the polymer may act hampering the interchain interactions and its three-dimensional arrangement, so that higher viscosity solutions are obtained by removing such impurities.[49]

Alginate Microcapsules: Different Ions and Coatings

Cross-Linking Ions

The affinity of alginates toward the different divalent ions has been shown to increase in the following order: Mn<Co<Zn<Cd<Ni<Cu<Pb<Ca<Sr<Ba.[66] Nonetheless, most of them cannot be used for therapeutic application of the microcapsules. In the practice, the most habitually employed divalent ions are Ca^{2+} and Ba^{2+}.

Since the first microencapsulation approaches, calcium has been usually used as the cross-linking ion.[13] That is mainly due to its incomparable physiologic and biocompatible qualities which provide excellent viability for the enclosed cells. However, in physiological solutions or once they are implanted, beads made of Ca-alginate show tendency to suffer osmotic swelling, giving rise an increase of permeability, destabilization and, finally, the breakage of the matrix.[24,73] This occurs as a result of the constant interchange between Ca^{2+} and other nongelling ions (i.e., Na^{+}, Mg^{2+}) and due to the fact that Ca^{2+} ion affinity towards some chelating agents such as phosphate and citrate is higher than that towards alginate. To solve this problem, resulting beads are usually coated with an additional polycation layer that provides strength to the matrix and adjust their permeability. Nevertheless, owing to the immunoreactivity of the polycation layer a second coating layer of alginate is necessary to avoid implant rejection.

The other possibility is to use barium instead of calcium. Ba^{2+} ions, present higher affinity towards alginate give better long-term mechanical stability.[47,74-76] Unlike Ca^{2+}, Ba^{2+} ions bind MM

blocks apart from GG blocks.[24] Assuming this and the higher affinity for the GG blocks, Ba^{2+} forms stronger gels with more reduced diameter, lower permeability and with no tendency to swell when used with high-G alginates. Nonetheless, in spite of the mentioned advantages, Ba^{2+} may result toxic. In fact, it has been reported that Ba^{2+} inhibits potassium channels in cell membranes at concentrations greater than 5-10 mM[77] and at cross-linking times greater than 15 minutes.[25] It has been shown that although lower concentration of Ba^{2+} is required to obtain stronger and more stable matrices, higher amounts of the ion are needed to achieve the permeability enough to avoid the entrance of IgGs within the capsule.[18] Thus, the risk of toxicity becomes elevated if the performance of efficient biosystems is intended.

In addition, another drawback of using Ba^{2+} is the inhomogeneity of the elaborated microcapsules (the core of the capsule is less gellified than the surface) as consequence of gradients occurred during the binding process.[78,79] This provokes irregular permeability profile across the axial plane of the capsule, obtaining wider porosity diameter at the surface that goes reducing toward the core. Moreover, mechanical stability results deteriorated comparing with homogeneously gellified ones. For overcoming that problem, Zimmermann et al developed the crystal gun method, thus, obtaining homogenous Ba-alginates beds.[25,80]

Coatings

In order to increase the mechanical stability and induce further restriction in the permeability of the microcapsules, the beads are coated with a polycation layer after gellification. As it is mentioned before, this positively charged membrane may result immunogenic and it is necessary to hinder the positive charges with an additional alginate coat. Therefore, up to few years ago it was assumed that microcapsules were formed by the core and two well differentiated layers. Nonetheless, studies have demonstrated that the polycation penetrates the matrix and, thus, the surface of the capsule is composed of a single mixed layer formed by a complex of polycation-alginate.[9,81,82]

One key issue is the choice of the polycation to cover the alginate beads. Different polycations such as poly-*L*-lysine (PLL),[83] poly-*L*-ornithine (PLO),[84] chitosan,[85] oligochitosan,[86] lactose modified chitosan[87,88] and photopolymerized biomaterials[89] have been employed for the coating of alginate matrices.[55] However, the selection of the most suitable material is still an issue of controversy.

Some groups support the use of PLO, due to the fact that provides higher mechanical stability, more restricted permeability and better biocompatibility than PLL.[90-92] Indeed, the group of Calafiore R. has used this polycation to elaborate capsules in a recent clinical trial.[93] The amino acids of PLO present one methyl group less in their backbone than PLL, resulting in shorter monomers.[94] It is suggested that this could probably permit a more efficient binding to the alginate and become coatings of increased thickness and strenght.[90] However, the same group admitted that the immunological effects of PLO on cell viability still needs for more extended studies.[90] In addition, PLO binds mainly to high-M alginates and, as it is referred before, this type of polymer provide weaker matrices than those formed by high-G ones.

The most broadly studied and the most frequently employed polycation is PLL, which is used to produce the classical alginate-poly-*L*-lysine-alginate microcapsules (APA) as designed by Lim and Sum.[13] Moreover, it is well known the biocompatibility problems arisen by the use of APA capsules.[95-98] Around this problem, several studies have been carried out to analyze charges distribution and the immune reactions induced by the polycation.[36,81,82,99-101] It has been shown that the external alginate layer does not effectively hinder (neutralizing) the immunogenic positive charges of this polycation and, therefore, PLL may contact with the host tissue.[95] De Vos et al reported an interesting approach to measure the electrical charges of the surface by means of Z potential with the aim of predicting biocompatibility of APA systems.[102] PLL binds better with intermediate-G alginates, since there are more available binding sites for an adequate linkage.[73,103] In high-G beads however, more incompletely binding molecules are resulted.[58,101,104] As some unbounded polycation chains remain, it is reasonable to think that these elements could probably diffuse out and prompt inflammatory response. This has been also recently confirmed by Tam et al that measured lower amount of opsonized immunoglobulin G in the surface of APAs made

of intermediate-G.[105] Even though, due to better elaboration features and lower rupture percentages, the use of high-G follows being more advisable.[36] At this point, it would be totally necessary to achieve an international consensus and define clearly each type of alginate depending on the percentage of G and M monomers.

With the aim of searching the most suitable polycation for cell microencapsulation and finishing with the existing discrepancies, Ponce et al performed a study in which capsules of different polycations were evaluated.[103] It was concluded that PLL coating resulted more stable and less immunogenic than PLO and poly-*D*-Lysine (PDL). Indeed, PLO and PDL showed lymphocytes in their surrounding area indicating that were responsible of a specific immunological response. Furthermore, PLO elicited significant protein adsorption when implanted in vivo.

Other proposed polycations apart from the already explained ones have been reported to provide alternative advantages over the classical alginate-PLL system. Nevertheless, so far there are no comparative in vivo studies to confirm such assumptions.[106,107]

Recently, modified oligochitosans (quaternary ammonium derivative oligochitosans) have been proposed as an interesting possible alternative to the currently PLL.[108] Results obtained both in vitro and in vivo demonstrated that modified oligochitosans are biocompatible and allow the elaboration of microcapsules mechanically stable at physiological pH.

Modifications and Innovations

A strategy that is gaining the interest of scientist as an alternative to avoid foreign response caused by exposed positive charges is the design of nonfouling surfaces.[43] Protein adsorption and cell adhesion onto microcapsule surface is sterically inhibited (Fig. 4). The coupling of poly-ethylene-glycol molecules (PEG) is one of the most known technique for that aim.[107,109] In addition to PEG, there are other several molecules that have been used with the same objective including phospholipid[110,111] and sacharide surfaces.[112]

Besides avoiding the foreign response caused by exposed positive charges, it is essential the improvement of the mechanical stability of cell-loaded microcapsules. Accordingly, the strategy of covalent cross-linking has been exploited over the last years both to levels of matrix and coatings. Covalent beads can be achieved by adding methacrylate moieties to the alginate chains and

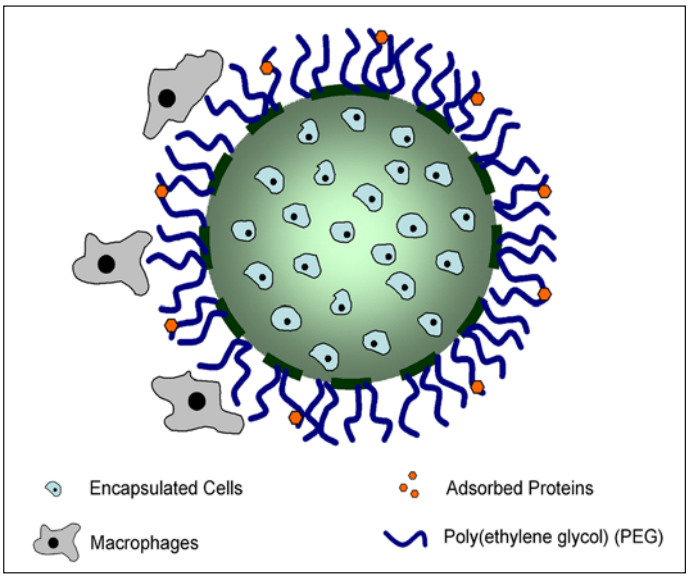

Figure 4. Proposed model for modified nonfouling surfaces.

their posterior photopolymerization.[113] However, in spite of the higher stability obtained, this technique is compatible with a narrow range of cell types. Other studies include the addition of phenol moieties or the development of alginate-tyramine conjugates, obtaining both ionically and covalently cross-linkable capsules.[114,115]

Several approaches have been described with the aim of reinforcing the layer that coats the beads. One method, for instance, relies on tailoring PLL particles with photoactivatable cross-linkers[116] or including additional polymer layers.[117] Overall, these strategies presented improved mechanical strength but, at the same time, compromised viability of enclosed cells and reduced biocompatibility compared with APA capsules. As modifying the coat of the capsules lead to changes in permeability, further studies should be done to characterize the mass transfer and the cut off resulted of those variations. Moreover, most of these approaches lack in vivo studies that would be necessary to ensure the correct behaviour of the capsules and to confirm all data. One possible use of cross-linked layers could be focused on avoiding the dissemination of enclosed cells in the case of working with cell lines that grow in an uncontrollable manner (hybridomas for example) or cells that could suffer tumoural transformation.[118]

Until recently, biomaterials have been considered as simple inert scaffolds in which cells were merely entrapped. Nowadays an increasingly important question is how those materials should interact with cells so that they were able to incorporate different biological cues.[119] In other words, acting as they were real extracellular matrices (ECM). The search of an ideal extracellular-like environment (trying to mimick the ECM of tissues) has led to the design and development of hydrogels that incorporate integrin-mediated cell adhesion sequences. Among others it can be mentioned RGD, IKLLI, IKVAV, LRE, PDSGR and YIGSR.[120-122] These moieties trigger a cascade of intracellular signalling events through the focal contacts providing tight control over cell-matrix interactions.[123]

The most widely employed peptide sequence is arginine-glycine-aspartic acid (RGD) derived from fibronectin, a natural protein presented in ECM.[124,125] The coupling of RGD sequences to alginate hydrogels has been extensively studied by Mooney et al. During the last decade, they have reported several approaches to characterize matrices functionalization and its possible applications. Among others, the most remarkable could be the direction of cell fate controlling RGD density,[126,127] the influence of different nanopatterned islands of RGD ligands on cell behaviour[128] and the development of tools that allow for quantifying the interactions between cells and presenting ligands.[139,130] In this context, they described a detailed study that makes a step forward in the understanding of cell-ECM interactions and how integrin expression varies depending on the stage of cell differentiation.[131]

Functionalization of 3D hydrogels has recently been transferred to the field of cell microencapsulation by Orive et al, significantly improving some of the most important features of this technology.[132] In this context, it has been shown that integrin mediated bindings to the RGD moieties act as additional cross-linkage molecule within the alginate matrix (Fig. 5), augmenting both their mechanical stability against swelling and their value of rupture force. In the same way, the addition of such oligopeptides results in a more natural environment for the enclosed cells that, as a consequence, improve their viability and long-term functionality in vivo. In fact, tailoring the alginates with RGD moieties prolonged the activity of the graft in more than 100 days compared with the conventional alginate matrices.[132]

Other modifications under evaluation are those focused on the control over the biodegradation rate of the alginate. Such feature becomes of the great appeal when more than drug delivery, the remodelling of damaged tissue is intended. In these cases, the capsules do not meet an immunobarrier role, but they acts as support scaffolds for a correct grafting of implanted cells. Once the biomaterial meets its function, it is necessary its clearance from the body. Thus, the degradation rate should be adjusted to the time required by grafted and host cells to replace the scaffold. Oxidation of alginate chains is one of the most employed strategies for this purpose, generating functional groups that are more susceptible to hydrolysis.[133,134] It is fundamental to take into account that

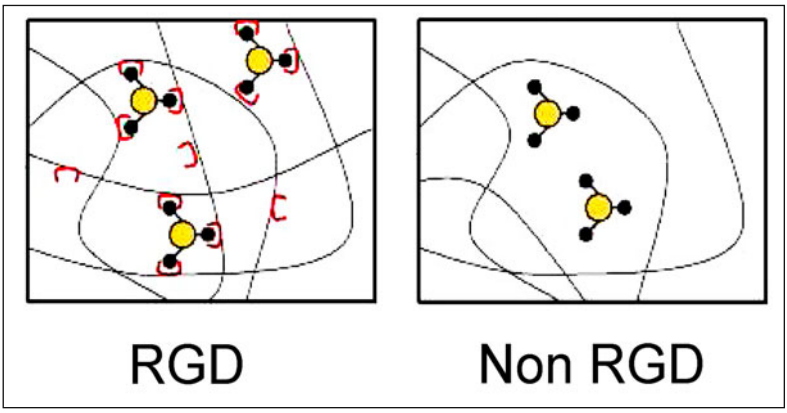

Figure 5. Illustration of RGD-functionalized and nonfunctionalized matrices. The diagram shows the attachment of cells to oligopeptides. Reproduced, with permission, from Orive G, et al. J Control Release.[132] © 2009 Elsevier B.V.

products resulted from the degradation must not be toxic neither for encapsulated cells nor for surrounding host tissue.[123]

Other Polymers and Type of Biomaterials

Other biomaterials have been investigated in cell microencapsulation field, although none of them is as much characterized and studied as alginates. On the way to obtain alternative cell-based therapeutic strategies, we could benefit from the advantages that other biomaterials could offer (Table 1).

In addition to hydrogels created by ionic interaction, biomaterials based on a cross-linked network formed by the presence of two or more polymerizable moieties, which is also known as radical cross-linking, have also been studied for cell encapsulation. Hyaluronic acid (HA) and poly(ethylene glycol) (PEG), functionalized with vinyl end groups, such as methacrylates and acrylates, are the most used polymers for this polymerization mechanism.[135] HA is a component of the extracellular matrix in mammalian connective tissues and participates in many important biological processes during wound repair. These properties make HA an interesting biomaterial for cell-based scaffolds, as it has been observed that cells (condrocytes, fibroblasts and murine embryonic stem cells) encapsulated into the HA beads proliferate and behave correctly.[136,137] On the other hand, PEG hydrogel beads formed by radical cross-linking have also been used to improve cell-based drug delivery. For example, islet cells and engineered fibroblasts have been encapsulated within PEG hydrogel devices with the aim of improving cell viability,[10,138] decreasing immunogenicity,[139-141] controlling the mass transport[142] and improving mechanical properties.[143] According to these preclinical studies PEG based microcapsules inhibit protein and cellular adhesion to the material and provide lower immune respond than nonpurified alginate based microcapsules.[8] However, in general PEG hydrogels present worse cell viability, mass transport control and mechanical properties than alginate beads. Besides, PEG hydrogels remain being a suitable option for tissue engineering. For instance, Benoit et al recently reported an approach in which they achieved differentiation of stem cells using different moieties attached to PEG hydrogels.[144]

Photoinitiated polymerizations are becoming increasingly popular because of the ability to form gels under physiological conditions, as in the case of HA and PEG based microcapsules.[135] Nevertheless, HA and PEG hydrogel beads fabricated by photopolymerization have several disadvantages in comparison with APA microcapsules. First, they need a highly reactive photoinitiator agent, i.e., 4-Benzoylbenzyltrimethylammonium,[136] which could provoque chain

Table 1. Clinical applications of hydrogels made of different biomaterials used for cell-based drug delivery

Biomaterial	Application	Type of Trial	Ref.
PEG	Insulin releasing islet cells for diabetes	Preclinical	10, 138
Alginate	Insulin releasing islet cells for diabetes	Preclinical	150, 109, 151, 152, 90, 153
PEG	Insulin releasing islet cells for diabetes	Preclinical	17, 140-142
PEG	Insulin releasing islet cells for diabetes	Clinical	Clinicaltrials.gov
Polyethersulfone	CNTF releasing engineered kidney cells for Huntington's disease	Clinical	154
Hyaluronic acid	Encapsulation of fibroblasts and ESCs	Preclinical	16
Alginate	Encapsulation of osteoblasts	Preclinical	71
HEMA	CNS diseases	Preclinical	18
Alginate	Insulin releasing islet cells for diabetes	Clinical	155
Alginate	VEGF releasing fibroblasts for ischemia	Preclinical	156
PEG	BMP releasing engineered fibroblasts for bone defects	Preclinical	143

PEG: poly(ethylene glycol); ESCs: embryonic stem cells; HEMA: 2-hydroxyethyl methacrylate; CNS: central nervous system disorders; CNTF: ciliary neurotrophic factor; VEGF: vascular endothelial growth factor; BMP: bone morphogenetic protein.

transfer to proteins and molecules on the cell membranes. Second, it is often necessary the incorporation of hydrolytically or enzymatically degradable compounds that reside within the backbone of the macromolecular monomer and may be harmful and cytotoxic for the enclosed cells.[145] Third, it is important to emphasize that fabrication process of photopolymerized hydrogel beads is more complex compared with APA microcapsules, due to temporal spherical molds, as calcium alginate beads or polydimethylsiloxane (PDMS) molds, are necessary in the fabrication process.[136,137] Finally, high molecular weight degradation products derived from the photoinitiated polymerization process decrease the biocompatibility of the scaffold, although, including thiol groups in the polymerization process lower molecular weight degradation products are obtained improving the biocompatibility.[146]

Another alternative developed for cell encapsulation are collagen based devices. It has been demonstrated that collagen, an excellent biocompatible natural biomaterial widely used in several biomedical applications, presents superior advantages for the microencapsulation of human mesenchymal stem cells (hMSC) for normal hyaline cartilage reproduction.[147] Self-assembled collagen microspheres devices are stable, can be injected and are able to provide a protective, growth- and migration-supporting to hMSC. Moreover, hMSCs preserve their stem cell nature upon self-assembling collagen microencapsulation technique and are localized with retained viability upon in vivo implantation.[147,148] Nevertheless, it is important to underline that in this case the main objective is the application of self-assembled collagen microspheres in cell delivery or grafting and not the drug delivery.

In addition, neural grafting represents a promising approach where different biomaterials have been used to obtain an effective treatment for central nervous system (CNS) disorders. For example, 2-hydroxyethyl methacrylate (HEMA) copolymers with ethyl methacrylates (EMA) present an optimum swellability and stability and minimum cytotoxicity in in vitro

tests. Furthermore, it has been shown that HEMA-co-EMA hydrogels beads were stable even after implantation into brains of adult rats lasting 9 months.[149]

Conclusion

The choice of the biomaterial employed for cell microencapsulation represents a key issue to obtain the most suitable technology for each treatment. Several variables have to be taken into account with the aim of achieving the most stable design. The selected biomaterial must show good biocompatibility both with the enclosed cells and with the host tissue. It has to permit the elaboration of strong and stable gels under physiological conditions that at the same time do not elicit any damage to the cells in the pregelled solution. Alginate has been demonstrated to be probably the best polymer for cell encapsulation due to its biocompatibility, easy manipulation, gel forming capacity and in vivo performance.

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Chapter 3

Development of Subsieve-Size Capsules and Application to Cell Therapy

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Abstract

Reduction in the diameter of cell-enclosing capsules has a practical application in cell therapy as it induces beneficial effects such as higher molecular exchangeability between the enclosed cells and the ambient environment, as well as higher mechanical stability and biocompatibility. Subsieve-size capsules are capsules of less than 100 μm in diameter, which are approximately one tenth the size of conventional cell-enclosing microcapsules. Such small capsules can be prepared using the emulsion system obtained via the jetting process in which a cell-suspending polymer solution is extruded into an ambient coflowing water-immiscible liquid from a needle several hundred micrometers in diameter. The capsule size can be controlled by changing the velocity of the polymer solution and the ambient water-immiscible liquid. The emulsification process does not significantly affect viability of mammalian cells enclosed in the resultant subsieve-size capsules. In this chapter we will review the technique of subsieve-size capsule production and the effects of diameter reduction on the enclosed cells and properties of the capsules.

Introduction

A large number of studies have been published regarding enhancement of molecular permeability, mechanical stability and biocompatibility of cell-enclosing microcapsules as these are crucial factors governing the successful therapeutic treatment of diseases using microcapsules.[1-4] These studies can be classified into two categories depending on research approach: advancement and optimization of the biomaterials used to produce the microcapsules[5-11] and adjustment and optimization of the size of the microcapsules.[12-17] In the latter approach, smaller microcapsules offer many advantages. Reduction in capsule size has been reported to be effective in the enhancement of molecular exchangeability between the enclosed cells and the ambient environment. Chicheportiche and Reach[14] revealed that the response time of encapsulated pancreatic islets to glucose stimulation decreased with a reduction in microcapsule size. Beneficial effects on cellular activity were also reported in hepatocytes.[12] In addition, reduction in capsule size should allow the use of immune-privileged sites for implantation such as spleen capsules, omental pouch, or liver via the portal vein. The most frequently used implantation site is the peritoneal cavity due to the large space, however, this is not the most efficient implantation site in terms of immune molecule diffusion and vascularization.[12] Moreover, smaller capsules would reduce the size of the capsule injection device, thereby reducing surgical trauma.[18] It has also been reported that a reduction in microcapsule size suppressed foreign body response to the implanted microcapsules.[15] All these reports suggest that the effects of a reduction in microcapsule size can be positively considered to

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further practical application in clinical studies and that more attention should be given to capsule size to ensure graft function in vivo. A variety of methods have been developed and used for the preparation of cell-enclosing microcapsules following the first successful report in 1980.[19] In this first study, calcium-alginate based microcapsules of ca. 500 μm were used to enclose pancreatic islets of 50-300 μm in diameter. In some studies, the successful preparation of microcapsules of 100-200 μm have been reported.[20,21] However, microcapsules of 300-500 μm prepared using the method originally developed for encapsulating pancreatic islets has been investigated most frequently. In this chapter, the preparation of cell-enclosing microcapsules of less than 100 μm in diameter with a narrow distribution in size, in addition to the effects of diameter reduction and investigations using the capsules enclosing mammalian cells are described.

Narrow Dispersed Subsieve-Size Capsule Production Via the Jetting Process

We classified cell-enclosing microcapsules of less than 100 μm in diameter as "subsieve-size capsules".[22] The term "subsieve-size" is often used to classify particles in the range 10-100 μm in diameter. Compared with conventional-size microcapsules of 300-800 μm in diameter, subsieve-size capsules are about a tenth the size of conventional-size microcapsules. Of course this is too small to enclose pancreatic islets but is large enough to encapsulate single cells, 10-30 μm in diameter and aggregates of several cells. The range of diseases which can benefit from encapsulated cell transplantation technology has expanded due to advancement in genetic engineering enabling the use of cells as reactors to provide the desired biological products.[23-25] This means that the potency of subsieve-size capsules could also increase with advancement of genetic engineering. The subsieve-size capsule preparation technique involves a process of droplet breakup in a coflowing water-immiscible liquid. The emulsification technique using a magnetic stirrer and a homogenizer are well-known and are effective for obtaining emulsions containing droplets of subsieve-size.[26,27] However, the droplets are heterogeneous in size. In contrast, the droplets produced via the droplet breakup method (coflowing method) show a narrow distribution in size.[22] The emulsification device of the coflowing method is composed of a syringe filled with a polymer aqueous solution containing cells equipped with an inner needle (Fig. 1A), an outer tubule (B) and a pump connected to a reservoir containing water-immiscible fluid (C). The inner needle is positioned upstream in the vicinity of a coaxial outer tubule in which water-immiscible fluid flows. The resultant droplets or gelated capsules are collected at the tip of the outer tubule (D). The principle of the method is based on a jet technique. Jet techniques are cutting-edge approaches that use micrometer-sized stretched jets generated through nozzles or channels. The technique is associated with a wide variety of applications, such as ink-jet living cell printing[28] and the production of tissue engineering scaffolds composed of ultra-fine fibers, less than several micrometers in diameter.[29,30] To obtain cell-enclosing subsieve-size capsules, cell-suspending polymer aqueous solution is extruded into the immiscible ambient coflowing liquid from the inner needle. The behavior of the extruded

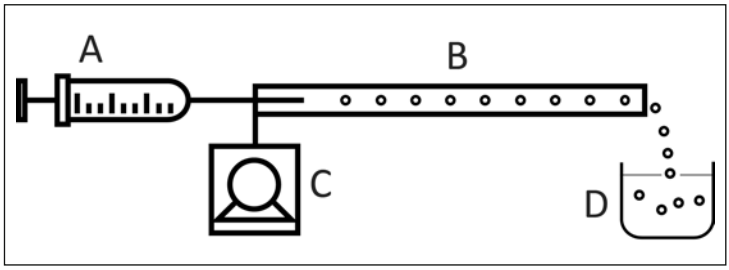

Figure 1. Schematic illustration of subsieve-size capsules generator composed of (A) a syringe equipped with a stainless steel needle, (B) a glass tubule, (C) a pump for flowing liquid paraffin and (D) a collector.

solution is affected by a complicated balance of three forces: the interfacial tension force, the drag force exerted by the ambient fluid[31,32] and the viscoelastic force of the polymer aqueous solution. Under appropriate balance of the forces, a stretched jet of polymer aqueous solution forms in the immiscible liquid. The jet subsequently become unstable and fragments into individual droplets.

Figure 2 shows the generation of droplets and jets of sodium alginate solution (194 mPa·s) extruded into a coflowing flow of liquid paraffin from a needle of 300 μm i.d. and 480 μm o.d.[22] The droplets having almost the same diameter as the inner needle formed at the tip of the needle without formation of a jet when we extruded sodium alginate solution at 1.2 cm/s into the liquid paraffin flow at 3.0 cm/s (A). This phenomenon is called "dripping". When a disperse phase is injected from a capillary into an immiscible liquid flow at laminar flow, two different droplet breakup modes are observed. Specifically, droplets either form close to the capillary tip, called dripping, or break up at the tip of a stretched liquid jet, called jetting.[33] The phenomenon of droplet breakup via jetting is explained by Rayleigh-Plateau hydrodynamic instability.[34] An increase in aqueous solution velocity to 4.7 cm/s induced the formation of a jet (B). Thinner jets resulted from an increase in drag force formed due to further increases in the differences in the velocities of sodium alginate solution and liquid paraffin (C,D). At the condition shown in Figure 2C, we obtained droplets of 44 ± 4 μm in diameter which was one-seventh of the inner diameter of the inner needle, 300 μm. It is easy to understand that the breakup of the stretched jets which are much thinner than the needle diameter results in such small droplet formation. The narrow distribution in size, ca. 10%, should also be noted. When air was used as the ambient fluid for obtaining the capsules smaller than the needle diameter, the resultant capsules had a heterogeneous size distribution rather than a narrow size distribution.[20,35] Sugiura et al[21] reported that sodium alginate aqueous solution had to be extruded from the nozzle with a 60 μm internal diameter for obtaining alginate-based microcapsules of ca. 150 μm in diameter with a narrow distribution in size. This is because the faster gas flow rate necessary for obtaining capsules smaller than the needle diameter results in a highly turbulent flow which loads a nonconstant force on the inner liquid when breaking into droplets. From a hydrodynamic point of view, a laminar flow resulting in a load of constant force to the inner fluid easily results from viscous fluid flow in a tube with a smaller diameter. In theory using the Reynolds number, an indicator expressing the state of flow, laminar flow of liquid paraffin is established under a velocity of less than ca. 150 m/s in a tubule of 2.5 mm in diameter. Viscosity of a polymer solution is also an important factor in the formation of stretched jets essential for obtaining capsules with a smaller diameter than the inner needle. For the development of suitable cell-enclosing capsules, knowledge of the effects of viscosity is

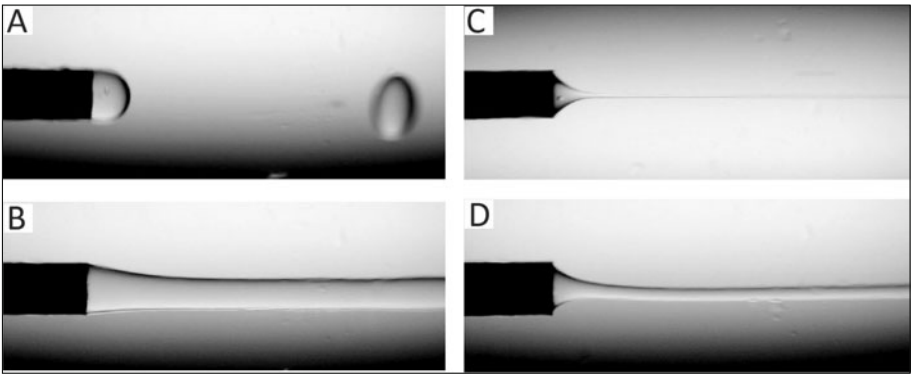

Figure 2. Sodium alginate aqueous solution extruded from needle of 300 μm i.d. and 480 μm. o.d. into the ambient liquid paraffin stream. The liquid paraffin flows from left to right. The velocities of the sodium alginate aqueous solution and liquid paraffin, respectively, were: (A) 1.2 cm/s and 3.0 cm/s, (B) 4.7 cm/s and 3.0 cm/s, (C) 1.2 cm/s and 23.5 cm/s and (D) 4.7 cm/s and 23.5 cm/s (adapted from Sakai et al, 2004[22]).

essential. This is because the viscosity of a polymer aqueous solution strongly correlates with the concentration, molecular weight and chemical composition of dissolving polymers governing the mechanical strength, molecular diffusivity and biocompatibility of the resultant capsules as well as the formation of stretched jets. In the droplet breakup process in the coflowing method, smaller droplets are obtained from a higher viscous polymer solution (Fig. 3).[36] This indicates that smaller cell-enclosing capsules can be obtained using a smaller quantity of water-immiscible fluid from the polymer aqueous solution with a higher viscosity. In other words, cell-enclosing subsieve-size capsules with a higher mechanical strength resulting from synergy between a reduction in diameter and a tightening of gel microscopic structure can be obtained using a smaller quantity of ambient fluid by using the coflowing method.

The reduction in the quantity of ambient fluid consumption is an important issue in several aspects of the development of a larger production process for practical use in clinical treatments. Using straight outer tubules with a smaller diameter may be a possible solution. However, practical difficulties in handling such equipment may arise as a result of the decreased distance between inner and outer needles. The introduction of a flow focusing technology is an effective solution. Flow focusing technology has attracted attention in a variety of fields and is an advantageous technique for the production of tuned micro- and nanoparticles.[37,38] In this process, the velocity of liquid flow in the tubule is greatly increased during flow focusing. Thus, the jet during flow focusing is further stretched by the accelerated ambient fluid during flow. By using the tubule with a reduced flow section (Fig. 4A) which tapers to 1 mm from 4 mm in diameter at a tubule length of 15 mm, we could prepare much smaller capsules from 4 wt% agarose solution at the same velocity of liquid paraffin at the tip of the needle compared with that using a straight tubule and retain a narrow distribution in size (Fig. 4B).[39] Subsieve-size agarose capsules of ca. 90 μm in diameter were prepared under a liquid paraffin velocity of ca. 1/30 of that necessary in the straight tubule.

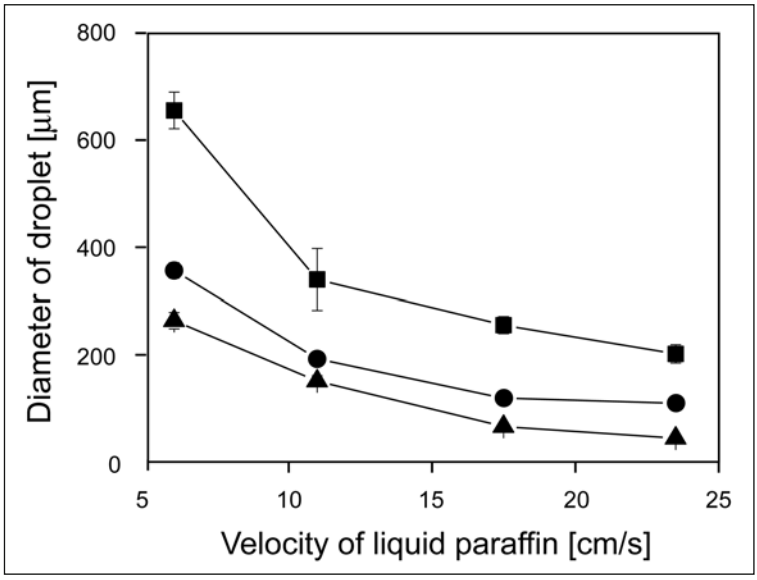

Figure 3. Droplet diameter as a function of the velocity of liquid paraffin for three aqueous solutions differing in viscosity: (■) 1.0, (●) 36 and (▲) 194 mPa·s. Aqueous solutions were extruded at a velocity of 1.2 cm/s from a needle of 300 μm inner diameter. Error bars represent SDs (adapted from Sakai et al, 2005)[36].

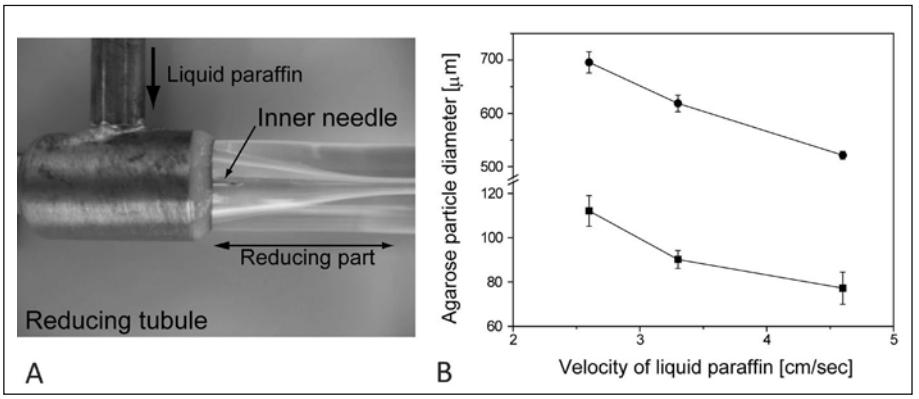

Figure 4. A) Photograph of reducing tubule. B) Agarose capsules diameter as a function of the velocity of liquid paraffin for two tubules with different configurations: (●) straight tubule and (▪) reducing tubule. Agarose solution was extruded at a velocity of 2.9 cm/sec. Error bars represent SDs (adapted from Sakai et al, 2006[39]).

Effect of Preparation Process on Mammalian Cells

It is well known that mammalian cells are easily damaged by forces exerted by the external environment, such as shear stress. In the coflowing method for subsieve-size capsule production, three forces, the interfacial tension force, the drag force exerted by the ambient fluid and the viscoelastic force of the polymer solution, play vital roles in droplet breakup in an immiscible coflowing stream. In particular, the drag force is the most influential force to affect the viability of cells suspended in the polymer solution. However, the results of our studies proved otherwise. Viability of mammalian cells retrieved from the nongelated subsieve-size droplets prepared by the coflowing method were ca. 95% independent of the morphology of tubules, whether straight or reduced tubules.[22,36,39] In addition, the retrieved cells showed the same proliferation profiles in tissue culture dishes as those seeded using a general subculture protocol.[36] These results suggest that the drag force necessary for droplet breakup in water-immiscible liquid resulting in subsieve-size capsules is insufficient to affect cell viability.

As the subsieve-size droplets exist in a water-immiscible fluid, a smart approach to obtain gelated capsules is to carry out gelation in the resultant emulsion system. Thermosensitive and photo-curable polymers are good candidates for this process. We have successfully prepared the capsules from agarose,[40,41] alginate-agarose composite[42] and an agarose-gelatin conjugate[43] by cooling the resultant emulsion system of cell-enclosing subsieve-size droplets. Agarose is a thermosensitive natural polysaccharide extracted from cellular walls and has been successfully used for cell encapsulation over the last couple of decades.[44-46] The mammalian cells enclosed in agarose subsieve-size capsules survived for more than 2 months in vitro. In addition, these cells survived and expressed their genetically modified function in vivo:[41] As a potential application of subsieve-size cell-enclosing capsules, we enclosed cells genetically modified to express cytochrome P450 2B1 enzymes (CYP2B1) in subsieve-size agarose capsules of ca. 90 μm in diameter and implanted these capsules into preformed tumors in nude mice for localized activation of the prodrug ifosfamide in or close to tumors. Ifosfamide is a prodrug that is metabolized into acrolein by CYP2B1 in the liver.[47] Due to a very short half-life of the activated compounds in plasma,[48] ifosfamide has to be given in relatively high doses despite severe side effects associated with such doses, such as leucopenia with granulocytopenia. Establishment of a second site of enzyme conversion near to or in the tumor using cells expressing CYP2B1 is an effective approach to reduce side effects without lowering response rates.[49-51] Compared with the conventional-size microcapsules of several hundred micrometers in diameter, subsieve-size capsules can be instilled with a low risk of blood vessel occlusion due to their much smaller size as well as reducing surgical trauma resulting from

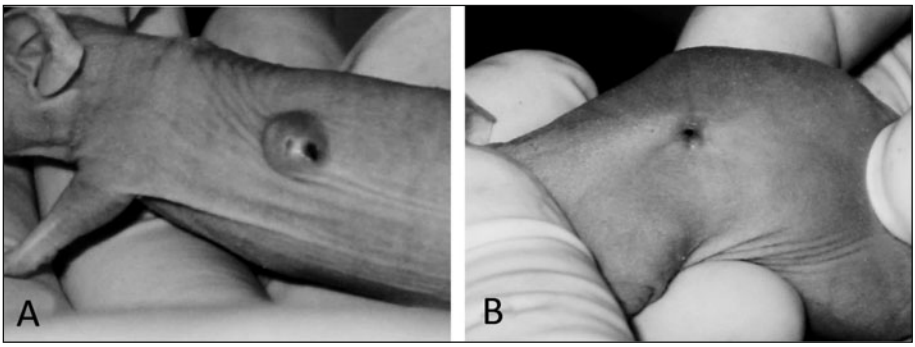

Figure 5. Subcutaneously xenotransplanted human tumors A) just after injection of CYP2B1 cells enclosed in subsieve-size agarose capsules and B) 26 days after treatment with ifosfamide (adapted from Sakai et al, 2005[41]).

the reduced size of the capsule injection device. In our study, the capsules could be injected into tumors using a 26-gauge syringe after suspending in saline. After 26 days of treatment, significant regression of tumors was observed in the recipients implanted with cell-enclosing subsieve-size capsules compared with those implanted with empty capsules (Fig. 5).

Effect of Reduction in Microcapsule Diameter

For conventional-size microcapsules, ithas been reported that a reduction in size is effective in the enhancement of molecular exchangeability, mechanical stability and biocompatibility. In theory, it is easy to understand that molecular exchangeability is enhanced by the reduction in capsule size from conventional-size to subsieve-size. Enhancement of mechanical stability was demonstrated by our findings: We compared two types of agarose capsules with diameters of 89 μm and 491 μm prepared from 1.0 wt% agarose by shaking their suspension in buffer solution vigorously at 37˚C for 7 days.[40] Despite damage to approximately 30% of conventional-size capsules, the subsieve-size capsules remained intact.

As well as the molecular exchangeability and mechanical stability, biocompatibility is also a crucial factor in the successful transplantation of cell-enclosing capsules. A pericapsular cellular reaction, i.e., cell adhesion resulting in cell layer formation on the capsule surface, caused by insufficient biocompatibility is highly undesirable because the cell layer not only serves as a diffusion barrier leading to an insufficient supply of oxygen and nutrients but also the cells themselves compete for this nourishment. One well-known factor that induces a pericapsular cellular reaction is the potency of cell-adhesive protein adsorption, which depends on the properties of capsule materials.[4] Robitaille et al.[15] revealed that microcapsule size also influenced the degree of cellular reaction. They showed that a reduction in size from 1200 μm to 350 μm reduced the reaction. In accordance with this report, we studied the effect of a further reduction in diameter to subsieve-size using agarose capsules by implanting cell-free agarose capsules into the peritoneal cavity of mice or the epididymal fat pads of rats.[52] Two weeks after implantation into the peritoneal cavity, the frequency of overgrown capsules decreased with a reduction in diameter from 925 μm to 387 μm similar to that reported by Robitaille et al[15] A further reduction in diameter to subsieve-size further reduced the frequency of overgrown capsules (Fig. 6A). Higher biocompatibility of subsieve-size capsules was supported by a reduction in floating cells in the peritoneal cavity after implantation (Fig. 6B). The implantation of cell-enclosing capsules in vivo is classified into two types as follows: implantation into a space in which the capsules float, such as the peritoneal cavity and into tissues, such as the liver and tumors. Thus, we also evaluated the effect of reduction in diameter to subsieve-size by implanting capsules into the epididymal fat pads of rats. The results show that subsieve-size capsules are more biocompatible

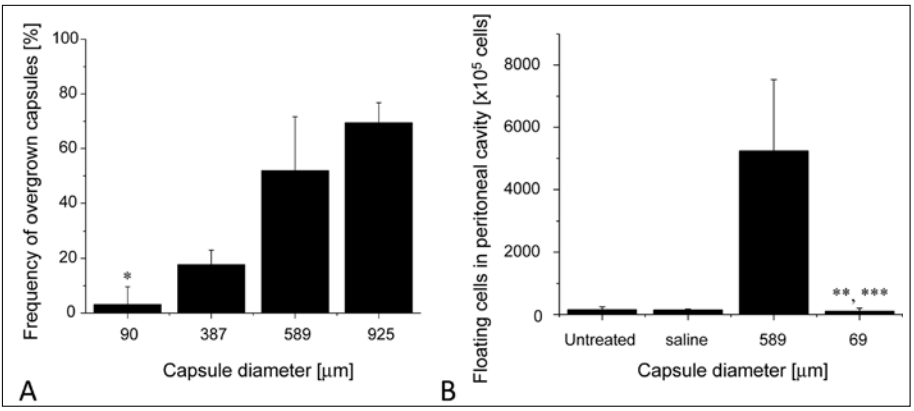

Figure 6. Effect of agarose capsules diameter (A) on the frequency of overgrown capsules retrieved from the peritoneal cavities of DDY mice and (B) on the number of floating cells in the peritoneal cavities 2 weeks after implantation.
$^{*}p < 0.01$ versus each capsules diameter, $^{**}p > 0.4$ versus untreated and sham control groups and $^{***}p < 0.01$ versus mice implanted with capsules 589 μm in diameter. Error bars represent SDs (adapted from Sakai et al, 2006[52]).

than conventional-size capsules. The mechanisms behind the enhanced biocompatibility of the reduction in diameter to subsieve-size are not clear. In these studies, we used capsules with an identical volume. This means that subsieve-size capsules have a larger volume-based surface area, thus have more potential to induce a cellular reaction than conventional-size microcapsules. As described by Robitaille et al,[15] one possible explanation is that greater stress induced by larger space-occupying objects stimulates the surrounding tissues and triggers a pericapsular cellular reaction. Higher biocompatibility as well as higher molecular exchangeability and mechanical stability and reduced surgical trauma during implantation, show that subsieve-size capsules are likely to become an invaluable cell-enclosing technique in cell therapy.

Conclusion

In this chapter, we describe the method of subsieve-size capsule preparation in water-immiscible liquid which was developed using the jet technique. Factors which are important in controlling capsule size and the less harmful effect on enclosed mammalian cells were also described. In addition, we showed the usefulness of subsieve-size capsules with higher molecular exchangeability, mechanical stability and biocompatibility than conventional-size microcapsules in cell therapy. These studies are still at an early stage regarding practical use and studies for the establishment of immunoisolatability and further enhancement of biocompatibility will be necessary. In addition, an increase in the kinds of advanced polymers which may be applicable for subsieve-size capsule production in water-immiscible fluid would be a key component for the further advancement of this technology. For example, we developed a novel alginate which can be crosslinked via an enzymatic reaction triggered by a substance dissolvable in liquid paraffin as well as a conventional crosslinking process with multivalent cations.[53]

In this chapter, we discussed the feasibility of cell-enclosing subsieve-size capsules in cell therapy. Mammalian cell-enclosing microcapsules have also been studied as a basic research tool and as a device for producing biological agents such as monoclonal antibodies, enzymes and peptides. More recently, this technology has attracted attention as a useful tool for investigating the differentiation pathway of embryonic stem cells in vitro and that in vivo.[54,55] We believe that subsieve-size capsules have a role to play in these fields due to the higher mechanical stability and molecular exchangeability of these capsules.

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Chapter 4

Regulatory Considerations in Application of Encapsulated Cell Therapies

J. van Zanten* and Paul de Vos

Abstract

The encapsulation of tissue in semi-permeable membranes is a technology with high potential and in due time several new therapies based on this technology will be tested in clinical trials. Recent, new legislation requires that these investigational medicinal products used in clinical trials Phase I must be produced according to Good Manufacturing Practice (GMP). Consequently, the activities of GMP are expanding to the field of research and researchers might need to change developed protocols in order to meet GMP legislation. This chapters gives an overview of the overall guidelines covering GMP and more specific guidelines dealing with cell based therapies and gene therapy.

Introduction

Immunoisolation involves the encapsulation of living tissues in semi-permeable membranes to protect the transplanted tissue against the host effects of the recipient's immune system. The technology has a high potential because no immunosuppression is needed and nonhuman cells can be used for transplantation, i.e., xenografts. Because of these benefits, the feasibility of transplanting cells in immunoprotective membranes is under study for the treatment of several (endocrine) disorders.[1-4]

The further improvement of the immuno-isolation technologies and the start of clinical trials have brought a new challenge which is not a scientific one. The majority of scientists have insufficiently realized during recent years that not only scientific impediments but also regulatory impediments have to be overcome.

So far, clinical trials have been conducted according to the principles of Good Clinical Practice (GCP). However, in the cases that investigational medicinal products were used no requirements existed to meet the standards of GCP or Good Manufacturing Practices (GMP). This has fundamentally changed since May 2004 when the EU Clinical Trials directive 2001/20/EC became law. Although this directive deals primarily with the implementation of GCP by laying down requirements for patient protection, article 13 of the directive defines that investigational medicinal products should be produced according to GMP legislation. Consequently, productions of biopharmaceuticals but also products of advanced cell-based therapies (i.e., gene therapy, somatic cell therapy and tissue-engineering) used in clinical trials should be produced according to GMP legislation.

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For clinical application of encapsulated cell therapies, the processing, screening and final application of therapeutic cells as well as the production of the biomaterials is subjected to many guidelines which have often not been met during the research phase. For instance, a major concern for products derived from cell lines of human or animal origin is the potential risk of contamination with viruses and steps should be taken to ensure that transmission of virus is limited. This all requires that during the developmental phase major changes in protocols are needed which may require years of optimalization.

The mandatory requirements that have to be met in order to perform clinical therapy with immuno-isolation will be discussed in the present review.

Background

The first government regulatory agency established was the US Food and Drug Administration (FDA). Due to severe conditions in the meat packing industry the American congress passed the Pure Food and Drug Act in 1906.[5] From this moment it became illegal to sell contaminated food or meat and a second enactment was that drugs containing selected dangerous ingredients should be labeled. During the years additional acts were accepted to fill the gaps of the first law, mostly as a result of fatal accidents. For example, in the mid thirties, an anti-freeze solvent was used in an oral application of an antibioticum. More than 100 people died and this resulted in a new Act (1938) where companies were required to prove that their products were safe before marketing them. In 1941 nearly 300 people were killed or injured by the use of a sulfa-drug contaminated with the sedative phenobarbital. As a result, the FDA revised the manufacturing and quality control requirements, leading to what would later be called GMPs. In the 1960's the thalidomide incident resulted in the requirement not only to test the safety of the products but also the effectiveness for their intended use. In 1963 GMP regulations came into effect in the USA. Nowadays, the US FDA has a wide range of responsibilities for drugs, biologicals, medical devices, cosmetics and radiological products. It consists of administrative, scientific and regulatory staff organised under the Office of the Commissioner and has several Centers with responsibility for the various products which are regulated.

In the European Community, the drug review is performed by the European Medicines Agency (EMEA). It has been established by the European Commission and began its activities in 1995. It is situated in London and its responsibility is the protection and promotion of public and animal health through the evaluation and supervision of medicines. EMEA co-ordinates the evaluation and supervision of medicinal products throughout the European Union. Companies submit one single marketing authorisation application to the EMEA which does not discriminate between biologicals and nonbiologicals like the FDA. A single evaluation is carried out through the Committee for Medicinal Products for Human Use (CHMP) or the Committee for Medicinal Products for Veterinary Use (CVMP). The European pharmaceutical legislation is stated in directives.

The International Conference on Harmonisation (ICH) is a joint initiative involving both regulators and industry in the scientific and technical disussions of the testing procedures which are required to ensure and assess the safety quality and efficacy of medicines. Six parties are the founder members of ICH which represent the regulatory bodies and the research based industry in the European Union, Japan and the USA. The ICH guidelines for GMP can be found in the Q7A document, derived from 21CFR and the EU GMP Directive 91/356/EEC.

Good Manufacturing Practice

Basically, GMP is an extended quality management system designed to assure constant production of therapeuticals with the required safety profile. GMP does not apply to the production of diagnostics.

The principles and guidelines of GMP are stated in the Directive 91/356/EEC for medicinal products for human use. Compliance with the principles and guidelines of GMP is a legal requirement. The European Guidelines to Good Manufacturing Practice are described in the European Community Guide to GMP and including annexes which interprets and expands on these principles and guidelines.[6] This publication brings together the main pharmaceutical regulations, directives

and guidance that apply to the European Union and European Economic Area. Changes in technical knowledge and in regulations result in additional and revised annexes. Legal requirements for investigational medicinal products are given in directives 2001/20/EC, 2003/94/EC, 2005/28/EC and annex 13 to the EU GMP guide.

The following aspects need to be considered in GMP and requirements concerning these aspects are given:

- Quality Management. In order to achieve the product quality that is conforming to GMP there must be a comprehensively designed and correctly implemented system of Quality Assurance (QA). The quality management portion is divided into Quality Assurance and Quality Control. QA is the sum of all the organised arrangements made with the object of ensuring that medicinal products are of the quality required for their intended use. GMP is that part of QA which ensures that products are consistently produced and controlled to the quality standards appropriate to their intended use and as required by the marketing authorisation or product specification. GMP is concerned with both production and QC. QC is that part of GMP which is concerned with sampling, specifications and testing and with the organisation, documentation and release procedures. Basic requirements of QA, GMP and QC are given in the guidelines.
- Personnel. Sufficient and qualified personnel are needed to carry out all the tasks which are the responsibility of the manufacturer. Individual responsibilities should be clear. Training of personnel and hygiene programmes are major issues in GMP.
- Premises and Equipment. Premises and equipment must be located, designed, constructed, adapted and maintained to suit the operations to be carried out. The layout and design must be such that risk of errors is minimal, effective cleaning is possible and cross-contamination can be avoided.
- Documentation. Good documentation is an essential part of the QA system. Specifications are given of the documents that are required.
- Production. Production processes must be according to clearly defined procedures. Items such as prevention of cross-contamination in production, validation as well as guidelines for starting materials, packaging materials and finished products are given.
- Quality control. The QC department should be independent from other departments. QC is not confined to laboratory operations but must be involved in all decisions which may concern the quality of the product.
- Contract manufacture and analysis. This chapter deals with the responsibility of manufacturers towards the competent authorities of the member states with respect to the granting of marketing and manufacturing authorisations.
- Complaints and product recall. All complaints and other information concerning potentially defective products must be reviewed carefully according to written procedures.
- Self-inspection should be conducted in order to monitor the implementation and compliance with GMP principles and to propose necessary corrective measures.

Other directives that are relevant for the production and testing of investigational medicinal products are depicted in Table 2 and described here.

Directive 2001/20/EC describes primarily the implementation of good clinical practice in the conduct of clinical trials on medicinal products for human use. Requirements for patient protection are described with special attention for minors and incapacitated adults. It defines minimum requirements concerning the organization of trials: commencement requiring involvement of Ethics Committees and Health authorities, conduct and conclusion. An essential article in this directive is article 13 where it is stated that the manufacture or importation of all types of investigational medicinal products should be according to GMP rules. Hence, a Qualified Person (QP) should be present and the QP is responsible for the manufacturing and check of each batch. If the product has been produced in a third country, each production batch should have been manufactured and checked in accordance with the standards of GMP applying to the respective EU member state.

Annex 13, Manufacture of Investigational Medicinal Products already existed before the Clinical Trials directive 2001/20/EC but it was not a binding document. With the implementation of the

directive it is and its principles are to be applied in all EU member states. The annex covers all aspects of the manufacture, control and labeling of investigational medicinal products. The principles and many of the detailed guidelines of Good Manufacturing Practice for Medicinal Products a well as some other guidelines on validation of virus inactivation/removal are relevant to the preparation of products for use in clinical trials.

Annex 13 specifically addresses those practices which may be different for investigational products which are usually not manufactured routinely but in an initial stage of clinical development. The product specifications and manufacturing instructions may vary during development. This requires a highly effective system of QA. For sterile products however, the validation of sterilising processes should be the same as for products authorised for marketing. An item that also does not differ from routine production is the separation of people responsible for production and quality control. All production operations should be carried out under control of a clearly identified responsible person. Personnel involved in release of investigation medicinal products should be trained in quality systems, GMP and regulatory requirements. They must be independent of the staff responsible for production. During manufacture of investigational medicinal products, it may be that different products are handled in the same premises and at the same time. This reinforces the need to minimise all risks of contamination, including cross-contamination and product mix-up. Campaign working may be acceptable in place of dedicated and self-contained facilities. In these cases, cleaning is of highly importance.

Aspects that should be kept in mind with respect to product development are for instance the culture media used (has the same medium been used that will be used during production) and stable production periods. Once a clinical trial has started it is not possible to change your production process anymore. It is important to deal with these issues at an early stage and before starting the manufacturing of cell banks. The preparation of cell banks is needed because quality of starting materials can influence the consistency of production. With respect to documentation it should be noted that history and source of the cells/cell lines should be well documented. Due to changes as development of the product progresses the documentation system should be flexible and effective and trace record is very important. During the development phase, validated procedures may not always be available. Provisional production parameters and in-process controls may usually be deduced from experience with analogues. Critical parameters should be considered.

Because manufacture, control and labeling of investigational medicinal products should now be according to GMP, a Qualified Person is responsible for batch certification and batch release. A Product Specification File is needed that contains all GMP documents relevant to manufacturing and controlling the product.

Directive 2003/94/EC is applicable to GMP's for both investigational medicinal products and marketed products. In general this directive describes the GMP legislation rules with respect to production and export. Conformity to GMP with respect to quality assurance system, personnel, premises and equipment, production, documentation, quality control, contract out, complaints and labelling are described. Sometimes, different requirements are defined in specific sections for investigational medicinal products. One difference concerns process validation. Some production processes of investigation medicinal products may not be validated to the extent necessary for a routine production, the validation of production processes should be appropriate to the stage of development. However, full validation should be performed of critical steps like sterilization. Another difference is that samples of investigational medicines should be stored for at least 2 years and batch documentation should be stored for 5 years after completion of the clinical trial and this is longer than for marketed products (1 year).[7]

Directive 2005/28/EC is dedicated for investigational medicinal products and lays down principles and detailed guidelines for good clinical practice (GCP) with respect to these products for human use, as well as the requirements for authorisation of the manufacturing or importation of such products. It gives guidelines with respect to GCP for the design, conduct, recording and reporting of clinical trials, manufacturing or import authorisation, batch documentation and archiving and inspections.

Table 1. Investigational new drug applications- regulatory agency responsibilities

Categories of Therapeutic Biological Products Transferred to CDER
Monoclonal antibodies for in vivo use
Proteins intended for therapeutic use
Immunomodulators, growth factors, cytokines and monoclonal antibodies intended to alter the production of hematopoietic cells in vivo
Categories of Therapeutic Biological Products Remaining in CBER
Cellular products, including products composed of human, bacterial or animal cells (such as pancreatic islet cells for transplantation) or from physical parts of those cells
Gene therapy products
Vaccines (products intended to induce or increase an antigen specific immune response)
Allergenic extracts used for the diagnosis and treatment of allergic diseases and allergen patch tests
Antitoxines, antivenins and venoms
Blood, blood components, plasma derived products, including recombinant and transgenic versions of plasma derivatives
[www.fda.gov/cber/transfer/transfer.htm].

FDA

When sponsors file an investigational new drug application (IND) with the FDA for approval to test the drug in the United States, this application can be submitted to the CDER (the Center for Drug Evaluation and Research) for most therapeutic proteins, peptides and small molecule synthetic drugs, or to the CBER (the Center for Biologics Evaluation and Research) for vaccines, gene and cell/tissue therapies, allergenics and blood products. Until 2003, therapeutic biological products should be submitted to CBER but the FDA transferred some of those products to the CDER. Table 1 shows a list of products that remain in the CBER and were transferred to CDER.

Clinical trials in the Unites States are regulated by the FDA under 21 CFR part 50, 56, 312. GMPs are codified in CFR part 210, 211, 600 and 610.[8]

Cell-Based Therapies

In the US, biopharmaceutical manufacturing occurs according to FDA legislation. Large scale production of monoclonal antibodies, blood derived products and pharmaceuticals fall under the cGMP guidelines. In the 1990's the FDA published a series of "points to consider" and guidance documents on somatic cell therapy, mainly to prevent transmission of communicable disease. In 2001 the FDA proposed guidelines for current Good Tissue Practice and finalized rules for the registration of US facilities that process cells and tissues in an extensive manner and further defines minimal and extensive cellular manipulation. Guidance for human tissue intended for transplantation is given in 21CFR1270 and for human cells, tissues and cellular and tissue-based products is given in 21CFR1271. With respect to the use of xenotransplantation products, a Guidance for Industry was published in 2003.[9]

European studies involving the use of investigational medicinal products, also cell-based therapies, are regulated by the European Clinical Trial Directive 2001/20/EC. It seeks to achieve this aim by setting down standards for the protection of clinical trial subjects. So, Good Clinical Practice is the basis of this directive and it states that manufacture of investigational products, including cell-based therapies, must be performed according to GMP standards.

For manufacture of cell based therapies in Europe it is of relevance that the starting material is of human origin and as such is subject to guidance provided in Annex 14 of the EU Guide to Good Manufacturing Practice. Quality assurance programmes need to cover all stages leading to the finished product that includes collection, storage, transport, processing, quality control and

Table 2. Directives covering the production of investigational medicinal products

Directive	Formulates
91/356/EEC	Principles and guidelines of GMP
2001/20/EC	Clinical trial directive
2003/63/EC	Cell and gene therapy
2003/94/EC	GMP rules for production and export
2004/23/EC	Safety and quality of human tissues and cells
2005/28/EC	Guidelines for good clinical practice for investigational medicinal products
2006/17/EC	Implementing directive 2004/23/EC

delivery. A system must be present that enables the path taken from each donation to be traced, both forward from the donor and back from the investigational medicinal product. Before any cell-based product is released it must have been tested using a validated method for suitable sensitivity and specificity for HbsAg, antibodies to HIV-1 and HIV-2 and antibodies to HCV.

Directive 2004/23/EC is on setting standards of quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells. This directive established a general framework of principles and common rules for the safety and quality and covers tissues and cells intended to be used for industrially manufactured products, tissues and cells including haematopoietic peripheral blood, umbilical-cord and bone-marrow stem cells, foetal tissues and cells and adult and embryonic stem cells. This directive excludes blood and blood products and human organs. Detailed implementing of these measures resulted in a first implementing directive (2006/17/EC) that covers all human cells and tissues and all manufactured products derived form them which are used for application to the human body, during the first phases of the process—donation, procurement and testing—in order to ensure their quality and safety. Donor selection criteria are given, specifications for testing, procedures for donation and obtaining of tissues and cells. Standards are given which establishments must meet in order to be authorised and accredited to procure human tissues and cells. Standard operation procedures must be followed for the donation and testing process, during transport and at the point of reception in tissue establishments. To this end, tissues and cells can be followed from donor to patient. A second directive will follow which will cover storage, processing and preservation criteria for tissues and cells.

Until recently, no general European regulation existed on gene, cell and tissue-based therapies. These therapies are regrouped under the term Advanced Therapy Products (ATP). Cell and gene therapy are regulated in Directive 2003/63/EC that has amended 2001/83/EC. So far, tissue-engineered products (like encapsulated cells) were still regulated by national regulatory legislation. Processing, preservation, storage and distribution of these tissues and cells are covered by Directives 2004/23/EC and 2006/17/EC and until new legislation will be implemented tissue-based therapies should follow the requirements of these directives and ask for an accreditation as a tissue establishment.

On 16 November 2005 the European commission published the draft *Regulation on Advanced Medicinal Therapies Products (ATP)* including its scope somatic cell therapy, gene therapy and tissue-engineered products. A tissue engineered product may contain cells or tissues of human or animal origin, or both. The cells or tissues may be viable or nonviable. It may also contain additional substances, such as cellular products, bio-molecules, bio-materials, chemical substances, scaffolds or matrices. An advanced therapy medicinal product containing both autologous and allogeneic cells and tissues is considered to be for allogeneic use.

According to this proposal, tissue-engineered products should be regulated under the EU pharmaceutical legislation and are thus regarded as medicines. Market approval occurs through the EMEA and to help evaluate ATPs, a special EMEA committee (the Committee for Advanced Therapies (CAT) will be created under the supervision of the Committee for Human Medicinal Products CHMP. The CAT's expertise will cover scientific areas including gene therapy and therapies, tissue

engineering, medical devices, pharmacovigilance and ethics. Specific guidelines are in preparation but Risk Management will be a major item in the ATP evaluation. The European Commission has chosen for a regulation instead of a new directive to prevent disparaties between member states. A regulation does not need to be transposed into national law to be implemented. Nevertheless, some discrepancy will occur because each state has the right to act in accordance with national ethical stances and the prohibition or restriction of use, sale, or supply of medicinal products containing or derived from human embryonic stem cells or xenogenic cells. It is therefore possible that advanced therapy products based on embryonic stem cells or xenogenic cells might be allowed in certain member states and not in others.

Moreover, the proposed regulation will have a major impact on companies and organisations that have developed tissue-engineered products under a "medical device like" legislation. This might also hold for medical devices combined with human cells or tissues.

To meet these companies, the EMEA has opened in December 2005 its SME Office for small and medium-sized enterprises. This office helps and encourages companies to reach compliance with regulatory requirements. Companies receive free regulatory assistance and 90% fee reduction on scientific advice. This should stimulate a frequent interaction between the companies and the EMEA toward better collaboration in achieving safe and efficient innovative products.[10]

Conclusion

Recent legislation layed down that investigational medicinal products, for instance gene therapy and cell-based therapies, should be produced according to GMP rules. Consequently, academic centres have to consider stringent process control systems. At first, the new guidelines gave much confusion and ignorance existed how to implement the new rules for their protocols. Building a new GMP facility is an option to handle cellular processing needs but such a plant should be embedded in a GMP system which leads to higher production costs. Eventually the positive effects will become visible. It is expected that in the years to come many cellular therapy protocols will be developed due to the many possibilities of advanced therapies with encapsulated, dendritic, or with stem cells. Also due to the new policy synchronization of protocols will be stimulated thereby avoiding a jungle of protocols, usage of media, materials and culture processes. This will result in better insights in the results seen in clinical studies using experimental biotherapeutics and better comparability of different clinical studies using similar products.

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Chapter 5

Treatment of Diabetes with Encapsulated Islets

Paul de Vos,* Milica Spasojevic and Marijke M. Faas

Abstract

Cell encapsulation has been proposed for the treatment of a wide variety of diseases since it allows for transplantation of cells in the absence of undesired immunosuppression. The technology has been proposed to be a solution for the treatment of diabetes since it potentially allows a mandatory minute-to-minute regulation of glucose levels without side-effects. Encapsulation is based on the principle that transplanted tissue is protected for the host immune system by a semipermeable capsule. Many different concepts of capsules have been tested. 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.

Introduction

The treatment of insulin-dependent diabetes with exogenous insulin 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 presently 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 nonglucocorticoid immunosuppression

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(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. This optimism has recently decreased after the publication of the long term survival rates.[6] Only 10% of the patients are still insulin independent 5 years after transplantation.[7] Many factors have been mentioned as causative. The most prominent one is insufficient means to prevent immunological attack of the cellular transplants.[8]

During recent years immunoisolation has revisited as a principle technology to overcome deleterious immune attacks of cellular islet transplants.[9,10] Immunoisolation is a technology in which islet-cells are enveloped in semipermeable membranes that are impermeable for the hostile effect of the host immune system but are permeable for nutrients, glucose and insulin. The technology is not new but many new insights have brought optimism that the area is closer to applications than ever. However, before large scale application can be proposed some obstacles have still to be overcome. The scientific obstacles are subject of this chapter.

Concepts of Encapsulation

The concept of immunoisolation is simple. It involves enveloping tissues in immunoprotective membranes in order to prevent graft rejection. The introduction of this concept dates back to 1933. Bisceglie et al[11] have replaced the endogenous pancreas by insulin producing tissue encapsulated in a semipermeable but immunoprotective membrane to study the effects of the absence of vascularization on the survival of tissues. Bisceglie et al[11] did not recognize the principle applicability of the approach for treatment of disease. It took until 1943 before Algire[12] 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,[13,14] Hemophilia B,[15] anemia,[16] dwarfism,[17] kidney[18] and liver failure,[19] pituitary[20] and central nervous system insufficiencies[21] and diabetes mellitus.[22]

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 immunoisolating membrane that can be implanted as extravascular and intravascular device. With microencapsulation, the islets are individually enveloped by their own capsule. These two approaches will be discussed in the next section.

Intravascular Designs

For application in the treatment of diabetes, the intravascular devices have a number of theoretical advantages over the extravascular approaches. 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 strict regulation of glucose levels. 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.[23,24] The device is implanted by vascular anastomoses to the blood stream of the host. The most intensively studied intravascular device is the modified diffusion chamber of Chick et al.[25] It is technically advanced and has been tested extensively in small[26] as well as in large animals.[26,27] 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.[28,29] This PAN-PVC ultrafiltration capillary design[20] 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,[26] dogs[27] and monkeys[26] 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

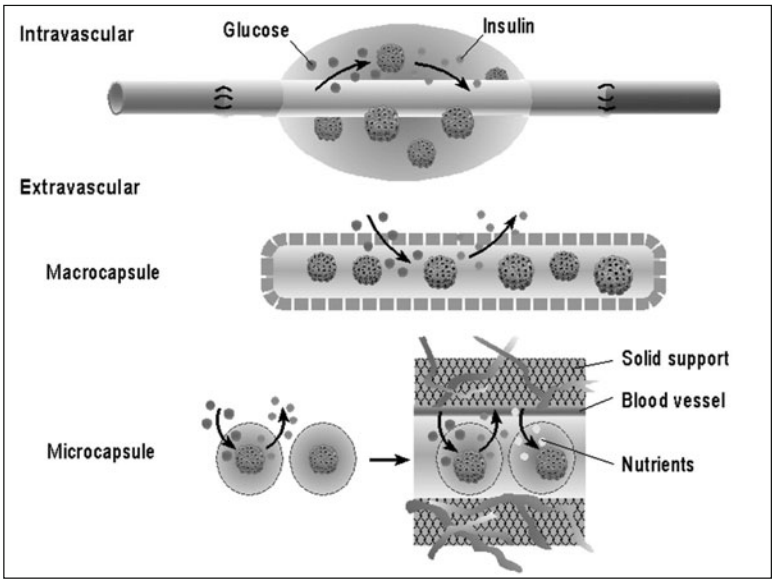

Figure 1. Immunoisolation approaches. In the intravascular device, islets are enclosed in a chamber surrounding a selectively permeable membrane. The device is implanted as a shunt in the vascular system. In the extravascular approach, islets are immunoisolated within membrane-diffusion chambers or enveloped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

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.[30] 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.[28]

Allo- and xenogenic islets in the high flow devices were successfully transplanted to diabetic dogs[31-36] 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.[37] 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,[20] 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) which was used for vascular anastomosis of the device has been shown to induce interleukin (IL)-1β production by macrophages,[38] which cytokine is lethal

for islets.[39,40] It is quite plausible that IL-1β causes loss of high numbers of islets during the period between implantation and complete integration of the prothesis since macrophages are usually the first cells to invade the implant.[41-43] 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 Macrocapsules

Because of the potential risks for the patient many groups have moved from the intravascular approach towards the extravascular approach in the past decade. Most extravascular approaches are based on the principle of diffusion chambers.[44] The majority of extravascular devices can be implanted with minimal surgery and are not associated with major risks such as thrombosis. Extravascular devices can be categorized into two different types of devices, i.e., the extravascular macrocapsules and extravascular microcapsules.

The macrocapsules can be implanted in different sites such as the peritoneal cavity,[45-48] the subcutaneous site,[49-55] or the renal capsule.[56] The geometry of macrocapsules varies. They may be planar in the form of a flat, circular double layer or tube-like as a so-called hollow fiber.[44]

Macrocapsules have been intensively studied in combination with many different biomaterials. Most researchers nowadays prefer the tube geometry over planar membranes for their higher degree of biocompatibility.[57] Tube-shaped fibers are usually produced from PAN-PVC.[28,29] They have been tested with a smooth or fenestrated outside. The design with the smooth outer layer provokes less reactions after implantation than the rough fenestrated surface. However, in some applications the rough surface is still preferred since it allows host tissue to grow into the spongy matrix which potentially is beneficial for vascularization and nutrition. Many modifications of this concept have been proposed in order to further improve the functionality and biocompatibility. A major threat for the biocompatibility has been protein adsorption. A modification to reduce this protein adsorption has been the coating of the membranes with poly-ethylene-oxide.[58]

During recent years 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.[59,60]

Different materials comprising hydrogels have been tested such as polyamide,[61,62] alginate,[63-65] agarose,[48,66] 2-hydroxyethyl methacrylate (HEMA)[67,68] and a copolymer of acrylonitrile and sodium-methallyl sulfonate, AN69.[69] Some supporting results have been shown with the hydrogel membrane AN69, which induced only minimal fibrosis in the peritoneal cavity of rats.[70,71] Surprisingly, 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.[72,73]

Some preclinical and clinical tests have been reported with extravascular macrocapsules. A commercial available macrocapsule, i.e., TheraCyt, allows, reportedly, for survival of pancreatic islets in monkeys for periods up to 8 weeks.[74] Also, some unpublished reports on meetings and the US-press mention successful treatment of Type I diabetic patients with macroencapsulated porcine islets. The researchers applied a two-step transplantation protocol. First 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. The rationally to insert sertoli cells is that these cells are considered to have a special ability to suppress the immune system. The researchers gave the cells to 12 children with Type 1 diabetes between the ages of 11 and 17 and did not apply any immunosuppression. Six of the 12 patients had functioning grafts and received an additional transplant of islets at 20 weeks. Prolonged function of the graft was reported. One child remained insulin-independent for a period of one year. Another was insulin-independent for six months and now requires 75 percent less insulin than before the procedure.

A number of issues have to be solved before macroencapsulation can be proposed as a realistic clinical option for the treatment of diabetes. 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 have been subject of not more than a few studies. Also the issue of the geometry of macrocapsules that is associated with an enormous diffusion distance for glucose and insulin has not been solved yet. Although many groups have shown that macrocapsules can induce normoglycemia, the presence of an adequate serum-insulin response upon elevation in glucose levels has never been shown. The same geometrical issue 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.[60,75,76] 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.[20,77-83]

Host Responses and Macroencapsulation

In contrast to other encapsulation approaches, host responses against extravascular macroencapsulation has gained not more than minor attention by the scientific community. This is surprising since it is predictable that the immunological responses interfere with the survival of the enveloped islet tissue. The macrocapsules initiate many different types of immunological reactions after implantation. These reactions can be categorized into at least three types. The first is the classical foreign body response against the capsules. For macrocapsules, 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.[28,29,61,73,84-86] As a consequence there is still insufficient insight in the pathogenesis of the foreign body response against macrocapsules and is it impossible to decide whether the responses are caused by e.g., geometry issues such as mechanical irritations in vivo or by physicochemical 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.[35,47,87] Most groups do not considered 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.[60,88,89] 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 for 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.

Extravascular Microcapsules

A system that is studied in more detail is microencapsulation of islets. Microencapsulation involves envelopment of individual islets by their own individual capsule. A number of considerations favor microcapsules over macrocapsules. The spherical shape of microcapsules offers better

diffusion capacity because of a better surface/volume ratio. Microcapsules are mechanically more stable than macrocapsules and do not require complex or expensive manufacturing procedures. Also, microcapsules can be implanted into the patient by a simple injection procedure.

As flexible and pliable characteristics are preferred for microcapsules almost all approaches use hydrogels. The authors of this chapter have like others[90-95] concentrated on alginate-based hydrogel 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.[96] 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.

Alginate-based capsules can be found as planar beads or coated with polymers that limit the permeability. The most commonly and extensively studied noncoated alginate-beads are the Barium-crosslinked alginate beads. The Barium-beads microcapsules have a molecular weight cut-off of 600 kD.[97] As a consequence of this high permeability the Ba-beads allow diffusion of IgG immunoglobulins which have a molecular weigh of 140 kD. According to some this is no problem when allotransplantation is performed[98] but when xenografts are applied the capsules should be able to withstand the diffusion of immunoglobulines and some cytokines.[98]

Capsules with versatile permeability properties due to a coating with polymers are preferred by most groups because of the broader potential application. The coating induces an increase in mechanical stability and a restriction in permeability which makes e.g., also xenotransplantation of islets a feasible option. Many different polycations have been applied and tested such as chitosan,[99] poly-L-ornithine,[100] poly-D-lysine[101] and poly-L-lysine.[22] In a recent collaborative effort, our group and that of Ponce et al,[101] compared the physicochemical properties and the biocompatibility of different types of polycations bound to alginate-matrixes of different composition. The capsules were evaluated at one month after implantation. Our results demonstrate that the different immune responses are the consequence of the variations in the interactions between the polycations and alginates rather than to the alginates themselves. In the experimental settings we applied, we conclude that poly-L-lysine is the best available option and that we should avoid using poly-L-ornithine and poly-D-lysine since strong inflammatory responses were observed against capsules coated with these polycations.[101]

Poly-l-lysine coating as applied in the preceding section was performed as follows. Alginate droplets are transformed into rigid gel-beads by collecting them in a calcium-solution. The beads are subsequently 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.[102,103] This induces the formation of complexes at the capsule surface surrounded by superhelically orientated polysaccharide chains.[103] 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.[104-110] 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[109,111] as well as on the temperature of an incubation.[107,109,111] In a final step, to provide biocompatibility, the capsules are suspended in a solution of alginate or other negatively charged molecules[107,111] to neutralize positively charged polylysine residues still present at the capsule surface.

Many modifications to this procedure have been described in literature. A number of this modification should be avoided. To improve the functional survival of cells that are susceptible for calcium some incubate encapsulated cells at 4°C instead of at room temperature. This lower temperature interferes with adequate coating and polymerization with less stable microcapsules as a consequence. Finally, most groups have abandoned EGTA or citrate[112] to liquify the inner core of the capsule. The reason for this modification of the original Lim and Sun method[22] was that we have observed many capsules loosing their integrity during the treatment. Also it is only a temporary liquification since after implantation or culture the capsules will meet concentrations of up to 2.5 mM calcium which concentration is sufficient to regelify the core.

Biocompatibility and Microcapsule Composition

In the preceding section we already illustrated the importance of the chemical composition of capsules and its relation with biocompatibility. Researchers have always 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 nonspecific foreign body reaction against the microcapsules and results in progressive fibrotic overgrowth of the capsules. This overgrowth interferes with adequate nutrition of the islets and consequently causes islet cell death. As a consequence, many have been the efforts to identify factors that are involved in determining the biocompatibility of microcapsules.

More than a decade ago, it was shown that pure alginate rather than commercially available crude alginates should be applied for encapsulation.[60] 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. Surprisingly and unnecessary, we can still find scientific papers than can not be correctly interpreted as the consequence of application of impure alginates.

Not only the purity of the alginate but also the composition in a specific application determines whether a biological response will occur. 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[113-117] and associated with less protruding islet cells[108,118] than the other types of alginate. 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.[113] When inadequately bound to alginate, polylysine can be a strong initiator of fibrosis. This was shown by others[119] and by us[113] 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 physico-chemical analysis of the capsules by applying X-ray photoelectron spectroscopy.[120-122] 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.[22,115,123] The data, which have lead to this model, were almost exclusively obtained by studying the chemical interactions of PLL with solved, 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.

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.[124]

We showed that with the current knowledge and insights we can produce fully biocompatible capsules which remain free of any significant biological response up to two years after implantation in rats, i.e., the life-span of a rat.[120]

Biology of Encapsulated Cells

It is only since recently that the scientific community recognizes the involvement of the enclosed cells in biological responses against encapsulated grafts. For many years it was assumed that improving the capsule's materials would bring about the ultimate goal of encapsulated-cell research, i.e., predictable long-term survival of the grafts.

The role of the enclosed cells in biological responses and longevity was painfully illustrated when allogenic islet grafts were implanted diabetic rat recipients with application of capsules with a proven biocompatibility for up to two years. With the new capsule type the engrafted immunoisolated cells did not suvive permanently but for periods up to 6 months.[125]

Initially, it was difficult to identify the factors causing failure since only 2-10% of the capsules was overgrown with inflammatory cells. This minor loss of functional islets can not explain the failure of the cells in the remaining 90-98% of the capsules.[111,126-130] However, 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-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. In a subsequent experiment we demonstrated that activated macrophages on the 2-10% of overgrown capsules do secrete the cytokines IL-1β and TNF-α when they were cocultured with islet-containing capsules and not with empty capsules.[131,132] This process was accompanied with a gradual loss of function of the encapsulated tissue.[132,133] 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. 2).

During recent years many have confirmed our observations. We also identified the event responsible for the initiation of the response.[122] It is not the implantation of the 'foreign' capsules as such but the required surgical procedure for implantation since we also observed the response in shams, in the absence of capsules.[122,134] For some this might be difficult to accept since it is only minor surgery. However, one should realize that the procedure of surgery is associated with tissue damage and release of bioactive proteins such as fibrinogen, thrombin, histamine and fibronectin.[135-137] These factors have chemotactic effects on inflammatory cells and induce influx of high numbers of granulocytes, basophiles, mast-cells, macrophages to the implantation site.[122]

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[136-140] and, more importantly, stimulate the cells in the capsules to produce graft-derived cytokines.

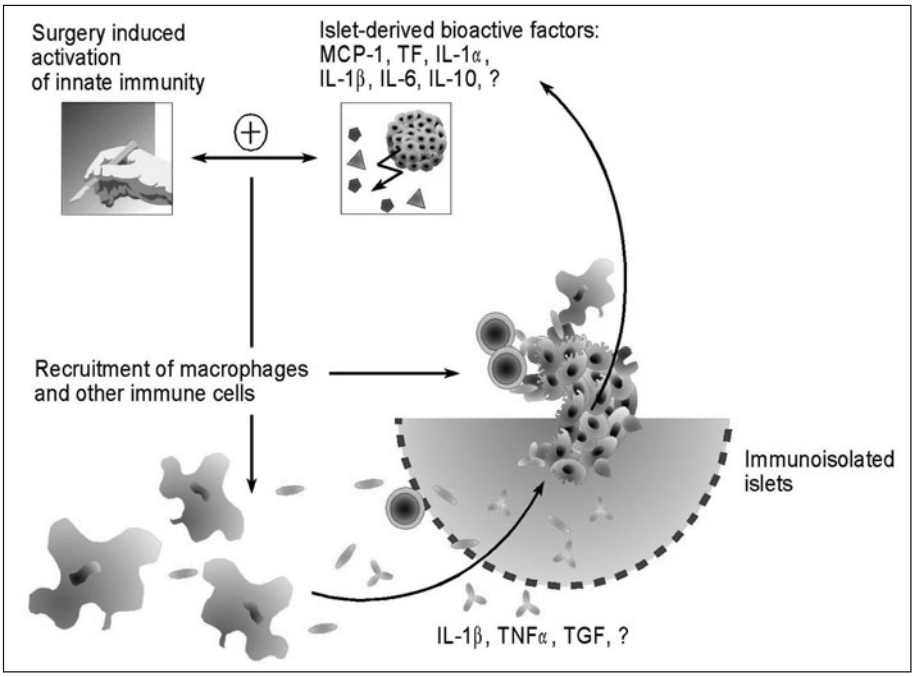

Figure 2. The vicious circle of activation causing failure of 60% of the islets in the immediate period after transplantation. Islets release cytokines which act in concert with cytokines released by a surgery induced activation of the immune system on the recruitment and activation of inflammatory cells in the vicinity of the graft.

We observed that within two weeks, basophiles and granulocytes gradually disappear from the graft site while macrophages and some fibroblasts remain attached to the portion of 2-10% the capsules.[122] These attached macrophages remain activated and, therefore, contribute to the vicious and deleterious circle of activation. Thus, although we and others[111,126-129] 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.

These observations have been the driving force for developing new approaches in which different types of anti-inflammatory or immunosuppressive agents[141] are released in the vicinity of the capsules. These approaches reduces the inflammatory responses in the immediate period after implantation and therewith also reduces loss of functional islets. This approach of reducing the inflammatory response is temporary rather than permanent since it involves a period of not more than two weeks.

The field of microencapsulation has entered the stage of clinical testing.[53,54,90,93,95,108,142-160] Calafiori et al[161,162] demonstrated the efficacy of the system by showing prolonged function in Type I diabetic patients in the absence of immunosuppression.[162] Notably, this is the first study ever presented in which functional survival of tissue was shown in humans in the absence of immunosuppression.

Conclusion

At present all signals point towards microencapsulation as the system of preference for implanting encapsulated pancreatic islets for the treatment of diabetes. During recent years many advances has been achieved with this technology, but as always, also some challenges have been exposed that have to be overcome before the technology can be proposed as a sound alternative for insulin therapy.

These challenges are not all scientific; also some political impediments hamper progress. A major political impediment during the past two decades has been the high expectations which have been a burden for researchers and now has turned into pessimism. Multidisciplinary areas such as immunoisolation require many research efforts on physicochemical, polymer-chemical and biological areas that are not very popular in the clinical area and are time consuming. This time is never available when high public expectations are on the shoulders of researchers. In the past two decades most groups did not go further than demonstrating the principle applicability of the technology in different animal models. Finding funding for focusing on true issues bringing progress to the field has been hard if not impossible due to the high expectations. Even now, it will be difficult to bring the field to large scale human application if additional support for fundamental research on capsule properties will not become available.

The scientific issues that currently deserve major attention are longevity of the graft and functional performance of the capsules in vivo. Many consider that indefinite survival of immunoisolated islets can not be achieved due to lack of entry of cells with capacity for continues renewal and due to lack of vascularization. This implies that means should be developed to replace the graft. A conceivable approach is the prevascularized artificial transplantation site. Also it is mandatory to determine the maximal survival time of an encapsulated graft. This however is connected to the following issue.

Most studies on encapsulated grafts have been performed with fresh capsules that have not been exposed to pathophysiological circumstances such as the low pH and the high (glyco)pro-

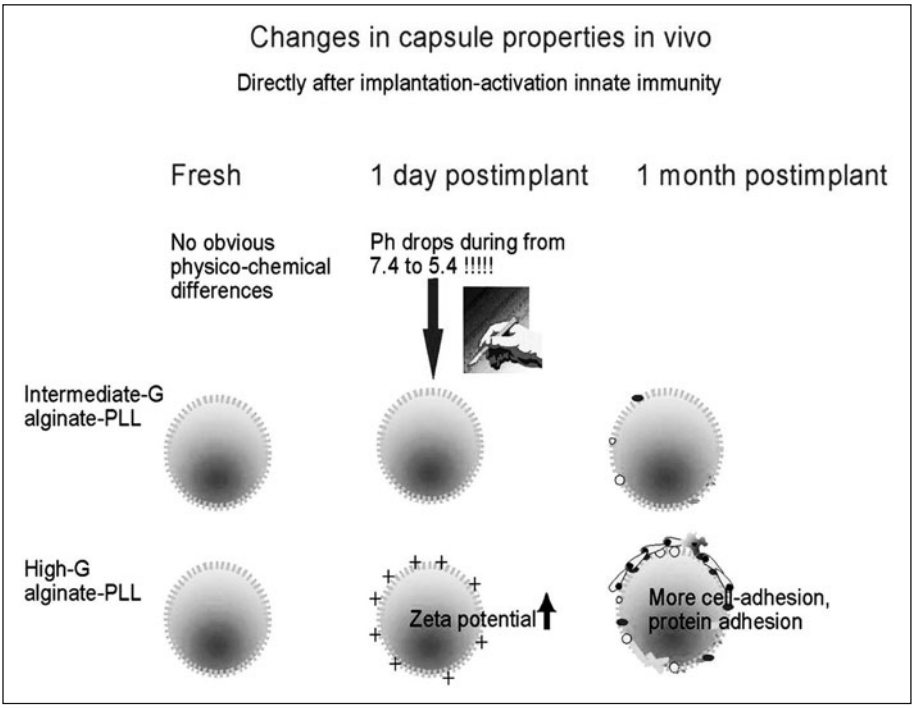

Figure 3. The pathophysiological circumstance in vivo may change the capsule properties. Recently, we showed that exposing high-G alginate capsules to a lower pH will induce an increase of the zeta-potential of the capsules surface. This higher zeta-potential is associated with more and other types of protein adsorption and therewith more likely to be subject of an inflammatory response. This does not occur with intermediate-G capsules that are not sensitive for pH changes.

tein concentrations directly after implantation. We recently showed that this might have been a mistake. We showed that exposing high-G alginate capsules to a lower pH will induce an increase of the zeta-potential of the capsules surface. This higher zeta-potential is associated with more and other types of protein adsorption[163] and therewith more likely to be subject of an inflammatory response (Fig. 3). This was a major step forwards in understanding why only minor changes in the capsule properties such as a change in guluronic acid content may have major consequences for the functional performance. Another observation we did was that irrespective of the type of capsule some (glyco)protein adsorption always occurs.[164] This adsorption is not necessarily associated with cellular overgrowth of the capsules[122,163] but will have an effect on the functional performance of the capsules such as permeability. What this implies for the functional survival of the cells on the long-term remains to be determined.

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Chapter 6

Epo Delivery by Genetically Engineered C_2C_{12} Myoblasts Immobilized in Microcapsules

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Abstract

Over the last half century, the use of erythropoietin (Epo) in the management of malignancies has been extensively studied. Originally viewed as the renal hormone responsible for red blood cell production, many recent in vivo and clinical approaches demonstrate that various tissues locally produce Epo in response to physical or metabolic stress. Thus, not only its circulating erythrocyte mass regulator activity but also the recently discovered nonhematological actions are being thoroughly investigated in order to fulfill the specific Epo delivery requirements for each therapeutic approach.

Introduction

The foundations of the present understanding of the hormonal role of Epo were laid by a succession of French scientists during the second half of the 19th century. Bert and his collaborator Jourdanet demonstrated that the physiological effects of gases depend upon their partial pressure and the relationship between tissue hypoxia and the production of erythrocytes was established.[1] In 1906 Carnot and De Flandre hypothesized that a circulating factor, namely "hemopoietine", was responsible for red blood cell production and that in certain situations such as anemia or high altitude, its concentration in the blood increased.[2] The term hemopoietine was replaced four decades later, in 1948, by the term erythropoietin introduced by Bondsdorff and Jalvisto, who linked Epo solely with red blood cell production.[3] As scientific and clinical experiments started to show promising results in the 1950s, Jacobson and his group established the kidney as the primary site of production of Epo.[4] The isolation and purification of Epo required huge efforts and finally in 1977 Miyake et al could successfully purify it to apparent homogeneity from urine collected from patients suffering from aplastic anemia.[5]

The cloning of the epo gene in 1983, initiation of human recombinant Epo (rHuEpo) therapy in 1985 and approval for its clinical use in 1989, gave rise to a greatly expanded understanding of the biology of Epo and has already been associated with several chronic states. Epo has been widely used in the treatment of anemia that is associated with various chronic conditions. These include end-stage renal disease, malignancy and HIV infection. Epo is also used before selective surgical procedures to reduce blood transfusions, especially in Jehovah's Witnesses. The scarcity and complications of allogeneic blood transfusions such as allergic reactions, immunosuppression,

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alloimmunization, graft-versus-host disease[6] and transmission of viruses and parasites should be carefully considered against the cost and benefits of rHuEpo.[7]

Epo is an acidic glycoprotein consisting of 165 amino acids and a molecular mass of 30-35 kD produced mainly by hepatocytes during fetal stage. After birth, almost all circulating Epo originates from peritubular fibroblast-like cells located in the cortex of kidneys[8-10] under the control of an oxygen-sensing mechanism as proposed by Erslev and Gabuzda[11] where a functional feedback links the rate of red blood cell production to the demand for oxygen by tissues.

The regulation of *epo* gene expression occurs mainly at the transcriptional level by DNA-dependent mRNA synthesis and gene activation. Tissue hypoxia is the main stimulus for Epo production[12] and this mechanism has been thoroughly investigated for many years although *epo* gene expression is not only stimulated when the O_2 capacity (corresponding to the Hb concentration) of the blood decreases, but also when the arterial pO_2 decreases (i.e., at high altitude residence) or when the O_2 affinity of the blood increases.

The mechanisms of degradation of the circulating Epo are still incompletely understood. To a minor degree, Epo may be cleared by the liver and the kidneys. However, there is evidence to assume that Epo is mainly removed from circulation by uptake into erythrocytic and other cells possessing the Epo receptor.[13]

Therapeutic Applications Beyond Erythropoiesis

Recently, advances in analytical techniques have enabled to demonstrate that Epo provides its effects not only in the erythroid compartment but also in other non-erythrocytic cells and organs carrying Epo receptors including the brain, spinal cord, retina, reproductive organs, cardiovascular system (cardiomyocytes, endothelium, vascular smooth muscle), skeletal muscle, liver, gastrointestinal tract (gut and pancreas), lung and the kidney,[14-26] which has led to a major revision of the biological role of Epo (Table 1). In addition, in the embryo, Epo is required for cardiac myocyte proliferation.[27] A chance observation by Anagnostou and coworkers[28] suggested the first extra-hematopoietic activities of Epo observing that Epo induced chemotaxis and mitosis of cultured endothelial cells. Endothelial cells respond to local ischemia by producing Epo. Therefore, these cells, distributed universally throughout tissues, could potentially provide Epo-mediated protective function globally.[29] These findings support the idea that in fact, Epo is a more pleiotropic growth and survival hormone than previously thought. Epo/Epo-R interactions have been reported to induce a wide range of cellular responses, including angiogenesis, chemotaxis, mitogenesis, mobilization of intracellular calcium and inhibition of apoptosis,[30] providing cell proliferation, differentiation and survival and consequently tissue protection. However, not only these beneficial effects should be mentioned. In fact, Epo seems to exert both positive and negative effects on tumor biology. As a consequence, further well-controlled studies carried out in xenogeneic models of different types of cancer are essential to determine the ability of Epo to regulate angiogenesis, apoptosis, chemoradiation sensitivity and tumor growth in the presence or absence of concomitant chemoradiotherapy.[31-37]

Novel Erythropoiesis Stimulating Strategies: Potential New Treatments for Anemia

Before recombinant human Epo became available for therapy, about 25% of patients with chronic kidney disease needed regular transfusions of red cells. In the light of the therapeutic value of rHuEpo it should be remembered that today's success has been based on a century of laborious research into the basics of erythropoiesis.[38]

Genetic engineering enabled to produce rHuEpo for the treatment of anemias of chronic renal failure and other diseases. Table 2 gives an overview of novel pharmacological approaches to stimulate erythropoiesis.[39-58]

Regarding clinical setting, a major drawback of this treatment is the requirement of repeated injections, twice or three times weekly. Various efforts have, therefore, been proposed to produce longer-acting erythropoietin analogues that could retain the biological activity of erythropoietin

Table 1. Extra-hematopoietic functions related to Epo

Organ	Outcome	Refs.
Brain	Neuroregeneration, neuroprotection	14,15
Cardiovascular system	Tissue protection	16,17
Retina	Angiogenic function	18
Kidney	Tissue protection	19
Spinal cord	Neuroprotection	20
Lung	Cytoprotection	21
Gastrointestinal tract		
Liver	Tissue protection	22
Gut	Tissue protection	23
Pancreas	Cytoprotection	24
Reproductive organs	Influence on male reproductive function	25
Skeletal muscle	Tissue bioenergetics improvement	26

(increasing the half-life of the analogue), requiring less frequent dosing. Biochemical modification of the erythropoietin molecule by addition of two N-linked glycosylation chains (Darbepoetin-α),[39] linking repeating units of ethylene glycol (pegylation),[40] addition of an erythropoietin fusion protein (EFP)[41] has been shown to prolong erythropoietin's half-life. Moreover, peptidic and nonpeptidic organic molecules that mimic the action of Epo have also been taken into consideration recently.[42-44]

Other nonpeptide strategies currently under investigation for their potential to stimulate endogenous *epo* gene expression include hematopoietic cell phosphatase inhibitors,[45] hypoxia inducible factor α (HIF-α) stabilizers, GATA antagonists[46] and gene-activated Epo (Epoetin-δ).[47,48] Among these, the most promising results have so far been observed using specific enzyme (prolyl hydroxylase) inhibitors that stabilize HIF-α and mimic the effect of hypoxia.[49,50] However, there

Table 2. Novel compounds and strategies for stimulation of erythropoiesis

Strategy	Refs.
Darbepoetin-α	39
Epo pegylation (CERA)	40
Epo fusion protein (EFP)	41
Peptidic and nonpeptidic molecules that mimic Epo (EMPs)	42-44
Haematopoietic cell phosphatase inhibitors (SHP-1)	45
GATA antagonists	46
Gene activated Epo (Epoetin delta)	47,48
HIF-α stabilizers (prolyl-hydrolases: FG-2216)	49,50
Epo gene therapy	53-57
Synthetic Epo protein (SEP)	58

is some concern about the ubiquitous nature of this gene upregulation due to the fact that this transcription factor may upregulate many other HIF target genes in addition to *epo*.[51]

Since its biological effect is easy to measure and can be observed in healthy organisms, erythropoietin gene therapy is also frequently used as a model system for delivery of secreted proteins. Last but not least, this strategy is an attractive alternative to the administration of erythropoietic drugs in anemic patients.[52] A wide range of strategies have been proposed for Epo delivery as evidenced by the many approaches outlined on Table 3.[53,55-57,59-74]

Cell Encapsulation Technology as an Alternative to Frequent Dosing Schemes

The development of polymer-based encapsulation devices where different types of cells can be immobilized in order to deliver the desired therapeutic product, in a controlled and sustained fashion over time, becomes a promising therapeutic alternative to the current administration schemes. The "moment-to-moment" precise regulation is often very difficult to mimic as well as the complex roles of the hormone, factor, or enzyme that is not produced by the body.[75] As a con-

Table 3. Epo therapy: strategies and therapeutic applications

	Description	**Refs.**
Naked DNA injection	Gene electro-transfer. I.m. naked DNA injection followed by electric pulses to mice, rabbits and monkeys Electroporation-based gene transfer, i.m., mice, rats Naked plasmid DNA (pDNA) injection into skeletal muscle of rat I.m. plasmid injection + electroporation in rats I.m. plasmid injection + electroporation in mice	53,59-62
Adenovirus	I.m. AAV injection to monkeys Subretinal AAV injection to macaques AAV in the eyes of primates I.m. AAV in mice I.m. AAV in β-thalassemic mice	53,63-66
Lentivirus	Single injection on human skin in mice Single injection on human skin in mice I.M. single lentivirus injection in rats I.m. single lentivirus injection in uremic rats	67-70
Gastro-intestinal patch	Intestinal implant—gel containing Epo. Patch with Eudragit. Rats	71
Human artificial chromosomes	HACs as vectors introduced in human fibroblasts	55
PTFE* chambers	L6 transfected with retrovirus enconding ratEpo. Chambers implanted under the stomach	72
Microspheres w/o/w	In vitro assays	73,74
Human dermal cores (Biopump)	Ex vivo transduction of the dermal core with adenovector dermal implantation.	57
Hollow fibers	Ex vivo manipulated transplantation of cells into biocompatible devices	56

*Polytetrafluoroethylene (PTFE).

sequence, serious secondary side effects arise, associated with a reduction in life quality of patients. Moreover, considering the elevated cost of current therapies, the search for alternative therapeutic strategies is essential. Immunoisolated transplantation (i.e., encapsulated cell therapy) is one of the most promising technological approaches to overcome the limitations of current treatment procedures.[76,77] To avoid a life-time use of immunosuppressive drugs and prevent an immune rejection from the host, transplanted cells require their immunoisolation in capsules or similar devices.[78] In addition to reducing sharply the frequency of administration and thus improving patient comfort, cell encapsulation strategy would improve the pharmacokinetics of easily degradable peptides and proteins, which often have short half-lives in vivo.[79]

Several immunoprotection devices have been tested in the last years. Macroencapsulation approaches include the use of hollow fibers elaborated with selectively permeable polymers and diffusion chambers.[56,80-83] Having a look at the many studies carried out by Aebischer et al, an improvement in their encapsulation strategy has been observed, evidenced indirectly by higher secretion rates from the immobilized scaffolds (hollow fibers). On the one hand, they succeeded in developing an appropriate cell line with a high Epo secretion rate in order to achieve a suitable in vivo therapeutic response. On the other hand, both for allogeneic and xenogeneic approaches, in order to avoid the undesired fibrotic response taking place around the implanted devices, that is, to become a fully biocompatible therapeutic strategy, transient immunosuppressive protocols have been applied, which demonstrated to have a positive effect on macroencapsulation systems as evidenced by improved outcomes in comparison with the non-immunosuppressed groups.[84] One important advantage of this macroencapsulation approach lies in the easy removal of the implanted devices.

Microencapsulation systems that are produced from polymer-based hydrogels offer potential advantages in comparison with the macroencapsulation approaches. First, because of their spherical configuration and their small size, microcapsules have much better surface-to-volume ratios. This fact improves the membrane's permeability (an outstanding advantage for the optimal product exchange) and thus cell viability. For instance, many growth factors which show reduced diffusion properties, would have difficulties to cross the macrocapsule's membrane. Second, microcapsules minimize the overall risk of immunoprotection failure by using thousands of them instead of a single large macrocapsule (Fig. 1). Third, they can be injected directly or transplanted with minimal-invasive surgery into the peritoneal cavity,[85,86] subcutaneous tissue,[87] or elsewhere.

Recently, we have studied the proof of principle of cell encapsulation technology by implanting Epo-secreting C_2C_{12} myoblasts immobilized in microcapsules in the peritoneum and subcutaneous tissue of syngeneic and allogeneic mice (Fig. 2). Capsules were elaborated using an electrostatic droplet generator which allows the elaboration of devices with very narrow size dispersion and a perfect spherical shape.[87] Myoblasts were used since it has been shown that C_2C_{12} myoblasts are particularly well suited to cell encapsulation technology.[83,88-89] C_2C_{12} myoblasts can be engineered by conventional transfection techniques to release high and sustained levels of the desired protein over time. Moreover, myoblasts present a relative lack of major histocompatibility complex expression on the surface which may decrease the stimulation of a humoral immune response.[90] Results showed that implantation of Epo-secreting cell-loaded microcapsules lead to high and constant hematocrit levels for more than 100 days in all implanted mice without implementing immunosuppressive protocols. As previously discussed, Epo acts as an angiogenic factor, thus, we hypothesized that in addition to its erythropoietic effect, Epo might be responsible for the formation of a vascularized network surrounding the microcapsule graft, improving the oxygen and nutrient supply to the encapsulated cells. Histological evaluation of the explanted microcapsules evidenced the angiogenic effect of the released Epo by means of a fully rich vasculature surrounding the capsule implant, especially in the case of capsules implanted subcutaneously. Capsules retrieved from the peritoneum were free-floating or forming small capsule clusters and only a weak fibroblast overgrowth in capsules adhered to organs was observed, whereas capsules explanted from the subcutaneous tissue turned out to show a richly vascularized structure with no signs of

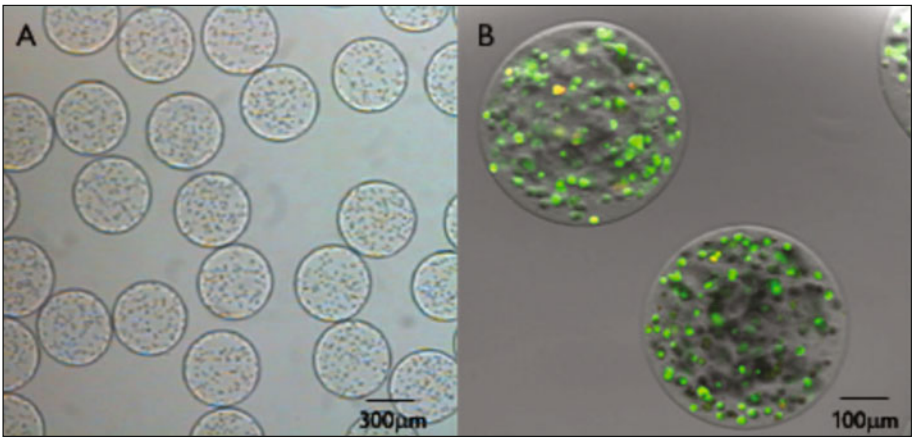

Figure 1. Morphology of microencapsulated Epo-secreting myoblasts. A) Optical microscopy. B) Fluorescence image of cells stained with Calcein-AM (Green: live cells) and Ethidium Homodimer (Red: dead cells). Reproduced, with permission, from Murua A, et al. Biomacromolecules.[110] © 2007 American Chemical Society.

major host reaction. These results highlight the feasibility of cell encapsulation technology for the long-term delivery of Epo independent of the method of administration and the mouse strain.

In agreement with other published reports[91] a moderate reduction in Epo release following encapsulation (66%) was observed compared with values obtained on a two-dimensional culture surface. Some important criteria were followed during the development of polymer microcapsules: biocompatible-grade and low-viscosity alginates were used. These alginates were purified to reduce the presence of the most common impurities, including endotoxins, proteins and polyphenols.[92,93] In addition, since biocompatibility of microcapsules seems to be affected by the microencapsulation process (mechanical factors related to the production process such as correct encapsulation of the cell suspension, uniform size of the polymer drops, optimized diameter etc.) uniform and regularly-shaped microcapsules with a diameter of 450 μm were prepared for this study. As previously hypothesized, the resulting small and uniform microcapsules offered many advantages, including a higher degree of biocompatibility,[94,95] enhanced cell-product pharmacokinetics,[96] improved cell oxygenation and nutrition[97] and last but not least a reduced total implant volume achieving potential access to different implantation sites.

As previously mentioned, the hematocrit levels of all recipients implanted with cell-loaded microcapsules increased during the first 3-4 weeks postimplantation, to a plateau level of around 80% mantained until the end of the study (100 days), independent of the mouse strain and the administration route. The results observed in the subcutaneous tissue were found to be particularly interesting due to the fact that previous trials in this space resulted in poor implant viability and lack of therapeutic response (hematocrit levels) to Epo release, even in syngeneic recipients.[81] It is possible that the angiogenic effects of the Epo molecule could have aided to the improved survival providing the necessary supply of nutrition, oxygen and growth factors to the immobilized cells.[98] Based on its evident advantages, the sustained subcutaneous administration of Epo should be taken into consideration for future potential clinical applications.[99] In fact, patients receiving constant subcutaneous release of Epo require lower doses and less frequent injections than patients treated with intravenous injections.[100,101]

One important consideration to improve the long-term Epo release from the cell-loaded devices may rely on studying and improving the biocompatibility of materials and capsules.[102,103] Previous analyses carried out by our research group evidenced that a careful selection and evaluation of purified alginates, selection of cell lines with adequate features and the development of small and

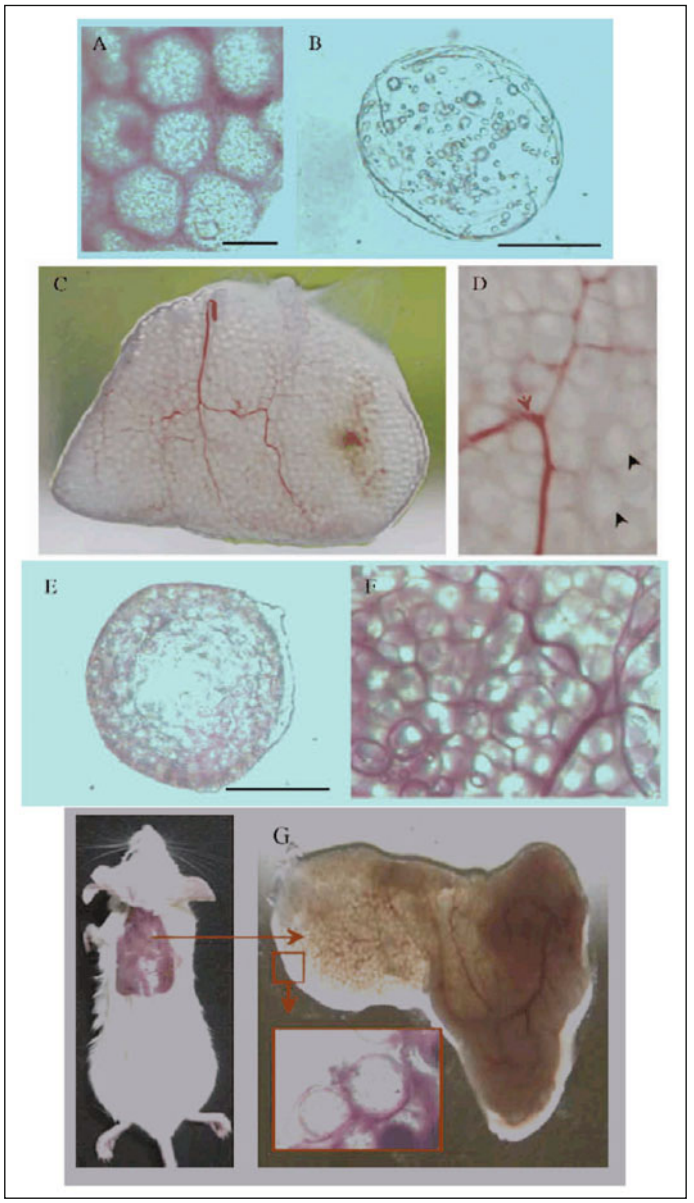

Figure 2. A-D) Photographs of explanted microcapsules 150 days postimplantation in syngeneic C3H mice. A,B) Microcapsules retrieved from the peritoneum. C,D) Microcapsules explanted from the subcutaneous tissue. E-G) Images of explanted microcapsules 150 days postimplantation in allogeneic Balb/c mice. E) One microcapsule retrieved from the peritoneum. F,G) Microcapsules explanted from the subcutaneous tissue. Note the presence of the capsules (black arrowheads) and the vascularization developed close to the capsule aggregate (red arrowhead). Scale bar: 250 μm. Reproduced, with permission, from Orive G, et al. Mol Ther.[87] © 2005 American Society of Gene Therapy.

uniform microcapsules are key requirements to ensure an optimal biocompatibility, long-term functionality and suitable zero-order kinetic releases of the therapeutic molecules.[104-107] However, little research has involved the study of parameters such as the implantation site of the encapsulated cells, the feasibility of using the same approach for syngeneic or allogeneic transplantation, or the application of a well vascularized immobilization device to permit close contact between the encapsulated cells and the bloodstream and thus improve the long-term efficacy of the graft.

Factors limiting the long-term efficacy of microencapsulated cells have been extensively studied.[103,108] In an effort to evaluate the importance of the biocompatibility of the biomaterials employed, a next step towards the optimization of our Epo-secreting C_2C_{12} microencapsulation methodology was taken and the long-term functionality of genetically modified cells immobilized in microcapsules elaborated with alginates of different properties (purification degree, composition and viscosity) was studied.[109] The aim of the work was to determine whether the main variables demonstrated to be key factors for the in vitro biocompatibility of alginates and alginate microcapsules were also responsible for the in vivo long-term functionality of these cell constructs. Based on the positive results obtained in the subcutaneous approach previously described, the same route was also selected for this study.

Alginates are the most employed biomaterials for cell encapsulation mainly due to their easy gelling properties and apparent biocompatibility. However, as natural polymers do, they contain different impurities. Our approach aimed at investigating the long-term functionality and biocompatibility of Epo-secreting C_2C_{12} cells entrapped in microcapsules elaborated with five different commercial types of alginates (different in purification degree, viscosity and composition) (Table 4). All microcapsules prepared had a small size and a uniform shape without irregularities on the surface. Small differences in size did not provoke significant differences in terms of cell viability and Epo secretion. Results showed that independently of the alginate employed, elevated hematocrit levels were maintained until day 130, showing values between 70-87% (Fig. 3). However, the histological evaluation of the explanted devices revealed increased overgrowth surrounding nonbiomedical grade alginate microcapsules. Interestingly, although the formation of a fibrotic layer around microcapsules could worsen Epo delivery by the encapsulated cells, the high Epo secretion of the cell lines together with the pharmacodynamic behaviour and the angiogenic and immune modulatory properties of Epo resulted in high hematocrit levels both for biomedical and nonbiomedical grade alginates.

As a whole, the vital importance of the biomedical grade alginates used in cell encapsulation technology might not be evidenced in terms of different in vitro cell viability or Epo secretion rates, but in vivo experiments do manifest the negative effects exerted by the use of nonbiomedical grade biomaterials in terms of a higher degree of fibrotic overgrowth detected in the implantation site surrounding microcapsules. However, mainly due to the pharmacodynamic behaviour and the

Table 4. Chemical compositions and sequences obtained by ^{1}H NMR spectra and SEC-MALLS measurements[a]

Alginate Type	F_G	F_M	F_{GG}	F_{GM}	F_{MM}	$M_w{}^b$ ($\times 10^{-3}$)	$M_n{}^b$ ($\times 10^{-3}$)	$M_w/M_n{}^b$
LVGP	0.68	0.32	0.57	0.11	0.21	89	56	1.59
LVMP	0.44	0.56	0.23	0.2	0.36	133	78	1.71
MVGP	0.69	0.31	0.58	0.11	0.2	263	141	1.87
MVMP	0.43	0.57	0.23	0.2	0.37	215	134	1.61

[a]Data was provided by Novamatrix, FMC Biopolymer (Oslo, Norway). [b]SEC-MALLS measurements. LVGP: Low viscosity, high guluronic acid content alginate. LVMP: Low viscosity, high mannuronic acid content alginate. MVGP: Medium viscosity, high guluronic acid content alginate. MVMP: Medium viscosity, high mannuronic acid content alginate. Reproduced, with permission, from Orive G, et al. Biomacromolecules.[106] © 2005 American Chemical Society.

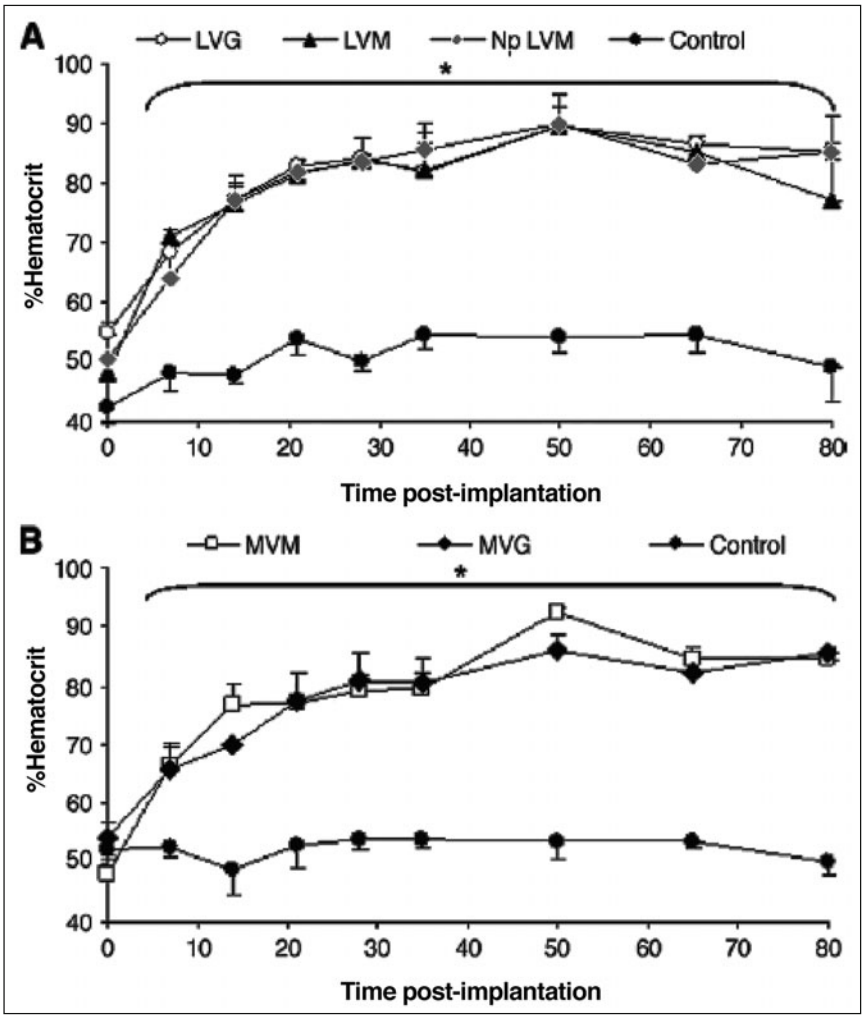

Figure 3. A) Hematocrit levels of Balb/c mice 80 days after subcutaneous implantation of mEPO secreting C_2C_{12} cells (2×10^6 cells/mL) immobilized in microcapsules elaborated with different types of alginates ($n = 3$). LVG: low viscosity high-G alginate; LVM: low viscosity high-M alginate; and Np LVM: nonpurified low viscosity high-M alginate. B) Hematocrit levels of Balb/c mice 100 days after subcutaneous implantation of mEPO secreting C_2C_{12} cells (2×10^6 cells/mL) immobilized in microcapsules elaborated with different type of alginates ($n = 3$). MVG: medium viscosity high-G alginate; MVM: medium viscosity high-M alginate. Values represent mean ± S.D. $^*P < 0.05$ versus the control. Reproduced, with permission, from Ponce S et al. J Control Release 2006.[109] © 2006 Elsevier B.V.

angiogenic and immunomodulatory properties of the Epo molecule, no correlation between the biocompatibility of the alginate and the therapeutic response obtained was observed. However, this interesting point should be taken into consideration due to the fact that the use of other molecules, with no remarkable immunomodulatory effects, could result in different in vivo outcomes when using different types of alginates.

Once allogeneic model approaches based on subcutaneous implantation of microencapsulated Epo-secreting cells had been suitably characterized and biomedical grade biomaterials selected as the most biocompatible polymers, our most recent approach comprised a complete morphological and mechanical evaluation of microcapsules containing Epo-secreting C_2C_{12} myoblasts. Furthermore, the in vitro characterization and the in vivo functionality and biocompatibility of the encapsulated cells during four months were studied and discussed.[110] The membrane's resistance to bursting forces was corroborated by high mechanical resistance against compression showed by the cell-loaded microcapsules. In addition, the swelling behaviour of microcapsules was found to remain stable after a citrate swelling treatment assay showing a slight increase in their diameter in approximately 10%. These results confirmed the high mechanical and chemical resistance of the microcapsules elaborated in this study. The data presented in this study demonstrated a proof-of-principle for cell encapsulation technology for the long-term delivery of Epo. Thus, it could be concluded that the correct characterization of the immobilization systems is of paramount importance to optimize the final cell encapsulation product.

As observed in our previous approaches, implanted mice showed high and sustained hematocrit levels during a 4-month period after a single subcutaneous administration of microcapsules and lacking immunosuppressive protocols. At removal, slight fibrosis was detected surrounding the microcapsule aggregate thus proving the effectiveness of the approach once more.

However, some challenges still remain unsolved in this approach of cell-based microencapsulated Epo delivery: to prolong the duration of the in vivo functionality of the system, to improve the biocompatibility in order to be able to reduce or even avoid the host response, thus resulting in an increase of the biosafety of the technology, to develop optimal retrievability strategies or alternatives and last but not least, to achieve a more physiological release of the protein to prevent undesired high hematocrit levels which may lead to polycythemia.

Conclusion

In overall, this "living drug delivery system" offers a safe and manufacturable method for the systemic delivery of biologically active products such as Epo from genetically engineered cells which can provide an unlimited drug source. As long as the cells are viable and functional, they are able to release the desired products in a more physiological manner. This technological approach, associated with the emergence of reliable cell sources for the constant or even regulated delivery of proteins, offers new perspectives in cell therapy approaches of numerous diseases such as anemia. Thus, immunoisolated cell transplantation holds promise for the controlled and sustained delivery of recombinant proteins such as Epo, offering an alternative to the repetitive administrations of the bioactive protein currently practiced.

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Chapter 7

Artificial Cell Microencapsulated Stem Cells in Regenerative Medicine, Tissue Engineering and Cell Therapy

Zun Chang Liu and Thomas Ming Swi Chang*

Abstract

Adult stem cells, especially isolated from bone marrow, have been extensively investigated in recent years. Studies focus on their multiple plasticity of transdifferentiating into various cell lineages and on their potential in cellular therapy in regenerative medicine. In many cases, there is the need for tissue engineering manipulation. Among the different approaches of stem cells tissue engineering, microencapsulation can immobilize stem cells to provide a favorable microenvironment for stem cells survival and functioning. Furthermore, microencapsulated stem cells are immunoisolated after transplantation. We show that one intraperitoneal injection of microencapsulated bone marrow stem cells can prolong the survival of liver failure rat models with 90% of the liver removed surgically. In addition to transdifferentiation, bone marrow stem cells can act as feeder cells. For example, when coencapsulated with hepatocytes, stem cells can increase the viability and function of the hepatocytes in vitro and in vivo.

Introduction

Stem cells from embryonic or adult sources have important roles in tissue and organ regeneration under physiological or pathophysiological states. Ethical controversy and availability of embryonic stem cells have directed attention toward the use of adult stem cells. Adult stem cells exist in various tissues including bone marrow, muscle, trabecular bone, dermis, adipose tissue, periosteum, pericyte, blood and synovial membrane.[1] Among these sources, the bone marrow, due to its easy accessibility and high yield, has been studied extensively in recent years. Adult stem cells are quiescent under normal physiological state, but when stimulated by specific factors, they can differentiate into the respective adult cell lineages, or can even transdifferentiate into other lineages. This plasticity of adult stem cells holds much promise for regenerative medicine, tissue engineering and cell therapy.

In this review, we briefly introduce use of artificial cell for cell encapsulation and also tissue engineering approaches using stem cells. We then illustrate this using the example of the encapsulation of bone marrow stem cells including in vitro and in vivo studies and therapeutic potentials in liver diseases.

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Table 1. Some examples of cell encapsulation and their applications. The microcapsules can contain various cells for different therapeutic purposes.

Liver failure, congenital liver diseases[3,4,8-15]
Diabetes[3-6,16-19]
Renal failure[20]
Secrete mGH for dwarfism[21]
Factor IX for haemophilia B[22]
Hybridomas antibody production[7]
Secrete erythropoietin for anaemia[23]
β-endorphin for pain killing[24]
Tumor suppression[25]
Cholesterol removing[26]
Huntington's disease[27]
Secrete hNGF for parkinsonism[28]

Cell Encapsulation

The principle, preparation and applications of artificial cells including applications for cell encapsulation are first reported in 1964.[2] One of the areas is its use for the encapsulation of living cells.[2-4] Since then there has been much basic and clinical studies as well as industrial developments.[5-7] Table 1 gives some examples of cells encapsulation and their applications.

Cell encapsulation provides a three dimensional environmental to enhance the cell-cell interactions, acts as niche-like microenviroment to maintain cells viability and function.[3-5,7,29-31] Candidate cells for encapsulation includes a wide range of cell types, such as hepatocytes, islets and many others.[5] Cells for encapsulation can be from autologus; allogeneic or xenogeneic source. Cells in microcapsules are immunoisolated from the host immune system, this renders the use of immunosuppressive unnecessary in allogeneic or xenogeneic transplantation. The original drop method for cell encapsulation[2-4] has been extended to become the alginate poly-lysine alginate (APA) drop method[6] that is now the most commonly used method for cell encapsulation.[5]

Adult Stem Cells and Their Plasticity

Adult stem cells are the immature tissue cells that are able to self-renew and differentiate into multiple cell lineages.[32] These adult stem cells may not be as 'powerful' or diverse as embryonic stem cells, but obviously they have the advantages of ease of isolation and expansion, stable phenotype and compatibility with different delivery methods and formulations.[33] The conventional concept of adult stem cells being restricted to their own tissue has been challenged in the recent years, numerous reports indicated that adult stem cells can jump lineage barriers and differentiate into cells outside their own tissue, this process is called stem cell transdifferentiation.[34]

Adult stem cells exist in various tissues, bone marrow is the most commonly used source of adult stem cells.[35] There are two main types of adult stem cells that reside in the bone marrow, the first category is hemotopoietic stem cell (HSCs), which are the stem cells of each kind of blood cell, although some studies show the HSCs can transdifferentiate into other cell linages, like hepatocytes, under either physiological or pathological conditions, this frequency is very low (10^{-4}), but they may provide cytokines and growth factors that promote other cells functions by paracrine mechanisms.[36] The second main category is mesenchymal stem cell (MSCs), which can differentiate into connective tissue cell types, such as adipocytes, chondrocytes, osteoblasts and hematopoiesis-supporting stroma cells. MSCs were first recognized by Friedenstein, who identified an adherent, fibroblast-like population that could regenerate rudiments of normal bone in vivo.[37-40] Due to lack of definitive markers in vivo, MSCs are not clearly characterized in vitro by now.[41]

The MSCs pharmacological and therapeutic importance is mainly related to these four aspects: they can secrete biologically important molecules and factors; they can express specific

Table 2. Plasticity of bone marrow MSCs

Plasticity	References
Liver	43-45
Neuron	46-49
Heart	50-53
Renal	54
Adipocyte	55,56
Astrocyte	49
Chondrocyte	55,57-59
Mesangial cell	60
Muscle	61,62
Osteoblast	55,63-65
synovial joint	33,66

receptors; they can be genetically manipulated; and they are susceptible to molecules that modify their natural behavior.[42] MSCs can differentiate in vitro and in vivo into nonmesodermal cellular linages such as hepatocytes, muscle cells, neurons and others. Table 2 shows the plasticity of the bone marrow MSCs.

Bone marrow MSCs are commonly isolated using adherent culture method. Although MSCs are rare in bone marrow, representing 1 to 5 in 10,000 nucleated cells, they can expand in vitro to hundreds of millions of cells from bone marrow aspirate [63,67-70] as the following procedure. Firstly, whole bone marrow cells suspension is overlaid onto a percoll gradient (1,073 g/ml) and centrifuged. Mononuclear cells layer is collected and pelleted, then inoculated in defined expansion medium and cultured in 5% CO_2, 37°C incubator. The non adherent cells (non-MSCs) will be removed when changing medium. As the adherent cells reach 80% confluence, they are passaged. Cells harvested after the third passage are usually used for further experiments. MSCs yield and phenotype after expansion vary with the age and condition of the donor and with the harvesting techniques.[63,71-73]

Tissue Engineering of Bone Marrow Stem Cells

MSCs can be injected or delivered directly to the recipients, but in many circumstances, these free stem cells would not function properly after transplantation, so certain kinds of tissue engineering approaches should be used to optimize the application of stem cells.

Tissue engineering implies the use of organ specific cells for seeding a scaffold in vitro[74] and is a multidisciplinary field combining biology and engineering along with clinical application to design, manufacture, modify, grow and maintain living tissue.

Table 3 shows some approaches of stem cells tissue engineering in recent years.

Microencapsulation is one of the main approaches for stem cells tissue engineering, the main purpose of microencapsulation of MSCs is to maintain their viability and their phenotype, or their transdifferentiation capacity. Human bone marrow MSCs encapsulated in alginate beads, cultured in serum-free medium with the addition of transforming growth factor (TGF) beta1, dexamethasone and ascorbate 2-phosphate, after two weeks in culture, cells were characterized with substance positive in Safranin-O staining and immunohistochemistry for collagen Type II at the periphery of cells. The reverse transcriptase-polymerase chain reaction (RT-PCR) revealed the expression of COL2A1 and COL10A1 which are the typical marker of chondrocytes and hypertrophy chondrocytes, respectively,[76] this indicates bone marrow MSCs can undergo endochondral ossification to begin mineralization in the alginate beads.[76,90] In another study with human adult MSCs, using a defined mixture of collagen Type I and agarose polymers, MSCs were encapsulated in a three dimensional microenvironments beads, the collagen Type I was

Table 3. Bone marrow stem cells tissue engineering approaches

Approaches	Applications	Study	Source of Stem Cells
Microencapsulaton[14,15,75-77]	Liver diseases, induction of chondrogenesis	In vivo, in vitro	Rat, human
Embedded in atelocollagen[78]	Degenerative disc disease	In vivo	Rabbit
Hydroxyapatite (HA) ceramics[79]	Bone tumor therapy	In vivo	Human
Three-dimensional poly-lactic-glycolic acid (PLGA) scaffolds[80]	Induction of chondrogenesis, Engineered cartilage	In vitro	Rabbit
Porous silk scaffold[81]	Cartilage tissue engineering	In vitro	Human
Alginate and collagen hydrogels[82]	Tendon repair	In vivo	Rabbit
Silk with covalently bound RGD sequences[83]	Engineering bone-like tissue	In vitro	Human
Collagenous matrix[84]	Cartilage defect repair	In vitro	Human
Polyglycolic acid (PGA) nonwoven mesh[85]	Chondrogenic differentiation	In vivo	Rabbit
Ascorbic acid-functionalized poly(methyl methacrylate)[86]	Osteogenic differentiation	In vitro	Human
Micropatterned polydimethylsiloxane (PDMS) substrates[87]	Connective tissue repair	In vitro	Human
3D nanofibrous scaffold[88,89]	Cartilage tissue engineering	In vitro	Human

incorporated in the matrix in order to promote cell spreading and osteogenesis. It was found there was clear effect of the collagen component of the bead matrix on the MSCs morphology. When collagen content increased, the MSCs increased spread morphologies over time in culture.[77] The cell shape can affect cell function[91] and differentiation.[92] Interestingly, when MSCs were allowed to spread on a 2D substrate, they underwent differentiation to osteogenesis, while they were kept rounded they became adipocytes. Using the 3D encapsulation microenvironment can control over cell shape and therefore regulate stem cell differentiation.[77] The microencapsulation system can guide MSCs differentiation and be used as cell delivery vehicle in tissue repair applications. The bead microenvironment technology also has other potential applications, such as cell-based diagnostics and therapeutic protein production via biotechnology.[77]

Coencapsulation of Bone Marrow Stem Cells with Hepatoyctes to Enhance Hepatocytes Viability and Function

Isolated primary hepatocytes can maintain viability and function in vitro for 1-2 weeks under conventional culture conditions.[93] Various approaches have been employed to increase the viability in vitro. One commonly used approach is to cocultivate hepatocytes with other types of cells, "feeder" cells. The most commonly used feeder cells are epithelial- and fibroblast like cells.[94-96] In recent years, we have used bone marrow stem cells as feeder cells to enhance hepatocytes viability. The unfractionated rat bone marrow stem cells are coencapsulated with hepatocytes and cultured, this significantly enhances the hepatocytes viability than that of encapsulated hepatocytes alone.[30,31] Figure 1 shows the protocol of using bone marrow stem cells as feeder cells in maintaining hepatocytes in coencapsulation.

In the in vivo studies, coencapsulated hepatocytes and bone marrow stem cells from wistar rats were transplanted into normal rats. The transplanted microcapsules were recovered at different

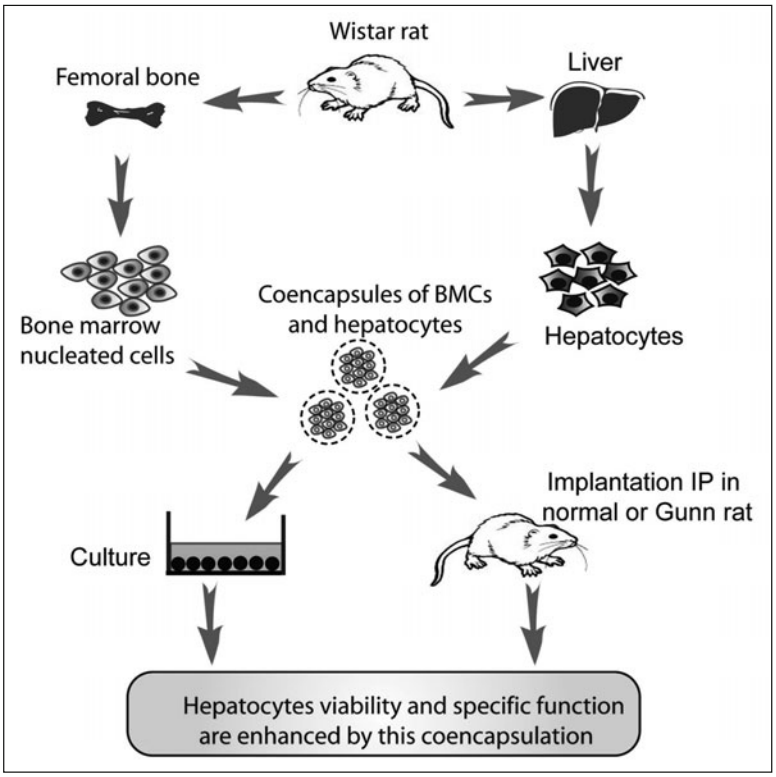

Figure 1. The schematic protocol of the use of bone marrow stem cells to enhance hepatocytes viability and specific function. The bone marrow stem cells and hepatocytes were coencapsulated, this enhanced the hepatocytes viability both in culture and in transplantation into rats. This coencapsulation also enhanced hepatocytes' specific function of ammonium conversion in culture and of lowering blood bilirubin after implantation into hyperbilirubinemia Gunn rats.

time intervals and hepatocytes viability was determined. From week 7 after transplantation, the coencapsulated hepatocytes viability is significantly higher than that of hepatocytes encapsulation alone.[31] The microcapsules could be recovered 4 months after transplantation, at this time the hepatocytes still maintained a high level of viability.[31]

We carried out further studies to investigate the maintenance of the hepatocytes specific liver function by coencapsulatation with bone marrow stem cells. These coencapsulated cells were cultured in the medium supplemented with different concentration of ammonium chloride solution to study the conversion of ammonium into urea by the hepatocytes. During the culture period, the ammonium chloride concentration was much lower from week 2 in the coencapsulation group than in the hepatocytes encapsulation group.[14] In the in vivo function test, coencapsulated hepatocytes and bone marrow stem cells were transplanted into Gunn rats. Gunn rats are allogeneic of wistar rats with hereditary deficiency in the enzyme, dridine diphosphate glucuronyltransferase (UDPGT). This enzyme is necessary for the conjugation of bilirubin to bilirubin diglucuronide, so Gunn rats have lifelong hyperbilirubinemia. After the coencapsulated hepatocytes and bone marrow cells transplantation, the Gunn rat blood bilirubin levels decreased significantly more than that in the single hepatocytes encapsulation transplantation. The control group of encapsulated bone marrow cells transplantation did not show any decrease in systemic blood bilirubin level.[14]

Hepatocyte transplantation are studied for the treatment of various liver diseases and thought to be an effective alternative to the orthotopic liver transplantation. The implantation of coencapsulated hepatocytes and bone marrow stem cells transplantation promises to be a new approach of promoting hepatocytes function in hepatocytes transplantation therapy.

The mechanism of longer term hepatocytes phenotype and specific function maintenance in the coencapsulation experiments in vitro and in vivo has not been elucidated yet. The possible mechanism may involve two aspects, firstly, the cell-cell direct interactions between hepatocytes and bone marrow stem cells, secondly, the growth factors or cytokines produced by bone marrow stem cells. Bone marrow stem cells have various types of cells including stromal cells and blood cells as well as their respective progenitor cells or stem cells. Whether the stromal cell types or the blood cell types or both play key roles needs to be further investigated.[30]

Therapeutic Effect of Encapsulated Bone Marrow Stem Cells on the Liver Failure Model

In 1980, Makowka et al intraperitoneally implanted syngeneic bone marrow cells into rat liver failure models induced by D-galactosamine, they found that the liver failure models survival was prolonged.[97] This early study indicated a new therapeutic potential of bone marrow cells for the liver failure, but no further detailed studies at that time on therapeutic mechanism. Injection of bone marrow cells obtained from another human or from animal sources would require immunosuppression to prevent rejection. We have therefore studied the use of encapasulating adult stem cells for the therapy of liver diseases.[14,15] Rat bone marrow stem cells were encapsulated and then transplanted intraperitoneally into 90% hepatectomy induced acute liver failure rat model.[15] In the two weeks follow up, survival of the encapsulated bone marrow stem cells transplantation group was significantly prolonged comparable to the hepatocytes transplantation group (Fig. 2H). However, unlike Makowka's findings,[97] we found that free bone marrow cells transplantation did not prolonged survival in our study.[15] We carried out histology and immunocytochemistry study of the recovered encapsulated bone marrow cells. Some cells showed evidence of glycogen synthesis as indicated by positive PAS staining (Fig. 2C). Some of the cells express albumin, cytokeratin 8, cytokeratin 18 and alpha-fetoprotein (Fig. 2D-G) that are typical markers of hepatocytes.[15]

The above findings indicate the transdifferentiation of the bone marrow stem cells to the hepatocyte-like cells in the 90% liver failure model. Transdifferentiation of the bone marrow stem cells is important to maintain the liver function and the life of the hepatecomized rats. However, they have another mechanism that is responsible for preventing the death of these rats in the first week. For most rat model, death occurred within 3 days post surgery in the hepatectomy control group, but the transdifferentation needs time and thus can not provide immediate liver support at the beginning. The possible mechanism might be that the growth factors or cytokines secreted from the bone marrow stem cells have effects on increasing the regeneration of remnant liver and also these growth factors and cytokines alleviate apoptosis of the remnant liver cells. Bone marrow MSCs can express HGF and HGF receptor/c-met.[98-101] In the microencapsulated MSCs transplantation in the 90% hepatectomy rat models, the plasma HGF level is significantly increased than in the control groups in the first three days post transplantation.[15] HGF is a potent mitogen for hepatocytes in normal liver. In vitro and in vivo studies show HGF promotes DNA and protein synthesis during liver regeneration after hepatectomy.[102] In addition, extensive hepatectomy can cause increased remnant liver cells apoptosis,[103,104] this is the main cause of mortality in extensive hepatectomy,[104-106] HGF can prevent hepatocytes apoptosis after liver injury, such as partial hepatectomy.[107] HGF have effects on the MSCs proliferation, migration and differentiation.[15,108] HGF can induce differentiation of bone marrow cells into a hepatocyte lineage in vitro.[109] The later transdifferentiation into hepatocyte like cells is the second mechanism that is important in the recovery of the liver function after the acute stage. Figure 3 shows the mechanism schematic of therapeutic effect of microencapsulated bone marrow stem cells on 90% hepatectomized liver failure models.

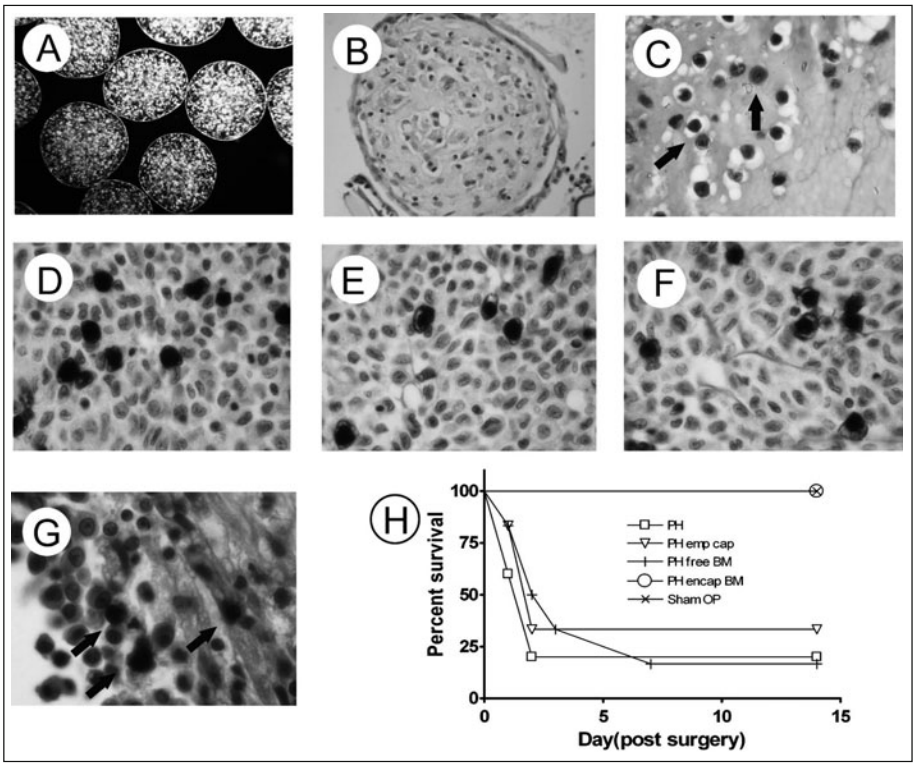

Figure 2. Histology and survival study of microencapsulated bone marrow stem cells transplantation into 90% hepatectomized rat model. A) microcapsules with bone marrow cells before transplantation. Original magnification × 40. B) HE staining of microcapsules recovered 2 weeks post transplantation. Original magnification × 400. C) PAS cytochemical glycogen stain for the recovered microencapsulated bone marrow stem cells. Positive cells showed pink staining in cytoplasm. Original magnification × 1000. D-G) Immunocytochemistry staining of the recovered microencapsulated bone marrow 2 week post transplantation, for albumin (D), cytokeratin 8 (E), cytokeratin 18 (F), alpha-fetoprotein (G). Dark brown cells indicate the positive cells. Original magnification × 1000. H) Kaplan-meier survival curve, at week 2 post transplantation, rats transplanted with microencapsulated bone marrow stem cells (encap BM) survived longer than the partial hepatectomy (PH) group, empty microcapsules (emp cap) group and the free bone marrow cells (free BM) group, comparable to the sham operation (sham OP) group, n = 6 in each group. (Reprinted with permission from John Wiley and Sons, Inc. Liu ZC, Chang TM. Transdifferentiation of bioencapsulated bone marrow cells into hepatocyte-like cells in the 90% hepatectomized rat model. Liver Transpl 2006; 12:566-572.)

Conclusion

Encapsulation of stem cells has four main implications, to provide a novel stem cells immobilization and delivery approach; to provide favorable microenvironment for stem cells survival and function; to be recovered easily for tracing and studying stem cells after transplantation; and to prevent stem cells from immunorejection after transplantation.

Cellular microencapsulation has been proved to be an effective tissue engineering approach in the past years. Adult stem cells have wide plasticity and are new source of cellular transplantation.

Our preliminary results show the promising application of stem cells encapsulation, however some questions should be answered to improve this approach before its full clinical application.

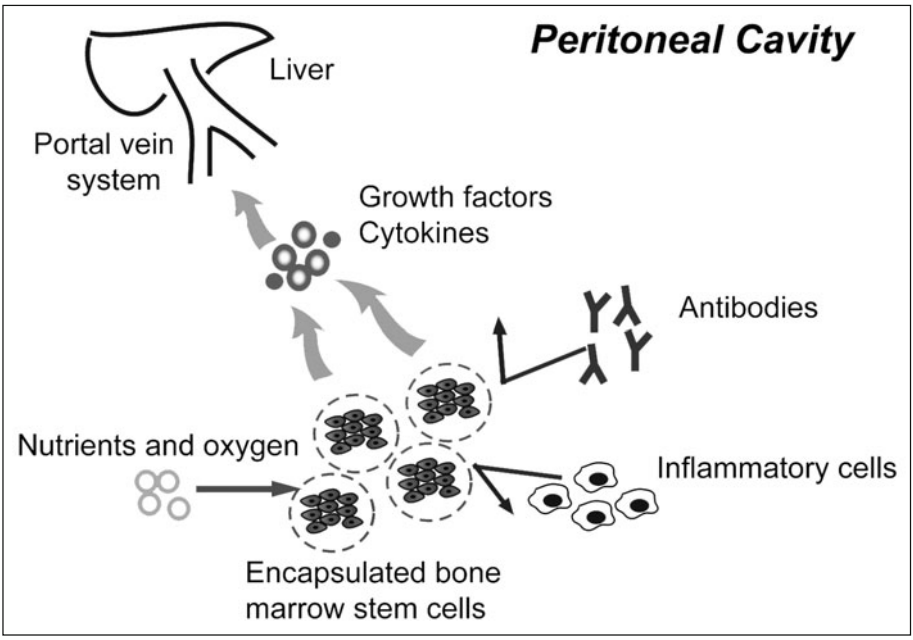

Figure 3. Mechanism of therapeutic effect of encapsulated bone marrow stem cells transplantaed into 90% hepatectomized rats. The encapsulated bone marrow cells are immunoisolated from imunorejection by antibodies and inflammatory cells, whereas the nutrients and oxygen can pass freely through the capsular membrane, growth factors and cytokines secreted by the stem cells can diffuse out the microcapsules into peritoneal cavity, then were drained into portal venous system into the liver to enhance the liver regeneration.

These include questions both from encapsulation technique as well as the stem cells research. What is the optimal encapsulation procedure for the stem cells, what is the stem cells density in encapsulation and what is the optimal passages of stem cells being encapsulated. If using xenogeneic stem cells, there should be no pathological agents transmitted during transplantation. Another issue is to develop high biocompatible polymeric membrane, with sufficient durability and appropriate permeability. Purification of the alginate used in alginate-PLL-alginate(APA) capsular membrane not only reduces the total amount of impurities, but also avoids antibody response when microcapsules are transplanted. More studies should focus on the molecular and cellular mechanism of microencapsulated bone marrow stem cells therapy.

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Chapter 8

Microencapsulated Choroid Plexus Epithelial Cell Transplants for Repair of the Brain

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Abstract

The choroid plexuses (CPs) play pivotal roles in basic aspects of neural function including maintaining the extracellular milieu of the brain by actively modulating chemical exchange between the CSF and brain parenchyma, surveying the chemical and immunological status of the brain, detoxifying the brain, secreting a nutritive "cocktail" of polypeptides and participating in repair processes following trauma. Even modest changes in the CP can have far reaching effects and changes in the anatomy and physiology of the CP have been linked to several CNS diseases. It is also possible that replacing diseased or transplanting healthy CP might be useful for treating acute and chronic brain diseases. Here we describe the wide-ranging functions of the CP, alterations of these functions in aging and neurodegeneration and recent demonstrations of the therapeutic potential of transplanted microencapsulated CP for neural trauma.

Introduction

The choroid plexuses (CPs) lie within the ventricles of the brain producing the cerebrospinal fluid (CSF) and forming an interface between the peripheral blood and the CSF. The CP plays pivotal roles in processes that establish, survey and maintain the biochemical and cellular status of the brain under normal and pathological conditions. It is also becoming increasingly clear that the CP has potential as a source of transplantable trophic and tropic factor-secreting cells with neuroregenerative and neuroprotective capabilities. Conditioned media from CP promotes neurite outgrowth and prevents the death of cultured embryonic neurons[1-3] while CP transplants promote regeneration of the damaged spinal cord and reduce the functional and structural consequences of brain trauma in animal models of stroke.[1,5] Neonatal transplants of encapsulated porcine CP are also neuroprotective in rat[6] and monkey models of Huntington's disease (HD).[3] Here we briefly review the role of the CP in development, normal brain functions, neuronal surveillance, aging and selected CNS disorders. We then provide a detailed overview of the data supporting the use of transplantable CP cells to repair the damaged brain.

Basic Structure and Function of the Choroid Plexus

Grossly, the CP appears lobulated with a single continuous layer of cells derived from the ependymal lining of the ventricles (Fig. 1). The fronds of the CP project into the fluid-filled ventricles, where their complex morphology allow them to perform specialized functions. Their structure roughly mirrors that of the GI tract, with successively smaller folds that aid in increasing surface

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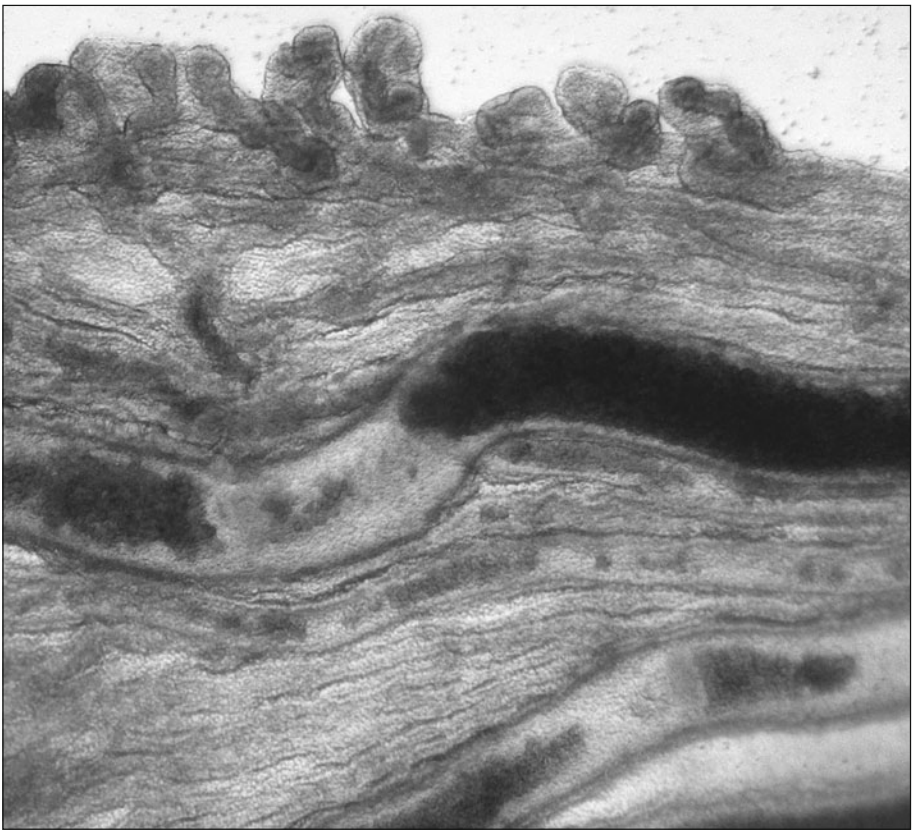

Figure 1. Photograph of freshly isolated neonatal porcine CP. Note the epithelial cells on the top portion overlying a dense highly vascularized stroma.

area and mixing. Within the projections lie a densely enriched bed of vascular supply interspersed between connective tissue and epithelium. The choroidal epithelial cells rest on a basal lamina, contain a large central spherical nucleus with abundant cytoplasm and possess numerous villi on their luminal surface (Fig. 2A). Adjacent epithelial cells are connected by tight junctions to physically restrict the movement of substances to and from the CSF (i.e., the blood-CSF barrier) (Fig. 2B). Due to the required secretory capacity of the CP, there are abundant organelles related to metabolism and protein synthesis (Fig. 2C). The capillaries of the vascular bed are large with thin fenestrated endothelial walls and bridging diaphragms overlying the fenestrations. The most recognized function of the CP is CSF production.[7] In humans, CSF volume is 80-150 mls and new CSF is formed at a rate of approximately 500 ml/day. CSF is produced mainly by active secretion with water entering the CSF from the blood along an osmotic gradient or by specific water channels such as aquaporin. Within the CP, the barrier function is shifted from the vasculature to the epithelium where tight junctions form between the epithelial cells to confer the permeability properties of the individual cells.[8]

A second principle role of the CP in brain functioning is to monitor the CSF for the presence of noxious compounds or potentially damaging cellular invasion.[9,10] The CP protects the brain by aiding or impeding the overall bio-distribution of drugs and toxic compounds by using a full compliment of metabolizing enzymes including Phase I-III enzymes for functionalization, conjugation and transport of drugs. The CP contains (a) high concentrations of glutathione, cysteine

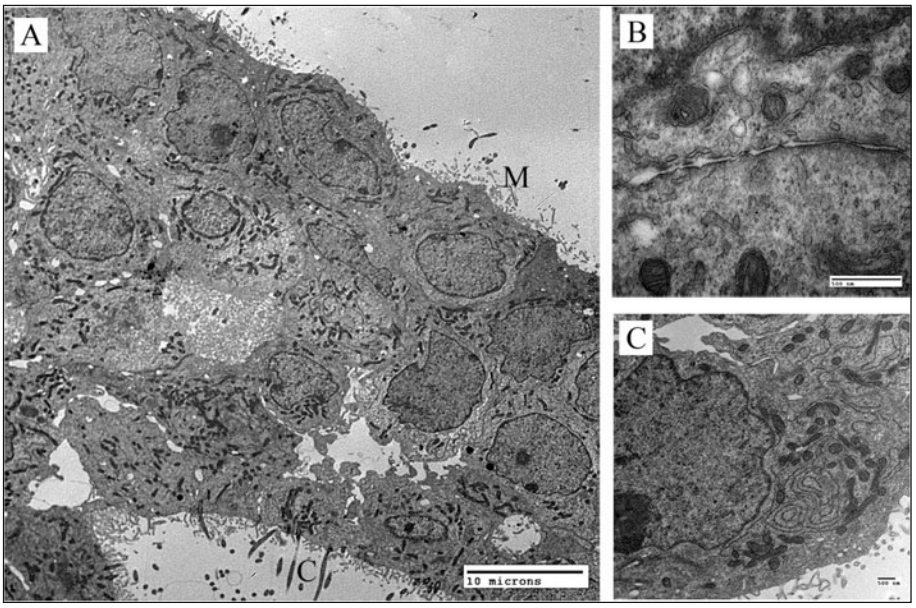

Figure 2. TEM images of CP. A) 2400X image of CP frond, with Microvilli (M) and Cilia (C) localized on the surface; B) 54,800X image of cell junction; C) 14000X image of cytoplasm, displaying densely packed organelles.

and metallothioneins that potently sequester toxic agents circulating in the CSF, (b) protective enzymes such as superoxide dismutase, glutathione-s-transferase and glutathione peroxidase and reductase to provide a barrier protecting against free-radical oxidative stress and (c) organic ion transport systems and multidrug resistance proteins for exportation of noxious compounds from the CSF. An equally important role of the CP is monitoring the immunological status of the brain.[11]

The Central Role of the Choroid Plexus in Brain Development

The CP is perfectly located for distributing molecules both locally and globally to the brain. The CP is also a major source of biologically active compounds (Table 1). These capabilities allow the CP to monitor and respond to the biochemistry of the brain by manipulating and maintaining baseline levels of the extracellular milieu throughout the CNS. The molecules secreted by the CP gain access to the brain parenchyma via volume transmission, convective distribution and intraparenchymal diffusion/receptor-mediated retrograde transport.

CP's form early during embryogenesis, helping to control the developing extra-cellular environment[12] by secreting morphogens, mitogens and trophic factors that guide and pattern both the general and specific growth of the brain.[10,13] For instance, the embryonic CP contains high levels of IGF-II. Based on the localized and high expression of IGF receptors in the floor plate of the hindbrain it has been hypothesized that CP-derived IGF-II diffuses to and binds to IGF receptors on the floor plate cells and activates their role in guiding spinal axon growth.[14] As pointed out by Strazielle and colleagues[15] additional support for the role of CP in morphogenesis comes from demonstrations that the radial migration of cerebral cortical neurons from the ventricular and sub-ventricular zone to the cortical plate is governed by gradients of soluble factors, such as CP-secreted Slit proteins.[16,17] Hu[17] demonstrated in vitro that a soluble factor related to Slit2 is secreted by the CP to diffuse through the CSF and aid in establishing a gradient of a repulsive cue guiding cortical neurons away from the ventricular surface. Along these same lines, Yamamoto and colleagues showed that the CP modulates neurite outgrowth in the developing cerebellum.[18]

Table 1. Gene array analysis showing examples of the diversity of genes expressed in high abundance within porcine CP

	Gene	Abundance
Selected therapeutic proteins of interest	Transthyretin	23478
	Connective tissue growth factor	20951
	Transforming growth factor β1	9545
	Neuronatin	8420
	Osteoclast stimulating factor	7242
	Tissue factor	6557
	Axotrophin	5179
	Vascular endothelial growth factor	4260
	Neuronal endocrine protein	3636
	Neuronal protein 3	2781
	Pigment epithelium derived factor	1352
Matrix and adhesion factors	Integrin, β1 binding protein 1	23753
	Laminin receptor 1	15791
	Matrix metalloproteinase 9	9399
	ICAM-1	8039
	Metalloproteinase inhibitor 1 (TIMP-1)	7983
	VCAM-1	7451
	Type III collagen	4709
	Integrin, β5	2776
	Integrin, β4	2430
	Fibronectin	1737
	Integrin, α_v	1847
Cytoskeletal components	β-actin	19715
	Vimentin	17416
	Tubulin, α2	15099
	Tubulin, β1	14403
	Villin 2	11168
	Dynein	10093
	Actinin, α1	8919

Using cocultures of explanted cerebellum and fourth ventricle CP from fetal and infant rat, it was confirmed that CP secretes a soluble neurite-growth factor that is biphasic and correlates with the major milestones of cerebellar morphogenesis. The importance of the CSF distribution of soluble factors during development is also highlighted by Miyan and colleagues[19] showing that hydrocephalus in rats impairs cortical development suggesting that the factors in circulating CSF are vital for development.

The Choroid Plexus in Aging

In humans, the height of CP epithelial cells decreases by about 10-11% during life.[20] The aged epithelial cell cytoplasm becomes rich with Biondi Ring tangles and lipofuschin deposits[21] and the nuclei appear irregular and flattened as the basement membrane thickens.[20] The stroma thickens and contains collagen fibers, hyaline bodies, calcifications and psammoma bodies and the infiltrating arteries become thicker and fragmented.[22,23] Similar changes occur in the aged mouse and rat choroid epithelial cells.[24,25]

The functions of the CP are energy-dependant and the aging CP cannot maintain its normal energy output. Synthesis of enzymes needed for anaerobic respiration and oxidative phosphorylation decline in aging rats with lactate dehydrogenase and succinate-dehydrogenase decreasing 9% and 26%,

respectively.[26] There are age-dependant increases in the number of epithelial cells deficient in cytochrome C oxidase, altering the respiratory mitochondrial chain and decreasing ATP production.[27] Reductions in Na^+K^+-ATPase and the $Na^+K^+–2Cl^-$ cotransporter also occur.[28] These anatomical and enzymatic deteriorations could lead to a diminution of CSF secretion which is decreased by up to 45% in animals.[29] Due to the decreased secretion and the simultaneously increased CSF volume caused by brain atrophy, CSF turnover is significantly longer in elderly rats (7.9 hours) than in young rats (2.2 hours). In man, CSF production has been reported to diminish with age, from 0.41 ml/minute at 28 years to 0.19 ml/minute at 77 years.[10,22] Coupled with age-related cerebral atrophy, the turnover of CSF decreases to less than 2 times daily in elderly subjects versus 3 to 4 times per day in young adults. Such dramatic alterations in the CP and CSF could lead to inadequate distribution of nutritive substances, additional cellular stress and reduced clearance of toxic compounds all of which could play a part in age-related cognitive and motor decline or the development of specific neurological disorders.

Choroid Plexus and Neurodegeneration: Alzheimer's Disease (AD) as an Example

The age-related deficiencies of the CP are exacerbated in AD. Epithelial cell atrophy is greater, with cell height decreasing up to 22% relative to age-matched controls.[20] Greater intracellular distribution of lipofuschin vacuoles and Biondi Ring Tangles occurs.[64,104] The epithelial basement membrane becomes very irregular and thickens an additional 28% beyond that seen in age-matched controls.[20] The stroma of the villi becomes fibrotic with extensive vascular thickening[21,27] and numerous hyaline bodies and calcifications are found with deposits of IgG, IgM and C1q along the epithelial basement membrane. Further decreases in CSF secretion occur with turnover requiring up to 36 hours in AD patients.[22]

The further atrophy of the choroidal epithelial cells in AD is associated with pronounced decreases in secretory activity and transport functions. Levels of transthyretin (TTR), a CP synthesized molecule that associates with β-amyloid peptide to form complexes, are >10% lower in AD.[30] Ascorbic acid and α-tocopherol levels, the two major scavengers of free radicals of CSF, are decreased in AD likely adding to oxidative stress.[31,32] CSF folate and vitamin B12 (important for methylation of numerous molecules) are significantly lower[33-35] while homocysteine, which mediates lipid peroxidation and increases the production of toxic (*E*)-4-hydroxy-2-nonenal, is increased in AD CSF. The impaired ability of the CP to clear molecules from the CSF has profound implications.[22] In rats, clearance of intraventricularly injected β-amyloid peptide decreases from 10.4 μl/minute at 3 months of age to 0.71 μl/minute at 30 months. Consequently, the brain content of amyloid peptide increases from 7% at the end of CSF perfusion in young rats to 49% in old animals.[29] The increase of β1-40 and β1-42 amyloid peptide levels in elderly humans could be related to decreased clearance from the CNS. Decreased CSF production could also enhance protein glycation and the formation of β-amyloid oligomers.[22] AD brain contains elevated levels of glycation products and deposits of amyloid peptide, senile plaques and fibrillary tangles contain advanced glycation products.[22] Glycation promotes protein aggregation, the polymerization of tau micro-tubule associated proteins and protein β-amyloid peptide aggregation. The decrease of CSF turnover, the increase of protein glycation and the diminution of β-amyloid clearance could also induce oligomer formation and retention. As pointed out by Serot and colleagues,[22] these changes could lead to an even greater impoverishment of the brain leading to methylation problems, increased oxidative stress and lipid peroxidation, decreased amyloid clearance, augmented tau protein polymerization and amyloid peptide oligomers and fibrillo formation.

Harnessing the Choroid Plexus for Transplantation Therapy: Preliminary Studies

The profound age- and disease-related changes in CP raise the possibility that replacing damaged CP or transplanting healthy CP into local areas of cell degeneration can be therapeutic.[10] The diminished function of the aged/diseased CP may be much like other diseases characterized by secretory

cell dysfunction where the principle of transplanting or replacing a failing organ (such as CP) or specific cell type is a logical means of restoring lost function. The role of the CP in growth factor and nutrient production makes these cells a potential source of stable, dose-controlled polypeptide delivery.[36] While the notion of delivering transplanted CP cells to the damaged brain regions is a new idea, the studies conducted to date support the concept and warrant further investigation.

CP isolated and maintained in vitro exerts potent neuroprotective effects.[1-3] Conditioned media from alginate encapsulated CP promoted the survival and extension of neurites from embryonic cortical neurons against serum deprivation-induced cell death. This effect was dose-dependent and nearly complete with 10% to 30% conditioned media. These data dovetail nicely with a study where mouse CP epithelial cells were cultured with dorsal root ganglion (DRG) neurons.[2] After 4-5 hours of coculture, the DRG neurons developed elongated neuronal processes with elaborate branching patterns over the surface of the epithelial cells. The ability of CP cells to provide a scaffold for the extension of neurites is consistent with its known production of extracellular matrices including laminin and fibronectin.[37,38] The trophic and tropic effects of CP establish potentially excellent circumstances for the protection and repair of damaged CNS architecture. The delivery of neurotrophic factors via CP transplants to the site of injury also offers theoretical promise for treating spinal cord trauma. Recently, Ide and colleagues[4] grafted syngeneic fragments of CP into the dorsal funiculus (C2 level) in a damaged rat spinal cord. At various times posttransplant, subsets of animals were evaluated histologically to confirm cell survival and determine any regenerative effect on the damaged spinal cord. Electron microscopy and fluorescence immunohistochemistry showed that epithelial cells of the grafted CP survived well and induced a robust regeneration of the damaged axons of the spinal cord. Injections of horseradish peroxidase into the sciatic nerve labeled regenerating fibers extending from the fasciculus gracilis into the graft within 7 days post transplant. This effect was evident for at least 10 months. Some axons elongated rostrally into the dorsal funiculus and long duration evoked potentials were recorded 5 mm rostral to the lesion 8 to 10 months after grafting.

Immunoisolation within Alginate Microcapsules Enables the Use of Xenogeneic Choroid Plexus Transplants

Immunoisolation is based on the observation that xenogeneic cells can be protected from host rejection by encapsulating, or surrounding them within an immunoisolatory, semipermeable membrane. Single cells or small clusters of cells can be enclosed within a selective, semipermeable membrane barrier which admits oxygen and required nutrients and releases bioactive cell secretions, but restricts passage of larger cytotoxic agents from the host immune defense system.

To date, all studies using encapsulated CP for CNS transplant studies have employed microcapsules formed using alginate. Alginate is one of the most frequently investigated biomaterials for cell encapsulation and is a polysaccharide composed of guluronic (G) and mannuronic (M) acid linked by (1,4)-α- and -β-glycoside bonds. The ratio of these monomers contributes directly to certain physical characteristics of the polysaccharide. Once cationically crosslinked, materials high in G, due to a more networked structure resulting from α(1-4) bonds, are more brittle while those high in M, with more linear β(1-4) linkages, exhibit decreased 3-D crosslinking and greater elasticity.

Characterization of Alginate and Encapsulated Choroidal Epithelial Cells

Prior to cell encapsulation, alginate powder is typically reconstituted in a suitable buffer and a variety of purification techniques are employed to rid the solution of proteins, endotoxin and polyphenols. These include solvent extraction, sequential filtration, charcoal extraction, dialysis and others. Contaminant removal is essential to maintaining the optimal balance of hydrophilicity as well as preventing inflammation related to endotoxin. It is the purification process that will ultimately determine the final physical and chemical characteristics of the encapsulated cell product, as fine variations in copolymer ratio, molecular weight and purity can all be controlled at this step. Following purification and reconstitution of the alginate solution at a suitable pH, quality

control analysis is carried out to maintain optimal operating specifications for encapsulation and subsequent in vivo longevity. The final purified alginate can be characterized both as a raw material and as a formed capsule using the analytical techniques shown in Table 2.

Prior to encapsulation, neonatal porcine CP (7-14 days of age) is isolated from the lateral ventricles and dissociated using conventional collagenase digestion procedures. The resulting cell clusters are groupings of epithelial cells ranging from 50-200 um in diameter. Prior to encapsulation, viability is confirmed by staining the cells with a vital dye and is typically >95%. The encapsulation process does not impact cell viability and these cells can be maintained in culture for months if needed or desired. The cultured CP clusters maintain the typical genotypic and phenotypic characteristics of the native, undigested tissue. Because the epithelium of the CP is rich in tight junctions and lined with microvilli we have used immunocytochemical techniques (*zonnula occludens*; ZO-1) to identify tight junctional complexes and the tubulin associated with the cytoskeleton of the microvilli (Fig. 3).

Following confirmation of cell viability and phenotype, the CP cells are encapsulated in alginate microcapsules by extruding a mixture of cells dispersed in 1.7% sodium alginate through a droplet-generating apparatus into a bath of cations. This process is typically performed at an encapsulation density of 10,000-50,000 clusters or 200,000-5M cells/mL alginate. The cells, entrapped in the calcium-alginate gel, are coated twice with PLO, followed by an outer coat of alginate. The central core of alginate is liquefied by chelation. The resulting microcapsules have a diameter of between 500-750 μm.

The alginate/PLO microcapsule appears is very stable when implanted into the brain relative to a commonly used transplant site such as the peritoneum. Using Fourier-Transform Infrared Spectroscopy (FTIR), the surface of explanted capsules (up to 6 months in the brain or peritoneum) was analyzed for the relative proportion of alginate (outer coat) and the PLO (middle coat). Using a mathematic relationship between FTIR peaks related to these two material components, an index was generated to compare the stability of the microcapsules. A notable difference was observed with rapid breakdown in the peritoneum while identical alginate capsules transplanted into the brain were completely stable for the 6 month study.[39,40]

Encapsulated Xenogeneic Choroid Plexus Transplants in Animal Models of Stroke

Stroke is the third leading cause of death and a leading health care burden in developed countries. There are no effective treatments for mitigating the neuronal loss following stroke although neural transplantation may be one means of repairing the stroke-ravaged brain. Delivery of therapeutic molecules via cell transplantation soon after stroke might be useful for reducing or preventing the disease pathology. Based on these considerations isolated CP obtained from rodents was tested for its neuroprotective effects in a conventional rodent model of stroke.[1,5] Rats received a one-hour middle cerebral artery (MCA) occlusion immediately followed by transplantation of alginate-encapsulated CP on the cortex overlying the brain region (striatum) that would be normally infarcted following the MCA occlusion. Behavioral testing on days 1-3 following surgery using the elevated body swing test and Bederson neurological examination revealed profound motor and neurological impairments in control animals that were significantly improved in animals receiving alginate-encapsulated CP transplants. Histological analysis revealed that the behavioral improvements were accompanied by a significant decrease (approximately 35-40%) in the volume of striatal infarction. This paradigm might have actually underestimated the therapeutic potential of CP grafts since the therapeutic molecules were required to diffuse out of the capsules and through several millimeters of cortical tissue. Accordingly, the concentration of the cocktail reaching the infarcted region was modest compared to local concentrations. Future studies should carefully consider alternative transplant sites as well as the possibility of using single cells suspensions of epithelial cells to potentially augment the benefits obtained to date.

Table 2. Analytical techniques for alginate characterization

Analytical Method	Purpose
Bulk material analysis	
^{1}H-NMR	Uronic acid ratio (M:G). Critical for physical and chemical stability of formed capsules
SEC-MALLS	Weight-average molecular weight (M_w) calculations for overall chain length and sample homogeneity (polydispersity)
Dynamic viscosity	Used to calculate intrinsic viscosity and molecular weight. Useful for controlling droplet formation and encapsulation procedure
Protein concentration	Bulk quantification of alginate purity and potential for degradation/host response
FTIR	Alginate purity and comparison against a standard. Useful for characterizing stability based on ratio of alginate to polycation peaks
Endotoxin	Quantification of endotoxin impurities per FDA guidelines and to minimize tissue response
Alginate-polycation microcapsules	
Viability	Vital dye for determination of dosing, vitality and biomass of encapsulated product
Diffusion	Predictor of isolation capability and pharmacokinetic potential
Burst	Bulk modulus of material and ultimate physical strength of microcapsule
Post-encapsulation phenotype	Confirmation of potential cell functionality and morphology
Microbiology/Virus Screening	Screen for product acceptance and lot release
Morphometry	Batch polydispersity and average size of capsules and their respective wall thickness

Encapsulated Xenogeneic Choroid Plexus Transplants in a Rat Model of Huntington's Disease

Huntington's disease (HD) is a devastating autosomal dominant neurodegenerative disorder characterized by an intractable course of mental deterioration and progressive motor abnormalities that invariably results in death. There are no effective treatments. Unlike many other neurodegenerative diseases, the polyglutamine expression in HD permits an unequivocal diagnosis of HD early in life, even in utero. The ability to identify presymptomatic individuals provides the opportunity to design interventions that could intercede before the development of substantial neurodegeneration and the expression of the behavioral changes. Accordingly, the preservation of the neuronal cytoarchitecture and physiology of the striatum could be maintained, while forestalling the debilitating consequences of the disease.

To determine if CP transplants have therapeutic potential in HD, neonatal porcine CP was encapsulated within alginate microcapsules and tested for its neuroprotective potential in a rat model of HD.[6] In these studies, the animals received stereotaxic transplants of either empty capsules or CP-loaded capsules directly into the striatum. Three days later, the same animals received unilateral injections of the excitotoxin quinolinic acid (QA; 225 nmol) into the ipsilateral striatum. Transplanted animals gained body weight post surgery more rapidly than controls. After surgery,

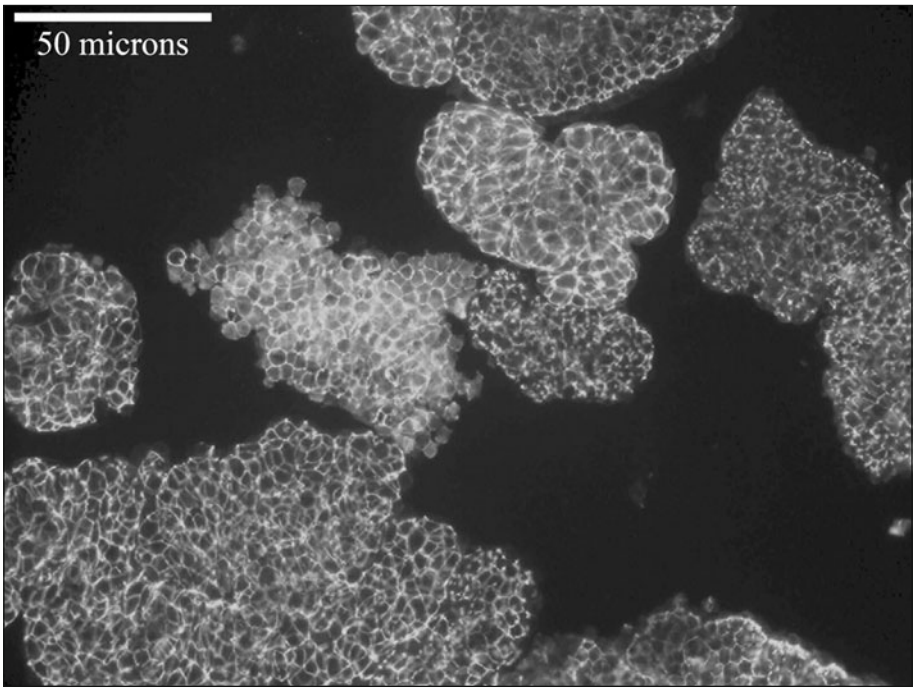

Figure 3. Porcine CP epithelial clusters, stained with ZO-1.

animals were also behaviorally tested for function of their forepaws using the placement test. When given 10 trials on the behavioral test, the control rats were only able to make 1-2 directed motor responses. In stark contrast the rats receiving CP transplants were virtually indistinguishable from normal animals on this task as they made greater than 9 out 10 correct responses. Nissl-stained sections further demonstrated that CP transplants significantly reduced the volume of the striatal lesion produced by QA by greater than 80%.

In Vitro and In Vivo Determinations of the Effect of Age on CP Function

Given the profound changes that occur in CP function during aging we conducted a series of studies to determine if (1) encapsulated CP can be maintained in vitro for extended periods of time without losing its therapeutic activity and (2) if encapsulated CP derived from aged animals is less potent than CP from young animals. To begin to answer the first question, neonatal porcine CP was encapsulated within alginate microcapsules and maintained in vitro for 1, 2, or 7 months.[41] The encapsulated cells remained viable (>80%) at all time points and were transplanted unilaterally into the rat striatum. Seven days later, the same animals received unilateral injections of QA adjacent to the implant site. Separate groups of animals served as controls and received QA alone. In controls, QA lesions produced a significant loss of body weight and impaired function of the contralateral forelimb. In contrast, implants of CP were potently neuroprotective as rats receiving CP transplants did not lose body weight and were not significantly impaired when tested for motor function. These benefits were independent of the length of time that the cells were held in vitro

A second set of studies determined whether age-related impairments occur in the neuroprotective capacity of CP. Choroid plexus was isolated from either young (3-4 months) or aged (24 months) rats.[42] In vitro, young CP epithelial cells secreted more vascular endothelial growth factor (VEGF) and were metabolically more active that aged CP epithelial cells. Additionally,

conditioned medium from cultured aged CP was less potent than young CP at enhancing the survival of serum deprived neurons. Finally, encapsulated CP was tested in the QA model of HD as described above. Animals were tested for motor function 28 days after CP implantation (21days post QA). In the control group, QA lesions severely impaired function of the contralateral forelimb. Implants of young CP again prevented the impairments in motor function. In contrast, implants of CP from aged rats were only modestly effective and were much less potent than young CP transplants. Together, these data demonstrate that the potential potency of alginate encapsulated CP cells can be retained for extremely long periods of time in vitro but they also directly link the natural aging process with a diminished neuroprotective capacity.

Encapsulated Xenogeneic Choroid Plexus Transplants in a Monkey Model of Huntington's Disease

Based on the benefits of CP transplants in the QA rodent model of HD a similar experiment was conducted using young adult cynomolgus monkeys.[3] Using stereotaxic techniques, 20 cell-loaded capsules were loaded into a cannula and implanted into the head of the caudate and the right putamen. A total of four monkeys received cell-loaded implants while three monkeys served as controls and received implants of empty capsules. Seven days following capsule implantation each monkey received an injection of QA (5 µl for a total of 900 nmol of QA) approximately 2 mm posterior to the previous implant site. All monkeys were sacrificed four weeks after the QA lesion. The brains were removed and frozen sections (40 um) were cut on a sliding microtome. A mouse anti–neuronal nuclei (NeuN) monoclonal antibody was used to label striatal neurons for determinations of striatal cell counts and lesion volumes. The number of NeuN immunoreactive (NeuN-ir) neurons within the caudate and putamen nuclei was estimated stereologically using an optical fractionator unbiased sampling design. The volume of intact striatum was also estimated on a series of equispaced NeuN-ir sections along the striatum.

The histological results paralleled those observed in the previously described rodent studies. In controls (animals receiving QA and empty capsule implants), QA administration produced a large lesion in both the caudate and putamen nuclei as shown in NeuN stained sections. The lesion site encompassed much of the caudate and putamen nuclei before the anterior commissure. With the exception of some occasional NeuN-positive debris and shrunken neurons the lesion core was virtually devoid of NeuN positive neurons. In contrast, the size of lesion was notably reduced in animals receiving implants of encapsulated CP. In these animals, the core of lesion was minimal and limited to a small, defined area at the tip of injection site. Immediately outside of this central core, but still adjacent to the needle tract, numerous healthy NeuN-ir neurons with dendritic NeuN immunoreactivity were observed.

Stereological counts of NeuN-ir neurons confirmed the gross histological assessment revealing that, relative to the intact striatum, QA produced a marked loss of NeuN-ir striatal neurons (43%) that was significantly prevented by prior implants of encapsulated choroid plexus (only an 8% loss of neurons (Table 3). Results from the volumetric analysis of intact striatum also paralleled the cell counts. Relative to the intact striatum, animals receiving QA and empty capsules exhibited large lesions characterized by a 40% decrease in striatal volume (745.508 mm^3 vs 446.825 mm^3). Conversely, the striatal volume was 672.228 mm^3 in animals previously implanted with encapsulated CP which did not differ significantly from the volume of the intact striatum. Together, these studies are the first demonstration that implants of CP can provide trophic influences to degenerating striatal neurons in the rodent and primate brain and suggest that this strategy may ultimately prove relevant for the treatment of HD.

Conclusion

Sometimes history repeats itself in unexpected ways. The earliest proposed function of the CP was that it provided a source of the pneuma or animal spirits that gave energy and motion to the entire body. This idea certainly seems odd today but it might turn out to effectively capture the essential and far-reaching role of the CP in enabling neural function and the spirit of its central

Table 3. Neuronal counts in QA-lesioned monkeys receiving choroid plexus transplants

Treatment	Intact Striatum	Lesion/Implanted Striatum	% Cell Loss
QA + empty capsule implant	41959437 ± 1309554	23965075 ± 1557936**	43%
QA + choroids Plexus implant	42031113 ± 409306	38615375 ± 6012797^{++}	8%

**P < 0.0001 vs intact striatum, $^{++}$P < 0.001 vs QA lesioned striatum.

role in the CNS. The few transplant studies conducted to date have been generally encouraging but have not yet focused on determining the means by which transplanted CP exert their beneficial effects. Future efforts will need to systematically approach each potential clinical indication with emphasis on optimizing the donor source and age of the transplanted cells, determining whether specific cell types within the CP (i.e., purified epithelial cells) are most beneficial, identifying the optimal post injury timing, transplant location and dosage of cells to be grafted, whether CP functions within parenchymal tissue in the same manner as within the CSF, the mechanism by which transplanted CP affords beneficial effects and whether these benefits are greater because of the native ability of CP to secrete a physiologically balanced and temporally adjusted cocktail of bioactive compounds versus delivery of single agents.

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Chapter 9

Therapeutic Application of Cell Microencapsulation in Cancer

Brian Salmons* and Walter H. Gunzburg

Abstract

Although cell encapsulation technologies were originally developed for the treatment of acquired and genetic diseases such as diabetes, they can also be applied to the treatment of a variety of solid tumours. There are a number of strategies aimed at treating tumours with encapsulated cells and most of these are reviewed in this chapter. Many of these strategies have shown promise in preclinical studies and clinical trials.

Introduction

The microencapsulation of cells and the subsequent implantation of these encapsulated cells into patients as a strategy to treat diseases was first pioneered over 30 years ago and was originally envisaged as a means to treat conditions like diabetes.[1-3] As encapsulated cell therapies have inched towards acceptance, their uses have been extended to the treatment of a multitude of diseases, including those of the central nervous system, infections and, importantly, for the treatment of various neoplastic disorders, particularly those of unmet medical needs.[4,5] The advantage to the use of encapsulated cells for the treatment of cancers (but also other diseases) is that therapeutic molecules can be delivered in a sustained manner from implanted cells since the cells are enclosed in microcapsules and are thus protected from host immune rejection.

In spite of the advantages of encapsulation of cells for the treatment of tumours, one potential problem that can be encountered is that such cells may show poor in vivo survival rates due to the highly hypoxic and acidic conditions found inside many tumours.[6] In order to improve the survival of the encapsulated cells in these arduous conditions, Hamilton and colleagues successfully selected a human HEK 293 cell line for its ability to survive in hypoxic conditions and thus this cell line can withstand the hostile environment found in tumours. Such selected cells may form the basis of a good platform for the treatment of tumours with encapsulated cells, regardless of which anti-tumour factor the cells are producing.

A variety of cells, including HEK 293, CHO and C_2C_{12} cells (Tables 1 and 2) have been encapsulated and used for the treatment of tumours. These cells have been genetically modified to express products that either directly or indirectly combat tumours and a number of these strategies are discussed in this review. Many of these studies have been preclinical proof of principle studies and these make up the main section of this review article but one system has been already tested in clinical trials. The review concludes with a discussion of combination therapies.

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Table 1. Preclinical studies of encapsulated cells for cancer treatment

Active Product	Cells	Encapsulation	Tumour Type	Reference
Endostatin	HEK293	Alginate	Glioma	Read, 2001
	CHO	Alginate	Melanoma	Teng, 2007; Zhang, 2007
			Leukaemia	Schuch, 2005
Angiostatin	C_2C_{12}	Alginate		MelanomaCirone, 2003
	C_2C_{12}	Alginate	Melanoma	Li, 2006
Endostatin	Porcine			
Sol. neuropilin	Aortic			
Thrombospondin-2	Endothelial	Alginate	Renal cell carcinoma	Bartsch, 2008
Cytokines				
TNF-alpha	J558	Alginate	Breast cancer (MCF-7)	Hao, 2005
Interleukin-6	CHO	Alginate	Hepatocellular carcinoma	Moran, 2006
Antibodies				
RM4-TNFalpha	VkCk	Alginate	Colon carcinoma	Shi, 2005
Combination				
Angiostatin				
Fusion protein#	C_2C_{12}	Alginate	Melanoma	Cirone, 2004

#Interleukin-2 linked to the Fv region of a humanized antibody with affinity to HER-2/neu.

Preclinical Studies of Treatments with Therapeutic Products Produced from Encapsulated Cells

Anti-Angiogenic Agents

Anti-angiogenic agents were first proposed as a treatment for tumours by Judah Folkman, since without angiogenesis, most solid tumours cannot grow past a critical size because of inadequate tissue oxygenation and nutrient supply.[7] Two factors that possess anti-angiogenic activity, angiostatin[8] and endostatin[9] showed promise when injected into animals in preclinical studies and even in clinical trials. However, these studies revealed that sustained, therapeutic effects requires repeated injections of such recombinant anti-angiogenic agents, resulting in highly variable "peaks and troughs" in serum concentrations and only transient achievements of levels that exceed the concentrations required for in vitro anti-angiogenic effects.[10-12] Production of anti-angiogenic factors from encapsulated cells has the advantages that constant and longterm production should be possible thus avoiding multiple administrations and the "peaks and troughs" effects as well as providing the possibility to achieve high local concentrations of the factors.

Endostatin

Two studies were published back-to back in Nature Biotechnology from two independent groups reporting the use of encapsulated cells producing endostatin for the treatment of cancer. One of these studies employed genetically engineered HEK 293 cells encapsulated in ultrapure

Table 2. Preclinical studies of encapsulated cells expressing suicide genes and the relevant prodrug for cancer treatment

Suicide Gene	Cell Line	Prodrug	Capsule	Tumour	Reference
Cytochrome P450	CrFK	Ifosfamide	Cellulose sulphate	Pancreatic cancer	Lohr, 1998
Cytochrome P450	HEK293	Ifosfamide	Cellulose sulphate	Pancreatic cancer	Karle, 1998
Cytochrome P450	CrFK	Ifosfamide	agarose	Squamous carcinoma	Sakai, 2005
Cytochrome P450	HEK293	Ifosfamide*	Cellulose sulphate	Pancreatic carcinoma	Ryschich, 2005
Cytochrome P450	HEK293	Ifosfamide	Cellulose sulphate	Colon cancer	Samel, 2007
Cytochrome P450 + cytosine deaminase	CrFK	Cyclophosph +5 flurocy-tosine	Cellulose sulphate	Mammary cancer[+]	Kammertoens, 2000
Herpes simplex virus Thymidine kinase[$]	NIH3T3	Ganciclovir	Alginate	Colon carcinoma	Div-Ginzberg, 2007

*Some animals received radiation in addition; [+]two different mouse models; [$]retroviral vector delivered from encapsulated cells to infect tumour.

sodium alginate for the local production of endostatin for treatment of malignant brain tumours in a rat model.[13] The study demonstrated that endostatin secretion was maintained in vivo for at least four months after intracerebral implantation of the encapsulated cells, with 70% of the cells in the capsules remaining viable. Rats that received transplants of BT4C glioma cells, together with encapsulated cells producing 0.2 mg/ml endostatin per capsule, survived 84% longer than control animals and this was accompanied by an induction of apoptosis, hypoxia and the appearance of large necrotic avascular areas in 77% of the treated tumours. In a follow-on study by the same authors, glioma growth was reduced by 35% in treated animals that had received C6 glioma spheroids implanted either ectopically or orthotopically and, interestingly, tumour cell invasion into the surrounding tissue was also inhibited.[14] In a later publication, the same group showed that while endostatin greatly affects tumour-associated microcirculation, it appears to have little effect on normal microcirculation. The local delivery of endostatin seems to specifically affect tumour-associated microvessels by reduction of the vessel density, diameter and functionality. Moreover, tumour cell migration and invasion was greatly reduced in the endostatin treated animals.[15] In the second study, published in Nature Biotechnology, baby hamster kidney (BHK) cells were stably transfected with a human endostatin expression vector and were encapsulated in alginate-poly L-lysine microcapsules for long-term delivery of endostatin. A single local injection of encapsulated endostatin-secreting cells in nude mice subcutaneously xenografted with the human glioma cell line (U-87MG) resulted in a 72% reduction in tumour weight 21 days post treatment.[16]

Encapsulated cells producing endostatin have also been used to inhibit the growth of B16 melanoma cells subcutaneously implanted into C57 mice. These studies employed chinese hamster ovary (CHO) cells transfected with a human endostatin gene expression vector and then encapsulated in alginate-poly-L-lysine microcapsules. Production of biologically active endostatin from the encapsulated cells was confirmed in the chicken chorioallantoic membrane bioassay. The encapsulated cells were then injected into the abdominal cavity of mice with subcutaneous B16 tumours and shown to inhibit tumour growth in this model.[17,18]

Leukemic cells of patients with acute myeloid leukaemia also show an elevated expression of pro-angiogenic factors and this is associated with increased vessel density in the bone marrow of patients, suggesting that angiogenesis also plays a critical role in haematological malignancies. Encapsulated cells producing endostatin have been shown to inhibit angiogenesis (reduced microvessel density and a reduced number of CD31-positive, putative endothelial progenitor, cells in the bone marrow) of the treated animals and improve survival in a mouse model of leukaemia.[19]

Angiostatin

Cells genetically modified to secrete angiostatin have also been encapsulated and implantation in tumour bearing mice. Angiostatin was detected in sera of the treated mice and this was accompanied by a suppression of palpable tumour growth as well as improved survival. The involvement of angiostatin in the improved outcome was underscored by the findings that (i) angiostatin was localized to residual tumour material (ii) high levels of angiostatic activity were detected in tumour extracts (iii) apoptosis and necrosis was much more pronounced in treated as compared to untreated or mock-treated mice and (iv) immunohistochemical staining for von Willebrand factor, an endothelial cell marker, showed that the neovasculature within residual tumour tissue was poorly defined as expected. However, the tumours eventually developed neovasculature independent of endothelial cells.[20] The same encapsulated C_2C_{12} myoblasts secreting angiostatin have also been used to treat melanoma in a mouse tumour model system and shown to reduce tumour volume by 70-80%.[21]

The production of a combination of multiple antiangiogenic factors may lead to an improved treatment response and minimize resistance since different pathways can then be targeted. Such a combination of the endogenous inhibitors endostatin, soluble neuropilin-1 and thrombospondin-2 produced from microencapsulated porcine aortic endothelial cells has been tested in a murine renal cell carcinoma. This model consisted of Renca cells that were applied either subcutaneously for local therapy or injected intravenously in a metastatic model. The study tested each factor either alone or delivered as a combination of all 3 factors. In cell culture, each factor alone released from

microbeads inhibited endothelial cell function but did not affect tumour cell proliferation. In the mouse model, tumour growth was inhibited to a similar extent by each of the angiogenic factors alone. The combination of all 3 inhibitors, however, resulted in a further decrease in tumour weight and, in the metastatic model, treatment with angiogenic inhibitors induced a significant reduction in the size and number of lung metastases with additive effects when factors were used in combination. Thus the combination of angiogenic inhibitors was superior to single factors, suggesting synergistic activity and support the strategy of combining angiogenic inhibitors to accomplish a complete angiogenic blockade.[22]

Cytokines

The production of cytokines, such as interleukins or tumour necrosis factor-alpha (TNF-alpha), from encapsulated cells as a means to stimulate an immune response against tumours is another potential strategy for the treatment of solid tumours. In one such study, a tumour cell line was genetically modified to produce functional tumour necrosis factor-alpha (TNF-alpha) and the cells encapsulated. In cell culture, the microencapsulated cells could be shown to release significant amounts of functional TNF-alpha, as evidenced by a cytotoxic effect on L929 indicator cells. These encapsulated cells were intratumorally implanted into athymic nude mice bearing tumours derived from the human breast cancer cell line, MCF-7. The treated tumours showed extensive tumour cell apoptosis and necrosis in response to TNF-alpha production and this resulted in significant tumour regressions as well as slower tumour growth than in the control groups.[23]

In another study, CHO cells producing interleukin-6 (IL-6), a cytokine with pleiotropic effects that plays a central role in normal and abnormal hepatic function and response, were encapsulated in alginate.[24] The encapsulated cells were implanted into the peritoneal cavity of rats bearing hepatocellular carcinoma (HCC). A significant increase in the circulating and intra-hepatic levels of human IL-6 could be measured up to 4 days post-implantation but, despite these significantly increased levels of IL-6, there was no significantly altered rate of net tumour progression, perhaps because a rodent HCC was used. However, Stat3 activity was significantly increased in both normal liver and HCC tissue resected from animals implanted with the IL-6 producing CHO cells. These data demonstrate the viability of using cell encapsulation technology to generate short-term high levels of active circulating and intra-hepatic cytokines and also raise the possibility of modifying specific signal transduction cascades that have been identified as being important during tumour progression.[24]

Antibodies

At the turn of the century, implanted, encapsulated cells were first used for the long term in vivo production of antibodies.[25-27] Such long term, continuous antibody production should avoid the peaks and troughs in the amount of circulating antibody that is a consequence of the classical means of delivery i.e., direct injection. The implantation of antibody producing cells would open up a new means to deliver some of the already well established monoclonal antibodies that are now on the market such as herceptin and avastin and might even improve their therapeutic efficacy. Moreover, new monoclonal antibodies or combinations of antibodies that induce immune mediated killing of tumours could be also be produced by such a means.

Shi and colleagues genetically modified a tumour cell line which secreted a fusion protein (RM4-TNFalpha).[28] This protein comprises the chimaeric anti-tumour antibody, RM4, which recognizes the tumour antigen TAG72, linked to TNF-alpha.[29] The genetically modified cells were encapsulated and shown to exert a cytotoxic effect on L929 cells in vitro. The antigen-specific binding-reactivity of RM4/TNF-alpha for the TAG72 antigen was confirmed by immunohistochemical staining of rat LMCR tumour cells which expressed TAG72 antigen. Implantation of encapsulated cells producing the RM4-TNF-alpha cells into LMCR tumours in rats induced tumour cell necrosis as well as tumour regressions, suggesting that microencapsulation of recombinant tumour cells secreting a tumour targeting antibody armed with a cytokine could hold promise for the treatment of cancer.

In another study of armed antibodies delivered by implanting encapsulated cells, the cell death inducing protein tumour necrosis factor related apoptosis inducing ligand (TRAIL) was linked to a recombinant engineered single chain antibody with specificity for the epidermal growth factor (EGF) receptor (scFv425) in order to facilitate TRAIL binding to tumour cells and to enhance the consequent apoptosis inducing effects. CHO cells that had been recombinantly engineered to produce the single chain anti-EGFR-sTRAIL protein (scFv425:sTRAIL) were encapsulated in alginate and implanted into mice carrying SW948 cell line derived tumours but significant tumour reductions were not observed in this study.[30]

Targeting Chemotherapy

In contrast to the more commonly used encapsulation of chemotherapeutics (i.e., novel formulations of chemotherapeutics), encapsulated cells over-expressing enzymes that can activate chemotherapeutic agents or prodrugs offers a promising means to treat tumours. Depending on the half life of the activated drug, this type of approach can either be used locally or systemically. The first demonstration that this method could be used to treat solid tumours was provided in 1998, in a mouse model of pancreatic cancer.[31] In this study, feline kidney epithelial cells genetically modified to over-express a cytochrome P450 enzyme (CYP) were encapsulated in polymers of cellulose sulphate, in contrast to the more commonly used alginate based encapsulation technologies. Capsules consisting of polymers of cellulose sulphate and polydiallyldimethyl ammonium chloride (pDADMAC) offer a number of advantages including relative ease to reproducibly produce the cellulose sulphate starting material, greater robustness of the capsules (permitting delivery by needle or by a catheter without bursting), good biocompatibility both for the cells in the microcapsule as well as with the surrounding tissue upon implantation and lack of an immune or inflammatory response. Moreover, production of an encapsulated cell medicinal product under good manufacturing practice (GMP) based on cellulose sulphate and pDADMAC has recently been established.[32] In the study, CYP overexpressing cells encapsulated in cellulose sulphate were implanted into xenograft tumours and this was then followed by multiple administration of the prodrug ifosfamide, a well known and widely used chemotherapeutic which is activated by CYP. This combined cell therapy product plus chemotherapeutic treatment was shown to give tumour reductions and, in some mice, even complete loss of the tumour. That the cytochrome P450 enzyme produced by the cells was involved was suggested by the fact that (i) encapsulated non genetically modified feline kidney cells did not give such striking antitumour effects and (ii) the data could be reproduced using encapsulated HEK 293 cells over-expressing the same CYP but not using nonmodifed HEK 293 cells.[33]

CYP expressing CrFK cells have also been encapsulated in subsieve-size agarose capsules. Viable cells were detected in vitro for more than 1 month after encapsulation and the cells were able to activate ifosfamide. The capsules were also implanted in preformed tumours resulting from the injection of the human tongue squamous carcinoma cell line SAS in nude mice and this was followed by administration of ifosfamide. Also in this study, a greater degree of regression of the tumours was observed in animals implanted with cell-containing capsules and receiving ifosfamide compared with those implanted with empty capsules and ifosfamide.[34] These authors more recently refined their encapsulation technology to produce capsules with hollow cores filled with cells and demonstrated even more pronounced anti-tumour effects (Sakai et al, submitted).

Another study combined the encapsulated cytochrome P450 expressing cells and ifosfamide treatment with low-dose irradiation and showed that such "add on" treatments are possible. This study was performed in Lewis rats inoculated with DSL6A tumour cells.[35] Microencapsulated, CYP expressing cells were injected peri-tumorally 10-12 weeks after tumour implantation and the rats then received (i) placebo (NaCl), (ii) ifosfamide (50 mg/kg, i.p., 3 times per week), (iii) local irradiation with 5 Gy or (iv) ifosfamide plus irradiation with 5 Gy. The best responses were observed in rats receiving the combined therapy (encapsulated cells plus ifosfamide plus radiation), where 67% of the animals showed an objective response to the therapy. In contrast,

only 55% of rats receiving ifosfamide alone and 33% receiving radiation alone, showed objective responses to their therapies. The mean tumour volume was significantly reduced after therapy with encapsulated cells plus ifosfamide plus radiation therapy already in the first week of treatment, whereas monotherapy with ifosfamide or radiation significantly decreased tumour growth only after 2 and 3 weeks, respectively. The relatively high TNF-alpha plasma level associated with this tumour type was significantly reduced after combined encapsulated cell/ifosfamide/irradiation treatment. In contrast, lymphocyte infiltration and tumour proliferation were not significantly different between the groups.

The potential use of encapsulated cells expressing CYP and ifosfamide for the treatment of peritoneal spread from gastrointestinal cancer has also been demonstrated in a mouse model.[36] To model peritoneal spread in a mouse system, adult Balb/c mice were inoculated i.p. with 10^6 colon cancer cells that had previously been transfected with the green fluorescent protein (GFP) gene to facilitate the detection of tumour spread in the peritoneal cavity as well as to enable facile monitoring of response to treatment. Two or five days after tumour cell administration, the mice were randomly subjected to either i.p. treatment with ifosfamide alone or ifosfamide combined with microencapsulated CYP expressing cells. Peritoneal tumour volume and tumour viability were assessed 10 days after tumour inoculation by means of fluorescence microscopy, spectroscopy and histology. Treatment with CYP expressing cells and ifosfamide at the earlier time point resulted in complete tumour responses whereas treatment starting on day five or treatment with ifosfamide alone (without encapsulated cells) resulted in only a partial response. This data suggest that targeted i.p. chemotherapy using a combination of a prodrug and its converting enzyme may be a successful treatment strategy for peritoneal spread from colorectal cancer.

The prodrug activating strategy using cytochrome P450 is not limited to the activation of ifosfamide since cyclophosphamide and related agents are also activated by CYP.[37] Anti-tumour activity of encapsulated CYP expressing cells and cyclophosphamide was observed in mouse preclinical models of mammary cancer,[38] leading to a clinical trial of this encapsulated cell therapy in dogs with spontaneously occurring mammary tumours.[39]

Clinical Trials of Cancer Treatment Using Encapsulated Cells to Target Chemotherapy

NovaCaps® is an encapsulated cell therapy product produced under GMP. It is analogous to the early generation product tested in mice discussed above[33] consisting of cells genetically modified to overexpress cytochrome P450 encapsulated in biologically inert cellulose sulphate polymers. Ten years ago, these cells were tested in a Phase I/II clinical trial in patients with pancreatic cancer. In contrast to the mouse preclinical experiments, where the encapsulated cells were directly injected into the tumour (which could potentially give metastatic spread along the needle track in patients), in the clinical trial the encapsulated cells were delivered by supra-selective catheterisation of blood vessels leading from the groin area to vessels feeding the tumour. Release of the encapsulated cells results in them being flushed into smaller vessels where they become lodged immediately adjacent to the tumour.[40,41] Each patient was instilled with 300 capsules (i.e., 3×10^6 cells) and this was followed by i.v. infusions of low doses of ifosfamide. The infused ifosfamide is carried by the blood stream into the encapsulated cells, where it is metabolised by the cells to the relatively short-lived, tumour toxic, product which is then released from the capsules and flows directly into the pancreas. Thus, in essence, NovaCaps® functions as a targeting device (Fig. 1), increasing therapeutic efficacy while at the same time reducing the side effects associated with the usual doses of chemotherapy (Table 3). The safe delivery of encapsulated cells by such an angiographic route was successfully demonstrated in advance of the clinical trial in a porcine model.[42,43] The results of the Phase I/II clinical trial, which involved the treatment of 14 patients suffering from pancreatic cancer with encapsulated cells, were quite promising. The major findings of the clinical trial were that (i) the application of the encapsulated cells by such an angiographic route is safe (ii) the encapsulated cells are well tolerated and there was no evidence of inflammatory or immune reactions (iii) there were no

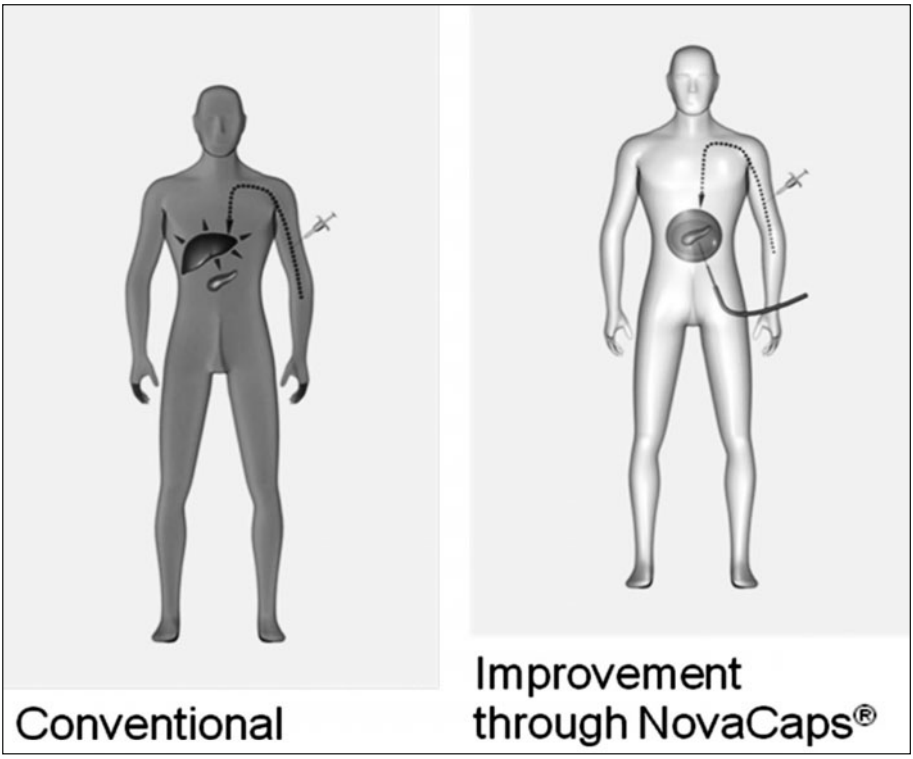

Figure 1. Encapsulated cells as a means to focus chemotherapy on tumours. Right hand panel: Conventional chemotherapeutic agents like ifosfamide or cyclophosphamide are usually given as a bolus injection i.v. These agents themselves are not toxic (i.e., they are prodrugs) and are metabolised to give the toxic, anti-tumour active form by cytochrome P450 enzymes in the liver. The toxic form of the chemotherapeutic is then distributed via the blood stream throughout the body. Although it is relatively short lived, it attacks all dividing cells (tumour cells, but also hair follicle cells, cells of the gastrointestinal tract and bone marrow and blood precursor cells) leading to tumour killing but also the typical side effects of chemotherapy. Left hand panel: Encapsulated cell products like NovaCaps® are delivered to a vessel feeding the pancreatic tumour using a supraselective catheter. Once released, the capsules are propelled by the blood flow into smaller vessels around the tumour until they eventually become lodged. Two days later, ifosfamide is given at low doses i.v. and arrives at the capsule in the blood flow, which forces the ifosfamide into the capsule where it enters the cells that are overexpressing a cytochrome P450 enzyme that is particularly active in metabolising the chemotherapeutic agent. The active, anti-tumour form is then released from the cells and leaves the capsule and, because it is relatively short lived, attacks preferentially the pancreatic cancer cells nearby.

major toxicities beyond grade 2 associated with the low dose of ifosfamide that was used.[40,41] Therapeutic benefit was also documented with respect to (i) the quality of life, which was improved in most patients (ii) tumour reductions which were observed in 4 patients with the other 10 patients showing stable disease (iii) a 100% improvement in median survival over a control group and (iv) 1 year survival rates, which were almost twice as high as those documented after treatment with the current gold standard treatment, gemcitabine.[40,41]

Table 3. Advantages of local implantation of encapsulated cells to focus chemotherapy

	Systemic Drug Alone	Cell-Based Therapy Targeting
Site of activation	In the liver	Next to the tumour (and liver)
Doses required	Relatively high	Relatively low
Toxic side effects	High and systemic	Low
Anti-tumour effect	Poor to moderate	Good anti-tumour effect

NovaCaps® has been designated an orphan drug in Europe by the European medicines agency (EMEA) as well as the first of a new class of therapeutics created by the EMEA called "advanced therapy medicinal products (particularly somatic cell therapy medicinal products)". [44]

Combination Therapies—The Way for the Future?

The development of resistance to therapeutic agents has been and still is, a major problem in cancer treatment. This remarkable propensity of tumour cells to evolve and escape from a given treatment is often blamed on the genetic complexity and instability of tumour cells and the multi-factorial nature of the disease.[45] Thus, effective long term treatment successes may require a multipronged attack of tumour cells where multiple pathways are targeted in order to improve treatment efficacy.[46] Although such combination therapies do not have to be limited to cell therapies, it has been shown that they can be a possibility. This can be illustrated by two examples.

In order to determine if multiple prodrugs and their activating enzymes can be employed for the treatment of tumours, cells have been genetically modified to express both the cytochrome P450 gene and cytosine deaminase. The resultant cells were then encapsulated in cellulose sulphate and tested into two mouse models of mammary cancer (TS/A and GR). Following implantation of the encapsulated cells, the mice were treated with (i) cyclophosphamide, which also is metabolised by cytochrome P450 to its' active anti-tumorigenic form, or (ii) 5 fluorocytosine which is converted by cytosine deaminase to anti-tumour metabolite 5 flurouracil or (iii) a combination of both cyclophosphamide and 5 fluorocytosine. The results revealed that additive toxicity was found when cyclophosphamide and 5 fluorocytosine were given together in both mouse models of mammary cancer.[10] Interestingly, the anti-tumoral effect mediated by cytochrome P450/cyclophosphamide was more efficient than that of cytosine deaminase/5 fluorocytosine in one of the mouse model systems (TS/A) whilst in the GR mouse tumour model, both systems worked equally well. This kind of multi-drug activating system can be expanded so that one cell is genetically modified to express three or more prodrug activating enzymes, opening up the possibility of tailoring treatment to individual patient's needs by only applying the prodrugs to which the tumour responds (Fig. 2). Another combination therapy that has been examined in animal models is the combination of immunotherapy with anti-angiogenic therapy. In this study, B16-F0/neu melanoma-bearing mice were intraperitoneally injected with alginate microencapsulated mouse myoblasts (C_2C_{12}) genetically modified to deliver angiostatin and an interleukin 2 fusion protein (sFvIL-2). The combination treatment resulted in improved survival, delayed tumour growth and increased apoptosis and necrosis. In addition to improved efficacy, the combination treatment also ameliorated some undesirable side effects that resulted from treatment with each of the agents individually e.g., inflammatory responses to IL-2 or vascular mimicry due to angiostatin.[47]

Retrovirus Vector Production from Encapsulated Cells

Encapsulated cells have also been employed as a launching pad for more traditional gene therapy using virus vector systems in two studies. Both publications report the encapsulation of retroviral vector producing cells and their implantation so that a local and sustained release of viral particles

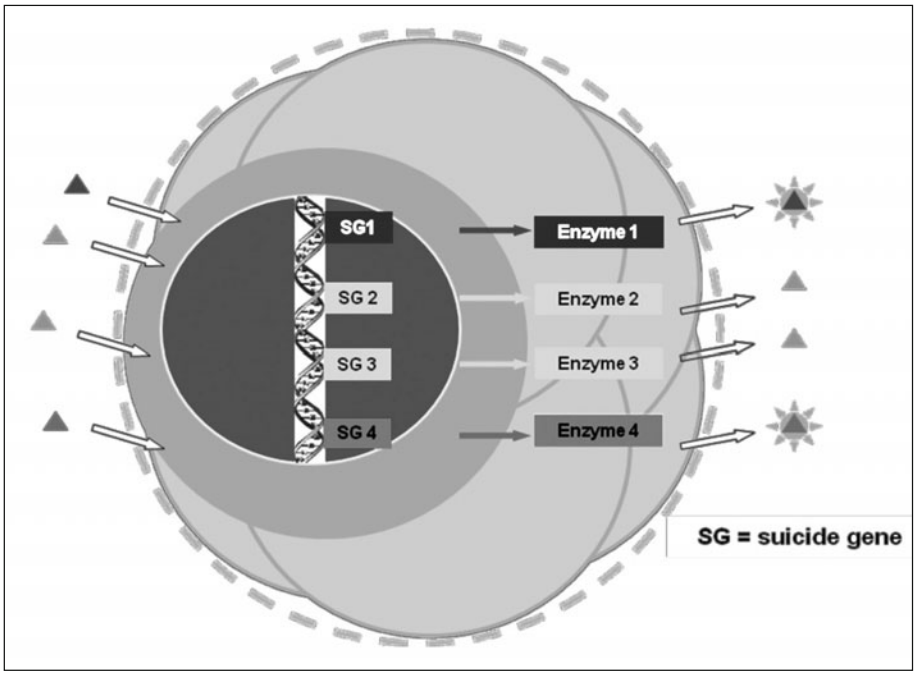

Figure 2. Multidrug, patient specific treatment of tumours. Cells are genetically modified to express more than one prodrug activating enzyme (enzyme 1-4) from the corresponding suicide gene (SG) and encapsulated. After implantation into the patient, the patient receives more than one prodrug. In the example shown here, the patient receives the purple and pink prodrugs which are activated by enzyme 1 and 4 respectively. Prodrug 2 and 3 are not given to this patient because the tumour is resistant to their activated forms. Patient specific treatment can be achieved in that, although the same cells expressing the same suicide genes are implanted, another patient may receive a different combination of prodrugs (for example 2, 3 and 4 or all four prodrugs). A color version of this image is available at www.landesbioscience.com/curie.

to the malignant milieu can be achieved. This is important since commonly used retroviral vectors like those based on murine leukaemia virus are only able to infect dividing cells and when given as a bolus injection, considerable amounts of vector virus may be inactivated before a tumour cell enters into the cell cycle where it is available for infection.[48] The first reported use of encapsulated cells for this purpose involved the encapsulation of such cells in cellulose sulphate capsules and implantation into mice. Infection of tumour cells in vivo was demonstrated in two different models. In the first, capsules were implanted in the fat pad of the mammary gland of Balb/c mice. The capsules were well tolerated for at least 6 weeks and a self-limiting inflammatory reaction without any other gross immune response was observed during this period. Furthermore, the virus-producing cells remained viable. In the second model, severe combined immune deficient mice were immunologically reconstituted by subcutaneous implantation of thymus lobes from MHC-identical Balb/c newborn mice and gene transfer into lymphoid cells was achieved by retroviral vectors released by co-implanted capsules.[49] A more recent study confirmed and extended these findings, using alginate encapsulated packaging cells producing a retroviral vector carrying the herpes simplex virus thymidine kinase (HSV-tk) gene. Once HSV-tk is expressed, it preferentially phosphorylates nucleoside analog prodrugs, such as ganciclovir (GCV) or *N*-methanocarbathymidine (*N*-MCT), to their active triphosphate metabolites that are incorporated into cellular DNA and cause cell death. Preliminary in vivo transplantation of encapsulated virus producing cells into the peritoneal

cavity of mice bearing intraperitoneal MC38 tumours with 2 weeks subsequent GCV administration resulted in a significantly higher survival rate relative to control groups.[50] Both of these studies demonstrate the feasibility of employing encapsulated cells for the long term production of retroviral vectors in vivo for cancer gene therapy.

Conclusion

Clearly the use of encapsulated cells has great potential as the basis for the treatment of a wide variety of diseases, including various forms of cancer. This chapter has attempted to summarise preclinical and clinical data from some of the more promising strategies involving encapsulated cells to treat tumours. The authors believe that at least some of these approaches will soon be tested in further clinical trials of encapsulated cell therapies, particularly for those tumours representing an unmet medical need.

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Chapter 10

Inorganic Nanoporous Membranes for Immunoisolated Cell-Based Drug Delivery

Adam Mendelsohn and Tejal Desai*

Abstract

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

Introduction

Cell-based drug delivery has been proposed as a treatment for diseases characterized by cell degeneration including Parkinson's disease,[1,2] testicular dysfunction and hypogonadal disorders[3] and liver failure.[4] However, the driving force behind cell-based drug delivery research has been to improve the treatment of insulin dependent diabetes mellitus (IDDM). IDDM is characterized by the loss of pancreatic β-cell function which normally regulates the blood-glucose concentration by the secretion of insulin. Without functional β-cells, chronic hyperglycemia can lead to complications including retinopathy, neuropathy, nephropathy and death. Healthy β-cells secrete insulin in quantities that are highly sensitive to the blood-glucose level and successful IDDM treatment requires the same sensitivity to avoid debilitating events.

The first major advancement in treating IDDM occurred in 1922 with the first successful clinical trial using insulin.[5] Unfortunately, while insulin-replacement therapy has saved countless lives, 82 years later in 2004 diabetes remained one of the most deadly diseases, ranking 6th in the United States.[6] The most common insulin-replacement therapy requires frequent blood-glucose measurement through finger pricks as well as multiple insulin injections per day. The most advanced insulin-replacement therapy is approaching its ultimate goal of a closed-loop artificial pancreas, consisting of an artificial glucose sensor coupled to an insulin delivery pump.[7] So far the development has fallen short of its goals for two reasons. First, a fully implantable long-term insulin pump has not yet achieved clinical success, requiring the user to wear an external pump. Second, development of a long-term artificial glucose sensor remains elusive in part because of protein adsorption causing measurement drift, thus requiring frequent sensor calibration through finger pricking. As a result, while the current

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technologies offer remarkable advances for insulin-replacement therapy when used appropriately, proper treatment requires constant user attention. Lastly, even if glucose-sensing technologies improve, the algorithms with which the sensor communicates information to the pump to modulate insulin delivery kinetics represents only an approximation of blood-glucose regulation in healthy patients. Several companies continue to research towards a closed-loop artificial pancreas, including Medtronic Minimed Inc. and Roche Diagnostic's Disetronic.

Cell-Based Drug Delivery

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

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

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

The only chance of avoiding the above immune responses without immunosuppression or immunoisolation is to transplant cells that are genetically identical to the patient. For Type I diabetics, these are the cells that have degenerated and are therefore not available as autografts. Furthermore, Type I diabetes is thought to have an autoimmune etiology and therefore even the transplantation of pancreatic β-cells that are genetically identical to the recipient will be subject to the same degeneration that originally caused the patient's disease. Recently in Brazil, one study demonstrated the potential of autologous bone marrow-derived pancreatic stem cell transplantation

following immune ablation.[12] Unfortunately, this study applied only to patients between ages 14-31 that were diagnosed with Type I diabetes within 6 weeks prior to treatment. Furthermore, patient hospitalization and isolation was required because of the temporarily weakened immune system caused by ablation. While a small subset of Type I diabetics may benefit from this treatment, more research is needed to determine whether it can be applied to a larger patient population.

Immunosuppressed Cell Transplantation

One approach to providing protection for islet transplants has been to chronically administer immunosuppressive medication. In this pursuit, whole organ pancreas transplantations are possible with immunosuppression. The complications are deemed worthwhile only for patients that are already undergoing transplantation of a life-sustaining organ such as a kidney. These complications include susceptibility to infection, decreased capability of healing wounds properly and increased risk for developing lymphoma.[13,14] Until recently, isolated islet transplantations had been much less successful than whole pancreas transplantations, with only 8% of patients maintaining insulin independence for up to one year in all procedures between 1990 and 1998.[15] More recently, isolated allogenic islet transplantation was validated using a medication regimen outlined in the Edmonton protocol that resulted in 7 of 7 patients who remained insulin independent one year after transplant.[16] However, a 5 year study of the same therapy resulted in only 10% of patients who remained insulin-independent.[17] Additionally, this therapy requires human donor pancreatic islets of which the supply is limited.[18] Currently, efforts are underway to differentiate pancreatic β-cells from human stem cell lines that could ultimately increase the supply for allogenic transplantations.[19] Unfortunately, insulin-independence for the patient has not been achieved through immunosuppressed xenogenic islet transplantation for which the current supply is much greater.[20]

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

Immunoisolated Cell-Based Drug Delivery

Origins

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

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

The treatment of IDDM by immunoisolated cell transplantation was made possible only after the β-cell containing Islets of Langerhans were isolated in 1965.[31] Several immunoisolated transplantation methods were subsequently developed, including intravascular chambers, microcapsules and extravascular chambers (Table 1).[32-34] Each of these will be addressed in the following sections.

Intravascular Chambers

Motivation

Intravascular chamber development was motivated by the need for transplanted cells to regulate the blood-glucose level in a timely manner. These chambers directly access the blood, being separated only be a semipermeable membrane. Such an approach offers an advantage over both extravascular chambers and microcapsules, which are also implanted in an extravascular space, often in the peritoneal cavity. Glucose from the blood must first diffuse through the mesothelium that lines the peritoneal cavity in order to access the cells. As a result, the cells receive blood-glucose information that is delayed. This delay is exacerbated in humans because of the greater thickness of human mesothelium compared with that of animals. For example, human mesothelium is 4-5 times thicker than that of a rat.[35] If the delay is significant in duration, the patient will experience peaks and valleys of blood glucose concentrations that will increase the chance of debilitating events. Therefore, the intravascular chamber approach avoids the increased delay and for this reason is a promising approach for immunoisolated cell-based drug delivery.

Development

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

Commercialization

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

Failure Modes

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

Microcapsules

Motivation

Nutrient availability is another factor that determines the viability of cellular implants over time. For this reason, one design consideration in the early development of islet transplantation chambers were insulin and glucose diffusion across the membranes.[42] In order to optimize these

diffusion rates, the surface area to volume ratio should be maximized. As a result, researchers began transplanting cells encapsulated in semipermeable microcapsules.[43,44] Furthermore, microcapsule implantation can occur through injection, offering a less invasive procedure than the surgery required for transplantation chamber implantation.

Development

Cell microencapsulation was first mentioned by Chang in 1964.[45] However, it was not until 1980 that Lim and Sun applied microcapsules to diabetes treatment, demonstrating prolonged isograft islet survival when microencapsulated in alginate-polylysine-polyethyleneimine microcapsules.[46] Initially, microencapsulated islet transplantation delayed the return to hyperglycemia compared with the transplantation of unencapsulated islets by only 10 days and failed due to a lack of biocompatibility of the microcapsule itself. The microcapsule material was improved in 1984 by O'shea and Sun who removed the polyethyleneimine component and designed alginate to be the outer layer of the microcapsule.[47] The improved material demonstrated significant improvement and in one of the five animals the microencapsulated islets remained viable for 365 days, when the experiment ended. An additional advantage of the new microcapsules was increased microcapsule strength. Efforts to further improve biocompatibility of alginate microcapsules involved decreasing the impurities and increasing the guluronic acid to mannuronic acid ratio.[48,49] Other researchers questioned the reproducibility of alginate-polylysine microcapsules and explored either their coating with a polyethylene glycol hydrogel or manufacturing the microcapsules from a different material altogether such as a polyacrylate[50,51] or silica.[52] In an optimization effort, Wang et al evaluated over 1,000 combinations of polyanions and polycations with regards to suitability for cell encapsulation.[53] The result was a polyelctrolyte complexation process using 5 different polymers enabling independent control over capsule size, wall thickness, mechanical strength and permeability. For further information, microencapsulation technology has been extensively reviewed elsewhere.[54,55]

Commercialization

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

Failure Modes

Despite significant activity, microencapsulation technology still has not achieved clinical success. Several experiments point to some key factors that may be playing a role in transplant failure. Originally, the lack of biocompatibility of the membranes was associated with cellular overgrowth

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

While microencapsulation technology is approaching human clinical success, there remain many disadvantages inherent with this approach. Microcapsule manufacturing processes have resulted in pore sizes with relatively broad distributions.[65] Even if cytokine-mediated cell death is limited, a broad pore size distribution presents a potentially insurmountable challenge in the attempt to isolate antibodies, complement and immune cells while allowing sufficient nutrient and insulin diffusion. An optimal membrane will completely isolate the encapsulated cells from the relevant antibodies and complement (IgG, IgM and C_{1Q}). Transport inhibition of such molecules is particularly necessary for xenotransplants because of increased indirect antigen presentation.[8] Additionally, microcapsule walls are susceptible to having embedded islets enabling a portion of the islet that is not protected by the membrane to stimulate an immune response.[66] Although this limitation can be overcome, doing so typically requires a larger diameter microcapsule or a double layer, increasing the blood-glucose diffusion time.[67] Efforts are underway to create ultrathin microcapsule walls without any exposed portion of the islet, but in vivo success has not yet been demonstrated.[68]

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

Extravascular Chambers

Motivation

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

Development

The extravascular chamber method developed by Algire, Weaver and Prehn, discussed earlier, in the 1950's for transplanting tissues was a natural starting point from which researchers could develop an extravascular chamber for immunoisolated islet transplantation.[28-30] During the 1970s, Millipore

Corporation produced a commercially available extravascular transplantation chamber using the Algire approach.[34] These membranes typically had pore sizes on the order of 450 nm, a size sufficiently small to prevent direct cell-cell contact and therefore promising for allotransplants. Studies by Algire and colleagues demonstrated improved cell viability when encapsulated in these membranes.[27,70,71] Although many of the initial experiments involved syngeneic cells, transplant failure occurred nonetheless due to fibroblastic overgrowth of the graft and chamber, highlighting the importance of biocompatibility of the chamber to transplant success.[34] Significant advances have been made since these early experiments and they have been reviewed extensively elsewhere.[34,72,73]

Commercialization

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

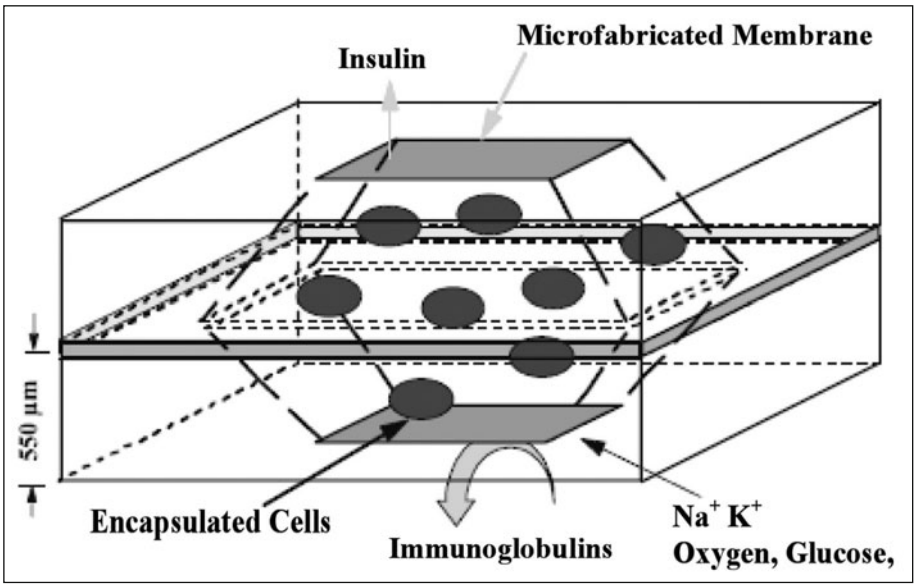

Figure 1. Extravascular Transplantation Chamber. A device encloses a collection of cells with an immunoisolative membrane. Shown here is a cross-section of a device with cells in between two nanoporous membranes. Reprinted with from Leoni L, Desai TA. Micromachined biocapsules for cell-based sensing and delivery. Adv Drug Deliv Rev 2004; 56(2):211-29, with permission from Elsevier.

Failure Modes

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

Inorganic Nanoporous Membranes

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

Silicon Nanoporous Membranes

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

Preparation

Silicon nanoporous membranes are prepared initially from silicon wafers. A comprehensive outline of the history and development of the silicon membrane was previously reviewed by Leoni.[81] Presented here is the most current manufacturing strategy, also previously described (Fig. 2).[82,83] First, a support ridge structure is photo-lithographically etched to provide mechanical support to the final structure.[80] A low-stress silicon nitride layer is deposited over the top surface of the wafer. The membrane structure will be formed on top of the silicon nitride, which will serve as an etch-stop for future processes. This etch-stop layer is very thin and small in comparison to the depth between support ridges. A polysilicon film, henceforth referred to as the base layer, is deposited on top of the silicon nitride layer, filling the remaining space between support ridges. The thickness of the base layer will determine the overall thickness of the nanoporous membrane.

Holes are then etched through the base layer but not through the nitride etch-stop layer. The geometry of the holes determines the shape of the pores. This geometry is defined by a thermally-grown

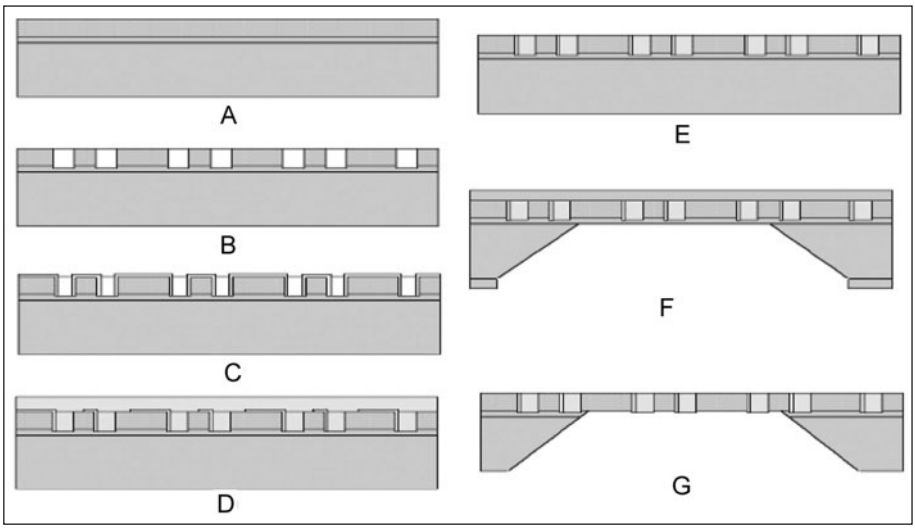

Figure 2. Silicon Nanoporous Membrane Fabrication. A) Support ridges are fabricated from a silicon wafer using lithography; Silicon nitride etch-stop layer is deposited; Polysilicon base layer fills the remainder of the space between support ridges. B) Holes etched through the base layer define the geometry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

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

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

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

Advantages

The silicon nanoporous membrane has the potential to overcome all of the limitations associated with polymer membranes discussed above. Pore widths of 18 nm have demonstrated significant diffusion resistance to IgG while allowing relatively unrestricted diffusion of insulin and glucose.[78,85]

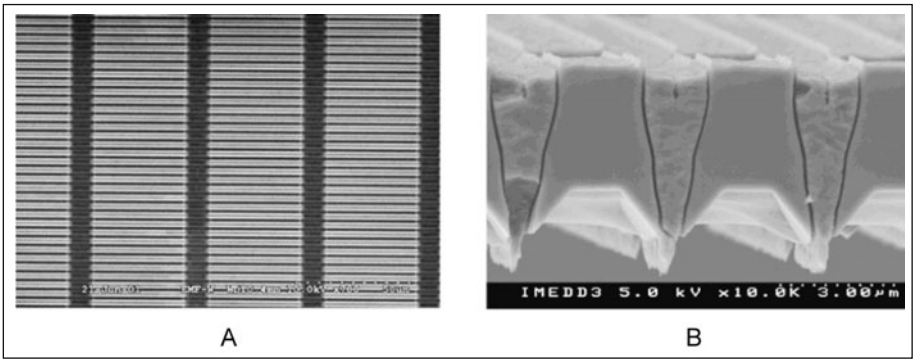

Figure 3. SEM Micrographs of Silicon Nanoporous Membrane. A) Top view detail. B) Side view detail. Reprinted with permission from Leoni L, Boiarski, Anthony et al. Characterization of nanoporous membranes for immunoisolation: Diffusion properties and tissue effects. Biomedical Microdevices 2002; 4(2):131-139.

Furthermore, the highly controllable pore channel width to within 0.5 nm[86] results in a substantially tighter pore size distribution of approximately 5% compared with the 30% distributions that can be associated with polymer membranes.[65] It has been suggested that if only 1% of the pore sizes exceed the desired cut-off, sufficient quantities of antibodies, complement and cytokines will diffuse to cause immune-mediated death.[43] In order for less than 1% of the pores to exhibit sizes above the desired cut-off, a broader pore size distribution necessitates a smaller nominal pore size. However, a smaller nominal pore size will result in decreased diffusion of insulin, glucose and nutrients, leading to a greater chance of nutrient starvation and poor insulin secretion kinetics. Additionally, 18 nm pore width membranes have demonstrated protection for islets when placed in a serum complement/antibody solution over a 2-week period as measured by improved glucose stimulated insulin secretion compared with unencapsulated islets.[87] Furthermore, in vivo studies have confirmed both short-term biocompatibility of the membranes and increased insulinoma cell viability.[77] All of these results support the potential that silicon nanoporous membranes have in providing adequate immunoisolation to encapsulated cells.

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

Disadvantages

The silicon nanoporous membrane possesses one disadvantage compared with polymer membranes as well as the alumina and titania membranes that will be discussed later. Currently, it is only feasible to manufacture silicon membranes with rectangular pores, whereby the width can be

in the nanometer range but the length is limited by that which traditional etching methods allow. The width of the pore and not the length serves to restrict antibody and complement diffusion. When considering diffusion of a protein through a pore, however, the 3-dimensional conformation of the protein must be considered. IgG is a relatively flexible y-shaped molecule that can assume conformations that minimize width and extend length, allowing enhanced diffusion through a rectangular pore compared with a circular pore. This phenomena has been demonstrated by the restricted diffusion of IgG through an alumina nanoporous membrane with 75 nm diameter pores compared with a silicon nanoporous membrane with 49 nm wide pores.[88] The alumina membrane also restricted glucose diffusion more than the silicon membrane; this was likely due in part to the larger alumina membrane thickness. However, the difference in restricted glucose diffusion was less than that for IgG. Therefore, at least part of the decreased IgG diffusion was due to the circular nature of the pores in the alumina, suggesting that a circular pore can provide improved immunoisolation. While currently not easily available, technologies for creating circular nanopores in silicon may someday become commercially available by using more advanced lithographic techniques such as electron-beam or nano-imprint lithography.[89] Until then, the silicon nanoporous membrane, while extensively studied and promising, possesses the disadvantage of containing rectangular-shaped pores.

Alumina Nanoporous Membranes

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

Preparation

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

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

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

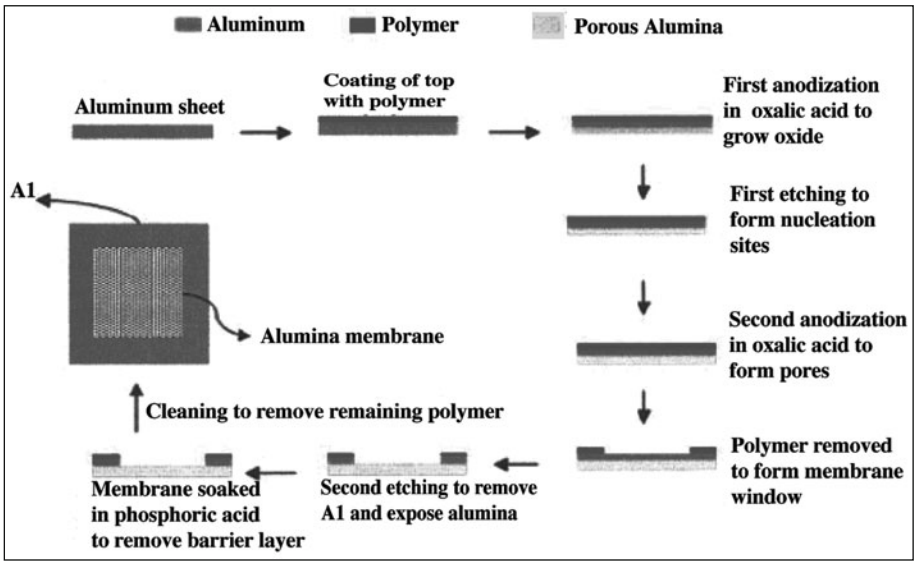

Figure 4. Alumina Nanoporous Membrane Fabrication. Reprinted with permission from Swan EE et al. Fabrication and evaluation of nanoporous alumina membranes for osteoblast culture. J Biomed Mater Res A 2005; 72(3):288-95.

In order to expose the nanoporous membrane to the outside of the tube, a window-area is created in the polymer film through the selective application of acetone and a cotton swab. A 10% NaOH solution can be poured for 15 minutes to completely remove the unwanted layer of alumina that is formed during the second anodization step. Parafilm or silicone plugs are capped on the tube ends to protect the inside of the tube from the subsequent etching step. After a thorough rinse in DI water, the unprotected aluminum in the window is etched using a 10% (w/w) HCl and 0.1 M ($CuCl_2$) solution, exposing the transparent alumina membrane. Finally, a 10% (w/v) phosphoric acid solution for 1 ½ hours at room temperature removes the barrier oxide layer on the outside of the nanoporous alumina. After the parafilm or silicone plugs are removed, the result is an aluminum cylinder with a nanoporous alumina membrane window.

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

Advantages

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

Additionally, the pore density of an alumina nanoporous membrane can exceed that for both polymer and silicon nanoporous membranes.[88] The ability to increase pore density offers a potential advantage in the design of a cell encapsulation device in the pursuit of balancing the requirements

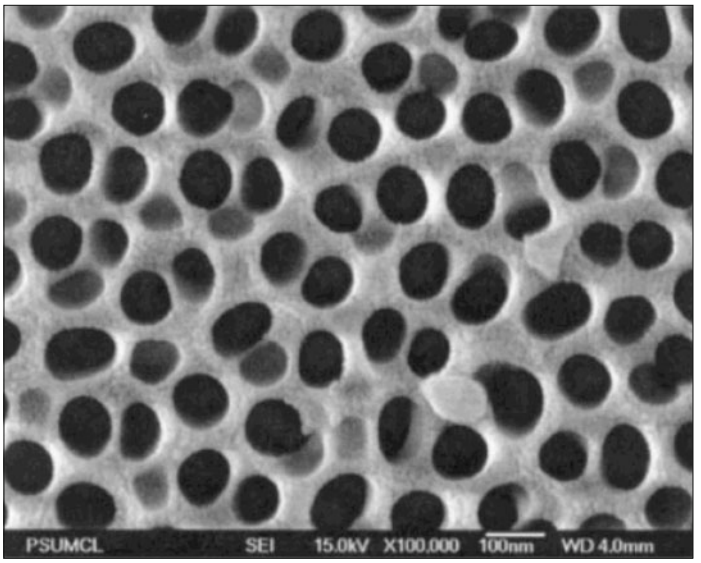

Figure 5. SEM Micrograph of Alumina Nanoporous Membrane. Reprinted with permission from Swan EE et al. Fabrication and evaluation of nanoporous alumina membranes for osteoblast culture. J Biomed Mater Res A 2005; 72(3):288-95.

for immunoisolation and nutrient availability. If the pore diameter sufficiently impedes antibody and complement diffusion, the larger pore density will increase the diffusion of insulin, glucose and nutrients more than it will increase the diffusion of antibodies and complement in a size specific manner. Additionally, alumina nanoporous membranes improve upon polymer membranes by offering greater control over membrane thickness.

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

Lastly, the studies evaluating the biocompatibility of alumina nanoporous membranes have been favorable. Alumina has demonstrated bio-inert characteristics in humans for certain applications, enabling its use in hip and knee replacements.[97] More recently, alumina nanoporous membranes have not caused fibroblast cytotoxicity nor complement activation in vitro. In vivo studies in the same report reveal that membrane-containing capsules are free from fibrous growth and membranes remain intact when implanted in the peritoneal cavity of rats for up to 4 weeks.[98] Tissue samples surrounding the implants do show signs of inflammation, but samples taken from tissue surrounding polyethylene glycol (PEG) coated alumina nanoporous membrane capsules exhibited less severe signs of inflammation which receded after 4 weeks.[93,99] These results suggest that the inflammation from PEG-coated capsules occurs from the surgery itself and not from the implanted capsule. In vivo studies with encapsulated cells have not yet been performed. In conclusion, the alumina nanoporous membrane offers many promising characteristics that can be applied to immunoisolated cell-based drug delivery.

Disadvantages

One limitation that the alumina nanoporous membrane has compared with the silicon nanoporous membrane is the thickness of the membrane. The alumina nanoporous membrane has been fabricated with thicknesses as small as 70 μm and although thinner membranes are possible, such modifications will negatively affect membrane strength. As discussed above, a thicker membrane results in delayed diffusion of glucose information to the cells and insulin secretion to the body.

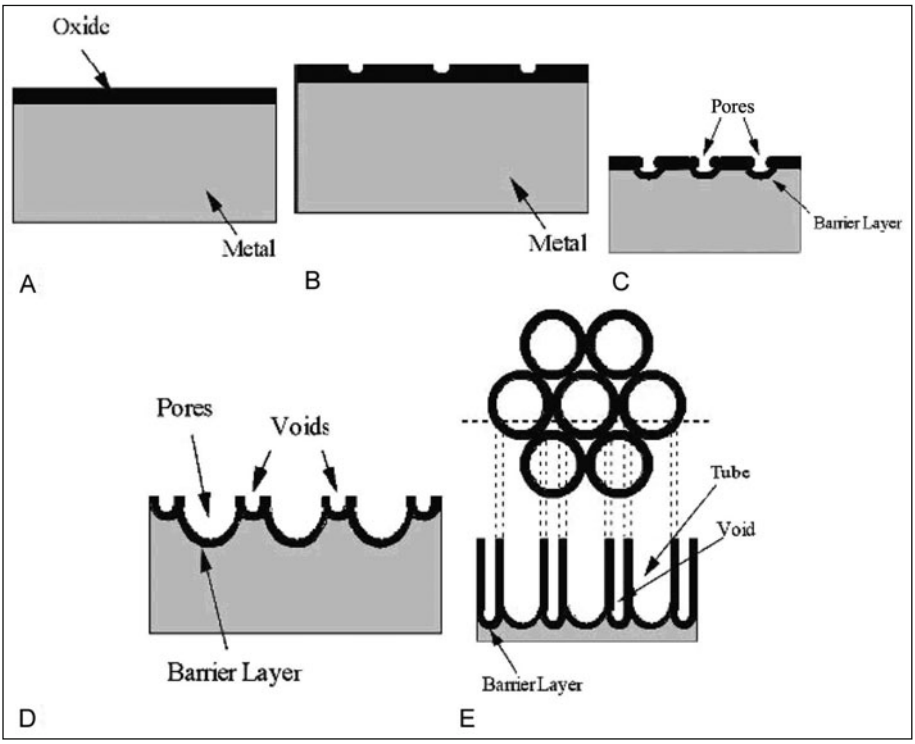

Figure 6. Titania Nanoporous Membrane Fabrication. A) Oxide layer formation. B) Pit formation on the oxide layer. C) Growth of the pit into scallop-shaped pores. D) The metallic part between the pores undergoes oxidation and field-assisted dissolution. E) Fully developed nanotubes with a corresponding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

The relationship between having an increased pore density but a thicker membrane needs to be more thoroughly evaluated. The advantage of increased pore density could potentially recooperate any diffusion loss due to membrane thickness in comparison to silicon nanoporous membranes. Regarding biocompatibility, it is unclear in vivo whether alumina nanoporous membranes can be as stable as either polymer membranes or silicon nanoporous membranes. As a result, despite promising results thus far, further evaluation will be necessary to determine whether the alumina nanoporous membrane is the ideal choice for immunoisolated cell-based drug delivery.

Titania Nanoporous Membranes

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

Preparation

The nanotubular titania can be grown from a titanium foil in several ways. The formation of nanotubular titania described here is adapted from previous reports (Fig. 6).[100-105,107] First, high purity titanium foil (99.97% or higher, thickness approximately 250 μm) is degreased by sonication

Table 1. Advantages and disadvantages of immunoisolation technologies

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

in acetone, ethanol and DI water, followed by a DI water rinse and nitrogen drying. The growth of the nanotubular titania occurs with a subsequent potentiostatic anodization in a 2-cell electrode electrochemical cell connected to a dc power supply, using platinum foil as the counter electrode at room temperature. Methods of controlling nanotube diameter and length have recently been elucidated, although this research is still in its infancy and greater optimization will likely occur in the future.

The first successful nanotubular titania growth occurred through anodization of titanium foil a 0.5% (w/w) HF solution.[102] Under these conditions, the nanotubular structure is formed at voltages greater than 10 V and less than 40 V. Nanotubes fabricated using this process have diameters ranging from 25-65 nm and thicknesses up to 500 nm.[102,103] The first event in the anodization process occurs within 10 seconds when the titanium film is covered by a compact oxide film of uneven height. At 30 seconds the oxide film begins to dissolve exposing a continuous nanoporous layer without the presence of any tubular structures. After 8 minutes of continued anodization, the oxide layer is completely removed, exposing discrete emerging nanotubular structures. It has been proposed that nanotubular structure formation occurs by the following mechanism: At sufficiently high anodization voltages, the electric field strength will mobilize titanium ions from the surface in between the pores and facilitate their migration to the oxide/solution interface, resulting in the growth of tubular structures from the titanium surface.[102]

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

Advantages

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

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

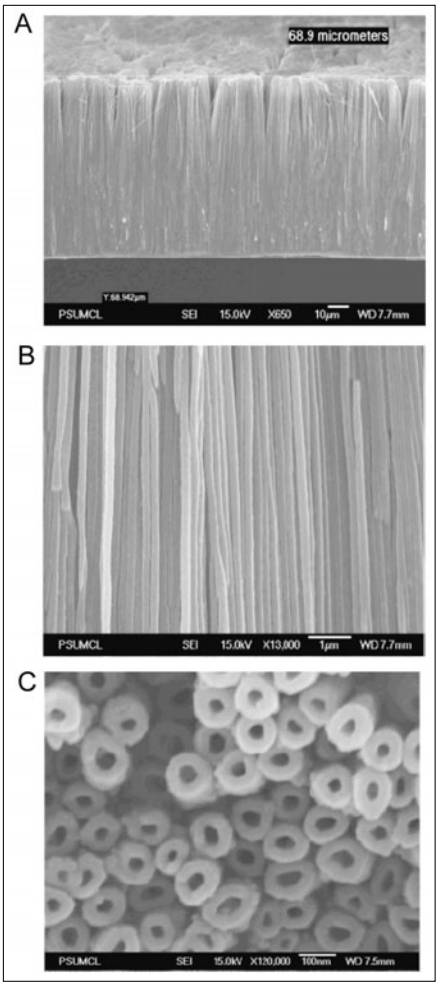

Figure 7. FESEM Micrographs of Titania Nanoporous Membranes. A) Cross-section at lower magnification. B) Cross-section at higher magnification. C) Top-surface image. Reprinted with permission from Paulose M et al. Anodic growth of highly ordered TiO2 nanotube arrays to 134 um in length. J of Phys Chem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

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

Disadvantages

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

Table 2. Advantages and disadvantages of polymer and inorganic nanoporous membranes

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

Conclusion

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

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Chapter 11

Cell Microencapsulation

Grace J. Lim, Shirin Zare, Mark Van Dyke and Anthony Atala*

Abstract

In the past several decades, many attempts have been made to prevent the rejection of transplanted cells by the immune system. Cell encapsulation is primary machinery for cell transplantation and new materials and approaches were developed to encapsulate various types of cells to treat a wide range of diseases. This technology involves placing the transplanted cells within a biocompatible membrane in attempt to isolate the cells from the host immune attack and enhance or prolong their function in vivo. In this chapter, we will review the situation of cell microencapsulation field and discuss its potentials and challenges for cell therapy and regeneration of tissue function.

Introduction

A major obstacle associated with the transplantation of non-autologous cells or tissue is graft rejection.[1-2] To overcome this problem, recipients are obliged to take immunosuppressive drugs for extensive period of time, which often causes major side effects such as losing resistance to infections, increasing the potential of spreading malignant cells, hypertension, anemia, hyperglycemia, peptic ulcers and nephrotoxicity. The immunosuppressive drugs also interact with other medicines and affect their metabolism and action.[3-6]

Microencapsulation of cells has significant promise for minimizing the need for such drugs when cells are transplanted. Microencapsulation is a technology to capsulate the transplanted cells with biocompatible materials to safely isolate lythe cells from the host immune system. Cell microencapsulation is probably the preferable system for cell transplantation or forming functional new tissues by the fact that the system enables the continuous delivery of various secreted factors emanating from the microencapsulated transplanted cells to the host. Due to the semi-permeable membrane of the capsule, antibodies or immune cells are unable to cross the membrane and destroy the graft.[7-10]

There has been a few successful clinical applications using microencapsulated cell systems and many preclinical trials are underway. We will review the progress of cell microencapsulation and discuss the potential of this cell capsulation system for efficiently utilizing the functions of transplanted cells or tissues. Understanding of the current status and limits of this technology will enable the development of new strategies and successful cell therapy and transplantation.

Why Is Microencapsulation Necessary?

Immune Protection by a Semipermeable Membrane

When nonautologous cells or tissue are transplanted, graft rejection occurs. As a way to prevent immune rejection, cell microencapsulation method has been used for minimizing or totally

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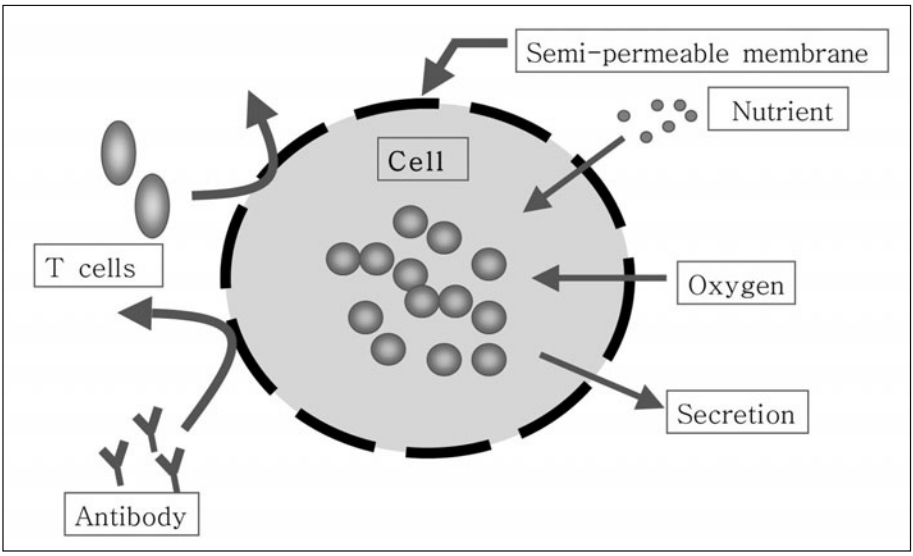

Figure 1. Semipermeable membrane protecting transplanted cells by microencapsulation with a polymer. Nutrients and oxygen diffuse across the membrane, whereas inflammatory cells, antibodies and immune cells are excluded.

overcoming the need for taking immunosuppressive drugs. Microencapsulation is the processing to isolate xenogenic or allogenic cells from the host's immune system by surrounding them in a semi-permeable membrane prior to implantation within the host. The semi-permeable membrane is relatively impermeable to large molecules, such as components of the host's immune system, but is permeable to small molecules. The membrane allows therapeutic molecules produced by the implanted cells to diffuse to host cells. Thus, the semi-permeable membrane enables the implanted cells to receive nutrients necessary for viability and allows metabolic waste to be removed.[11,12] The use of an immuno-protective, semi-permeable membrane now allows transplantation of encapsulated cells from one species into a host from a different species without the risk of immune rejection or use of immunosuppressive drugs (Fig. 1).

Membrane permeability is a function of both transport and thermodynamic properties, which are dependent upon the molecular characteristics of both the membrane and solute population. The use of different membranes allows for variations in permeability, mass transfer, mechanical stability, buffering capability, biocompatibility and other characteristics. A balance, however, has to be maintained among the physical properties of capsule membranes so as to support the entrapped cells' survival.

Molecular Weight Cutoff for Control Permeability

Ordinarily the desired capsule permeability is determined by the molecular weight cutoff (MWCO) of an encapsulating material and is application-dependant. The MWCO is the maximum molecular weight of a molecule that is allowed passage through the pores of the capsule membrane. For transplantation, the MWCO must be high enough to allow passage of nutrients but low enough to reject antibodies and other immune molecules. For example, molecular weight cutoff of an alginate-poly(L-lysine)-alginate membrane which is widely used in cell microencapsulation lies in approximately 60-70 kd through which leukocytes and various immunoglobulins such as IgM (950 kd) and IgG (150 kd) are not permeable but lower molecules necessary for cell survival such as glucose (180 d) and carbon dioxide (44 d) and secretary proteins from cells such as albumin (66 kd), growth factors (6-50 kd) and insulin (6 kd) can easily pass. Figure 2 shows

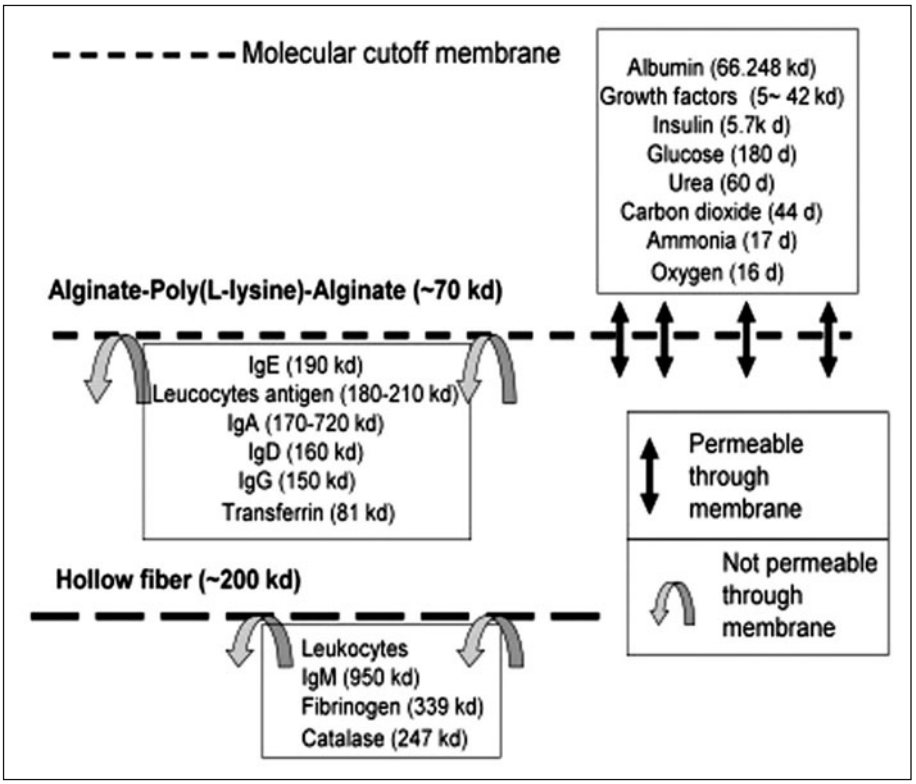

Figure 2. Diagram of molecular weight cutoffs of semipermeable membranes.

the molecular cutoffs of capsule membranes and molecules and their molecular weights which control the passage through the barrier.

Materials for Cell Microencapsulation

Since Lim and Sun published their results on the Langerhans islet encapsulation in the way of mild electrostatic crosslinking of sodium alginate and its complexation by poly(L-lysine) to treat diabetes, which is now the most commonly used cell encapsulation technique,[13] a great number of techniques for cell encapsulation have been proposed by using various polymeric materials.

The use of biologically compatible polymeric materials in construction of encapsulation devices is critical to a successful cell encapsulation. The cell encapsulation material plays important roles in not only by providing immune protection by isolating encapsulated cells from host tissue but by keeping the cell well distributed in capsule and maintaining the phenotype of cells by providing a proper 3D environment and subsequently enhancing the production of therapeutic biologics from cells. The optimal material for a particular cell encapsulation device is highly dependent on the cell type. For example, alginate is a family of polyanionic copolymers derived from brown sea algae and comprises 1, 4-linked ß-D-mannuronic (M) and a-L-guluronic (G) residues in varying proportions. Sodium alginate is soluble in aqueous solutions and forms stable gels at room temperature in the presence of noncytotoxic concentrations of certain divalent cations (i.e., Ba_2^+, Ca_2^+) through the ionic interaction between the guluronic acid group. This enables three dimensional shapes to be formed, with viable cells embedded in the gel by crosslinking in noncytotoxic conditions.[14,15] An investigation on the effect of material on proliferation and

differentiation of rat bone marrow cells found that alginate purity and composition are critical in determining both rat marrow cell proliferation and mechanical property of capsules. High purity and high G-type (guluronic content) were able to support proliferation of rat marrow cells and their differentiation along osteoblast lineage by retaining 27% of its initial strength after 12 days in culture and that comparable levels of proliferation were observed on this material and tissue culture plastic.[4] The results highlight the importance of using well-defined biomaterials. A study on the cell type and encapsulating material investigated the impact of various types of alginate microcapsules, which were fabricated (1) with or without the Poly(L-lysine)—alginate laminated surface, (2) crosslinked with Ca_2^+ or Ba_2^+ and (3) retaining a gelled or solubilized alginate core on the secretion of recombinant gene products from different cell types and reported that unlike the epithelial cells and myoblasts, the fibroblasts favored the Ca_2^+ linked spheres with a solubilized core over Ba_2^+ and solid gel core. Another study indicated that while adherent cells often prefer a solid surface on which to adhere, suspension cells may prefer a hydrophilic lightly cross-linked hydrogel as a matrix material.[16,17]

One approach has been the use of hollow fibers made of synthetic polymers including polyacrylonitrile/polyvinylchloride (PAN/PVC), polyurethane and polypropylene within which cells can be placed and the implanted fiber could be conveniently retrieved when the cells are no longer needed or caused a problem in vivo. Loss of cells viability due to a relatively small surface area, however, has limited the supplying nutrients and oxygen.[18-20]

Table 1 lists the widely used materials for cell encapsulation and target functions using the encapsulated cells.[21-40]

Geometry of Capsules Matters?

Capsule geometry critically affects the cells within. The mass transport properties of a capsule membrane are critical since the influx rate of molecules, essential for cell survival and the outflow rate of metabolic waste ultimately determine the viability of entrapped cells. A spherical geometry is mostly made by drop method and advantageous because of the high surface area to volume ratio. An islet (~150 μm in diameter) microencapsulated in an alginate bead (600~800 μm in diameter) was shown to be less susceptible to oxygen mass transfer than a tubular or planar diffusion chamber.[41] Long-term viability or functionality of encapsulated cells in association with capsule size investigated by Canaple and Chicheportiche[42,43] revealed that the reduction in capsule size from 1 mm to 400 μm was effective in improving capsule quality, mechanical stability, diffusion properties and in vitro activities of the encapsulated cells. The capsule's mechanical stability was largely dependent on the volume ratio of the capsule over the membrane and microcapsules are more durable and stable than macrocapsules. The in vitro cellular activities, for both primary cultures of rat islets and murine hepatocytes, were improved for cells encapsulated into the 400 μm capsules compared with those in the 1 mm capsules. All of the experimental findings suggest that the smaller capsules present better properties for future clinical applications, at the same time widening the choice of implantation site and strengthen the notion that slight changes slight changes in the capsular morphological parameters can largely influence the graft function in vivo.

Recent study in our laboratory to formulate a novel type of cell capsules for cell transplantation indicated that a doughnut shaped capsule having a hole in the center demonstrated more effective compared to conventional spherical shaped capsule in producing secretary proteins from the same numbers of cells encapsulated (Fig. 3). An experiment using primary porcine chromaffin cells which are known to secret neurotransmitters such as catecholamine and opioid peptides was performed to investigate the influence of geometrical structure of capsule on cell function. Cells were encapsulated with a keratine hydrogel[44] and assembled into doughtnut shaped capsules (Fig. 4A). As a control, alginate-poly (L-lysine)-alginate was used to capsulate the same number of cells as used for doughnut capsule into spherical shape. The neurotransmitter, catecholamine secretion from the encapsulated cells upon nicotine stimulation measured by HPLC showed that significantly higher amount of catecholamine was secreted from doughnut

Table 1. Materials used for cell encapsulation

Materials	Encapsulating Cells	Clinical Application
• Alginate-poly(L-lysine)-alginate[21]	Pancreatic islets	Diabetes
• Alginate-aminopropylsilicate-alginate[22]		
• Alginate-poly(L-ornithine)[23]		
• Alginate-cellulose sulfate-poly (methylene-*co*-guanidine)[24]		
• Agarose-poly(styrene sulfonic acid)[25]		
• Poly(N-isopropylacrylamide-*co*-acrylic acid)[26]		
• Alginate-poly(L-lysine)-poly(ethyleneimine)-Protamine-heparin[27]		
• Alginate-chitosan-polyethylene glycol[28]		
• Alginate-chitosan[29]	Hepatocytes	Liver transplantation
• Hydroxyethyl methacrylate-methyl methacrylate[30]		
• Alginate-poly(L-lysine)-alginate[31]	Kidney cells	
• Alginate[32]	Parathyroid cells	Parathyroid hormone
• Alginate-poly(L-lysine)-alginate[33,34]	Chromaffin cells	Neuropathic pain
• Alginate[35]	Chondrocytes	Chondrocyte transplantation
• Alginate-agarose[36]	Hybridomas	Antibody production
• Polyethersulfone hollow fiber[37]	Embryonic cells	Epilepsy
• Poly(acrylonitrile-vinyl chloride) (PAN-PVC)[38]	Baby hamster kidney (BHK) cells	Human nerve growth factor (NGF)
• Polyethersulfone[39]	Mouse C2C12 myoblasts	Adenosine
• Alginate-poly(L-lysine)[40]	iNOS-expressing cells	Inducible nitric oxide synthase gene (iNOS) for tumor suppression

shaped capsule compared to conventional type of spherical capsule (Fig. 4B). This stimulating phenomenon may be attributed from the existence of the hole in the center which played a role of overcoming the diffusion limit of oxygen and nutrient normally faced in spherical capsule environment where the cells in the core of the capsule had lower rate of getting oxygen and necessary nutrients and facilitated the transport them more efficiently to the cells. The mechanical stability of the doughtnut capsule in vitro exhibited strong for an extended period of time.

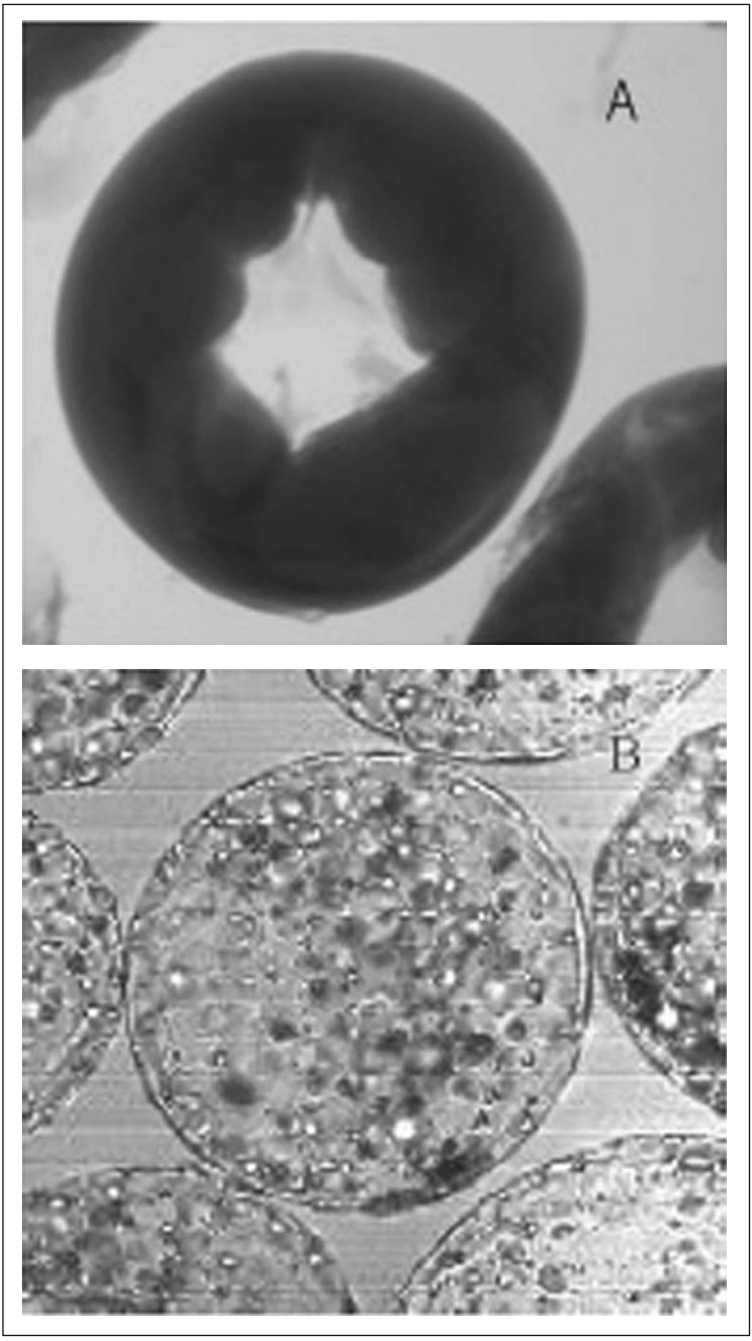

Figure 3. The observation of microencapsulated chromaffin cells. Optical microscopy image. Magnification: × 100. A) Keratiene hydrogel based doughnut shaped capsule. B) Alginate based spherical shaped capsule.

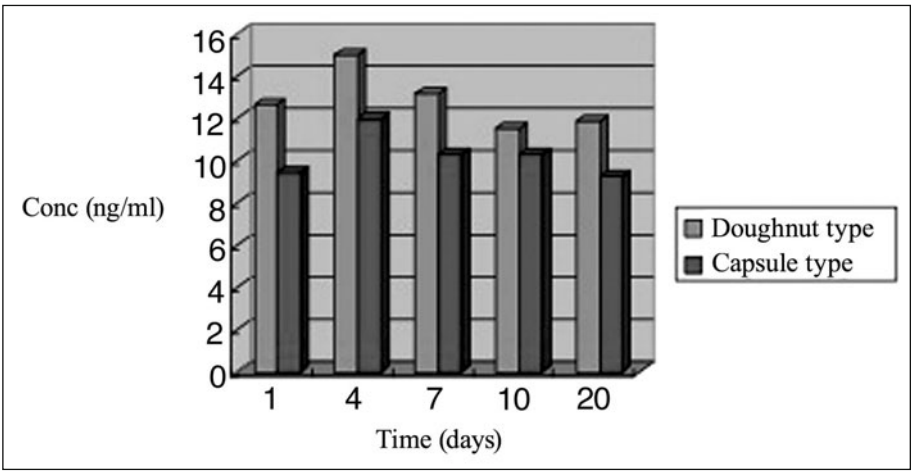

Figure 4. In vitro catecholamine analysis of secretion from procine chromaffin cells cells encapsulated in different shapes of capsule upon nicotine stimulation (HPLC, Mobile phase: 50 mM sodium phosphate, 50 mM citric acid , 20 mM octhan sulphonic acid, Flow rate: 0.3 ml/min, C18 reversed-phase 4.6 × 250 mm column. 25˚, Catecholamine monitored by electrochemical detection at a total potential of +770 mV.) For 20 days in vitro, catecholamine secretion is significantly higher in doughnut shaped capsules compared to spherical shaped capsules.

Clinical Impact of Microencapsulation Technology

Cell encapsulation is a probably the preferable system for cell transplantation and represent an exciting biotechnological approach for both organ replacement and continuous delivery of drugs.[45] This rising concept of cell-based therapy requires advances in cell encapsulation technology and there have been successful efforts in applying this technology for the treatment of human diseases including diabetes, hemophilia, renal failure, neurological disorders, cancers, ischemia and liver diseases.[46-52] There has been some success in the use of these microcapsules in animal models of diabetes and Parkinson's disease. Specifically, the production of dopamine and catecholamine and opioid peptides by microencapsulated chromaffin cells have been shown to reverse behavioral deficits in animal models of Parkinson's disease[49] and chronic pain, respectively.[53-55] For example, patients presenting severe and disabling chronic pain, especially among patients with terminal cancer, remain a therapeutic challenge. Transplants of capsulated chromaffin cells localized in the medullary portion of the adrenal glands produce and release high levels of opioid peptides and catecholamines into the CNS, thus providing a local source of neuroactive substances for reducing pain sensitivity. This strategy has resulted in a long-term or permanent source of pain-reducing agents without development of significant tolerance, eliminating the need for repeated narcotic administration.

A co-encapsulation approach is widely used to increase the duration of viability and function of cells. For example co-encapsulated hepatocytes with bone marrow stem cells resulted in increased viability of the hepatocytes in vitro and in vivo and also significantly prolonged the lowering of high systemic bilirubin levels in congenital Gunn rats with defects in the liver enzyme uridine diphosphate glucuronosyltransferase (UDPGT).[47]

Microencapsulation of recombinant cells is a novel alternative approach to tumor gene therapy. Therapeutic protein delivery can be sustained for systemic treatment of tumors. Peritoneal administration of encapsulated endostatin-CHO cells microcapsules as small as 200 microm in diameter inhibited melanoma growth to 66.4% and enhanced the survival of treated mice to 80% by 27 days posttreatment. Continuous systemic release of endostatin from microcap-

sules offers an effective therapeutic strategy to eradicate solid tumors.[56] Allotransplantation of microencapsulated parathyroid tissue is a promising approach to the treatment of permanent hypoparathyroidism. Preoperative assessment of the quality of microencapsulated parathyroid tissue could facilitate selection of the optimal bioartifical graft for human parathyroid allotransplantation. Microencapsulated parathyroid single cells showed significantly better function than the tissue cluster encapsulation.[57,58] Various types of cells can be used. The simplest and safest sources of cells are autologous cells which in reality often limit in sourcing. The advantage of genetically engineered cells is that a steady and more physiologic concentration of a therapeutic compound may be achieved without the complication of systemic side effects. For instance, baby hamster kidney (BHK) cells can be transfected with a human nerve growth factor fusion gene and transplanted into brains after encapsulating them in a semi-permeable polymeric membrane.

Several companies are working on polymeric encapsulation systems for clinical trials. For example, Biohybrid Technologies, Novocell, Inc. and Islet Sheet Medical Inc. (USA) and Neurotech (France), have developed encapsulation system for allografts to treat diabetes. Layton Biosciences (USA) has developed system for treating CNS disorders such as stroke, Parkinson's disease and Alzheimer's disease.

Challenges in Cell Microencapsulation

In spite of a great promise of cell encapsulation technology, there have been continuous challenges in cell therapy using microencapsulated cells. The major challenge is long term cell survival or prolonged cell viability in capsules. Cell survival in capsules has a limitation due to the supply of nutrients and oxygen. Nutrients typically include low molecular weight solutes such as glucose, macromolecules such as albumin and transferrin for iron uptake. Growth factors may also be required. Although the transport limitations for macromolecules have not yet been quantified, it is likely that oxygen supply limitations are the most serious.

A class of microporous membranes that induce neovascularization membrane is in direct contact with the bloodstream at an arterial pO_2, of ~100 mmHg. By contrast, extravascular devices implanted intraperitoneally or in subcutaneous tissue are exposed to the average pO_2, of the microvasculature (~40 mmHg). Implantation in soft tissue is further disadvantageous if a foreign-body response occurs, in which an avascular layer of typically ~100 μm thick is produced adjacent to the membrane. This fibrotic tissue increases the distance between blood vessels and the implant and the fibroblasts in the avascular layer consume oxygen. As a solution to deal with the limitations in oxygen transport, our lab recently attempted forming a new shaped capsule having an opening in its center that could enhance diffusion rate and simultaneously induce neovascularization in the vicinity of the implant. Another approach is to formulate oxygen producing particle or matrix which can prolong the release of oxygen to the transplanted cells and site.

In the absence of a proper cell encapsulating material, adherent cells aggregate to form clusters. When the clusters grow too large, they typically develop a central necrotic core. Dying cells accumulate around the core and, upon lysing, release factors detrimental to the health of neighboring cells. The lysed cell fragments are also transported to the host environment, there eliciting an antigenic response in the long run.

Cell sourcing is a challenge in cell microencapsulation approaches for cell based therapy. The attributes of engineered cells for cell sources are their higher capacity for in vivo survival. Problems associated with engineering cells involve gene transfection efficiency, risk of viral vectors, related safety and multiple purification processing. However with advances in genetic engineering techniques, application of genetically modified cells for therapeutic delivery is improving and promising. Stem cells have potential for cell-based therapeutics because of their ability of being virtually unlimited donor source for transplantation and the advantage of being flexible to a wide spectrum of genetic manipulations. However, extensive studies are to be done for long-term safety and efficacy of encapsulated stem cells on host tissue.

Conclusion

Cell microencapsulation is a core technology which enables long term delivery of biological products from living cells in response to biological need which is a promising method for cell therapy and organ function regeneration. However, issues on long term viability, risk of immune development, related safety and retrieval of the unwanted cells should be addressed to further explore their possible clinical applications.

There have been successful business and clinical attempts by using various types of cells to treat human diseases. Development of novel biomaterials and biotechnologies along with exploring the cell sourcing will accelerate the clinical translation of cell microencapsulation technology.

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Chapter 12

Commercial Applicability of Cell Microancapsulation:
A Review of Intellectual Property Rights

Stephan M. Meier*

Abstract

Maturity and applicability of a distinct field of science can be estimated, using a review of current filing statistics of patent applications. Therefore, hereby a search based on international classifications has been directed to the world intellectual property organization (WIPO) to determine the amount and content of patent applications related to the field of cell microencapsulation. The search was evaluated with regard to the distinct indications envisaged and an evaluation of possible technology gaps for fostering further progress was conducted.

Introduction

Approaching the scientific field of cellular microencapsulation, as illustrated in this book in further detail, is not only triggered by a general scientific curiosity, but additionally applicability in a larger scale so as to reach a certain commercial maturity is considered a major aim of modern scientists as well.

While the editors of this book have laid certain focus on reviewing the scientific literature in the recent past,[1,2] a contribution directed to intellectual property literature is currently still lacked. Therefore and for directing efforts into fields of science which might not only yield an enrichment of the academia but also might facilitate a later commercialization of such discoveries, scientists need to be provided with information with regard to already existent or possible arising intellectual property rights of third parties, which might block their or their partners ambitions of later commercialization, thereby interfering with their freedom to operate. Considering these boundary conditions, the given scientist is additionally provided with information with regard to obviously still unsolved problems in a given field of science, by identifying the gaps within published patent literature.

Furthermore such search results can be resolved with regard to the given applicants and/or assignees and might therefore yield in an identification of possible industrial partners for scientists being active in the field. In the following, no reference will be made to distinct applicants and/or assignees to avoid any impression of the author wanting to foster or to impair with a certain enterprise or organization. The respective interested reader is kindly requested to scan through the bibliographic information of applications discussed herein to identify possible partners himself.

Nowadays patent literature is being published in rather any language, by rather any state and by a multitude of trans-national organizations. Therefore an exhausting search can never be obtained and with regard to the completeness of such searches one can easily compare these

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searches with the Einstein homology between mass and energy: A fully resolved search will require infinite energy while an incompletely resolved search will yield in a certain undiscovered mass.

Retrieval of the Data Base

For the purpose of this contribution the author has decided to choose a compromise between the above extremes and thereby accepts a certain blur of the result, by selecting only the patent literature published under the patent cooperation treaty (PCT).

Even though this restriction imposes a rather strong constraint on the obtainable information, it may be assumed that the information and results obtained are rather representative for the commercial maturity of the field of science being searched.

The fact of being representative is backed by the assumptions that applicants filing for an international application desire to achieve a rather worldwide protection of their invention which again can be argued to be based on the opinion of the applicants that the claimed subject matter is profitable.

Furthermore the WIPO offers a search interface open to public which can be publicly used. Usage of such interface for said search requires some background information on patent literature which shall be provided herewith as well, to enable the academia active in the field of cell microencapsulation to conduct further searches after the publication of this contribution.

Currently, the WIPO references a number of approximately 1.5 billion published international patent applications. Besides from the fact that these applications can be searched content based using distinct words in the application, published patents usually offer the interesting possibility of searching them by their international patent classification. The International patent classification (IPC), established by the Strasbourg Agreement 1971, provides for a hierarchical system of language independent symbols for the classification of patents and utility models according to the different areas of technology to which they pertain. The IPC-based search is favorable due to the fact that false usage of certain words like "encapsulation" in a non-interesting field is prima facie excluded, as the invention can be assumed to belong to another class.

For cell microencapsulation certain IPC classes are applicable. These govern certain aspects of such technology as referred to in Table 1.

Two sets of binary combinations of subclasses are picked for determining in more detail the progress of state of the art in the field of prostheses implantable into the body (as to subclass A61F-2/02) which still defines one of the most interesting applications for encapsulated cells as well as in the field of making microcapsules (as to subclass B01J-13/02) which is of general relevance in the field of cell microencapsulation.

It is considered that the binary combinations possible with A61F-2/02 and with B01J-13/02 are reasonable choices, as both have proven to be one of interest in the recent past.[1,2]

The Teaching of Bibliographic Data

A simple number based representation of the search results can already roughly elucidate the maturity of a certain technical field if compared to overall filing statistics or within each IPC class.

If compared to the overall filing statistics at the WIPO, one can derive that only 0.6% of the overall 1,437,667 published patent applications fall into anyone of the above referenced subclasses. Which teaches that compared to all other possible fields of science, cell microencapsulation represents rather a specialty. As a comparison published applications drawn to telephonic communication (represented by the IPC Class H04M) already represent 1.2%.

Comparing published applications within the subclasses A61K-9/16 and A61K-9/50 to the overall amount of published applications within IPC class A61K already yields a proportion of 9.16%. This teaches that within the preparations for medical purposes (which also encompass drugs and drug formulations) already a significant proportion of published applications are drawn to a particulate form or microcapsule composition. This result can be misleading as the constraint of existence of living matter is not yet imposed on that result. Nevertheless a certain awareness of needs for encapsulated formulations can be derived.

Table 1. Certain IPC classes and subclasses relevant to cell microencapsulation

IPC Class	Sub Class	Full Class	Aspect of Technology (Among Others)
A61F	-	-	Prostheses
	2/02	A61F-2/02	Prostheses implantable into the body
A61K	-	-	Preparations for medical purposes
	9/16	A61K-9/16	Medicinal preparations characterized by particulate form, especially agglomerates; granulates; microbeadlets
	9/50[1]	A61K-9/50	Medicinal preparations characterized by preparations in capsules, especially microcapsules
A61L	-	-	Surgical materials
	27/38	A61L-27/38	Materials for implants, comprising animal cells
B01J	-	-	Chemical or physical processes, their relevant apparatuses
	13/02	B01J-13/02	Making microcapsules or microballoons
	13/22	B01J-13/22	After-treatment of capsule walls via coating
C12N	-	-	Micro-organisms or enzymes; compositions thereof; Propagating, preserving, or maintaining micro-organisms; mutation or genetic engineering
	5/00[1]	C12N-5/00	Undifferentiated human, animal or plant cells, e.g., cell lines; Tissues; Cultivation or maintenance thereof

[1]This does not represent the most detailed sub classification; some more detailed subclasses do exist.

It's also important to note that the IPC subclass A61K-9/50 comprises fewer applications than A61K-9/16 even though it contains further subclasses. This fact can be attributed to the often used agglomerated form of drug formulations which are contained in the A61K-9/16 as well.

With regard to the applicability of cellular based implants, of which microcapsules form a certain proportion, a comparison of the totally filed applications in the subclass A61L-27/38 to the IPC class A61L, which comprises all surgical materials is more elucidating, as a reference can be made to the amount of cell based implants per total amount of surgical materials. Comparison yields a proportion of 4.4%. Therefore a certain development and maturity of the field of cell-based transplants within the entire field of surgical materials might be argued to exist.

Besides from the fact that such preliminary conclusions might be interesting for determining generally known fields of science, the possibility of deriving conclusions for cell microencapsulation is rather limited.

More detailed information can be derived by binary combination of the aforementioned subclasses, as referred to above. Such a representation of binary combinations is given in Figure 1 for the chosen subclasses A61F-2/02 and B01J-13/02.

Such binary combination as depicted in Figure 1 (upper part) can be read by finding that obviously a proportion of 15 published applications are drawn to implantable prostheses involving undifferentiated human, animal or plant cells (see C12N-5/00). Interestingly, no process of coating after an encapsulation for implantation is disclosed (see B01J-13/22), while the preparation of capsules for transplantation purposes seems to be of relevant use (see B01J-13/02). The most relevant innovations seem to relate to implantable prostheses comprising animal cells (see A61L-27/38, 58 published applications).

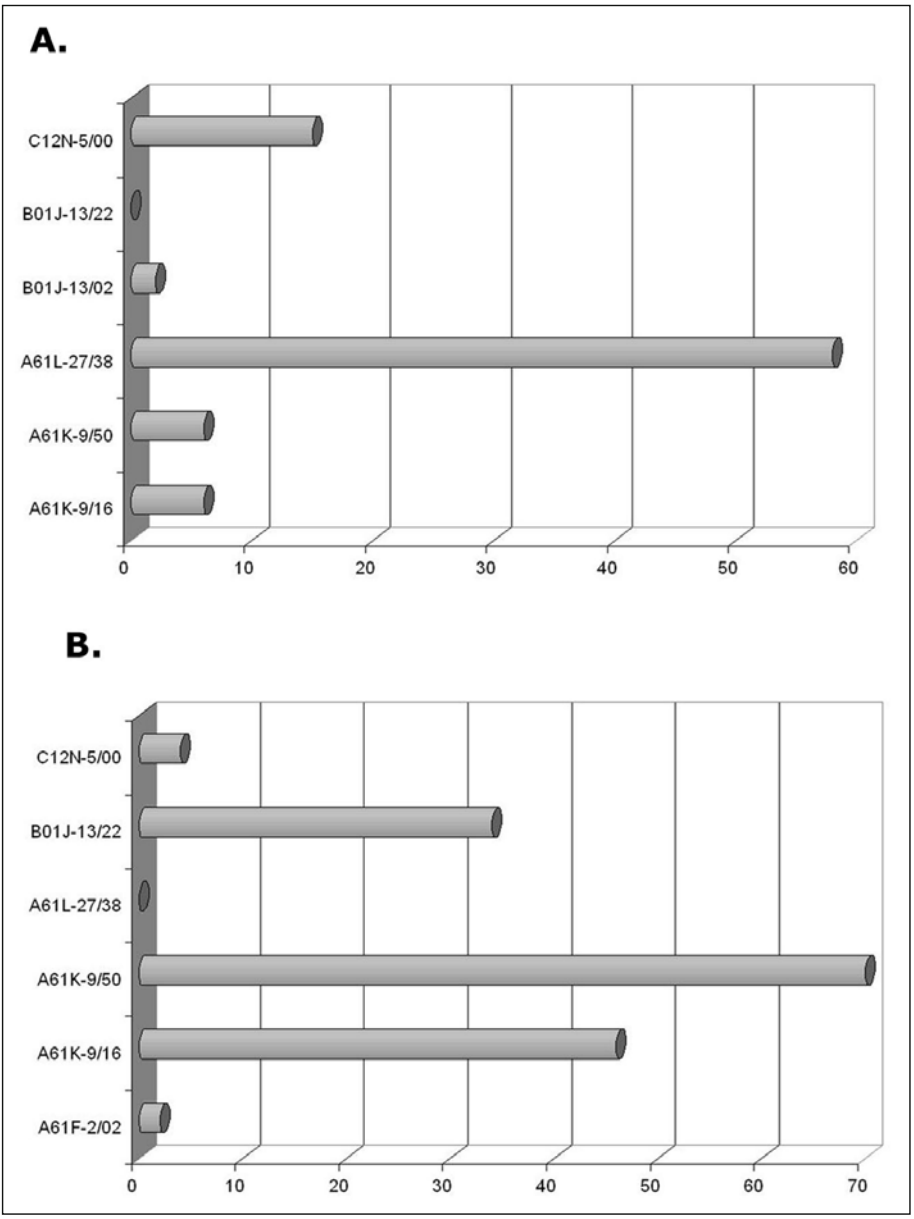

Figure 1. Results of binary combination of A61F-2/02 (upper) and B01J-13/02 (lower) with other subclasses.

Lower part of Figure 1 provides insight into the relevant art of processes of production of microcapsules in a similar way. This has already been reviewed several times from a more scientific point of view.[1,2]

For those 72[3-74] published applications it can be found that these have been applied for from and with 1987-11-17[3] up to 2006-05-31.[74] A statistic of said applications with regard to

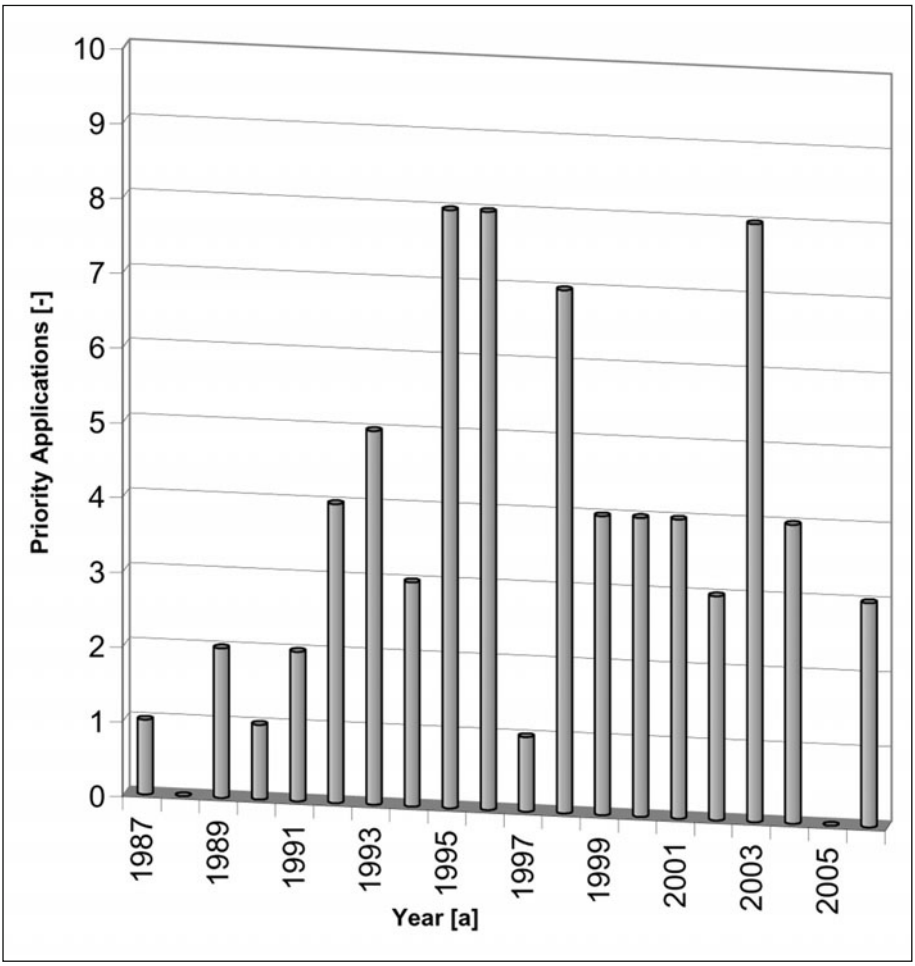

Figure 2. Priority filing statistics of applications falling being binary combinations with A61F-2/02.

their priority date which can be considered closest to the date of the invention is depicted in Figure 2. It should be noted that the fact that the latest application displays a priority date of 2006-05-31[74] can be transferred to the statement that there has not been a recent development. In contrary thereto one has to bear in mind that the publication of said applications is always earliest eighteen month after their priority date which yields that said application dated 2006-05-31[74] was laid open on 2008-01-10. Furthermore in Figure 1 a total amount of 84 published applications seems to prevail while Figure 2 teaches only 72 of those. This is to be contributed to the fact that some applications share some subclasses by providing not a binary but a ternary combination. Some of those applications can be considered to be the most interesting and will be exemplarily later.

Nevertheless the statistics of Figure 2 teach that applications drawn to prostheses implantable into the body have come to industrial applicability beginning with 1987-11-17[3] and maturity was reached throughout to 1995. Thereafter a more or less constant commercial interest can be stated up to today.

More Detailed Discussion of Relevant Prior Art

In the following a closer look will be laid upon the particular disclosure and teaching of those published applications being derived from binary combinations as depicted in, therefore drawn to transplants into the human body, which should be considered to be the most promising field of application for cellular microencapsulation in the upcoming future.

Therefore, of course the binary combination of the A61F-2/02 and the B01J-13/02 subclasses will be inspected in most detail, as well as the binary combination of B01J-13/02 and C12N5/00, which comprises the technical field of microcapsules aggregated with use of cells. It should be noted that the combination of A61L-27/38 and B01J-13/02 results in no hits which also will be subject to a later discussion herein.

Above said binary combinations do yield an overall amount of only five published applications; wherefrom four are derived from the B01J-13/02 and C12N-5/00 last named four are being from the same applicant.[3,75-77]

Even more interestingly is that the oldest one of the published applications[3] is related to "In Vivo Delivery of Neurotransmitters by Implanted, Encapsulated Cells".

In said patent application[3] the examples are drawn to a mouse model, wherein mouse ventral mesencephalon pieces are collected and encapsulated into polymer tubes which thereon are transplanted into the parietal brain cortex of rats. Same was done with immobilized dopamine secreting cells, which were implanted into rats with induced Parkinsonism. In the first experiment sufficient nutrition of the transplanted cells in the immobilisate could be proven, while in the later experiment a significant improvement of behavior of the rats could be found. Even though the experiments were drawn to encapsulation into polymer tubes which were thereon transplanted, the claims relate to a method of delivering neurotransmitters using encapsulated cells, whereby said method comprises the step of encapsulating cells into a semipermeable membrane. Thus said claim would comprise for example encapsulated cells in Alginate/Poly-L-Lysine thus forming a semipermeable membrane as well. According to claim 3 thereafter, said membrane may comprise polyalginates.

This application therefore already discloses one of the promising applications of encapsulated cells in medical therapy in an animal model. Unfortunately the author is not aware of later inventions/articles from the same applicant, proving applicability to humans.

The other patent applications[75,76] of the same applicant are drawn to a system and method for encapsulation as well as the resulting "vehicles", or to improved polymeric microbeads, whereby the concept of cell immobilization seems to have been abandoned[77] for the purpose of using biomolecules directly instead of cells producing the same.

Another interesting application[31] found via the A61F-2/02 and B01J-13/02 binary combination discloses a "Novel Artificial Pancreas", which again is an application of microencapsulation already focused by other authors. The pancreatic islet cells producing insulin were encapsulated in a semipermeable spheroidal membrane comprising agar gel, which also claimed in Claim 1 of the application. It is further claimed in claim 5 that another immunoisolating membrane is present and that according to claim 6-7 that said immunoisolating membrane is mechanical or synthetic and part of a diffusion chamber. Its envisaged that said immobilized cells should be implanted into a mammalian body, while the diffusion chamber is connected to blood vessels to allow perfusion with nutrients and release of insulin into the hosts body.

There is no explicit example given that might prove the workability of that invention even though the application can be read as that there is evidence on at least a prove of principle.

Again this application discloses one of the promising applications of cell microencapsulation, but later disclosures of the applicant in that field of science proving that said invention has come to commercial application were not found by the author.

Conclusion

Before drawing final conclusions certain boundary conditions need to be considered, for not giving a wrong impression.

Most patent systems in the world (e.g., the European patent convention) exclude methods of medical therapy from being patentable for not having industrial property rights interfere with medical care of humans or animals. Even though certain macerations might apply, such principles are still kept. Therefore industrial property rights related or directed to the actual use of encapsulated cells might be scarce. Above analysis interestingly displays only few applications using said macerations and further mostly lacks possible industrial property rights on preliminary stages of these methods, such as disclosed in WO/1989/004655.[3]

Nevertheless, from the afore mentioned analysis one can interpret that research in the field of cell microencapsulation and its most prominent fields of application (e.g., transplantation for medical purposes) has made some progress in the time since first applications for industrial property rights have been made, but obviously general applicability of such techniques has not yet reached a commercial maturity.

There is a whole bunch of knowledge already available in related fields of science, while aggregation to a complete, industrially exploitable technique seems to be still an open question unanswered.

One can argue that those enterprises being active in that field will most probably be mostly still working on their animal model studies; meaning these studies are still preclinical and therefore risk of loss of advance due to the inevitable disclosure by a patent might outweigh possible protection by a patent. Still, such argumentation further proves that a certain leap is still needed in some aspects to enable filing for patents and thereafter commercial use of these. Finally it should be encouraged to invest further efforts into cell microencapsulation, as some relevant applications seem to be still undiscovered or at least free from third party protection, offering opportunities of still being able to exploit that field commercially.

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